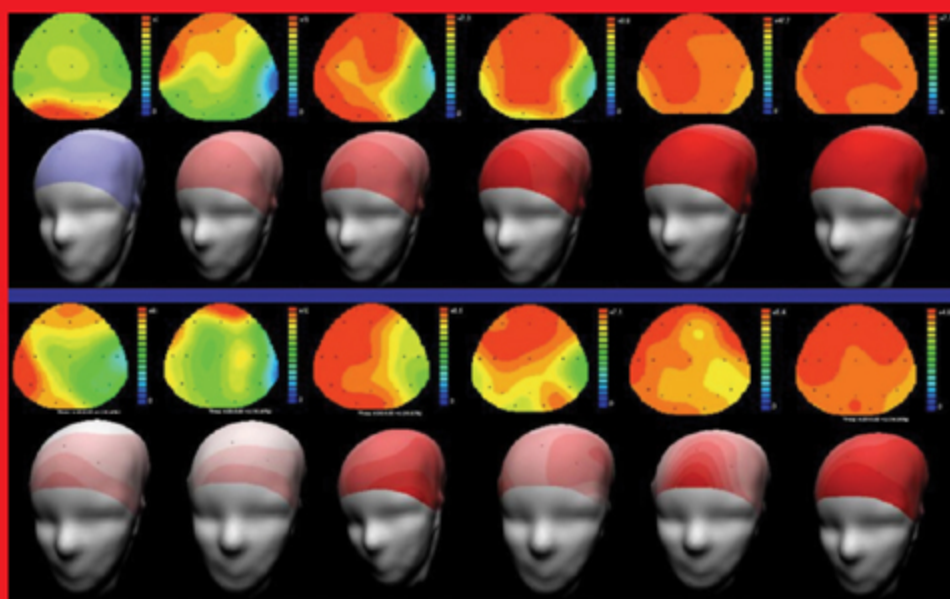


Pharmacogenomics in Drug Discovery and Development

Edited by
Qing Yan



Pharmacogenomics in Drug Discovery and Development

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Preface

Each human is genetically distinctive, and responds differently to disease-causing factors as well as to drugs. Mechanisms inside human bodies that control drug responses are complex and multifactorial. Pharmacogenomics arose in response to such recognition of the necessity of personalized medicine, a medicine that deals with the complexity of the human body. The development of pharmacogenomics represents the evolution of biomedicine from treating the general disease itself to treating the malfunction of an individual person, the “root” of diseases. With the change of focus from diseases to humans, pharmacogenomics brings hope for the transformation from disease treatment to disease prevention.

Pharmacogenomics is considered the future of drug therapy. For the drug development industry, pharmacogenomics is useful in identifying drug targets to obtain optimal drug efficacy for certain patient populations. Because of the diversity of patients’ biological backgrounds, the same disease may be caused by genetic variations in different people, who will respond differently to the same drug. Such situations require individualized treatment that avoids adverse drug responses and ensures the best possible results.

However, many challenges need to be resolved before pharmacogenomics can be applied in the clinic. These challenges include the identification of biomarker genes and pathways, the understanding of interactions between genes and drugs, and the correlation of genotypes to disease and drug response phenotypes.

In this book, we approach these challenges from three aspects. We first introduce some important cutting-edge technologies that are useful for the development of systems-based pharmacogenomics to solve the complexity; these technologies include bioinformatics, microarray, and association studies. These technologies can help us with the identification of biomarker genes and pathways and in understanding the associations among genes, drugs, and diseases.

These systems-based approaches use bioinformatics methods for studies in pharmacogenomics and systems biology to manage, organize, and understand the overwhelming information. Integrated methodologies and procedures for applying bioinformatics analysis in pharmacogenomics are presented in this book, as bioinformatics has become indispensable for almost all biopharmaceutical studies today. Pharmacogenomics-related resources, including databases and tools, are collected and provided.

Microarrays and biochips are powerful technologies for high-throughput (HTP) analysis that may enable systematic understanding of genomics and proteomics as well as large drug response data sets. The applications of microarrays in pharmacogenomics, genotyping, and clinical diagnosis, as well as the evolution and development history of the technology, are introduced in this book. Different techniques, platforms, and tests are also discussed.

Association study is a useful method in pharmacogenomics for investigating how individuals with unique genetic variants respond to a drug treatment. Confounding caused by population structure and admixture can contribute to the lack of replication of association study results. Methods for detecting and adjusting confounding are explained, as are their advantages and disadvantages.

The second aspect of this volume includes approaches to studying gene–drug interactions, that is, how drugs act and how they are processed in the human body, including drug absorption, distribution, metabolism, and excretion. Biomarkers and molecules such as ion channels, membrane transporters, receptors, and enzymes are playing increasingly essential roles in drug design and pharmacogenomics studies. These biomarkers provide critical links between drug discovery and diagnostics efforts. Updated introductions and detailed methods about studies in these molecules are provided in this book.

For example, membrane transporters are profoundly involved in drug disposition through transporting substrate drugs between organs and tissues. Investigations of genetic variations, genotyping methods, and substrate identification of membrane transporters are helpful for drug design and development. Different methods for assessing functional significance of transporter polymorphisms *in vitro* and *in vivo* as well as the application of transporter genetics in clinical pharmacology are described. Clinical significance of pharmacogenomics studies in drug-metabolizing enzymes and drug transporters for certain treatments, such as chemotherapy, is discussed in detail.

Studies of G protein-coupled receptors (GPCRs) may provide insight into disease pathways, such as the involvement of the regulator of G protein signaling (RGS) protein polymorphisms in hypertension. Pharmacogenomics of GPCR studies the involvement of genetic variations in structural and functional roles, such as GPCR activation and inactivation, their relationships with diseases, and their potential uses in defining optimized novel drug targets. These investigations can be useful for refining drug discovery as GPCR disorders are associated with a wide variety of human diseases, including retinal diseases, thyroid diseases, obesity, diabetes, asthma, cardiovascular diseases, cancer, and infectious diseases.

The third aspect composes a large part of this book: a focus on how pharmacogenomics can be used in therapeutics of diseases. These diseases include cardiovascular diseases, cancer, neurological diseases, gastrointestinal disorders, autoimmune diseases, and infectious diseases. Comprehensive information for each disease system is discussed, including biomarkers involved in the disease and the associations among genes, drugs, diseases, drug response phenotypes, and the environment.

For example, epigenetics and environmental factors may play important roles in major psychiatric disorders. Detailed methods for studying these factors are given to provide a prototype model system for better diagnosis and management of

mental diseases. Asthma is another disease caused by interactions among multiple causes, including demographic, social, environmental, and genetic factors. The most common biological pathways targeted by asthma therapy and the genetic contributions to varied therapeutic responses are described.

Drug treatment in Alzheimer's disease (AD) accounts for more than 10% of direct costs, while fewer than 20% of AD patients are fair responders to conventional drugs. Pioneering pharmacogenomics studies have shown that the therapeutic response in AD is genotype specific as pharmacogenomics factors account for more than 60% of drug variability in drug disposition. This book provides a comprehensive and detailed discussion of the pharmacogenomics of AD, from functional genomics to therapeutic strategies. The integration of these pharmacogenomics protocols with AD drug discovery and clinical practice can help promote therapeutics optimization and develop cost-effective pharmaceuticals to improve both drug efficacy and safety.

For cardiovascular diseases, methods for choosing candidate genes and single-nucleotide polymorphisms (SNPs) and the association with functional studies are discussed. These mechanistic studies are particularly important when it comes to pharmacogenomics associations. These studies provide significant and clinically relevant insights into the variable drug responses in cardiovascular disease management.

In gastroenterology and hepatology, genetic variations involved in drug metabolism or disease pathophysiology have been found to have an impact on drug responses. Discussions in this book focus on clinical pharmacogenomics of inflammatory bowel disease, *Helicobacter pylori* infections, gastroesophageal reflux disease, irritable bowel syndrome, liver transplantation, and colon cancer.

For rheumatoid arthritis, the pharmacogenomics of three major disease-modifying antirheumatic drugs (methotrexate, azathioprine, and sulfasalazine) and one class of biologic antirheumatic drugs (the tumor necrosis factor antagonists) are discussed in detail.

Cancer pharmacogenomics includes studies on biomarkers such as thiopurine methyltransferase (TPMT) and epidermal growth factor receptor (EGFR). Research methods such as germline and tumor DNA studies, polymorphism selection, and biomarker screening as well as genotyping systems are described.

Using array technology in pharmacogenomics, efficacy and systemic toxicity can be evaluated for the improvement of the design and development of preclinical vaccines. Methods of applying pharmacogenomics in the evaluation of efficacy and adverse events during clinical development of vaccines are also discussed.

By covering topics from individual molecules to systemic diseases, from fundamental concepts to advanced technologies, this book intends to provide a practical, state-of-the-art, and integrative view of the application of pharmacogenomics in drug discovery and development. I would like to thank all of the authors for their contributions to this exciting new field. I also thank the series editor, Dr. John Walker, for his help with the editing.

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Contents

Chapter 1	The Integration of Personalized and Systems Medicine: Bioinformatics Support for Pharmacogenomics and Drug Discovery	1
	Qing Yan	
Chapter 2	Applications of Microarrays and Biochips in Pharmacogenomics.	21
	Gary Hardiman	
Chapter 3	Confounding in Genetic Association Studies and Its Solutions	31
	Donglei Hu and Elad Ziv	
Chapter 4	Pharmacogenetics of Membrane Transporters: A Review of Current Approaches	41
	Tristan M. Sissung, Erin R. Gardner, Rui Gao, and William D. Figg	
Chapter 5	Pharmacogenomics of Drug-Metabolizing Enzymes and Drug Transporters in Chemotherapy	63
	Tessa M. Bosch	
Chapter 6	Pharmacogenomics of G Protein-Coupled Receptor Signaling: Insights from Health and Disease	77
	Miles D. Thompson, David E. C. Cole, and Pedro A. Jose	
Chapter 7	G Protein-Coupled Receptors Disrupted in Human Genetic Disease	109
	Miles D. Thompson, Maire E. Percy, W. McIntyre Burnham, and David E. C. Cole	

Chapter 8	G Protein-Coupled Receptor Pharmacogenetics	139
	Miles D. Thompson, Katherine A. Siminoivitch, and David E. C. Cole	
Chapter 9	Epigenetic Alterations of the Dopaminergic System in Major Psychiatric Disorders	187
	Hamid Mostafavi Abdolmaleky, Cassandra L. Smith, Jin-Rong Zhou, and Sam Thiagalingam	
Chapter 10	Pharmacogenomics in Alzheimer's Disease	213
	Ramón Cacabelos	
Chapter 11	Pharmacogenetics of Asthma.	359
	Gregory A. Hawkins and Stephen P. Peters	
Chapter 12	From SNPs to Functional Studies in Cardiovascular Pharmacogenomics.	379
	Sharon Cresci	
Chapter 13	Pharmacogenomics in Gastrointestinal Disorders	395
	Michael Camilleri and Yuri A. Saito	
Chapter 14	Pharmacogenomics in Rheumatoid Arthritis	413
	Prabha Ranganathan	
Chapter 15	Cancer Pharmacogenetics	437
	Sharon Marsh	
Chapter 16	Pharmacogenomics in the Preclinical Development of Vaccines: <i>Evaluation of Efficacy and Systemic Toxicity in the Mouse Using Array Technology</i>.	447
	Karin J. Regnström	
Chapter 17	Pharmacogenomics in the Evaluation of Efficacy and Adverse Events During Clinical Development of Vaccines.	469
	Lennart J. Nilsson and Karin J. Regnström	
Index		481

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Chapter 1

The Integration of Personalized and Systems Medicine

Bioinformatics Support for Pharmacogenomics and Drug Discovery

Qing Yan

1.1	Introduction.....	2
1.2	Methods.....	5
1.3	Notes	18
	References.....	19

Summary Pharmacogenomics may have a deep impact on every drug treatment protocol to bring the right drug to the right patient. While pharmacogenomics can help achieve individualized medicine, the study of systems biology can help us understand the key issues in pharmacogenomics at different levels. These key issues include the associations between structure and function, the correlations between genotype and phenotype, and the interactions among gene, drug, and environment. Utilizing bioinformatics in pharmacogenomics that is conducted in a systemic way can help integrate information from different levels. At the molecular level, the detailed features of a gene and the relationship between genetic structure and function need to be explored. These detailed features include sequence analytic information such as sequence retrieval and structural modeling, sequence variation information, and sequence patterns that can correlate sequence structure to functional motifs. At the cellular level, the interactions and networks among those molecules should be examined. Higher degrees of understanding at the tissue and organism levels can help establish the correlations between genotype and phenotype. The application of bioinformatics methods in pharmacogenomics and systems biology should enable a more profound understanding of diseases at different levels and lead to both individualized and systems medicine. To facilitate up-to-date bioinformatics support, an integrated search engine and updated collections of tools are freely available at <http://sysmed.pharmtao.com>.

Keywords Bioinformatics; database; disease; drug; function; genotype; interaction; pathway; pharmacogenomics; phenotype; single-nucleotide polymorphism (SNP); software; structure; systems biology.

1.1 Introduction

1.1.1 Pharmacogenomics, Systems Biology, and Drug Discovery

With the completion of the Human Genome Project and advancements in genetics and protein engineering, biology is entering the postgenomic era. Meanwhile, increasing development costs of new drugs and high-profile drug withdrawals are seen together with fewer approvals of new drugs (1). In addition, with more than 30,000 genes in the human genome, the study of all the information has become a compellingly complex problem.

Biomedical science is facing the need for a new approach, a transformation from reductionism toward a holistic paradigm, from one-size-fits-all drugs toward personalized medicine. The emerging disciplines, pharmacogenomics and systems biology, provide hope for answering the questions and lighting the future of drug therapy. To encourage pharmacogenomic tests during drug development, the Food and Drug Administration (FDA) published the *Guidance for Industry: Pharmacogenomic Data Submissions* in 2003 to provide guidelines on using genetic data to make better and safer drugs (2).

Pharmacogenomics studies the genetic basis of individual variation in response to therapeutic agents (3). The investigation of genetic diversity in humans can make it possible to tailor optimal drug prescription and to bring the right drug to the right person. Pharmacogenomics may have a deep impact on every step of medical care, from diagnosis to drug prescription and from drug design to clinical trials. In an ideal condition, the application of pharmacogenomics based on the patient's genetic profile would enable the prediction of a patient's response to particular drugs and empower physicians to make right decisions for the treatment.

The effective approach to pharmacogenomics requires the integration of different disciplines, including structural genomics, functional genomics, proteomics, disease pathogenesis, pharmacology, and toxicology (3). While pharmacogenomics may help achieve individualized medicine, the study of systems biology may help us understand the key issues in pharmacogenomics at different levels (see Fig. 1.1). These key issues include the associations between structure and function, the correlations between genotype and phenotype, and the interactions among genes, drugs, and the environment (3). Systems biology investigates the roles biological molecules play in the context of complicated pathways and interactions and enables the understanding of disease and drug mechanisms at the system level (4). Using computational methods, systems biology may help us simulate large networks of interacting components, organize biological principles, and create predictive models.

As shown in Fig. 1.1, the integration of pharmacogenomics and systems biology can help elucidate the mechanisms of diseases and drug actions at various levels and connect information between different levels. For example, altered genetic structure may cause malfunctions at the molecular level, which would influence the downstream interactions, pathways, and networks at the cellular level. Such changes may then lead to tissue or organ disorders that are disease phenotypes reflected as

Environment

Humans/Populations

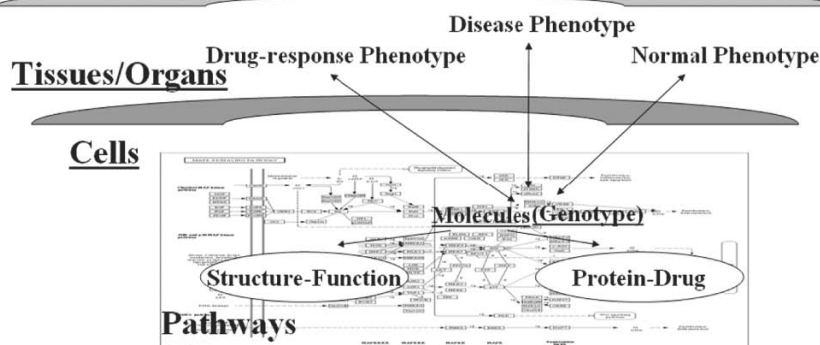


Fig. 1.1 Systematic understanding of pharmacogenomics at different levels

symptoms of the whole body. In addition, varied genetic structure and altered functions may influence the interactions between genes and drugs, which ultimately affect drug–response phenotypes. On the other hand, interactions among genes, drugs, and the environment at higher levels may also affect the structure and function of genes at the molecular level, which would in turn change downstream reactions and phenotypes, forming a feedback loop. The understanding of such an interwoven network may be the ultimate key to accurately identifying drug targets and to avoiding adverse reactions.

Handling such interwoven networks and complex feedback loops is beyond the capability of common laboratory methods, not to mention that just the complexity of scientific literature itself is already beyond measure. Help from computers and bioinformatics has become a must in today's biomedical research. In fact, bioinformatics methods have become indispensable for each step in biomedical research, from high-throughput data collection to clinical decision support. This chapter focuses on the application of bioinformatics methods in the study of pharmacogenomics, drug discovery, and systems biology.

1.1.2 Application of Bioinformatics in Pharmacogenomics and Systems Biology

Bioinformatics is using computational approaches to solve biomedical problems and to improve the communication, understanding, and management of biomedical information (5). Because of the information overflow, how to integrate biomedical

data and bioinformatics resources and how to make the best use of them have become a real challenge. Data integration is the process to transform data into information, then into useful knowledge (5). In this chapter, the integrated bioinformatics methods are presented, with those underlying tools and sources for carrying out these methods collected for systemic studies of pharmacogenomics information (see **Notes 1** and **2**).

As shown in **Fig. 1.1**, bioinformatics approaches in pharmacogenomics are conducted systematically. The lowest level in the system is at the molecular level. At this level, it is necessary to understand the detailed features of a gene and the relationship between genetic structure and function (see **Subheading 1.2.1**). These detailed features include sequence analytic information such as sequence retrieval and comparison, sequence variation information such as about single-nucleotide polymorphisms (SNPs), and sequence patterns that can correlate sequence structure to functional motifs.

When a time dimension is added at this level, evolutionary or phylogenetic trees can be built to compare these genetic sequences of different times. For example, tools such as the Basic Local Alignment Search Tool (BLAST) (see **Subheading 1.2.1.1**) and ClustalW (see **Subheading 1.2.1.1**) are commonly used in comparing genetic sequences and evolutionary relationships. SNP databases such as dbSNP (see **Subheading 1.2.1.4**) can provide information for individual genotype data.

Tools for sequence pattern analysis, including PROSITE and Pfam, are useful for correlating sequence structure to functional motifs (see **Subheading 1.2.1.2**). Three-dimensional (3-D) modeling of the sequence structure, such as using the database Protein Data Bank (PDB) and the program SWISS-MODEL (see **Subheading 1.2.1.3**), will also provide us better understanding of the structure–function relationship at this level.

In addition to sequence structural information on molecules themselves, these molecules have been categorized and classified according to their functions and interactions with drugs or other molecules. For example, some Web sites and databases can be used for studying such specific genetic molecules, including receptors and transporters.

With comprehensive examination at the molecular level, we can then scale up to the higher level to gain a more complete view of how the system works. At the cellular level, the interactions and networks among those molecules are examined. Protein–protein interaction databases and gene network and pathway databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) are usually used for this level of study (see **Subheading 1.2.2.1**). Resources are also available for studying even higher levels, including the tissue level and the organism level, and for making the connection between different levels. For example, some databases supply linkages between sequence variation genotypes and disease phenotypes, such as the Online Mendelian Inheritance in Man (OMIM) database (see **Subheading 1.2.3**).

In the following, the application of bioinformatics methods in pharmacogenomics studies is described at different levels of study to enable a relatively holistic understanding.

1.2 Methods

In this subheading, bioinformatics methods based on database and software tools for pharmacogenomics research at different levels are described. Most of the Web sites, databases, and software tools discussed in this chapter are freely available online, and universal resource locators (URLs) are provided (*see* [Note 2](#)). Many of the systems are not limited for use at only one level but provide information and connection about multiple levels. These multilevel systems, such as the National Center for Biotechnology Information (NCBI)'s Entrez system, allow us to have an integral view from different aspects while processing detailed information at a specific level. [Figure 1.2](#) shows the workflow of commonly used bioinformatics analyses in pharmacogenomics and systems biology.

1.2.1 *Methods to Study Pharmacogenomics at the Molecular Level*

1.2.1.1 Sequence Searching and Similarity/Homology Comparison

Genetic sequence searching and retrieving may be one of the most common methods used for genomics research. A useful searching system is NCBI's Entrez system (*see* [Table 1.1](#)). NCBI has a variety of databases and tools. [Figure 1.3](#) shows a screen shot of the Entrez cross-database searching system, through which a researcher can input keywords and look for results from different databases through just one query interface. These databases include the GenBank nucleotide and protein sequence databases, the structure database about 3-D structures, the SNP database, and the OMIM database about genetics and diseases. Various levels of information can be found in these databases.

For example, for sequence retrieval, the full gene definition, accession number, source, organism, and references are provided on nucleotide or protein database pages in the Entrez system. Two relatively new query systems in Entrez are especially useful, Entrez Gene and dbGaP (database of Genotype and Phenotype). Entrez Gene is an integrated environment that organizes and links relevant information about a specific gene, including general gene and protein information, genomic context, interactions and pathways, bibliography, and links to other systems such as dbSNP and Gene Ontology (GO). Entrez Gene can be queried with keywords, symbols, accessions, publications, chromosome numbers, Enzyme Commission (EC) numbers, and other features associated with genes. Entrez's dbGaP stores information on study results about the interaction of genotype and phenotype.

To identify the relationship between a new gene or the query sequence and those genes that are already known or stored in public databases and to elucidate the functions the new gene may have, programs in BLAST are usually used (*see* [Table 1.1](#)). BLAST provides a similarity search for both nucleotide and protein sequences against genomic sequence information available in the databases (*see* [Note 3](#)).

Table 1.1 Bioinformatics sources for pharmacogenomics research at the molecular level

Category	Name	URL	Explanation
General	NCBI	http://www.ncbi.nlm.nih.gov/	Bioinformatics databases and tools.
	Entrez	http://www.ncbi.nlm.nih.gov/Entrez/index.html	A cross-database searching system.
	Basic Local Alignment Search Tool (BLAST)	http://www.ncbi.nlm.nih.gov/BLAST/	Comparison of novel sequences with known genes.
Sequence alignment	ClustalW	http://www.ebi.ac.uk/clustalw/	Sequence alignment for their identities, similarities, and differences.
Gene prediction and sequence annotation	GENSCAN	http://genes.mit.edu/GENSCAN.html	Gene prediction.
	ORF Finder (Open Reading Frame Finder)	http://www.ncbi.nlm.nih.gov/gorf/gorf.html	Finding ORFs.
Pattern and motif analysis	Motif Scan	http://myhits.isb-sib.ch/cgi-bin/motif_scan	Finding motifs in a sequence.
	PROSITE	http://us.expasy.org/prosite/	Protein families and domains.
	Pfam	http://www.sanger.ac.uk/Software/Pfam/	Protein families database of hidden Markov models (HMMs).
Protein 2-D and 3-D structure	GeneBee	http://www.genebee.msu.su/services/rna2_reduced.html	RNA secondary structure prediction.
	PredictProtein Protein Data Bank (PDB)	http://www.predictprotein.org/ http://www.rcsb.org/pdb/	Protein secondary structure prediction. Biological macromolecular structure data.
	SWISS-MODEL	http://swissmodel.expasy.org/	Homology modeling for protein 3-D.

2-D, two dimensional; 3-D, three dimensional; NCBI, National Center for Biotechnology Information.

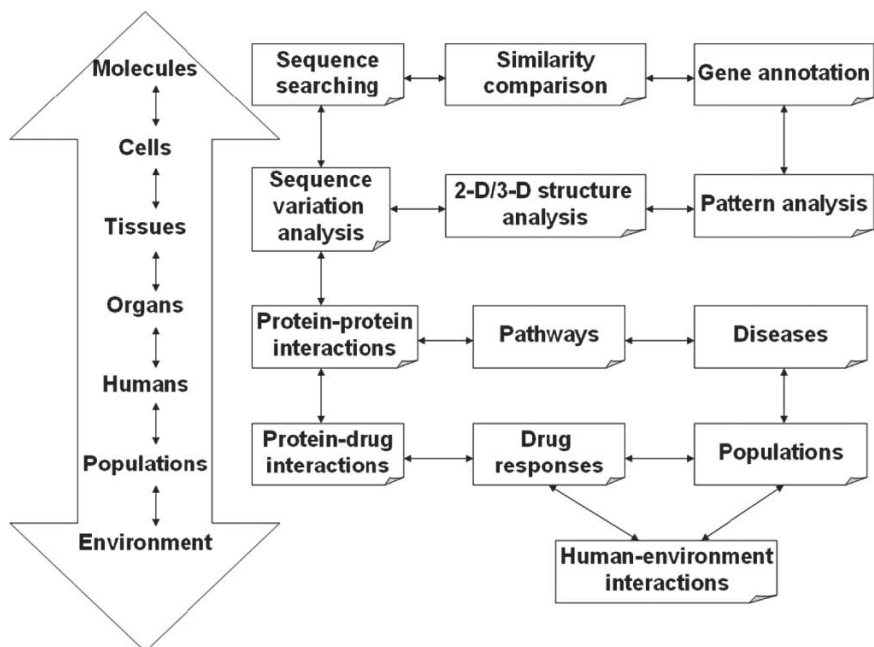


Fig. 1.2 The workflow of commonly used bioinformatics analyses in pharmacogenomics and systems biology

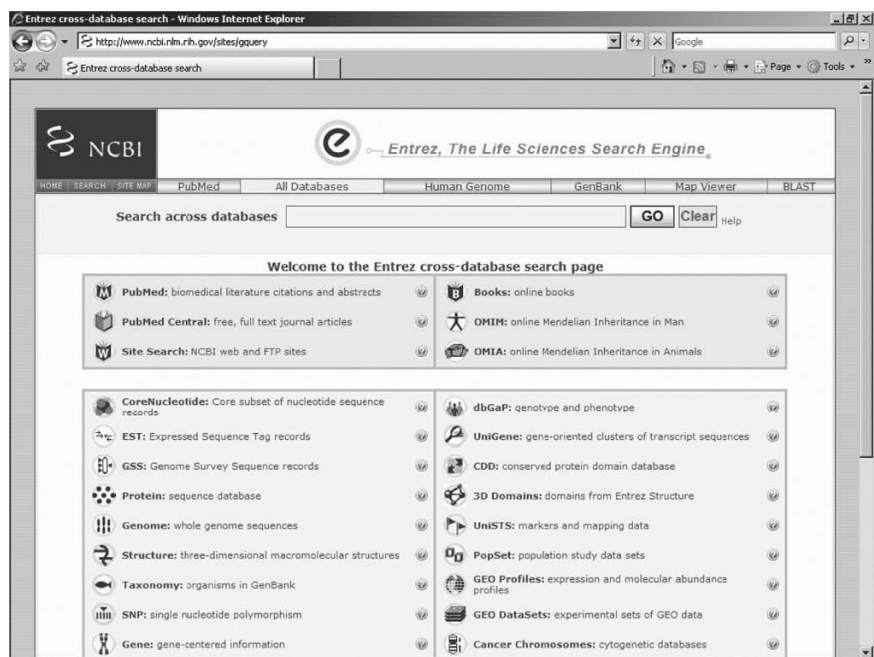


Fig. 1.3 The Entrez cross-database searching system

The BLAST searching output can help users deduce functional and evolutionary information for their query sequences. BLAST can also be used for comparisons in certain categories, such as SNP BLAST and Immunoglobulin BLAST (IgBlast).

For structure–function analysis at the molecular level, the comparison of available sequences is especially useful. Sequence comparison serves as the basis of many analyses that need a homology search, such as the construction of protein family databases. Multiple alignment tools such as ClustalW (*see Table 1.1*) can be used for customized sequence comparison and phylogenetic analysis. Sequences to be compared can be pasted or uploaded into the program. On the output page, conserved or different sequences are highlighted in different colors. Phylogenetic tree diagrams can also be shown to indicate the evolutionary relationships.

1.2.1.2 Gene Annotation and Pattern Analysis

The program GENSCAN can be used for gene identification and annotation in genomic DNA sequences (*see Table 1.1*). The program can predict exons, poly A signals, and the peptide sequence. To find open reading frames (ORFs) in a sequence, the Open Reading Frame Finder (ORF Finder) at NCBI can be used (*see Table 1.1*).

In addition to BLAST, alignment tools, and phylogenetic trees, another way to extract functional information on genetic sequences is analyzing patterns in protein sequences. A *pattern* in a protein sequence is a characteristic motif or domain that corresponds to the common structural or functional features of a protein family. A pattern usually has certain functional meanings (5). For example, a pattern in the format [V]-x(2)-{AE} represents [Val]-any-any-{any but Ala or Glu}. To search for motifs in a query sequence or the protein family to which the query sequence belongs, the program Motif Scan (*see Table 1.1*) can help. The program compares similarities between the query sequence and motifs in databases, including PROSITE and Pfam (*see Table 1.1*).

1.2.1.3 Secondary and Tertiary Structure Prediction

Programs such as the one in GeneBee can be used to predict RNA secondary structure (*see Table 1*), which can help in understanding how RNA secondary structure influences diverse functional activities. Using a sequence similarity search and neural network algorithms, the program PredictProtein can help in protein secondary structure prediction (*see Table 1.1*). The searching result of PredictProtein includes the predicted secondary structure, possible transmembrane helices, and the expected accuracy of prediction methods.

Programs for the prediction of 3-D structure usually use available protein domains from PDB (*see Table 1.1*) as templates. Tools that are usually used include SWISS-MODEL (*see Table 1.1*). The query result includes the template used, the alignment between the query sequence and the template, and the predicted 3-D model.

1.2.1.4 Sequence Variation Analysis

One of the most important analyses in pharmacogenomics is the identification of associations between a disease and genetic variations in a population. As listed in [Table 1.2](#), some resources contain comprehensive information about genomic variations, such as the International HapMap Project and the site of the Human Genome Variation Society (HGVS). The HapMap provides information about common genetic variants that occur in humans, including the description of the variants, their locations, and their distributions among populations. The HGVS site provides links to different genetic variation databases and relevant information, including locus-specific mutation databases, disease-centered central mutation databases, and central mutation and SNP databases.

The NCBI's database dbSNP includes information about Fasta sequences, GeneView via analysis of contig annotation, population diversity, and links to other databases (*see* [Table 1.2](#)). Researchers can query the database using keywords and database IDs such as GenBank accession numbers. [Figure 1.4a](#) shows a screen shot of the query result page from dbSNP with a search for a human gene using the keyword "human TAP1." The search retrieved more than 80 entries. [Figure 1.4b](#) is a sample page showing the location of one polymorphism, rs28986263, in a chromosome map, the MapView. [Figure 1.4c](#) shows the GeneView of the SNP, including the gene model, the region, the contig position, the amino acid position, functional changes, and other SNPs in nearby genomic regions.

The Human Gene Mutation Database (HGMD) provides information on the genetic mutation type, the genetic mutation data, and disease phenotypes (*see* [Table 1.2](#)). The program SNP-Fasta can be used to identify SNPs with homology and for similarity searching against sequence variation databases (*see* [Table 1.2](#)). The program query results are similar to general BLAST searches and include sequence alignments.

Several programs are available for SNP visualization. The Expression-Based SNP Imagemaps of the Cancer Genome Anatomy Project (*see* [Table 1.2](#)) can be searched by chromosome, tissue, and histology. The program Gene Viewer shows SNP information in the context of transcripts, ORFs, and motifs (*see* [Table 1.2](#)). The program can be queried through gene names, mRNA accession numbers, protein motif names, or sequences. The query results include predicted significant SNPs, protein sequence alignments, relevant motifs, statistics about how SNPs affect the fit of protein domains to motif models, and 3-D structures of motifs if they are known.

The importance of studying SNPs lies in their impact on the structure and function of a protein. Using physical and comparative mechanisms, the program PolyPhen can be used to predict the possible influence of an amino acid substitution on the structure and function of a human protein (*see* [Table 1.2](#)). For example, SNPs that have structural influences on buried sites may cause hydrophobicity disruption. SNPs may also have functional influences, such as damage on protein interaction sites and changes in downstream interactions.

The information about allele frequency in an anthropologically defined human population is important for pharmacogenomics studies as different population

Table 1.2 Bioinformatics sources for sequence variation studies

Category	Name	URL	Explanation
Sequence variance portal	Human Genome Variation Society (HGVS)	http://www.genomic.unimelb.edu.au/mdi/	Links sequence variation databases
	dbSNP	http://www.ncbi.nlm.nih.gov/SNP/	NCBI's database of SNPs
	International HapMap Project	http://snp.cshl.org/	Databases, linkage maps, features of sequence variation
Mutation	Human Gene Mutation Database (HGMD)	http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html	Gene lesions responsible for inherited disease
SNP homology and similarity searching	SNP-Fasta	http://www.ebi.ac.uk/snpfasta3/	Searching SNPs with the Fasta server for homology and similarity
SNP visualization	Gene Viewer	http://gai.nci.nih.gov/cgi-bin/GeneViewer.cgi	View SNPs in the context of transcripts, ORFs, and motifs
SNP/mutation: functional impact	Expression-Based SNP Imagemaps	http://gai.nci.nih.gov/html-snp/ts.html	SNPs based on chromosome, tissue, and histology
	PolyPhen	http://genetics.bwh.harvard.edu/pph/	Prediction of structural and functional effects of SNPs
	Allele Frequencies Database	http://www.allelefrequencies.net/	On human populations
Population	The Distribution of the Human DNA-PCR Polymorphisms	http://www.uniduesseldorf.de/WWW/MedFak/Serology/database.html	Population polymorphisms
Environment	Environmental Genome Project (EGP)	http://www.niehs.nih.gov/envgenom/home.htm	Human genetic susceptibility to environmental exposures

SNP, single-nucleotide polymorphism.

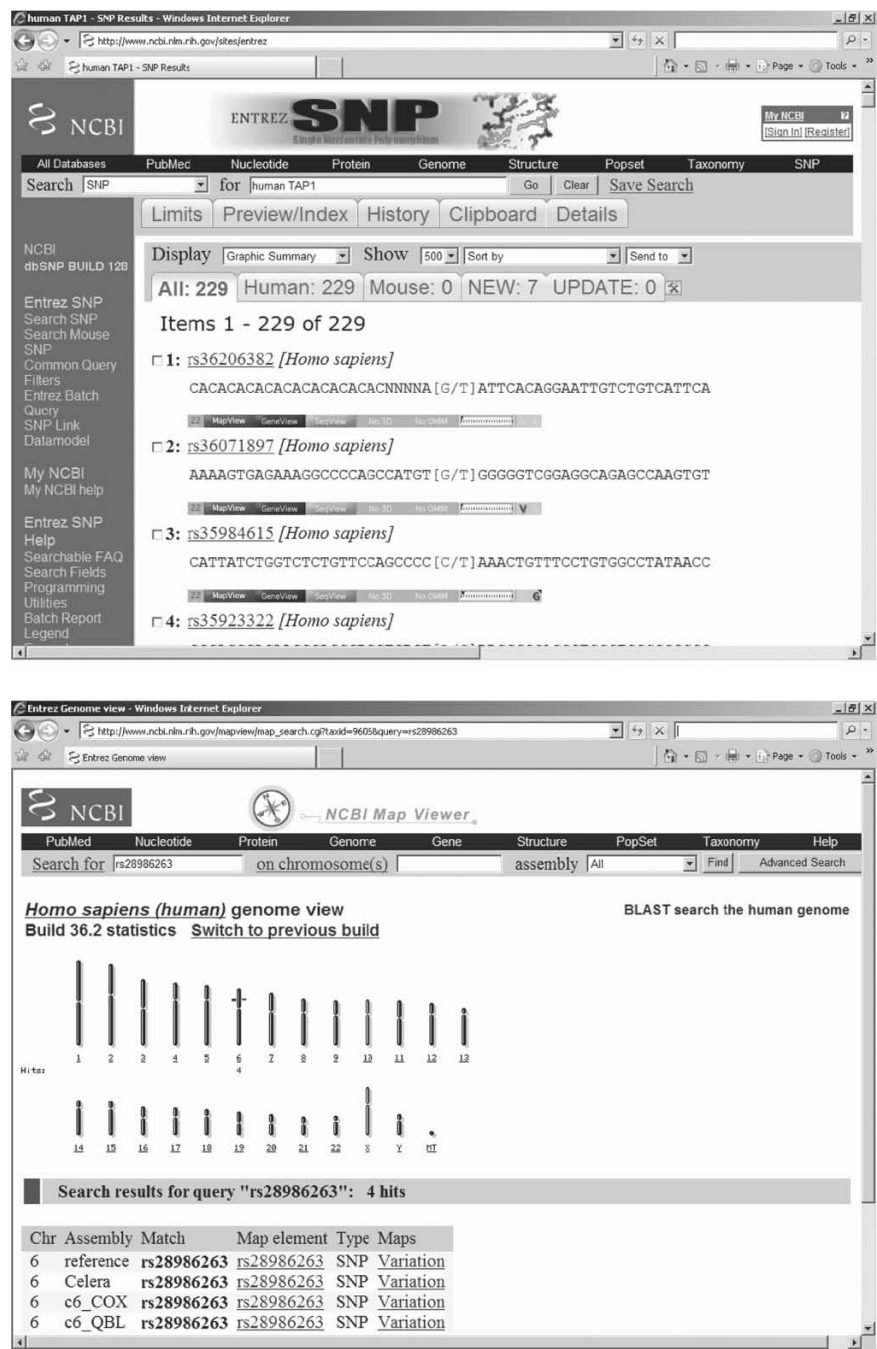


Fig. 1.4 a Searching results of dbSNP using the keyword “human TAP1.” **b** MapView of the SNP rs28986263 from dbSNP

(continued)

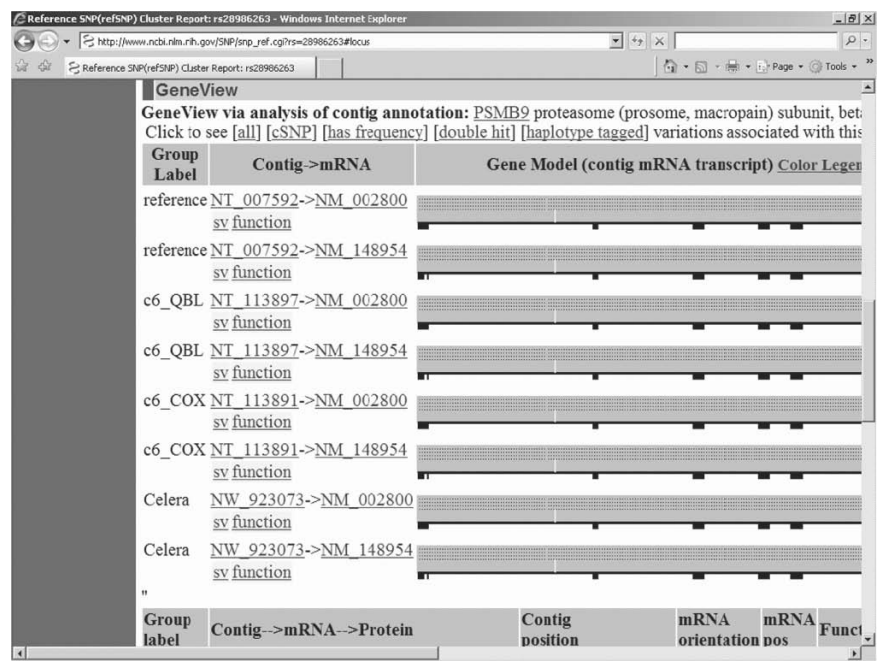


Fig. 1.4 (continued) c GeneView of the SNP rs28986263 from dbSNP

groups may have different drug responses. The Allele Frequencies Database can be used to search for polymorphic regions of different populations in fields of histocompatibility and immunogenetics (see Table 1.2). Such information provides direct connections of information from the molecular level to the population level.

Besides drugs, humans also have different susceptibility to environmental agents (see Fig. 1.1). The Environmental Genome Project (EGP) aims to improve understanding of human genetic susceptibility to environmental exposures (see Table 1.2).

1.2.2 Methods to Study Pharmacogenomics from the Cellular Level and Above

1.2.2.1 Protein-Protein Interactions and Pathways

For better understanding of structures, functions, and relationships between them at the molecular level, we need to move up to the cellular level to have a more comprehensive view. The understanding of changes in molecular and cellular pathways and interactions can be useful for finding new drug targets and designing effective drugs. One of the most comprehensive pathway databases is the Kyoto Encyclopedia of Genes and Genomes (KEGG) (see Table 1.3), which includes detailed graphical pathway maps. Figure 1.5 shows a screen shot from KEGG about pathways in

Alzheimer’s disease. Some other pathway databases, such as the Human Protein Reference Database (HPRD) and Reactome, are also listed in [Table 1.3](#).

The Database of Interacting Proteins (DIP) stores information about experimentally determined interactions between proteins (*see* [Table 1.3](#)). Users can search the

Table 1.3 Bioinformatics sources for pharmacogenomics research at the cellular level

Category	Name	URL	Explanation
Pathways	Kyoto Encyclopedia of Genes and Genomes (KEGG)	http://www.genome.ad.jp/kegg/kegg2.html	Pathway maps, ortholog group tables, catalogs
	Human Protein Reference Database (HPRD)	http://www.hprd.org/	Pathways and protein interaction networks
	Reactome	http://www.reactome.org/	Pathways
	GenMAPP	http://www.genmapp.org/	Pathway tools
	BioCyc	http://www.biocyc.org/	A collection of pathway/ genome databases
	Pathguide	http://www.pathguide.org/	The pathway resource list
Interactions	Database of Interacting Proteins (DIP)	http://dip.doe-mbi.ucla.edu/	Interactions between proteins
Protein localization in cells	PSORT	http://psort.nibb.ac.jp/	Prediction of protein localization sites in cells

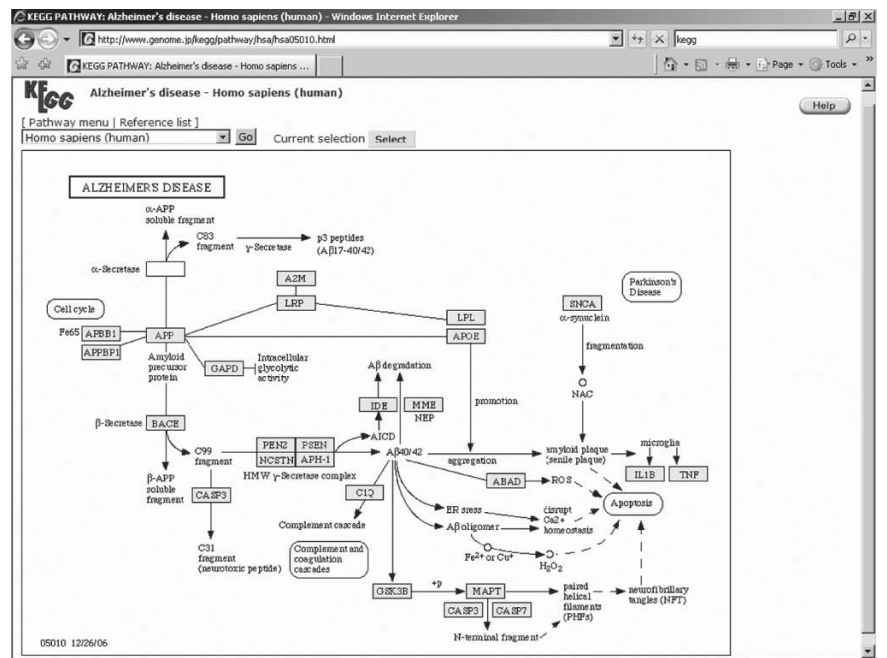


Fig. 1.5 A screen shot from the Kyoto Encyclopedia of Genes and Genomes (KEGG) about pathways in Alzheimer’s disease

Table 1.4 Bioinformatics sources for microarray analyses

Name	URL	Explanation
European Bioinformatics Institute (EBI)	http://www.ebi.ac.uk/	Databases and software
Microarray Informatics at the EBI	http://www.ebi.ac.uk/microarray/	Microarray data management, storage, and analysis
NHGRI (National Human Genome Research Institute) Microarray Project	http://research.nhgri.nih.gov/microarray/main.html	Protocols, databases, and tools
Gene Expression Omnibus	http://www.ncbi.nlm.nih.gov/geo/	Gene expression and array database
Bibliography on Microarray Data Analysis	http://www.nslj-genetics.org/microarray/	Links and publications related to data analysis
Stanford MicroArray Database	http://genome-www5.stanford.edu/	Microarray database and analysis tools

database with protein sequences, motifs, and keywords. For the prediction of protein localization sites in cells, the tool PSORT can be used (*see* [Table 1.3](#)). The current version of the program, WoLF PSORT, can analyze sequences from fungi, animals, and plants. The program analyzes the input amino acid sequence by applying the stored rules for features of known protein sorting signals and reports the possibility of localization sites for the input protein.

Microarray has been used extensively for protein–protein interaction and pharmacogenomics studies ([6](#)). Various sites are available for protocols, databases, and data analysis tools, such as the site of the National Human Genome Research Institute (NHGRI) Microarray Project (*see* [Table 1.4](#)). Another site, Microarray Informatics at European Bioinformatics Institute (EBI) provides resources for microarray data management, storage, and analysis (*see* [Table 1.4](#)). Gene Expression Omnibus is useful for gene expression and array information browsing, query, and retrieval. [Table 1.4](#) also lists some other sources for microarray analysis (*see* [Note 4](#)).

1.2.2.2 Protein–Drug Interactions

The study of protein–drug interactions is one of the most important parts of pharmacogenomics. Various databases are available about drug information, such as RxList, which is an Internet drug index that contains information about side effects, drug interactions, and full prescribing information (*see* [Table 1.5](#)). Users can search the database with drug names, keywords, pill ID, imprint codes, and medical terminology. Another useful site is FDA Drug Information, which provides information about products that the FDA regulates, including new prescription drug approvals, detailed information about drugs, drug safety and side effects, clinical trials, public health alerts, and relevant reports and publications. The Adverse Event Reporting System (AERS) of the FDA can be used to study specific phenotypes or drug responses, especially adverse drug events (*see* [Table 1.5](#)). This system stores adverse drug reaction reports for all approved drugs and therapeutic biologic products.

Table 1.5 Bioinformatics sources for pharmacogenomics research of protein–drug interactions

Category	Name	URL	Explanation
Drug	RxList	http://www.rxlist.com	Drug index
	FDA Drug Information	http://www.fda.gov/cder/drug/default.htm	Information about drugs
	FDA Adverse Event Reporting System (AERS)	http://www.fda.gov/cder/aers/default.htm	FDA regulates Safety reports
Adverse drug events (ADE)			
Protein–drug Interaction	Drug ADME (Absorption, Distribution, Metabolism, and Excretion)	http://xin.cz3.nus.edu.sg/group/admeap/admeap.asp	Drug ADME-associated proteins
	Associated Protein Database		
Pharmacokinetics/ pharmacody namics (PK/ PD)	Pharmacokinetic and Pharmacodynamic Resources	http://www.boomer.org/pkin/	Searchable site with links to software and relevant resources
	PK/PD software links	http://www3.usal.es/~galenica/climpkin/software.htm	A collection of links to software
	Stanford PK/PD Software Server	http://anesthesia.stanford.edu/pkpd/	Software and relevant resources
QSAR	QSAR datasets	http://www.cheminformatics.org/	QSAR data sets

Table 1.6 Bioinformatics sources for pharmacogenomics research of specific molecules

Category	Name	URL	Explanation
Cytochrome P450	Human Cytochrome P450 (CYP) Allele Nomenclature	http://www.imm.ki.se/cypalleles/	Data for the human polymorphic CYP genes
Receptors	GPCRDB	http://www.gpcr.org/7tm/	G-protein coupled receptors (GPCRs)
	Low Density Lipoprotein Receptor (LDLR) gene in familial hypercholesterolemia	http://www.ucl.ac.uk/fh/	Database and map of variations, primers, and oligonucleotides of LDLR
	Androgen Receptor (AR) Gene Mutations Database	http://androgendb.mcgill.ca/	Mutations and phenotypes of ARs
Membrane transporter	Human Membrane Transporter Database	http://lab.digibench.net/transporter/	Transporter pharmacogenomics
Antibody	international ImMunoGeneTics information system (IMGT) databases	http://imgt.cines.fr/	Sequences, structures, tools about proteins of the immune system

To study interactions between proteins and drugs, an available tool is the Drug Absorption, Distribution, Metabolism, and Excretion (ADME) Associated Protein Database (*see* [Table 1.5](#)). The database contains information about relevant proteins, functions, similarities, substrates and ligands, tissue distributions, and other features of targets. For the understanding of pharmacokinetic (PK) and pharmacodynamic (PD) features, some available resources are listed in [Table 1.5](#). For example, the Pharmacokinetic and Pharmacodynamic Resources site provides links to relevant software, courses, textbooks, and journals (*see* [Note 5](#)). For quantitative structure–activity relationship (QSAR), the QSAR Datasets site collects data sets that are available in a structural format (*see* [Table 1.5](#)).

Some specific molecules that are important in protein–drug interactions have been studied extensively, including cytochrome P450 (CYP450), receptors, membrane transporters, and antibodies (*see* [Table 1.6](#)). Databases about these molecules may also contain information about SNP effects, tissue distribution, and interacting substrates.

1.2.3 Genotype–Phenotype Interactions and Diseases

The association between genotype and phenotype connects information at different levels, from the molecular level to the whole organism. Many of the sources mentioned also provide information about direct connections between genotype and phenotype, such as dbSNP, HapMap, and OMIM (*see* [Table 1.7](#)). The OMIM program provides information about genetic disorders, allelic variants, biochemical and clinical features, clinical diagnosis, inheritance information, genomic mapping, genotype/phenotype research, population genetics, and references. It has links to

Table 1.7 Bioinformatics sources for pharmacogenomics research of diseases

Category	Name	URL	Explanation
All diseases	Online Mendelian Inheritance in Man (OMIM)	http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM	A catalog of human genes and genetic disorders
	Frequency of Inherited Disorders Database (FIDD)	http://archive.uwcm.ac.uk/uwcm/mg/fidd/introduction.html	For medical and epidemiological studies
	NetPharmacology	http://lysine.pharm.utah.edu/netpharm/	Cardiovascular pharmacology
Cardiovascular	Hypertension Candidate Gene SNPs	http://cmbi.bjmu.edu.cn/genome/candidates/snps.html	SNPs of hypertension candidate genes
Cancer	System for Integrative Genomic Microarray Analysis (SIGMA)	http://sigma.bccrc.ca	Visualization and analysis of data from high-resolution array comparative genomic hybridization (CGH) platforms about cancer genomes
	SNP500Cancer	http://snp500cancer.nci.nih.gov	Sequence verification of SNPs
	Anti-cancer Agent Mechanism Database	http://dtp.nci.nih.gov/docs/cancer/searches/standard_mechanism.html	Compounds with anti-cancer activity
	Anti-cancer Standard Agent Database	http://dtp.nci.nih.gov/docs/cancer/searches/standard_agent.html	Compounds designated standard agents
	AlzGene	http://www.alzforum.org/res/com/gen/alzgene/default.asp	Genetic association studies in the field of Alzheimer's disease
Alzheimer disease	National Alzheimer's Coordinating Center (NACC) Database	http://www.alz.washington.edu/	A database about Alzheimer's disease
Diabetes	Type 1 Diabetes Genetics Consortium (T1DGC)	http://www.t1dgc.org	An international, multicenter program about type 1 diabetes
	Canadian Diabetes Database System (CDDS)	http://www.harborsoft.com/CDDS/cdds.html	A database system about diabetes

other databases, including DNA and protein databases, the Human Genome Database (GDB), and Medline. Two relatively new query systems in OMIM, OMIM Gene Map and OMIM Morbid Map, provide cytogenetic map locations of diseases and gene lists organized by diseases, respectively.

Extensive pharmacogenomics studies have been done for various diseases, such as cardiovascular diseases, cancer, and infectious diseases, and for pain management (7–15). Databases and sources available for some specific diseases are listed in **Table 1.7**. For example, the database Hypertension Candidate Gene SNPs provides information that correlates SNP with the hypertension phenotype (*see Table 1.7*). Users can browse the database through gene symbols or functional classes. Information in the database includes map location, heterozygosity class, types of change, OMIM number, sequence data, and expression data for each gene.

For cancer pharmacogenomics, the System for Integrative Genomic Microarray Analysis (SIGMA) provides visualization and analysis of cancer genomic data. The system SNP500Cancer is a central resource for sequence verification of SNPs. AlzGene is a database that stores all available genetic association studies in the field of Alzheimer's disease and can be used to track the most relevant gene candidates.

With the advancement of pharmacogenomics and systems biology research, more databases and tools should be available for various diseases. The application of these bioinformatics methods should assist us in having a more profound understanding of diseases at different levels and lead us to both individualized and systems medicine.

1.3 Notes

1. This chapter is mainly about the application of bioinformatics programs and tools in pharmacogenomics. Some other informatics methods are beyond the scope of the chapter, such as data mining methods, including data clustering, artificial neural network, decision trees, genetic algorithm, rule induction, and data visualization. Detailed applications of bioinformatics techniques in certain areas can also be found in other chapters of this book.
2. Many of the bioinformatics programs contain redundant information, so we try to differentiate them based on their applications. Only some of those most commonly used tools are discussed because of space limitation. Web sites mentioned in this chapter were accessed in October 2007. The most recent advances in the field can be updated through browsing these sites regularly as new databases and tools are often added. To facilitate up-to-date bioinformatics support, an integrated search engine and updated collections of tools are freely available at <http://sysmed.pharmtao.com>.
3. The correct BLAST program for the analysis of nucleotide or amino acid sequences should be selected. A guide for BLAST program selection is available at <http://www.ncbi.nlm.nih.gov/BLAST/producttable.shtml>.
4. Because of the limit on chapter length, detailed discussion of microarray data analysis methods is not included, such as those comparison clustering analyses that include hierarchical clustering, k-means clustering, and self-organization maps (16). Some of the most commonly used microarray data analysis tools include Spotfire, GeneSpring, Cluster and TreeView, dChip, and GenMAPP (Gene Map Annotator and Pathway Profiler) (16).
5. Because of space limitations, PK/PD data analysis methods are not described in detail here. Some of the most commonly used PK/PD tools are listed in **Table 1.5**.

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Chapter 2

Applications of Microarrays and Biochips in Pharmacogenomics

Gary Hardiman

2.1	Introduction.....	21
2.2	Pharmacogenetic Testing and Health Care.....	22
2.3	Important Pharmacogenetic Targets	23
2.4	Evolution and Development of Microarrays	25
2.5	Microarrays and Genotyping	26
2.6	Microarrays and Clinical Diagnostics.....	27
2.7	Microarray Technology Limitations and Challenges	28
2.8	Conclusion	29
	References.....	29

Summary The complete sequence of the human genome and subsequent intensive searches for polymorphic variations are providing the prerequisite markers necessary to facilitate elucidation of the genetic variability in drug responses. Improvements in the sensitivity and precision of DNA microarrays permit a detailed and accurate scrutiny of the human genome. These advances have the potential to significantly improve health care management by improving disease diagnosis and targeting molecular therapy. Pharmacogenetic approaches, in limited use today, will become an integral part of therapeutic monitoring and health management, permitting patient stratification in advance of treatments, with the potential to eliminate adverse drug reactions. In this chapter, the current state of biochip technology is discussed, and recent applications in the arena of clinic diagnostics are explored.

Keywords AmpliChip; biochips; microarrays; P450; pharmacogenetics.

2.1 Introduction

The sequencing of the human genome has been widely touted as a critical scientific milestone that will revolutionize the process of drug discovery. The continuing analysis of the human genetic code will provide the scientific framework on which

it may be possible to identify novel potential drug targets, the common genetic factors that can affect drug metabolism and toxicity, and the genetic factors that contribute to the wide variability in pharmacological treatment responses routinely observed in clinical settings. The ever-increasing utilization of genetic techniques, including microarray technologies, has provided a means by which geneticists, biologists, and pharmacologists have begun to bridge the gap between gene sequence and function. These newer approaches are currently under integration into multiple aspects of the drug discovery process. The use of genetic polymorphism analysis has been applied to target validation, pharmacokinetics and toxicology, and clinical pharmacogenomics, while microarray technologies have been utilized in target validation, *in vitro* pharmacology, and toxicology (1).

A DNA microarray (also referred to as gene or genome chip, DNA chip, or biochip) is a collection of microscopic DNA features attached to a solid support, commonly glass, plastic, or silicon. The array features or “spots” contain DNA probes that are used to interrogate individual genes or polymorphisms. Most arrays in use today contain hundreds to thousands of probes. The value of this technology is that it permits highly parallel measurements. In the case of gene expression profiling, the massive number of data points obtained from a single experiment provides insight into the state of a transcriptome in, for example, healthy and diseased cells or cells before and after exposure to a therapeutic treatment. The knowledge obtained from such comparisons is incredibly compelling as it permits the identification of gene families and pathways pertinent to the malady or drug treatment in addition to those that remain unaffected. Similar expression profiles may infer that genes are coregulated, enabling the formulation of hypotheses about genes with hitherto unknown functions by comparison of their expression patterns to well-characterized genes (2).

The applicability of microarrays in genomics research has expanded with the evolution and maturation of the technology. Biochips have found utility in exon-based gene expression analyses, genotyping and resequencing applications, comparative genomic hybridization studies, and genomewide (epigenetic) localization (3). Biochips are widely applied to improve the processes of disease diagnosis, pharmacogenomics, and toxicogenomics (4–7). In this chapter, the evolution of biochip platforms is reviewed; I compare and contrast platforms currently in use and discuss biochips in the context of pharmacogenetic testing.

2.2 Pharmacogenetic Testing and Health Care

Pharmacogenetics is the discipline that studies the relationship between a patient’s inherited genetic makeup and that patient’s response to pharmaceutical drugs. Pharmacogenetic testing aims at determining the underlying genotypic and phenotypic differences in the pharmacodynamics and pharmacokinetics of drug metabolism. Whereas pharmacogenetics refers to genetic differences (variation) in drug metabolism and response, *pharmacogenomics* refers to study of the multiplicity of

genes that ultimately determine drug behavior. Pharmacogenomics is in essence the whole-genome application of pharmacogenetics, correlating gene expression or single-nucleotide polymorphisms (SNPs) with drug efficacy and toxicity. Genetic variability in drug response occurs as a result of molecular alterations in the enzymes involved in the metabolism of a particular drug in addition to the drug receptors and transport proteins (8).

A recent advance and fundamental shift in health care has been the emergence of personalized medicine. Drug–drug interactions (DDIs) can have serious consequences, such as adverse drug reactions (ADRs), and extreme outcomes, including death. DDIs have become a serious issue, particularly in the care of elderly patients, who are often prescribed a wide variety of medications (9). ADRs are presently the fourth leading cause of death in the United States, resulting in 106,000 deaths per year, and the fifth leading cause of illness, resulting in 2.2 million hospitalizations annually. At present, approx. 28% of adults and 17% of children hospitalized have drug-related ADRs. The economics of drug-related morbidity and mortality has become a pressing issue, with current costs estimated at \$177 billion annually (10).

Pharmacogenetic approaches, in limited use today, will in the near future become an integral part of the therapeutic monitoring and health management of patients. A major advantage of pharmacogenetic testing over classical therapeutic drug monitoring (TDM) approaches is that patient genotyping and stratification can be carried out in advance of drug treatments, thereby eliminating or reducing adverse effects. Testing can generally be performed in a noninvasive manner using DNA obtained from saliva, hair root, or buccal swab samples. Another benefit over traditional methods is that patient compliance with a particular treatment regimen is not required. In addition, the results remain constant over the lifetime of an individual, regardless of disease or aging. Finally, a major advantage of pharmacogenetic testing is that it can provide predictive value for many drugs rather than a single drug (8).

2.3 Important Pharmacogenetic Targets

The most relevant pharmacogenetic targets as defined by the American Association of Clinical Chemists (AACC) include the Cytochrome P450 enzymes CYP2D6, CYP2C9, CYP2C19, CYP3A5, CYP2B6 and thiopurine s-methyltransferase (TPMT), N-acetyltransferase 2 (NAT2), UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), multi-drug-resistance (MDR1) gene and methylenetetrahydrofolate reductase (MTHFR). Drug metabolism occurs largely in the liver and involves cytochrome P450 (CYP450), a large family of oxidative enzymes. The name derives from “pigment at 450 nm” as the majority of family members possess red coloration owing to the presence of heme at the active site. Although CYP450 plays an important role in the synthesis and breakdown of hormones, cholesterol synthesis, and vitamin D metabolism, from a health care perspective its role in drug metabolism is its most pertinent. Most common variations in drug metabolism

between individuals can be explained by polymorphisms in the *cypP450* genes. One of the best characterized of the CYP450 enzymes, CYP2D6, is responsible for metabolizing the majority of pharmaceuticals currently in use. These include an extensive range of therapeutic agents encompassing β -blockers, antidepressants, antipsychotics, and opioids. A poor metabolizer (PM) phenotype has been observed among 7–10% of the Caucasian population, with many suffering toxicity from normally prescribed doses. This is explained by adverse reaction to drugs prescribed in standard doses or undesirable DDIs when using multiple-drug therapeutics.

Warfarin (Coumadin) inhibits the synthesis of clotting factors, thus preventing blood clot formation. Although it remains the most frequently prescribed oral anticoagulant, it can cause severe bleeding that can be life-threatening and cause death. Successful management of warfarin therapy is problematical owing to the wide variation in drug response among patients. Variation in the vitamin K epoxide reductase complex 1 (*VKORC1*) gene affects the response to warfarin (*II*). Pharmacogenetic analysis of a patient's *CYP2C9* or *VKORC1* can provide information that allows fine-tuning of the appropriate warfarin dosage. Cytochrome P450C19 metabolizes 15% of all prescribed drugs and is involved in the metabolism and clearance of antidepressants (tricyclic antidepressants [TCAs] and selective serotonin reuptake inhibitors [SSRIs]), anticonvulsants, anxiolytics, and benzodiazepines (*12–14*). For 2C19, two phenotypes with variable metabolic activity have been defined, the extensive metabolizer (EM) and poor metabolizer (PM). The PM phenotype is associated with low enzyme activity. East Asians are most likely to exhibit the PM phenotype, with 2C19 PM rates observed in up to 25%. CYP450A4/3A5 is the most abundant CYP450 isoenzyme in humans and is responsible for the metabolism of the widest range of drugs. It is involved in the metabolism and clearance of calcium channel blockers, benzodiazepines, human immunodeficiency virus (HIV) protease inhibitors, HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors, and antithrombotics.

Thiopurine S-methyltransferase (TPMT) catalyzes the S-methylation or inactivation of the thiopurine drugs mercaptopurine, azathioprine, and thioguanine, which are commonly used to treat leukemia, rheumatic diseases, and inflammatory bowel disease. *TPMT* testing serves to detect patients at risk of developing side effects if treated with thiopurine drugs (*12*). *N*-Acetyltransferase 2 (*NAT2*) is of clinical importance as rapid or slow acetylation of therapeutic and carcinogenic agents is explained by variability at the *NAT2* locus. Interethnic variations in distribution of the acetylation phenotype are significant.

UDP glucuronosyltransferase 1 family, polypeptide A1 (*UGT1A1*), is a hepatic enzyme associated with the colorectal and small lung cancers. *UGT1A1* metabolizes irinotecan, an antineoplastic agent utilized for the treatment of colorectal cancer. Pharmacogenetic testing for *UGT1A1* will help the optimization of therapeutic approaches with antineoplastic agents that inherently have a low therapeutic index and will spare patients from excessive toxicity resulting from therapy with irinotecan.

P-Glycoprotein (P-gp), a member of the adenosine triphosphate (ATP)-binding cassette family of membrane transporters, is encoded by the human multidrug-resistance (*MDR1*, *ABCB1*) gene (*15*). This integral membrane protein serves as an

energy-dependent drug efflux pump and reduces the intracellular concentrations of a wide range of drugs and xenobiotics. The overexpression of MDR1 is associated with resistance to doxorubicin, taxanes, and vinca alkaloids, which are used to treat cancer. Resistance to chemotherapy has become a major obstacle in anticancer treatment. Methylenetetrahydrofolate reductase (MTHFR) is a cytoplasmic enzyme that plays a role in the conversion of homocysteine (a potentially toxic amino acid) to methionine. A common 677TT genotype predisposes individuals to mild hyperhomocysteinemia (high blood homocysteine levels), which can lead to neural tube defects in offspring, arterial and venous thrombosis, and cardiovascular disease.

Currently, the methods employed for genetic testing are labor intensive and intricate and demand the concurrent analysis of multiple nucleic acid markers. Microarray technology is undeniably the most practical approach to multiplex and analyze biomolecular markers.

2.4 Evolution and Development of Microarrays

The origin of the microarray or biochip can be traced to a seminal publication by Edwin Southern over 30 years ago. Southern described a method by which DNA could be attached to a solid support following electrophoresis and interrogated for sequences of interest by hybridization with a complementary DNA sequence (16). The complementary DNA sequence, termed a *probe*, was labeled with either a radioactive or a fluorescent marker and hybridized to the DNA target sample, which was immobilized on a solid support, such as a nitrocellulose filter membrane.

The biochips widely in use today owe their existence to innovations in miniaturization, DNA synthesis and attachment chemistries, and improvements in image acquisition. Key pioneers in the early innovation and development of this technology were Hyseq (Sunnyvale, CA); Affymetrix (Affymax) (Santa Clara, CA); Oxford Gene Technologies (Oxford, UK); and Stanford University (Palo Alto, CA). Hyseq exploited oligonucleotide arrays to permit sequencing of target nucleic acid sequences. The complementary oligonucleotide probe sequences overlapped, permitting the discrimination of perfect match DNA hybrids from hybrids that contained a single-nucleotide mismatch (17). Affymetrix utilized very large scale immobilized polymer synthesis (VLSIPSTM) substrate technologies for the synthesis of both peptides and oligonucleotides on solid supports. They successfully applied this technology to DNA sequencing, DNA fingerprinting, chromosomal mapping, and specific interaction screening (18). Spotted microarrays, yet another widely utilized application of this technology, were pioneered at Stanford University by Patrick Brown and colleagues. These arrays are fabricated using a capillary dispenser, which deposits DNA at specific array positions. Spotted microarray production is highly automated, utilizing either capillary pin-based or ink-jet microdispensing liquid-handling systems (19,20).

The major commercial microarray platforms in use today, over ten years after their first description, include those from Affymetrix, Illumina, Agilent, and

Applied Biosystems. A detailed comparison and contrast of the salient features of each of these platforms has been described previously (21,22). The Affymetrix GeneChip™ has been the most extensively used owing to its extensive genome coverage, its ease of use, and its high level of reproducibility. It is comprised of short single-stranded oligonucleotides and is fabricated via a combination of photolithography and solid-phase DNA synthesis. Illumina (San Diego, Ca) has established a bead-based technology that was utilized initially for SNP genotyping and subsequently for gene expression profiling. These arrays are comprised of thousands of tiny etched wells, into which thousands to hundreds of thousands of 3-μm beads randomly self-assemble. Then, 50-mer gene-specific probes linked with “address or zip code” sequences are immobilized on the bead surface and are used to facilitate a decoding process, which maps a specific bead type containing a particular sequence to a given location on the array.

Applied Biosystems Expression Array System (Foster City, CA) has devised a chemiluminescence-based microarray platform utilizing 60-mer oligonucleotides which are validated offline by mass spectrometry and are subsequently printed onto a derivatized nylon substrate. Agilent Technologies (Palo Alto, CA) also utilizes 60-mers, which are synthesized in situ by ink-jet printing using phosphoramidite chemistry.

2.5 Microarrays and Genotyping

Single-nucleotide polymorphisms are highly abundant, with over 10 million present in the human genome, and they serve as valuable markers of genomewide variation. A chromosome region may contain many SNPs, but just a few “tag” SNPs are required to provide information on the pattern of genetic variation. The high costs associated with most SNP detection strategies have until recently made genomewide approaches impractical.

Illumina bead-based technology has been applied to both SNP genotyping and gene expression profiling applications and utilizes two distinct substrates, the Sentrix LD BeadChip and the Sentrix Array Matrix (which multiplex up to 8 and 96 samples, respectively). Genomewide genotyping of defined sets of hundreds of thousands of SNPs can be performed using one of two array types, the Infinium I 109 K SNP arrays or the Infinium II 317 K SNP arrays. A whole-genome amplification step is initially employed to enrich the target DNA up to 1000-fold. Once amplified, the DNA is subsequently fragmented and mobilized by hybridization to SNP-specific primers present on the array. In the case of the Infinium I assay, which utilizes an allele-specific primer extension approach, the DNA is hybridized to allele-specific primers that are extended with multiple labeled bases only if a perfect match exists between the target and SNP-specific probe (23). The Infinium II assay differs in that it is based on single-base extension (SBE). An oligonucleotide primer is hybridized adjacent to the SNP site and is extended with a single labeled dideoxy-nucleotide terminator corresponding to the minor or major allele. Genotyping calls can then be made based on the dye-labeled terminator that is incorporated (24).

2.6 Microarrays and Clinical Diagnostics

Microarrays are today applied in the clinical diagnostics and genotyping arenas. Their successful utilization and survival in the clinic will depend on the ability of the technology to meet the rigorous requirements applied to human diagnostics in a cost-effective manner.

2.6.1 Roche Diagnostics AmpliChip

The first pharmacogenetic microarray-based test approved for clinical use is the AmpliChip CYP450 from Roche Diagnostics (Basel), which measures genetic variation, both deletions and duplications, for the *CYP2D6* and *CYP2C19* genes. The AmpliChip is a marriage of expertise in polymerase chain reaction (PCR; Roche) and microarray (Affymetrix) technologies. The AmpliChip has been approved for *in vitro* diagnostic use in the United States and Europe. The test determines the associated predictive metabolizer phenotype (poor, intermediate, extensive, or ultra) and can aid physicians in individualizing patient treatment and dosing for drugs metabolized through these *P450* genes. It detects a total of 27 polymorphisms and mutations for the *2D6* gene and 3 polymorphisms for the *2C19* gene.

Once patient genomic DNA has been extracted, the test involves a series of five steps, and the analysis time from start to finish is 8 h. A minimum of 25 ng of input genomic DNA is required for the assay, and the preferred tissue source is blood, although buccal swab-derived DNA would also suffice. First, PCR amplification is carried out to amplify the genes of interest using gene-specific primers. This is followed by fragmentation and biotin labeling of the amplicons at their 3' termini with terminal transferase (TdT). The biotin-labeled amplicon is subsequently hybridized to the AmpliChip DNA microarray. Following washing and staining via a streptavidin–phycoerythrin conjugate, the chip is scanned on an Affymetrix GeneChip Scanner 3000Dx, the data feature is extracted and analyzed, and genotyping calls are made.

2.6.2 Autogenomics BioFilm Microarrays

The Infiniti Analyzer, an automated, continuous-flow microarray platform for clinical applications has been developed by Autogenomics (Carlsbad, CA) (25). The underlying component of the Autogenomics technology is the BioFilm™, which consists of multiple layers of porous hydrogel matrices 8- to 10-μm thick on a polyester solid base. This provides an aqueous microenvironment that is highly compatible with biological materials. The BioFilm microarray is configured with 15 × 16 arrays (240 spots) per chip, suitable for current diagnostic applications, and permits analyses of both nucleic acid and proteins (26). It can be tailored to clinical genetic testing for custom polymorphisms of interest.

The analyzer integrates all the discrete processes of sample handling, reagent management, hybridization, and detection. A confocal microscope has been integrated into the analyzer; it has two lasers (red and green). In addition, a thermal stringency station and a thermal cycler for denaturing nucleic acids for primer extension studies or hybridization reactions in solution have been incorporated. A *CYP2D6* assay has been designed to detect the most prevalent and informative *CYP2D6* allele variants (25). The target regions of the *CYP2D6* gene are amplified via a multiplex PCR reaction with specific primer and reaction conditions that can discriminate *CYP2D6* from its pseudogenes. The PCR multiplex reaction is followed by the incorporation of fluorescently labeled nucleotides via primer extension and hybridization of the labeled targets to immobilized oligonucleotides on the BioFilm. Other pharmacogenetic specific tests that can be carried out on this platform include, *CYP2C9*, *CYP2C19*, *TPMT*, *CYP3A4/5*, and *NAT2*.

2.6.3 Nanogen NanoChip™

An interesting development has been that of electronic chip technology. Nanogen (San Diego) developed the NanoChip™, which exploits the charged nature of biological molecules. Electronic charges can rapidly shift molecules from one location to another and concentrate them at defined sites on an array. The concentration of biological materials with electronics enables rapid hybridization reactions; instead of the 12 to 16 h traditionally required for passive hybridization, electronic hybridization reactions can be performed in 2 min. When a test site on the NanoChip is charged, a nucleic acid target rapidly moves to that site. Other sites, which are not charged, do not attract the target. Each site or feature can be individually charged electronically via platinum wires and can contain an individual assay or experiment. Electronic hybridization and stringency can be carried out with single-base resolution.

Nanogen has developed pharmacogenetics research reagents for the analysis of *CYP2C9* and *VKORC1*, mutations of which have relevance to warfarin dose optimization. The reagents can be used to rapidly determine genotypes for up to 78 patient samples. In November 2007, Nanogen announced it would be closing its microarray business and repositioning of the company with a focus on real-time PCR and point-of-care testing units.

2.7 Microarray Technology Limitations and Challenges

The commercial microarray platforms in use today have established efficiencies regarding signal dynamic range, the ability to discriminate related messenger RNA (mRNA) species, the reproducibility of the data (raw data, fold change and expression levels). However, technological and standardization limitations exist with

biochip technologies. Expression microarrays facilitate the analysis of the relative levels of mRNA species in one tissue sample compared to another. Although a measure of transcript abundance is achieved, biochips do not provide absolute quantification of the specific mRNA. Microarrays are further limited by the certainty that the data obtained merely indicate whether a given mRNA is above the system's threshold level of detection. If the signal is significantly above the background intensity, then one can say with confidence that the transcript is expressed in that tissue. However, the absence of signal does not indicate the lack of expression. It merely indicates that it is below the detection capability of the system, and there is still a probability that the mRNA is expressed, albeit at basal levels, and this low-level expression may be of biological relevance.

Expression analysis using DNA microarrays analyzes only the transcriptome; it should be mentioned that mRNA abundance in a cell often correlates poorly with the amount of protein synthesized (27). Important regulation takes place at the levels of translation and enzymatic activities. The only effect of a signal transduction pathway that is observed in a gene expression experiment is that at the endpoint of a given pathway. DNA microarrays currently have little value in determining post-translational modifications, which influence the diversity, affinity, function, cellular abundance, and transport of proteins.

2.8 Conclusion

Currently, the methods employed for genetic testing are both labor intensive and highly complex and require the simultaneous analysis of multiple nucleic acid markers. Microarray technology is without doubt the most practical approach to multiplex and analyze biomolecular markers. Although widely used in the research setting, adaptation of microarray technology to the clinical environment has been slow.

The success of microarrays in the clinical laboratory will depend on their ability to adapt to the rigorous environment of routine usage while providing high-quality, reproducible, and robust results. The clinical environment stretches the limits of this technology as it measures performance criteria in a different manner compared to the research environment. One difference from an economic standpoint is that the cost per reportable result is more important than the cost per data point. Other key factors are the requirements for automation from sample processing to end result, precision, accuracy of results, and the ability to process large volumes of tests under strict regulatory guidelines and compliances.

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Chapter 3

Confounding in Genetic Association Studies and Its Solutions

Donglei Hu and Elad Ziv

3.1	Introduction.....	31
3.2	Population Structure and Admixture in Human Populations.....	32
3.3	Confounding Effect.....	33
3.4	Methods to Deal with Confounding Effect.....	35
3.5	Conclusions.....	38
	References.....	38

Summary An association study can be used to investigate how individuals with unique genetic variants respond to a drug treatment. In an association study, individuals may come from different ethnic groups or an admixed population. The heterogeneity of genetic backgrounds among individuals in association studies may lead to false-positive or false-negative results. Confounding caused by population structure and recent admixture may be one major factor that contributes to the lack of replication of association study results. Confounding can be detected and adjusted. Major methods that adjust for population stratification are described and explained in this chapter. Their advantages and disadvantages are discussed.

Keywords Admixture; association study; confounding; human genetics; population structure.

3.1 Introduction

The goal of pharmacogenomics research is to develop personalized medical treatment. As each person's genome is unique, it is conceivable to speculate that specific genetic variants are related to a specific phenotype or the response to a drug treatment. The first step of achieving this goal is investigating the relationship between phenotypes and genetic variants.

Linkage analysis is a traditional method to identify the relationship between phenotypes and genes. Application of this method can be difficult because the

required sample size for enough statistical power is too large (1). Furthermore, when studying a trait that is only revealed after a particular exposure (e.g., an adverse reaction to a medication), it may not be feasible to find families who have all had the exposure. As an alternative approach, association studies in unrelated populations bypass many of these hurdles. These studies are more feasible for pharmacogenetic studies in which obtaining relatives who have been exposed to the medication may not be feasible. Furthermore, association studies in unrelated individuals have higher power to detect subtler genetic effects.

Thus, an association study is commonly used in high-throughput studies in which the whole genome is scanned. An association study directly tests the association between the phenotype and the genetic variant, which is either the trait-affecting variant or in linkage disequilibrium (LD) with the trait-affecting variant. Case-control design is commonly used in association studies. In this type of design, the frequency of a genetic variant in the case group is compared with that in the control group. A test statistic (e.g., χ^2) is computed, and the null hypothesis is tested.

One major concern of the association study is that spurious associations may occur if the genetic background in cases is different from that in controls. Genetic heterogeneity in a population can be a result of population structure and recent admixture. In this chapter, we review the problem of population structure, demonstrate how that structure affects association study, and discuss solutions to this problem.

3.2 Population Structure and Admixture in Human Populations

Humans are different in their genetic backgrounds. The entire human population can be divided into subgroups based on the similarity of genetic background of those subgroups. Nonrandom mating between subgroups in a population results in population substructure.

Tree-based algorithms have been applied to genetic data and have discovered subgroups in human population. Bowcock et al. studied 148 individuals in 14 populations from five continents (2). Using 15–30 microsatellite markers, they constructed a neighbor-joining tree from the pairwise distances between individuals. The algorithm discovered five major branches, which represent African, European, Asian, Oceanian, and American individuals. A similar algorithm was used by Mountain and Cavalli-Sforza to infer relationships between populations (3). With 75 markers, the inferred tree clustered 144 individuals from 12 human groups in a way that most individuals were grouped with other members from the same region. Both studies found highest diversity in the African population, supporting the hypothesis of African origin for humans.

A thorough study by Rosenberg et al. (4) examined human population structure using 377 markers in 1056 individuals from 52 populations around the world. Without prior information about the origins of individuals, these authors used a Bayesian algorithm to identify six major genetic clusters: (1) sub-Saharan Africans;

(2) Europeans, Middle Easterners, and most Central and South Asians; (3) East Asians; (4) groups indigenous to the Americas; (5) Pacific Islanders; and (6) a group consisting of the Kalash, an isolated population in northwest Pakistan.

When the algorithm tried to identify more than six subgroups, it generated inconsistent results. The authors also found large variation within Native Americans, Oceanians, and sub-Saharan Africans. The variation was the least in European subpopulations. In a follow-up study, the authors found that the genetic clustering of individuals was not influenced by number of markers, sample size, number of clusters, geographic dispersion of the sample, and assumptions about allele frequency correlation (5).

The studies described found that individuals can be partitioned into clusters based on their genetic backgrounds. Those genetic clusters in general match major geographic subdivisions in the world. The contemporary migration of populations from different geographic regions has resulted in admixture between two or more ancestral populations. The admixture of populations has increased the complexity of human population structure. In the United States, self-described African Americans usually have European ancestry, with the degree varying among different regions in the United States (6). It has also been shown that Latino Americans are an admixed population with European, Native American, and African ancestries (7).

In summary of previous findings, human populations can be divided into subgroups based on genetic background. There is also substructure within major groups. Recent admixed populations of different ancestries have made the human population structure more complicated. All these have provided challenges in association studies, which usually involve large data sets from high-throughput experiments.

3.3 Confounding Effect

3.3.1 *Confounding Effect Caused by Population Structure*

In a case-control design of association studies, the frequency of a genetic variant in the case group is compared with that in the control group by an appropriate statistical test. If two or more populations are involved in the study, then false-positive or false-negative results can occur because of population stratification. For example, if subjects are randomly selected, then the case group would have a larger portion of subjects who are from a subpopulation with higher prevalence of disease. In this situation, any allele with a higher frequency in that subpopulation will be identified as associated with the disease trait. This is an example of *confounding*, which means the association between an allele or genotype and disease is confounded by the association of the trait with one or more of the subpopulations. In association studies, a confounding factor causes false-positive or false-negative results if both the prevalence of the disease trait and the allele frequency are different in subpopulations. The discussion is summarized in [Table 3.1](#).

Table 3.1 Scenarios of confounding

Scenario	Prevalence	Allele frequency	Confounding
1	Same for each population	Different for each population	No confounding
2	Different for each population	Same for each population	No confounding
3	Prevalence is higher in one population	Allele frequency is higher in the same population	Positive confounding
4	Prevalence is higher in one population	Allele frequency is lower in the same population	Negative confounding

The case-control groups involve two or more subpopulations. Subjects are randomly selected.

The confounding effect has also been quantified (8). If allele frequency differences are large between two subgroups, then a 50% increase of population relative risk in one population may result in an odds ratio of approx. 1.5.

3.3.2 *Confounding Effect Caused by Admixture*

In addition to population structure, population admixture can also lead to confounding. In incomplete admixture, mating within subgroups is preferential instead of random. This could generate distinct subpopulations and population substructure. As a result, confounding can exist and can cause false results in association studies.

In complete admixture, mating between populations is random. If the admixture is recent, then confounding is still a concern. Assuming there is random mating between two populations of equal size with different risks of disease, after two generations 1/16 of individuals would have all four grandparents from the high-risk population, and 1/16 of individuals would have all four grandparents from the low-risk population. The remaining individuals would have a disease risk in between based on their ancestry. If this type of recently admixed population is sampled in a case-control study, then individuals with the high-risk population ancestry would have a higher probability to be sampled. As a result, any allele that has a higher frequency in the high-risk population would be identified in the association study no matter whether it is biologically related to the disease or in LD with any disease-causing allele.

Population structure and admixture are two major factors that cause a confounding effect in association studies. One straightforward solution to the confounding problem would be to use ethnically homogeneous populations. In fact, confounding can still be a concern if there is assortative mating over time in ethnically homogeneous populations. *Assortative mating* is mate selection based on specific phenotypes. Redden and Allison (9) examined the effect of nonrandom mating on association studies. Their results, based on simulated data, showed that assortative mating can generate spurious association within a genetically homogeneous population. The type I error rate of an association study increases with increased degree of assortative mating. Methods that deal with population stratification (described in

Subheading 3.4.2) can correct for spurious association. These methods were recommended to adjust for population stratification even when a genetically homogeneous population is used in association studies.

The confounding effect has been demonstrated in clinical studies. Knowler et al. tested the association between a single haplotype at the HLA locus and type 2 diabetes in Pima Indians (10). The χ^2 test identified a strong negative association between the haplotype and type 2 diabetes when Native American (or Amerindian) ancestry was not taken into consideration. Because the same haplotype is more prevalent in Caucasians than in Pima Indians, they investigated the effect of proportion of Pima ancestry on the association. When the analysis was stratified by Pima Indian ancestry, the association was no longer significant. They concluded that the spurious association between the haplotype and type 2 diabetes was a result of different fractions of Caucasian ancestry among cases and controls.

3.3.3 *Detecting a Confounding Effect*

Population stratification in case-control design can cause systematic differences in genetic background between cases and controls. This type of genetic difference is detectable by using unlinked markers. Pritchard and Rosenberg compared frequencies of independent markers between cases and controls with the usual Pearson χ^2 statistic (11). Their method successfully detected population stratification in simulated data. The power of this method increases with the number of markers used and with the degree of stratification. About 15–20 microsatellites are sufficient to detect stratification with the type I error rate of 0.05. More loci are necessary if biallelic markers are used. This method has also detected population substructure caused by assortative mating (9).

3.4 Methods to Deal with Confounding Effect

3.4.1 *Family-Based Studies*

One conventional approach that tackles the confounding effect is family-based design. A typical family-based design involves genetic information for both parents and an affected child. A transmission disequilibrium test (TDT) can be used to examine the association of an allele with a phenotype by testing whether the allele is over- or undertransmitted to the affected offspring with a χ^2 statistic (12).

Confounding is not a concern in TDT because the allelic transmission within families is unaffected by population structure and admixture. Information on haplotypes is available in family-based design and may help increase the test power. There are two major drawbacks in family-based design. First, if the disease occurs

late in life, parents are usually unavailable for the study. Although siblings can be used in this situation, the test power is reduced. Second, in societies in which the proportion of single-parent families is high, it is not feasible to involve a large number of families in which both biological parents are available for the study. If a significant number of single-parent families are used in the study, then the test power would be substantially diminished.

Because of the difficulties in family-based design, an association study with a case-control design is more and more commonly used by investigators. Because of the concern of confounding caused by population structure and recent admixture, appropriate adjustment of stratification should be used in association studies. A number of approaches have been developed for this purpose.

3.4.2 *Adjustment of Population Stratification*

Several approaches have been proposed to deal with population stratification by using unlinked markers. In general, these methods fall into two categories: model-based and non-model-based approaches. We briefly describe and explain three major methods and discuss their advantages as well as disadvantages. The basic understanding of these model-based and non-model-based methods is necessary and helpful when users apply them to analyze genetic data.

Genomic control (GC) (13) is a nonparametric method and provides control similarly to genetic epidemiology's family-based designs (12,14). The idea behind GC is that the Cochran-Armitage test statistic would not be in χ^2 distribution with one degree of freedom if population substructure exists. With a set of control markers, GC estimates the variance inflation factor and adjusts the test statistic with it. The *adjusted test statistic*, which is the observed test statistic divided by the variance inflation factor, would approximately follow the χ^2 distribution with one degree of freedom. Random markers should be used as control markers to represent the overall distribution of genetic differences between cases and controls. Markers that are informative about ancestry and thus may better detect differences between cases and controls may cause overcorrection. In contrast, markers that are less different from average random markers may still cause false-positive results.

Genomic control has been applied in genetic association studies. In a cardiovascular study that involved subjects with different ethnic backgrounds, Dries et al. used GC to detect association of genetic variants with hypertension. They found that a single minor corin I555 (P568) allele was common in African Americans and was associated with increased risk for prevalent hypertension (15). In a simulation study, spurious association was caused by assortative mating in an ethnically homogeneous population. This type of spurious association could also be corrected with GC if loci that were involved in the assortative mating were included in the procedure (9).

Clayton et al. found that, in addition to population substructure, false-positive association was caused by bias in genotype scoring between case and control DNA

samples. To avoid excluding samples, they modified the GC model by adopting a log-linear regression model to estimate the variance inflation factor. In this model, the variance inflation factor is no longer a constant but is dependent on the accuracy of genotyping. The extended GC method corrected the bias effect caused by differences in sample preparation (16).

Marchini et al. examined the consequences of population structure on association studies and evaluated the effectiveness of GC in correcting for population structure (17). In simulated scenarios that involved two or three populations, GC did not remove the effects of population structure in association studies when a small number of loci were used. When a large number of loci were used, the correction by GC became conservative. This problem of GC became severe with small p values and large sample sizes.

In summary, GC adjusts for population stratification without the assumption or estimation of parameters such as the number of subpopulations involved in the study. It provides control of false-positive results caused by population structure as well as by multiple testing. One possible drawback of this method is that the correction of the test statistic is constant across the genome. As a result, GC may have less power in certain situations.

With the availability of many null markers, a non-model-based approach (EIGENSTRAT) applies principal components analysis to tackle the problem of population structure (18). This method first finds the axes of genetic variation in genotype data with principal components analysis. The axes of genetic variation usually represent geographic changes of samples with ancestry differences. It then adjusts the genotypes and phenotypes with the individual ancestry along a given axis of variation. Finally, EIGENSTRAT conducts an association test with adjusted genotypes and phenotypes. Principal components may be a promising approach to deal with population stratification in populations in which substructure is suspected but not well known and somewhat subtler and therefore more difficult to detect (e.g., European Americans). However, the need to select the number of principal components to include in an adjusted analysis and the need to decide how to model the association between the principal components and the trait may mean that the method requires careful application and may not solve all problems caused by stratification.

A widely used model-based method (STRUCTURE) was developed by Pritchard et al. (19). This method can be applied when both the number of subpopulations and the allele frequencies in those subpopulations are unknown. With a Bayesian clustering approach, this approach assigns each individual to a population based on the individual's genotype of unlinked markers. The population allele frequencies of markers are estimated at the same time. An extension of the original model, the linkage model, allows for linkage between loci in admixed populations (20). This modified model can detect admixture events and make inference to the population of origin of chromosomal regions. The method can be applied to microsatellites, restriction fragment length polymorphisms, or single-nucleotide polymorphisms.

This approach has been used to detect subgroups in both simulated data and human populations (4). The test for association can then be adjusted for the information

on subpopulation. The confounding effect caused by population stratification can then be corrected. It has also been shown that the power of this method is comparable to that of the TDT (21).

Compared with non-model-based methods, model-based approaches work better when no adjustment is needed or negative confounding exists. However, success of the algorithm depends on accurate subgroup classification. If random markers are used, then a large number of markers are required for the subgroup classification.

In addition to these three major methods mentioned, several other computational approaches can also be used to deal with population stratification. For example, ADMIXMAP (22–26) is a model-based method that estimates the individual history of admixture. It can be applied to an admixed population with two or more ancestral populations. It also tests the association of a trait with ancestry at a marker locus with control for population structure. Wu et al. developed a software package in R (PSMIX) for the inference of population stratification and admixture (27). PSMIX is based on the maximum likelihood method. It performs as well as model-based methods such as STRUCTURE and is more computationally efficient.

3.5 Conclusions

Association studies are widely used to assess individual response to drug treatment and correlation of a disease with genetic variants. Population structure and recent admixture may confound the results of association studies. Confounding can be detected and adjusted by using unlinked markers. Several model-based and non-model-based methods have been developed to adjust for population stratification. Each has specific advantages and disadvantages. A solid understanding of these methods helps users choose the right ones for their studies.

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Chapter 4

Pharmacogenetics of Membrane Transporters

A Review of Current Approaches

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4.1	Introduction.....	41
4.2	Genetic Variation and Genotyping Methods	46
4.3	Substrate Identification	50
4.4	Assessing Functional Significance of Polymorphisms In Vitro	51
4.5	Assessing Functional Significance of Polymorphisms In Vivo.....	53
4.6	Transporter Genetics in Clinical Pharmacology	54
4.7	Transporter Genetics in Clinical Endpoint Analysis	57
	References.....	57

Summary This chapter provides a review of the pharmacogenetics of membrane transporters, including adenosine triphosphate-binding cassette (ABC) transporters and organic anion transporting proteins (OATPs). Membrane transporters are heavily involved in drug disposition by actively transporting substrate drugs between organs and tissues. As such, polymorphisms in the genes encoding these proteins may have a significant effect on the absorption, distribution, metabolism, and excretion of compounds. The techniques used to identify substrates and inhibitors of these proteins and subsequently assess the effect of genetic mutation on transport, both in vitro and in vivo, are outlined and discussed. Finally, studies linking transporter genotype with clinical outcomes are discussed.

Keywords ABCB1; ABCC1; ABCC2; ABCG2; OATP1B1; OATP1B3; polymorphisms; transport.

4.1 Introduction

The fate of a drug in vivo is dictated by a variety of physiochemical properties, including size, lipophilicity, and charge. These properties determine how a drug is absorbed into the blood, distributed throughout the body, metabolized, and eventually eliminated. While movement of a drug molecule can occur through simple diffusion, there are many transporter proteins expressed on cell membranes to assist

with influx or efflux via active transport. As such, these transporters can significantly affect drug disposition. For example, influx of a drug from the blood to the liver, where it is subsequently metabolized and excreted, may increase the rate of elimination. These proteins and the genes that encode them are essential to drug uptake, bioavailability, targeting, efficacy, toxicity, and clearance. The genes encoding these transporters are polymorphic—phenotypically resulting in transporters with different expression levels and transport efficiency. Consequently, polymorphisms in transporters are often responsible for some variability in drug pharmacokinetics and response to treatment.

Many drugs undergo transport that is mediated by the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters. There are a total of 48 known ABC genes, including *ABCB1* (P-glycoprotein, multidrug resistance [MDR] 1), *ABCC1* (MRP1), and *ABCG2* (BCRP, MXR, ABCP), all of which utilize ATP to move substrates across membranes (1–3). These transporters generally limit drug uptake through the intestinal wall, move substrates out of tissues into the systemic circulation, and eventually mediate the clearance of drugs from the body. The ABC family of proteins are known as efflux transporters and move substrates across the cell membrane, out of the cell.

The most characterized polymorphic transporters to date are *ABCB1* and *ABCG2* (4). Many current drugs approved by the Food and Drug Administration (FDA) are substrates of these transporters, although both transporters also efflux a plethora of other compounds, including naturally occurring toxins. *ABCB1* and *ABCG2* are expressed in the enterocytes, colon, intestinal epithelium, canalicular plasma membrane of hepatocytes, and proximal renal tubule (5–9). *ABCB1* and *ABCG2* play a role in drug uptake, distribution, and elimination at these sites, and thus they often mediate bioavailability and exposure to their substrate drugs, as mentioned in Table 4.1 (10,11). In addition, *ABCB1* and *ABCG2* have been shown to be expressed in hematologic tissues, including hematopoietic stem cells, and endothelial cells composing blood–tissue barriers of the brain, heart, nerves, testes, and placenta, where they efflux substrates out of these tissues into the systemic circulation (6,12–17). An exception includes the expression of *ABCB1* in the choroid plexus, where it transports molecules from the circulation into the cerebrospinal fluid (16,18). It is believed that the evolutionary role of these transporters is to limit the penetration of toxic molecules into critical organs, thereby serving a protective role in blood–tissue barriers.

Two other efflux transporters, *ABCC1* and *ABCC2* (MRP2) are also involved in drug disposition. *ABCC1* is expressed ubiquitously and is localized to the basolateral, rather than apical, membranes of epithelial cells. Because of its basolateral localization, *ABCC1* pumps drugs into the body rather than into the bile, urine, or intestine. For this reason, it is thought to serve mainly as a protective barrier in epithelial cells of tissues rather than as a classic drug efflux pump (19). *ABCC2* is similar in function to *ABCB1*. It is expressed on the apical domain of epithelial cells and is involved in luminal excretion in organs such as liver, intestine, and kidney but also serves a role in blood–tissue barriers (20). Both *ABCC1* and *ABCC2* primarily secrete drugs that

Table 4.1 Selected substrates of ABCB1, ABCG2, ABCC1, ABCC2, OATP1B1, and OATP1B3

Transporter	Transporter substrates and inhibitors
ABCB1	
Anticancer drugs	Paclitaxel Docetaxel Vinblastine Vincristine Tipifarnib Diflomotecan Irinotecan Doxorubicin Daunorubicin Etoposide Tenoposide Tamoxifen (i)
Antibiotic	Erythromycin
Antifungal	Ketoconazole (i)
Antihistamine	Terfenadine
Antihypertensives/antiarrhythmics	Digoxin Talinolol Quinidine (i)
Antituberculosis agent	Rifampin
Calcium channel blockers	Verapamil (i)
CNS drugs	Quetiapine Risperidone Olanzapine Chlorpromazine Fluphenazine Clozapine
HIV-1 protease inhibitors	Ritonavir Aquinavir Saquinavir Indinavir Nelfinavir
Immunosuppressants	Cyclosporine (i) Tacrolimus Dexamethasone Rapamycin Valspodar (PSC833) (i)
Sedative	Midazolam (i)
Statins	Lovastatin

(continued)

Table 4.1 (continued)

Transporter	Transporter substrates and inhibitors
ABCG2	
Antibiotics	Ciprofloxacin Norfloxacin Ofloxacin Novobiocin (i)
Anticancer drugs	Daunorubicin Topotecan 9-Aminocamptothecin SN-38 (irinotecan metabolite) Epirubicin Mitoxantrone Methotrexate Imatinib Gefitinib (i)
Antiplatelet	Dipyridamole
Calcium channel blockers	Nicardapine (i) Nimodipine (i) Nitrendipine (i)
HIV protease inhibitors	Ritonavir (i) Saquinavir (i) Nelfinavir (i)
Immunosuppressants	Cyclosporine (s, i) Sirolimus (s, i) Tacrolimus (s, i)
Specific inhibitors	GF120918 (i) Ko143 (i) Tariquidar (XR9576) (i)
ABCC1^a	
Antibiotics	Difloxacin Grepafloxacin
Anticancer drugs	Methotrexate Doxorubicin Daunorubicin Epirubicin Etoposide Vincristine Vinblastine Paclitaxel Irinotecan
SN-38 (irinotecan metabolite)	

(continued)

Table 4.1 (continued)

Transporter	Transporter substrates and inhibitors
Flutamide	
HIV protease	
Saquinovir	
Ritonavir	
ABCC2	
Anticancer drugs	
Doxorubin	
Etoposide	
Methotrexate	
Mitoxantrone	
Cisplatin	
Vincristine	
Vinblastine	
Irinotecan	
Antibiotic	
Ampicillin	
HIV protease inhibitors	
Indinavir	
Ritonavir	
Saquinavir	
Adevovir	
Cidofovir	
OATP1B1	
Anticancer drug	
	SN-38
Antibiotics	
	Rifampin
Antitubercular	
	Rifampin
Antifungal	
	Caspofungin
Antihistamine	
	Fexofenadine
Blood glucose-lowering drug	
	Repaglinide
Statins	
	Pravastatin
	Atrovastatin
	Cervistatin
	Pitavastatin
	Rosuvastatin
Miscellaneous	
	Bromosulfophthalein
OATP1B3	
Anticancer drug	
	Irinotecan
Blood glucose-lowering drug	
	Repaglinide
Statins	

(continued)

Table 4.1 (continued)

Transporter	Transporter substrates and inhibitors
Antihistamine	Pravastatin
	Pitavastatin
	Rosuvastatin
	Fexofenadine

(i) denotes the drug as an inhibitor.

(s, i) denotes the drug as an inhibitor that may also be a substrate.

*See (21) for a complete list of Glutathione (GSH) and glucuronide conjugates.

have undergone phase II metabolism into glutathione, glucuronide, or sulfate conjugates, but both efflux a wide range of drugs (21).

There are also several classes of “influx” or “uptake” transporters that mediate the cellular uptake and reabsorption of drugs by moving substrates against a concentration gradient. The main uptake transporters are the organic anion transporting proteins (OATPs), organic cation transporters (OCTs), concentrative nucleoside transporters (CNTs), dipeptide transporters (PEPTs), and monocarboxylate transporters (MCTs) (22). For brevity, we discuss only two members of the OATP1B family of proteins as these are well-characterized influx transporters. OATP1B1 and OATP1B3 are expressed in liver tissues and are responsible for hepatocellular uptake of drugs from blood across the basolateral membrane where metabolism occurs (23). As such, the OATP1B family is important in regulating the pharmacokinetics of several substrate drugs.

There is significant variation in the genes encoding all of the aforementioned transporters. Several of these genetic variants result in alterations in messenger RNA (mRNA) expression levels (e.g., promoter variants); translational efficiency (e.g., alterations in mRNA folding); and protein function (e.g., coding polymorphisms). Such genetic variability in transporters often explains a component of the interindividual variability in drug disposition, ultimately resulting in differences in clinical endpoints, including toxicity and response. The field of transporter pharmacogenetics is thus concerned with elucidating the mechanisms by which genetic variation in transporters determines individual differences in drug transport, with a goal of eventually personalizing treatment with substrate drugs based on genotype. This chapter provides an overview of the methods by which investigators have discovered and characterized such associations in the ABCB1, ABCG2, ABCC1, ABCC2, OATP1B1, and OATP1B3 transporters. This methodology could be readily applied to the study of many additional transporters.

4.2 Genetic Variation and Genotyping Methods

More than 50 polymorphisms, three insertions/deletions, and several promoter alterations that modify gene transcription have been described in the *ABCB1* gene. There are three single-nucleotide polymorphisms (SNPs) that are common in most racial populations and demonstrate strong linkage disequilibrium: the synonymous

transition at nucleotide 1236C>T (Gly411Gly) in exon 12, the nonsynonymous triallelic 2677G>T/A (Ala893Ser/Thr) in exon 21, and the synonymous 3435C>T (Ile1145Ile) in exon 26. Of these SNPs, the 2677G>T/A (Ala893Ser/Thr) transition causes an amino acid change within a structurally important transmembrane domain of the translated protein, although the effects of this transition are controversial and drug specific (24–28). The 3435C>T SNP is associated with decreased mRNA stability and lower expression levels (29). The effect of the 1236C>T polymorphism is currently unclear, although it is in approx. 90% D' linkage with the 2677G>T/A polymorphism and by virtue of that linkage may be only artificially associated with interindividual ABCB1 transport alterations.

While there are many polymorphisms in *ABCG2*, the common *ABCG2* 421C>A allele in exon 5 is by far the most well characterized. This SNP results in an amino acid change of Gln to Lys at codon 141 and has been associated with low *ABCG2* expression levels (30–34). The variant allele (i.e., 421A and 141L) has also been associated with lower adenosine triphosphatase (ATPase) activity as compared with wild-type *ABCG2* (35). Thus, the *ABCG2* 421C>A SNP, much like the *ABCB1* 2677G>T/A allele, may alter both expression and activity of the encoded protein. The frequency of this mutation varies significantly by race, and while it occurs at 35% frequency in Chinese populations, the mutation is rare in African Americans (1%) (36). Another SNP exists at nucleotide 34, resulting in a V12M amino acid change. This mutation results in poor localization of the *ABCG2* protein (35) but does not change protein expression levels (37). Surprisingly, this mutation does not appear to modify substrate transport (38). A nonsynonymous SNP resulting in an amino acid change at 482 has been identified in numerous cancer cell lines (presumably a mechanism of multidrug resistance) but has never been found in humans. This mutation affects both transport and substrate specificity (39–42).

There are several polymorphisms in *ABCC1*, 11 of which are nonsynonymous: C43S, T731I, S92F, T117M, R230Q, R633Q, R723Q, A989T, C1047s, R1058Q, and S1512L. None of these SNPs has been shown to alter the functionality of the expressed protein and are unlikely to significantly influence the expression (43). Others have evaluated nonsynonymous polymorphisms to assess their impact on mRNA expression but have found no significant results (44). The *ABCC2* gene also contains several polymorphisms that have not been significantly associated with any differences in functionality or expression (20).

There are many polymorphisms in *OATP1B1* that have been associated with a decreased transport phenotype toward several endogenous substrates and drugs (see Table 4.1) (23). In vitro assays have consistently validated altered transport efficiency in at least 13, both synonymous and nonsynonymous, polymorphisms, whereas clinical outcome has been shown to be influenced by at least three SNPs, the –11187G>A, the 388A>G (*OATP1B1**1b), and the 521T>C (*OATP1B1**5). *OATP1B1* polymorphisms are frequent in some races and rare in others. For example, while the *OATP1B1**5 polymorphism is present in approx. 14% of the Caucasian population (45), only approx. 1% of Japanese subjects carry this allele (46). For this reason, studies evaluating associations between *OATP1B1**5 and clinical outcome in Caucasians have been more statistically powered and have resulted in clearer clinical outcomes. The *OATP1B3* gene has four polymorphisms (334T>G, 699G>A,

Table 4.2 Primers used to amplify selected polymorphisms in the ABCB1, ABCG2, OATP1B1, and OATP1B3 genes

Transporter polymorphism	Forward primer	Reverse primer	Reference
<i>ABCB1</i> 1236C>T			
PCR amplification	5'-GGCACAAACACAGATAATATTAAGG-3'	5'TATCCTGTCCATCAACACTGACC-3' ^a	
Nested PCR	5'-GTTCACTTCAGTTACCCATCTCG-3'	5'-TCCTGTCCATCAACACTGACCTG-3' ^a	
Sequencing PCR	5'-GTCAGTTCCTATATCCTGTGTCTG-3'	5'-TCGCATGGGTTCATCTCACCATC-3' ^a	
<i>ABCB1</i> 2677G>T/A (A893S/T)			
PCR amplification	5'-AGGGTATAGGTTCCAGGCTTGC-3'	5'-AGAACAGTGTGAAGACAAATGGCC-3' ^a	
Nested PCR	5'-CCCATCATTCGAATAGCAGGAG-3'	5'-GAACAGTGTGAAGACAAATGGCCT-3' ^a	
Sequencing PCR	5'-ATCCTTCATCTATGTTGGCAAC-3'	5'-TGAGTCCAAAGAACTGGCTTTGCG-3' ^a	
<i>ABCB1</i> 3435C>T			
PCR amplification	5'-ATCTCACAGTAACTTGGCAGTTTC-3'	5'-AACCCAAACAGGAAGTGTGGCC-3' ^a	
Sequencing PCR	5'-GCTGGTCTCTGAAGTTGATCTGTG-3'	5'-AAACAGGAAGTGTGGCCAGATGC-3' ^a	
<i>ABCG2</i> 421C>T			
PCR amplification	5'-TGGCAAATCCTTGTATGAAGCAG-3'	5'-TTCACGTACAACACCACATTGGCC-3' ^a	
Sequencing PCR	5'-GCAGGTTTCATCATTAGCTAGAAC-3'	5'- CCTACTTATGCTGATCATGAGC-3' ^a	
<i>OATP1B1</i> *1b			
PCR amplification	5'-GCAAAATAAGGGGAATATTCTC-3'	5'-AGAGATGTAATTAATGTATAC-3'	(45)
RFLP enzyme	<i>ClaI</i>		
<i>OATP1B1</i> *5			
PCR amplification	5'-GTTAAATTGTAAATAGAAATGC-3'	5'-GTAGACAAAGGGAAAGTGATCATA-3'	(45)
Allele-specific PCR primers	WT 5-CATACATGTGGATATATGT-3'	N/A	(45)
MT 5-CATACATGTGGATATATGC-3D			
<i>OATP1B3</i> 334T>G (S112A)			
PCR amplification	5'-CCTTCACAGTTAAATACATGGTC-3'	5'-TATTTCATTTCATATAAAAACGTATACC-3' ^a	
Sequencing PCR	5'-GGGCATATTGCAATTCATTGGG-3'	5'-CATGATAAAATAAAGAAATACATGATG-3' ^a	

<i>OATP1B3</i> 699G>A (M233I)	5'-TCCTTGTATTAGGTAACGTACAG-3'	5'-TCAAGTTTGGTTATTTGGATCAAG-3' ^a
PCR amplification	5'-GATCTACCCCTTGAAATAATAATGTC-3'	5'-GTAAAAGCAAAAGTATATAAATAGGAGC-3' ^a
Sequencing PCR		
<i>OATP1B3</i> 1564G>T (G522C)	5'-ATATACAGAAATTCATACACTAATTTC-3'	5'-AATTCTAAGAAAAATGCATTCTCAAAG-3' ^a
PCR amplification	5'-TATTTTGCCTTCACTATTAGCAAC-3'	5'-AATATGAATTTGAGCTCAAAAATACAG-3' ^a
Sequencing PCR		

Primers are used for direct sequencing following amplification from genomic DNA, unless otherwise indicated.
PCR, polymerase chain reaction.
^a Previously unpublished primer.

1564G>T, 1748G>A) that have been associated with altered transport; the relevance of these polymorphisms *in vivo* remains to be reported.

Many of the recent publications regarding transporter genotyping have utilized restriction fragment length polymorphism (RFLP) analysis or direct sequencing, although several other methods of genotyping are available, such as resequencing, allele-specific polymerase chain reaction (PCR), TaqMan PCR, fluorescence resonance energy transfer (FRET), and so on. [Table 4.2](#) includes PCR and direct-sequencing PCR primers that are currently used in the field to amplify polymorphic regions of DNA.

Genetic sequence variation may provide useful information to assist in making clinical decisions about drug treatment. Like all possible prognostic markers, the effect of polymorphisms on clinical endpoints must be validated through several preclinical and clinical processes mentioned in the following subheadings. Studies of the pharmacogenetics of transporters should (1) establish theoretical and experimental evidence that a transporter polymorphism is associated with interindividual variability in drug treatment; (2) establish drug interaction with a transporter; (3) establish that a polymorphism results in differential drug transport *in vitro*; (4) verify that transporter function is potentially important *in vivo* using animal models; (5) validate that the polymorphism is associated with clinical interindividual variability of drug treatment in specific drug-treated populations with specific measurement methods of specific endpoints; and (6) validate the precision, reproducibility, and accuracy for clinical endpoint measurement ([47](#)). Only then are these polymorphisms useful in predicting clinical outcome.

4.3 Substrate Identification

ABCB1 and ABCG2 substrates (*see* [Table 4.1](#)) are typically hydrophobic molecules and include lipids, peptides, steroids, and xenobiotics—such as anticancer, human immunodeficiency virus (HIV), atypical antipsychotic, and immunosuppressant drugs. There is often broad overlap between ABCB1 and ABCG2 substrates. The ABCC proteins are multispecific anion transporters. ABCC1 is known to be involved in anthracycline transport ([48](#)), but ABCC2 effluxes a wider range of drugs, such as cyclosporine, cisplatin, vinblastin, and camptothecin derivatives ([19,49](#)). OATP1B1 and OATP1B3 interact with a wide range of substrates (not only organic anions as the nomenclature implies), including bilirubin, bile acids ([50](#)), peptides, eicosanoids, hormones, and prescribed drugs, including fexofenadine ([51](#)). However, each transporter has distinct substrate specificity, so some compounds are transported by one transporter but not another in the same family.

Substrates are usually identified using transfected MDCK, Caco-2, or endothelial cell lines that express the transporter of interest. These cell types are grown in a monolayer on a membrane separating two chambers of culture medium (i.e., the Transwell Cell Culture Assay, Corning Costar Corp., Cambridge, MA). Drug is administered into one chamber, and drug transport across the monolayer is

evaluated by sampling from the other chamber. The experiment is then repeated by applying drug to the opposite chamber. Because of the directionality of the transporters, these experimental systems allow investigators to assess the basolateral-to-apical (B:A) and apical-to-basolateral (A:B) transport of drug. If the drug is a substrate for the transporter, then the A:B and B:A ratios will differ significantly. Several other methods also exist to evaluate the transport capabilities toward individual drugs, such as ATPase assays, transport of fluorescent or radiolabeled compounds into and out of cells that are native expressing, drug-selected expressing, or transfected with a transporter of interest (52,53).

4.4 Assessing Functional Significance of Polymorphisms In Vitro

4.4.1 Cell-Based Assays

Polymorphic efflux of ABCB1 substrates was initially evaluated using flow cytometry, although such assays are limited in that only fluorescent compounds can be assayed, and differences in polymorphic transporter expression and function are not made clear. To date, the influx of rhodamine 123, calceine, doxorubicin, and daunorubicin has been evaluated using such methods and are still used in drug–drug interaction studies. The same technique has been used with mitoxantrone to assess transport by, and inhibition of, ABCG2. Such assays were initially used in the field of transporter pharmacogenetics to show that rhodamine 123 transport is lower in 3435TT human CD56⁺ cells (54).

As the pharmacokinetics of many other drugs could potentially also be differentially altered based on polymorphic ABCB1 expression and function, with ensuing clinical implications, many have evaluated ABCB1 efflux using other in vitro assays. Some have used transfected cell lines to evaluate the functional significance of nonsynonymous polymorphisms in ABCB1 and have demonstrated that differences in activity exist between proteins carrying a single amino acid difference brought on by these SNPs. For example, using this technique, it was found that the 2677G>T/A (893S>T/A) polymorphism results in activity differences toward vincristine such that $V_{\max}$ 893T > 893S > 893A, while K_m 893S > 893T/A (55). Other investigators have employed ATPase assays to evaluate the ATP-dependent active transport of substrates. In this assay, vesicles obtained from Sf9 cells transfected with *ABCB1* variants were studied and validated the finding with ABCB1 (56). The effect of different polymorphisms on substrate transport by ABCG2 has been assessed using stably transfected HEK293 cells (57). Following incubation of the cells with the drug, concentrations can be measured via flow cytometry (58), liquid scintillation counting if radiolabeled drug is available (59), or liquid chromatography/mass spectrometry (LC-MS) (60). In vitro analyses of *OATP1B1* functional polymorphisms were evaluated similarly (45,46,61–64).

Interestingly, these assays have been employed to address the functional consequences of polymorphisms in the ABCC family of transporters but no notable alterations in transport capacity have been found (43). It seems that although ABCC transporters contain several potentially important polymorphisms and are important in drug transport overall, functional variability is actually quite low. This is perhaps the reason for the multiple negative studies that have assessed ABCC polymorphisms as they relate to drug bioavailability (20).

4.4.2 Assessing the Cause of Phenotypic Differences

Polymorphic differences that result in altered transporter kinetics, and possibly subsequent changes in drug disposition, can affect this change via multiple mechanisms, including modulated tissue expression. For example, the *ABCB1* 2677TT genotype was associated with decreased mRNA expression in several human tissues as compared to the wild-type allele (54,65,66), and thus the functional consequences of the 2677G>T/A polymorphism may be explained by expression alterations alone and not necessarily by altered substrate binding or transport efficiency of the protein. Some postulate that polymorphisms encoding rarer codons for the same amino acid (a synonymous or silent mutation) result in decreased translation efficiency of the mRNA, resulting in lower protein levels, and that it is possible that alterations in polymorphic mRNA secondary structure could also result in inefficient translation. This mechanism has been suggested as one possible explanation for the effects seen with a synonymous mutation in *ABCB1* because the 3435C>T transition does not result in an amino acid change but is still associated with differential drug efflux capability. An alternate, although not mutually exclusive, explanation has also been proposed; the 3435C>T SNP is in linkage with the nonsynonymous 2677G>T (893T>S) transition; therefore, it may be associated with a protein product with attenuated efflux capacity through lowered efflux efficiency.

The first hypothesis has been evaluated using mRNA expression measurements in human tissues, and it was found that *ABCB1* is generally expressed at higher levels with the 3435C (54,65–67). These observations were replicated with cotransfection of equal amounts of plasmid, and it was concluded that the 3435T allele lowers mRNA stability and is therefore responsible for decreased efflux capacity (68). In the case of *ABCG2*, the effect of the 421C>A polymorphism has been debated. Originally, the resulting amino acid change was believed to reduce protein expression because of instability (37), but this finding was not confirmed by human intestinal samples, which did not reveal a difference (69). Subsequently, it was shown that the transport efficiency of the protein is decreased. This was demonstrated by measuring ATPase activity in wild-type and mutant cells, normalizing for expression (35).

When *OATP1B1* variants were expressed in HeLa cells, it was noted that *OATP1B1**2, *3, *5, *6, *9, *12, and *13 alleles were associated with reduced transport

toward OATP1B1 substrates (45). Others noted that when the *OATP1B1*15* variant was expressed in HEK293 cells and *Xenopus laevis* oocytes, these cells also had reduced transport capability (61,63). The reasons for the reduced transport capacity of these alleles were made clear after it was demonstrated that the plasma membrane localization of many of these polymorphic transporters was impaired because of a cell surface trafficking defect (45). It was also shown that some polymorphisms encode for impaired protein maturation that results in retaining the encoded OATP1B1 protein intracellularly (62). Studies evaluating *OATP1B3* polymorphisms are currently undergoing similar validation, but no significant results have yet been reported.

Despite the encouraging results of these investigations, not all studies using the experimental systems have consistently validated these observations in other tissues and cell types. For example, associations between genotype and expression seem to be tissue specific as lymphocytes and the small intestine both express ABCB1, but expression levels were not associated with polymorphic variants, and it is often the case that reports evaluating the same tissues conflict (4). Furthermore, some tissues, such as cardiac endothelium, actually express ABCB1 at greater levels in patients carrying variant alleles, which is the direct opposite of data generated in other tissues (67). Others have used nonhuman in vitro expression systems in an attempt to validate the effect of *ABCB1* polymorphisms, although transfected variant alleles do not seem to influence ABCB1 transport in some of these experimental systems, perhaps because of differences in mRNA-processing membranes in different cell lines and between species (70).

4.5 Assessing Functional Significance of Polymorphisms In Vivo

Mice carry two homologs of *ABCB1* (*Abcb1a*, *Abcb1b*), and viable single- (*Abcb1a*) and double-knockout mice are commercially available (Taconic Laboratories). In addition, triple-knockout (TKO) mice have become available in which homologous genes encoding *ABCB1* and *ABCC* family members have been removed from the mouse genome. An *Abcg2* (the mouse homolog of *ABCG2*) knockout mouse has also become commercially available from Taconic Laboratories, in addition to a triple knockout, null for *Abcb1a*, *Abcb1b* and *Abcg2*. Many have utilized such mice to evaluate the influence of ABC transporters on the pharmacokinetics and toxicity of drugs. Based on data obtained from these mice, ABCB1 has been shown to play a major role in detoxification and serves as a protective barrier against the toxic effects of xenobiotics (71). These knockouts have been used as animal models of compromised blood–brain barrier function (8,72), intestinal drug absorption (73), fetal drug exposure (74), and drug-induced damage to testicular tubules, choroid plexus epithelium (18), oropharyngeal mucosa (16), and peripheral nervous tissues (17).

Mice lacking the expression of a transporter generally have less ability to eliminate substrate drugs except if compensatory pathways are upregulated that circumvent transporter-mediated clearance (75,76). Alterations in plasma pharmacokinetics result from the lack of transporter expression in gut, liver, and renal tissues, where several transporters are involved in the elimination of substrate drugs through hepatobiliary pathways and glomerular filtration. Such mice generally also demonstrate increased uptake of oral substrate drugs as efflux transporters are involved in the excretion of toxic substances back into the gut lumen in normal mice. As such, bioavailability and exposure are usually increased in knockout mice, while clearance is decreased. This can have both positive and negative effects and can allow translational researchers to make clinical decisions based on the outcome of these drug-treated mouse models. However, this is not necessarily always the case. Compounds that are highly bioavailable in wild-type mice are unlikely to show great increases in absorption when the transporter protein is impaired. Also, as mentioned, many drugs have alternate routes of elimination, which may become more important when the primary transport mechanism is not functioning. As such, it is critical that *in vivo* testing is carried out for each compound rather than assuming that because a drug is a substrate, it will be greatly affected by these polymorphisms.

Mice that do not express a specific transporter are generally more likely to experience benefit from treatment with a substrate drug because bioavailability and exposure to the drug are usually increased along with the beneficial aspects of treatment. Lack of transporter function may also allow penetration into tissues that were previously impermeable to the agent. For example, *Abcb1* knockout mice with brain metastases can be successfully treated with drugs that otherwise would not penetrate the blood–brain barrier, such as paclitaxel (77). *ABCB1a*^{-/-} mice also showed ten times more brain–serum ratios of both risperidone and its active metabolite 9-hydrorisperidone than control mice (78), and most central nervous drugs showed 1.1- to 2.6-fold greater brain-to-plasma ratios in double-knockout mice compared to wild-type mice (79).

Although the efficacy of drug treatment may increase, this is counterbalanced by increases in toxicity through routes other than increased plasma concentrations as blood–tissue barriers are disrupted, allowing increased penetration of drugs into organs, especially the brain, where ABCB1 is an important mediator of drug exposure. In drugs with a narrow therapeutic window (e.g., many anticancer agents), the toxicity can outweigh the beneficial aspects of drug treatment. Following the example, *Abcb1* knockout mice treated with paclitaxel are more susceptible to treatment-related peripheral neuropathy because of increases in drug concentrations in nerve cells (17).

4.6 Transporter Genetics in Clinical Pharmacology

Initially, investigators determined that the *ABCB1* 3435C>T transition was associated with lowered ABCB1 expression and higher digoxin levels *in vivo* (65). The association was stronger when the *ABCB1* 2677G>T/A and 3435C>T

polymorphisms were evaluated together as a haplotype—those patients variant at both alleles having both the lowest ABCB1 expression and the highest digoxin area under the concentration vs time curve (AUC) (80,81). Since then, many investigators have found similar associations between these polymorphisms and plasma concentrations of several other drugs, although these observations have not been consistently confirmed (13,20,82). Often, it is found that a polymorphism is potentially important to drug treatment based on in vitro and in vivo evidence, but when clinical studies are conducted, the polymorphism is not associated with clinical parameters.

Table 4.3 provides examples of transporter polymorphisms that have had an effect on certain substrates in vitro or in vivo that have explained interindividual variability toward these drugs in clinical pharmacology or have not. It should be mentioned that just because a polymorphism has no effect on a substrate drug in the clinic, a potential gene–drug interaction could not be found following treatment with another substrate drug.

These relationships are often dependent on route of administration, drug dosage, and schedule and can also be largely dependent on drug metabolism. For example, polymorphisms in the cytochromes P450 (CYPs) are often associated with pharmacokinetics of transporter substrate drugs, whereas *ABCB1* polymorphisms are not. This is believed to occur because metabolism by the CYPs may be the rate-limiting step in drug clearance, and variation in *ABCB1* expression levels plays only a small deterministic role in interindividual variability. If an impaired transporter limits transport out of the liver, then it is also possible that more metabolism occurs. However, the mechanistic relationship between transporter polymorphisms and drug plasma levels remains largely unclear, and the reasons that several drugs are more or less associated with *ABCB1* polymorphisms across multiple studies in various racial populations also remain unclear.

More recent evidence suggests that polymorphic *ABCB1* expression influences not only plasma pharmacokinetics but also the degree to which drugs are able to penetrate into tissues that express *ABCB1* (e.g., tumors, brain, HIV-infected cells, etc.) (83,84). As mentioned, drug penetration into tissues can be both efficacious (i.e., by increasing therapeutic effect) and deleterious (i.e., by increasing toxicity). *ABCB1* is also deterministic of the intracellular concentration of drugs as it effluxes drugs from the cytoplasm into the extracellular matrix in several cell types. Based on these findings, the hypothesis can be formed that *ABCB1* expression levels are associated with pharmacokinetic parameters. Because the efficacy and toxicity of drugs are ultimately determined by plasma pharmacokinetic parameters and by the degree to which drugs are able to penetrate into tissues, studies investigating *ABCB1* polymorphisms as they relate to drug administration are likely to become increasingly important in the clinical setting. The effect of *ABCG2* polymorphisms on clinical pharmacology has only recently begun to be evaluated. Thus far, associations between the 421C>A mutation and plasma pharmacokinetics have been evaluated for several drugs, including topotecan, irinotecan, and imatinib. The polymorphism was shown to increase bioavailability of topotecan (17), but the findings for other drugs were less deterministic.

Table 4.3 Examples of studies that have either verified or detracted from the importance of transporter polymorphisms to drug treatment after in vitro or in vivo validation

Transporter	Drug	In vitro or in vivo verification of polymorphic effects	Reference	Clinical verification of interindividual variability	Reference
ABCB1	Flexofenadine	Increased transport with 893Ser	(88)	Decreased AUC in patients with 2677TT and 2677AA genotypes	(28, 89)
	Docetaxel	Increased transport with 893Thr Decreased expression of ABCB1 in liver with 3435TT genotype Docetaxel is transported by ABCB1	(65, 66) (93)	Docetaxel AUC and overall survival unchanged	(90–92)
	Topotecan	Increased accumulation of drug in cells transfected with 421A (Q141K)	(94)	Increased bioavailability in heterozygous C421A patients	(94)
ABCC2	Imatinib	Increased accumulation of drug in cells transfected with 421A (Q141K)	(95)	No significant difference in AUC or C_{max} in heterozygous C421 patients	(95)
	N/A	None to date			
OATP1B1	N/A	None to date			
	Pravastatin	Reduced membrane expression of OATP1B1*5 variant	(45)	Increased AUC in OATP1B1*5	(85, 96)
OATP1B3	N/A	None to date in prescribed drugs	(23)		

AUC, area under the concentration vs time curve; N/A, not applicable.

The clinical consequences of OATP1B1 are still under investigation, although several good studies have confirmed that *OATP1B1* polymorphisms are associated with interindividual variability in drug treatment (23). This is especially true for pravastatin. It has been suggested that reduced hepatic uptake of pravastatin resulting from decreased transport through OATP1B1 is responsible for the increased plasma AUC of pravastatin (85), the unfavorable plasma pharmacokinetics of cholesterol synthesis biomarkers (86), and the resulting decreased efficacy of the drug (i.e., lowered total cholesterol). Interestingly, an OATP1B1 allele that was associated with lower pravastatin AUC (and presumably greater liver uptake) was also associated with increased efficacy (87), although in vitro assays did not confirm an altered transport efficiency (63).

4.7 Transporter Genetics in Clinical Endpoint Analysis

The ultimate research goal of transporter pharmacogenetics is to further our understanding of the ways in which transporter genetics influence clinical endpoints so that current drug treatment can be made safer and more efficacious and investigational therapies can be better developed. The literature consists of a multitude of studies that have evaluated drug efficacy and toxicity and have made associations between these parameters and polymorphisms in drug transporters (4,20,23). The FDA recommends several endpoints to evaluate specific diseases, and those endpoints should be evaluated when making associations between a genetic variation and the treatment of diseases with drugs (see www.fda.gov/cder/guidance; accessed November 22, 2006). In pharmacogenetic studies, these endpoints should be evaluated in a standard fashion in similar populations to establish the predictive value of a polymorphism. Unfortunately, the literature has not typically been consistent, mainly because of the low availability of samples for analysis, and perhaps this is the reason that transporter polymorphisms have not been consistently validated. Thus far, all studies linking pharmacogenomics of membrane transporters with clinical outcome have been retrospective, taking place in eclectic populations with relatively low statistical power. It is essential that prospective studies are undertaken, prior to any treatment modification, to assess the true effects of these polymorphisms and determine whether the effect is drug specific or disease related.

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Chapter 5

Pharmacogenomics of Drug-Metabolizing Enzymes and Drug Transporters in Chemotherapy

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5.1 Introduction.....	64
5.2 Drug-Metabolizing Enzymes.....	64
5.3 Drug Transporters.....	69
5.4 Conclusions.....	71
References.....	72

Summary There is wide variability in the response of individuals to standard doses of drug therapy. This is an important problem in clinical practice, where it can lead to therapeutic failures or adverse drug events. Polymorphisms in genes coding for metabolizing enzymes and drug transporters can affect drug efficacy and toxicity. Pharmacogenomics aims to identify individuals predisposed to high risk of toxicity and low response from standard doses of anticancer drugs. This chapter focuses on the clinical significance of polymorphisms in drug-metabolizing enzymes and drug transporters in influencing efficacy and toxicity of anticancer therapy. The most important examples to demonstrate the influence of pharmacogenomics on anticancer therapy are thiopurine methyltransferase (TPMT), UGT (uridine diphosphate glucuronosyltransferase) 1A1*28, and DPD (dihydropyrimidine dehydrogenase) *2A, respectively, for 6-mercaptopurine, irinotecan, and 5-fluorouracil therapy. However, in most other anticancer therapies no clear association has been found for polymorphisms in drug-metabolizing enzymes and drug transporters and pharmacokinetics or pharmacodynamics of anticancer drugs. Evaluation of different regimens and tumor types showed that polymorphisms can have different, sometimes even contradictory, pharmacokinetic and pharmacodynamic effects in different tumors in response to different drugs. The clinical application of pharmacogenomics in cancer treatment therefore requires more detailed information regarding the different polymorphisms in drug-metabolizing enzymes and drug transporters. A greater understanding of complexities in pharmacogenomics is needed before individualized therapy can be applied on a routine basis.

Keywords Drug-metabolizing enzymes; drug transporters; oncology; pharmacogenomics.

5.1 Introduction

Patients who are treated with standard doses of chemotherapy exhibit large inter- and intraindividual variability in the development of severe toxicities. In addition, there is a wide variation between patients with the same tumor type and stage in the likelihood of response after standard chemotherapy.

The factors responsible for the individual pharmacokinetic and pharmacodynamic variability are manifold and include drug–drug interactions, ethnicity, patient's age, renal and liver function, concomitant diseases, nutritional status, smoking, and alcohol consumption. In many cases, however, genetic factors have an even greater influence on drug efficacy and toxicity (*1*). Such variation (i.e., as seen in genes coding for drug-metabolizing enzymes and drug transporters) can influence pharmacokinetic and pharmacodynamic parameters of anticancer drugs.

Pharmacogenomics is the study of the inherited basis of interindividual differences in drug response. One approach is to search for genetic variants that are associated with severe adverse effects, which in turn can be used to screen for individuals who should not receive the drug in question or should receive an adjusted dose of the drug. Another approach is to identify markers that predict drug efficacy (*2–4*).

Understanding the variable response to drugs seems particularly pressing in the field of oncology, in which stakes are high, drugs commonly have a narrow therapeutic index, and toxicities can be severe (even lethal). Pharmacogenomic screening before the start of cancer chemotherapy may thus enable the identification of patients at increased risk of toxicity or low likelihood of response. The promise of pharmacogenomics is that it could lead to tailored drug therapy, also called *individualized medicine*. This chapter focuses on the clinically significant role of genetic polymorphisms of selected drug-metabolizing enzymes (cytochrome P450 [CYP450], dihydropyrimidine dehydrogenase [DPD], uridine diphosphate glucuronosyltransferase [UGT] 1A1, thiopurine methyltransferase [TPMT]) and adenosine triphosphate-binding cassette (ABC)–drug transporters (P-glycoprotein [P-gp], breast cancer resistance protein [BCRP]) and their possible influence on toxicity or response to chemotherapy.

5.2 Drug-Metabolizing Enzymes

5.2.1 Cytochrome P450

Among the human CYP enzymes involved in metabolism of anticancer agents, genetic polymorphisms have been reported in CYP isoforms 2C8, 2C9, 2C19, 2D6, and 3A4. The effects of polymorphisms in these enzymes on the metabolism of anticancer drugs *in vivo* are not described, or pharmacogenomic data are not consistent (*5*). Further research has to be performed to evaluate the influence of polymorphisms in these enzymes on the efficacy and toxicity of specific anticancer drugs separately. Pharmacogenomic data for CYP2D6 and CYP3A4 are discussed in more detail.

5.2.1.1 CYP2D6

With respect to anticancer drugs, CYP2D6 is only involved in the conversion of tamoxifen to the 50 times more potent antiestrogen 4OH-tamoxifen (6). Plasma endoxifen concentrations (a metabolite of tamoxifen) after 4 mo of tamoxifen therapy were statistically significantly lower in subjects with a CYP2D6 homozygous variant genotype or a heterozygous genotype than in those with a homozygous wild-type genotype. Besides, in subjects taking a potent CYP2D6 inhibitor (such as paroxetine), the plasma endoxifen concentration was reduced substantially (7). Although the role of CYP2D6 in tamoxifen treatment is evident, most anticancer agents are not subjected to CYP2D6 metabolism at clinically relevant concentrations (8).

5.2.1.2 CYP3A4

The allelic variants of CYP3A4 in humans have been investigated thoroughly (see <http://www.imm.ki.se/CYPalleles/cyp3a4.htm>). All of the coding single-nucleotide polymorphism (SNP) allele frequencies of the *CYP3A4* gene are relatively low in most of the populations studied, and no homozygotes for these mutations have been reported (9). It was estimated that only 14% of Caucasian, 10% of Japanese, and 15% of Mexican individuals carry a CYP3A4 allele with at least one coding change. With such low allele frequencies, it will be exceedingly difficult to link genotype to pharmacokinetics and pharmacodynamics of CYP3A4 substrates. A possible explanation can be given by polymorphisms in the pregnane X receptor (*PXR*) gene that regulates the transcription of CYP3A4. Several studies have described polymorphisms in the *PXR* gene and their influence on CYP3A4 expression (10–12), but allele frequencies are low, and variant proteins showed varying degrees of reduction in transactivation. Another possibility to clarify the variability in pharmacokinetics and pharmacodynamics of CYP3A4 substrates is linkage of CYP3A4 and CYP3A5 polymorphisms. Strong linkage disequilibrium (LD) has been confirmed between CYP3A4 and CYP3A5, but so far no effects on pharmacokinetics and pharmacodynamics have been described.

Overall, no major pharmacokinetic consequences for the identified CYP3A4 mutations have been observed for the metabolism of anticancer drugs. Greater understanding of the complexities of multiple gene modifiers will be needed before individualized therapy can be applied for CYP3A4 anticancer substrates.

5.2.2 Dihydropyrimidine Dehydrogenase

Dihydropyrimidine dehydrogenase is the first and the rate-limiting enzyme in the three-step metabolic pathway involved in the degradation of the pyrimidine bases uracil and thymine. In addition, this catabolic pathway is the only route for the synthesis of β -alanine in mammals.

5-Fluorouracil (5-FU) is a major drug in the treatment of advanced colorectal cancer. Of the administered 5-FU, 70–80% is degraded by DPD in vivo to dihydrofluorouracil.

Therefore, the activity of the enzyme DPD is one of the main factors determining exposure to 5-FU.

Patients with partial deficiency of this enzyme are at risk of developing severe 5-FU-associated toxicity as DPD is responsible for detoxification of pyrimidine-based antimetabolite analogs, such as 5-FU and capecitabine. The onset of toxicity occurs, on average, twice as fast in patients with low DPD activity compared with patients with normal DPD activity (13–16).

Another reason for the variation in 5-FU-related toxicity may be the detection of mutations in the *DPYD* gene. Most patients with 5-FU-related toxicity have multiple mutations in the *DPYD* gene. However, only a few patients with a low DPD phenotype have a molecular basis for reduced activity. Although novel *DPYD* variants have been identified in studies, the *DPYD* mutations now described do not entirely explain polymorphic DPD activity and toxic response to 5-FU.

Analysis of the *DPYD* gene of a cancer patient that exhibited grade 4 toxicity 10 d after 5-FU treatment revealed the presence of a splice site mutation IVS14+1G>A, which led to the skipping of exon 14 directly upstream of the mutated splice donor site during DPD pre-messenger RNA (mRNA) splicing (17). These results are in line with other study results. Among 25 patients with severe 5-FU-related toxicity, 5 were heterozygous and 1 was homozygous for this mutation. All of these patients had experienced grade 4 leukopenia, and lethal outcome was seen in the homozygous and two of the heterozygous patients (18).

A study by Maring et al. (19) indicated that the inactivation of DPD by one heterozygous allelic mutation of IVS14+1G>A in exon 14 can result in a strong reduction in 5-FU clearance, causing severe 5-FU-induced toxicity. The allele frequency of this mutation is 0.91% in a Dutch Caucasian population (20). In patients with low DPD activity, 42% were heterozygous and 3% were homozygous for this mutation (21). Almost similar results have been described by another study by van Kuilenburg et al. (22).

In addition, other variants, such as T85C, A496G, G1601A, A1627G, T1679G, and A2846T, were also related to the reduced enzyme activity in patients (23,24), although other studies showed no association of these polymorphisms with DPD activity (25–27).

These data emphasize the complex nature of the molecular mechanisms controlling polymorphic DPD activity in vivo. The clinical utility for genetic polymorphism testing to date is not optimal because of its low sensitivity and unknown specificity (28). Overall, it can be remarked that the splice site mutation IVS14+1G>A causes severe, even lethal, 5-FU-related toxicity. Unfortunately, the roles of other polymorphisms in the *DPYD* gene in the severe 5-FU-related toxicity are not clarified.

5.2.3 Uridine Diphosphate Glucuronosyltransferase

Uridine diphosphate glucuronosyltransferase, more specifically UGT1A1, is capable of glucuronidating bilirubin. The clinically relevant polymorphisms related to genetic abnormalities in the UGT1A1 enzyme are those associated with familial

(un)conjugated hyperbilirubinemia syndromes such as Crigler–Najjar (types I and II) and Gilbert’s syndromes. More than 50 genetic lesions in UGT1A1 have been reported; many are found in patients with Gilbert’s syndrome.

UGT1A1 has an important role in the metabolism of irinotecan, etoposide, epirubicin, and tipifarnib. Irinotecan is a camptothecin derivative used in the treatment of metastatic colon cancer. Irinotecan is a prodrug since it is activated to Ethyl-10-hydroxycamptothecin (SN-38) by carboxyl esterase to exert its antitumor activity mediated by the inhibition of topoisomerase I. SN-38 undergoes UGT1A1-catalyzed glucuronide conjugation to form the inactive SN-38 glucuronide (SN-38G).

In the promoter region of the *UGT1A1* gene, a microsatellite mutation has been found, (TA)₇TAA instead of (TA)₆TAA (UGT1A1*28), resulting in reduced UGT1A1 expression levels and activity. The frequency of the (TA)₇ allele was 32–39% in Caucasians, 40–43% in Africans, and 16–33% in Asians (29,30). Alleles with five and eight TA repeats, that is, (TA)₅ and (TA)₈, have also been identified (respectively, UGT1A1*33 and 34), although almost exclusively in an African population, with an allele frequency of 3.5% and 6.9%, respectively (29,31).

Homozygosity for UGT1A1*28 is usually associated with Gilbert’s syndrome, a mild chronic, unconjugated hyperbilirubinemia that often remains undiagnosed. Two patients with metastatic colon cancer and Gilbert’s syndrome were treated with irinotecan-based chemotherapy. Both patients presented grade 4 neutropenia or diarrhea in every treatment cycle (32). A different metabolic ratio, SN-38/SN-38G, is a possible explanation for interindividual variability of the pharmacokinetic profile of SN-38 and its glucuronide. A significant trend toward a decrease in SN-38 and bilirubin glucuronidation rates was found as the number of TA repeats increased ((TA)₆/(TA)₆ > (TA)₆/(TA)₇ > (TA)₇/(TA)₇) (33–36). More severe grades of diarrhea and neutropenia were reported in patients heterozygous or homozygous for the (TA)₇ sequence (35,37,38). Standard dosing regimens given to patients with Gilbert’s syndrome with mild hyperbilirubinemia displayed an increased area under the concentration vs time curve (AUC) of SN-38/SN-38G, a factor that is linked to leukopenia (37,39).

In a study by Marcuello et al. (38), no statistical significance could be detected between UGT1A1 genotype and clinical response or overall survival.

In black children with acute lymphocytic leukemia (ALL) who were treated with etoposide, the wild-type UGT1A1*28 genotype (TA)₆ has been associated with a higher clearance of etoposide, also indicating that the mutant genotype is responsible for less-potent glucuronidation (40).

In conclusion, the homozygous or heterozygous genotype for UGT1A1*28 may be a significant risk factor for severe irinotecan toxicity. The example of irinotecan demonstrates clearly how polymorphisms in an inactivating metabolic pathway may affect the adverse events and therapeutic outcome in cancer chemotherapy.

5.2.4 Thiopurine Methyltransferase

Thiopurine methyltransferase is involved in the methylation reactions of 6-mercaptopurine (6-MP) and azathioprine, its pro-drug (41). Since 1953, 6-MP has been

administered in the treatment of childhood ALL. More experience with this drug and azathioprine has been obtained in the treatment of rheumatoid arthritis and inflammatory bowel diseases.

The activity of TPMT is influenced by genetic polymorphisms that can alter the rate of 6-MP metabolism by TPMT. The enzyme activity of TPMT varies among patients; 86.6% of the Caucasian population has high TPMT activity, 11.1% has intermediate activity, and 0.3% are deficient in TPMT (42–44). The TPMT found in patients with normal TPMT activity is classified as the wild-type TPMT*1.

Eight TPMT alleles have been identified. Three of these alleles, TPMT*2 (45), *3A, and *3C (G460A), account for 80–95% of intermediate or low enzyme activity cases. High concentrations of “variant” TPMT (TPMT*3A, *3B, and *3C) are found in patients with decreased TPMT activity: Patients with TPMT*3A have a complete loss of TPMT catalytic activity, patients with TPMT*3B have a 9-fold reduction, and those with TPMT*3C have a 1.4-fold reduction (43,46). The frequency of loss of TPMT activity (TPMT*3A) appears to vary with ethnicity but not with gender or age. In addition, haplotyping methods have been developed to discriminate the genotypes TPMT*1/*3A (intermediate metabolizer) and TPMT*3B/*3C (poor metabolizer) (47).

The TPMT genotype correlates well with in vivo enzyme activity within erythrocytes and leukemic blast cells and is clearly associated with risk of toxicity (44,48,49). The cumulative incidence of 6-MP dose reduction because of toxicity was highest among patients homozygous mutant for TPMT, was intermediate among heterozygous patients, and was lowest among wild-type TPMT patients (50,51). Unfortunately, in the published studies often no distinction has been made between the different polymorphisms of the *TPMT* gene. The concentration of 6-thioguanine (6-TG) nucleotides in erythrocytes is directly correlated with risk of development of leukopenia and inversely with risk of relapse in patients treated with thiopurine drugs. Dose reduction in patients with inactive TPMT is associated with lower 6-TG concentrations and thus a higher risk for relapse (52). The TPMT genotype has a substantial impact on minimal residual disease after administration of 6-MP in the early course of childhood ALL (53). In addition, there are emerging data that the TPMT genotype may influence the risk of secondary malignancies, including brain tumors and acute myelocytic leukemia (AML). High-dose 6-MP in wild-type TPMT patients with ALL has been associated with a higher occurrence of infectious episodes, which could not be influenced by dose adjustment in homozygous and heterozygous mutant patients during maintenance therapy (54).

Tests for the TPMT genotype and phenotype are commercially available. Attention should be paid for those patients who test negative for TPMT status. Patients with poor or intermediate TPMT activity may tolerate only 1/10 to 1/2 of the average 6-MP dose. A pharmacoeconomic model has been developed to analyze the potential cost of screening to prevent azathioprine toxicity. In this model, it was assumed that TPMT deficiency is present in 0.3% of the population, that intermediate activity is present in 11%, and that both groups have an increased risk of developing myelosuppression. Under these circumstances, the model predicted that the costs per Caucasian patient for the first 6 mo of therapy with screening are lower

compared to not screening (55,56). In addition, the genotype-based dosing strategy was also shown to be less costly and more effective in Korea as screening was associated with a marked reduction in the number of serious adverse events (57).

Overall, genetic variation in the *TPMT* gene has clearly shown its importance for the clinical response in anticancer treatment in ALL. This results not only in a clinical benefit for the patient but also in a significant impact on the economics of medical practice.

5.3 Drug Transporters

5.3.1 *P-Glycoprotein*

Genetic polymorphisms in the *P-gp* gene may be important in influencing the outcome of pharmacotherapy (58), and several SNPs in the *ABCB1* gene have been described. The three most mentioned SNPs are P-gp*6 (C3435T), *7 (G2677T/A), and *8 (C1236T).

In Caucasian populations, a frequency of 22–56% for the mutant T allele in P-gp*6 was observed (59–61). In African populations, the frequency of the C allele was significantly higher in comparison with Caucasians (respectively, $\pm$ 80% and 50%) (62).

A common haplotype was found to contain three SNPs simultaneously (P-gp*2): *6, *7, and *8, with a frequency of 62% among European Americans (63). Direct sequencing of DNA from subjects homozygous for all of these three SNPs strongly suggested that they were linked to polymorphic positions at regulatory sites of the P-gp promoter and may account for different regulatory kinetics.

P-gp is responsible for transport of the carboxylate form of irinotecan (64). The homozygous mutant *8 polymorphism has been associated with significantly increased exposure to irinotecan and its active metabolite SN-38 (65). Furthermore, significantly decreased docetaxel clearance was found in patients homozygous mutant for P-gp*8 (66), although Goh et al. (67) did not find a significant effect of this polymorphism on docetaxel pharmacokinetics. Also, a trend to an increased AUC of tipifarnib in patients with the homozygous mutant allele compared to patients with only one or no mutant alleles of *8 was found in a study by Sparreboom et al. (68). In a study by Kishi et al. (40), the mutant allele for *6 was also correlated with a lower clearance of etoposide in children with ALL.

P-gp polymorphisms are also reported to affect the outcome of therapy in patients with AML. Illmer et al. (69) compared the clinical course of AML treatment with the anticancer drugs etoposide, mitoxantrone, or daunorubicin among patients with various P-gp genotypes and demonstrated that patients homozygous for the wild-type allele at any locus investigated (exons 12, 21, and 26, respectively, for P-gp*8, *7, and *6) exhibited a significantly decreased overall survival with a higher probability of relapse. Theoretically, a reduced intracellular concentration of

anticancer drugs attributable to the action of P-gp in AML blasts may be related to failure of AML therapy and disease resistance. In a study by Goreva et al. (70), individuals with homozygous mutations for *6 and *7 are at highest risk of drug resistance in the treatment for lymphoproliferative diseases. Another study showed that the response to preoperative chemotherapy in breast cancer patients was higher in patients with the homozygous mutant *6 genotype (71). Efferth et al. (72) did not find a difference in overall survival for wild-type and mutant *6 genotype alleles in ALL after doxorubicin treatment.

In conclusion, contradictory data have been published for P-gp genotypes. The studies discussed are evaluating different regimens and tumor types. The same protein can have different pharmacokinetic and pharmacodynamic effects in different tumors and in response to different drugs. Therefore, every tumor type and drug has to be investigated independently.

5.3.2 Breast Cancer Resistance Protein

The role of BCRP (or ABCG2) seems to be to protect tissues by actively transporting toxic substances and xenobiotics out of the cells. Cancer cells overexpressing ABCG2 show multidrug resistance to mitoxantrone-, methotrexate-, doxorubicin-, and camptothecin-based anticancer drugs, such as topotecan and SN-38. Large interindividual differences have been observed in oral availability and clearance of drugs that are substrates for ABCG2, especially topotecan (73). Genetic variation in the *ABCG2* gene could possibly explain the variability in pharmacokinetics of ABCG2 substrates. SNPs have already been reported in the *ABCG2* gene (74–76).

Mizuarai et al. (77) analyzed the effect of the polymorphisms G34A and C8825A, leading to an amino acid change of V12M and Q141K, respectively, on the transporter function of the protein. Drug resistance to indolocarbazole, a topoisomerase I inhibitor, of cells expressing V12M or Q141K was less than 1/10 compared to wild-type ABCG2-transfected cells and was accompanied by increased drug accumulation and decreased drug efflux in the variant ABCG2-expressing cells. A possible explanation for this altered function of the ABCG2 enzyme is the fact that the ABCG2 transporter is not localized to the apical membrane in the V12M clone. However, it is not known if the altered transport function of ABCG2 influences drug transport in vivo.

In a study by Imai et al. (78), Japanese volunteers with the mutant C8825A polymorphism (allele frequency of 46%) expressed a low amount of the Q141K ABCG2 protein. The pharmacokinetics of diflomotecan have been affected by this polymorphism (79). Patients heterozygous for this allele showed statistically significant increased plasma levels in comparison to patients with wild-type alleles. De Jong et al. (80), however, showed that no significant changes in irinotecan pharmacokinetics in vivo were observed in relation to the ABCG2 C8825A genotype, although one of two homozygous variant allele carriers showed extensive accumulation of SN-38 and SN-38G.

In AML cells, *ABCG2* is overexpressed; however, the association between the expression and clinical resistance to anticancer drugs remains undetermined (81,82). The identification of functional sequence variation in the *ABCG2* gene could also be of interest in the field of prognosis of disease.

In conclusion, to date no *in vivo* effects of BCRP polymorphisms have been detected in relation to efficacy and toxicity of anticancer drugs that are substrates for BCRP.

5.4 Conclusions

Conventional anticancer drugs have been used for more than 50 years in the treatment of a variety of cancers. An important limitation associated with use of these drugs is the unpredictable interindividual variability in efficacy and toxicity. Potential causes for such variability in drug effects include the pathogenesis and severity of the disease treated, the occurrence of unintended drug interactions, and impairment of hepatic and renal function or both. Despite the potential importance of these clinical variables in determining drug effects, it is recognized that inherited differences in metabolism and excretion into feces and urine can have an even greater impact on the efficacy and toxicity of drugs.

In common with many new technologies, the generalizability and clinical application of pharmacogenomics has proved more challenging than expected. Several polymorphisms have been detected in genes coding for drug-metabolizing enzymes and drug transporters, but published results are conflicting. Difficulties include, in many examples, a modest clinical effect relative to genotype; therapy-specific, not broad, applicability; and the very major challenge of unraveling the complexity of gene–gene interactions. Besides, the effect of noncoding polymorphism on pharmacokinetics and dynamics of drugs has not been identified. Polymorphisms in noncoding areas can influence mRNA stability and can be linked to coding polymorphisms or cause gene–gene interactions. In addition, ethical and economic challenges to the application of pharmacogenomics have moved to the fore in recent years, particularly in the context of racial differences in outcome of therapy. Genomic, rather than candidate gene, approaches to identification of relevant loci are increasingly under exploration, and significant progress is being made.

The clinician will be faced with assessing the overall risk for adverse effects, which have to be weighed against potential benefits as well as the availability of alternative therapies and the costs. The reality is that clinicians will have to make their decisions based on the risk assessment gathered from large, population-based studies. However, this information is not available at this moment.

The Food and Drug Administration (FDA) recently approved label changes for 6-MP and irinotecan, to include pharmacogenomics testing as a potential means to reduce the rate of severe toxic events. Currently, one specific recommendation can be made to optimize anticancer therapy for patients by genotyping drug-metabolizing enzymes and drug transporters: That is, in patients with reduced TPMT activity

(based on pharmacogenomic screening of the *TPMT* gene or activity analysis), the dose of 6-MP should be reduced to 1/2 or 1/10. Comprehensive evaluation of clinical benefit and cost-effectiveness of screening strategies has not been completed for irinotecan. The most promising polymorphism for pretreatment screening in the future is the IVS14+1G>A mutation in the *DPYD* gene because pharmacogenomic research has shown significant relationships between pharmacokinetic variability in this gene on the one hand and drug-related toxicity on the other. However, well-designed and well-executed studies will help demonstrate convincing links between genetic variation and toxicity and responses in oncology.

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Chapter 6

Pharmacogenomics of G Protein-Coupled Receptor Signaling

Insights from Health and Disease

Miles D. Thompson, David E. C. Cole, and Pedro A. Jose

6.1	Introduction.....	78
6.2	GPCR Signaling.....	80
6.3	Accessory Proteins.....	86
6.4	Inactivation of GPCRs.....	89
6.5	Conclusion.....	97
	References.....	98

Summary The identification and characterization of the processes of G protein-coupled receptor (GPCR) activation and inactivation have refined not only the study of the GPCRs but also the genomics of many accessory proteins necessary for these processes. This has accelerated progress in understanding the fundamental mechanisms involved in GPCR structure and function, including receptor transport to the membrane, ligand binding, activation and inactivation by GRK-mediated (and other) phosphorylation. The catalog of $G_s\alpha$ and $G\beta$ subunit polymorphisms that result in complex phenotypes has complemented the effort to catalog the GPCRs and their variants. The study of the genomics of GPCR accessory proteins has also provided insight into pathways of disease, such as the contributions of regulator of G protein signaling (RGS) protein to hypertension and activator of G protein signaling (AGS) proteins to the response to hypoxia. In the case of the G protein-coupled receptor kinases (GRKs), identified originally in the retinal tissues that converge on rhodopsin, proteins such as GRK4 have been identified that have been subsequently associated with hypertension. Here, we review the structure and function of GPCR and associated proteins in the context of the gene families that encode them and the genetic disorders associated with their altered function. An understanding of the pharmacogenomics of GPCR signaling provides the basis for examining the GPCRs disrupted in monogenic disease and the pharmacogenetics of a given receptor system.

Keywords Accessory proteins; G protein-coupled receptor; G protein-coupled receptor kinases; hypertension; pharmacogenomics; signaling.

6.1 Introduction

Pharmacogenomics—the genomics of pharmaceutical targets such as the G protein-coupled receptors (GPCRs)—involves the classification of the genes encoding the proteins that are necessary for a pharmaceutical target, such as a GPCR, to function. With respect to the GPCRs themselves, there are three subclasses of receptors that are of particular importance in the study of GPCR pharmacogenomics. The class A receptors share sequence similarity to rhodopsin and the calcitonin receptor. The class B receptors consist of secretin/glucagon-like receptors that share little structural similarity to the other classes of GPCRs. The class C receptors are related in structure to the metabotropic receptors (*1–3*). The genomic classification of GPCRs allows more accurate prediction of the changes in receptor function that might result from sequence variants—those that occur in nature or in vitro.

The way in which GPCRs are able to regulate subtle physiological processes, however, suggests that the specificity of GPCR signaling is also determined by which of the family of accessory proteins is recruited. The structure and function of GPCRs therefore may often be as important as the families of accessory proteins involved in receptor inactivation. Genetic variations in accessory proteins that disrupt receptor function have been identified. Examples include (1) the variants of the regulators of G protein signaling (RGSs) that may confer risk for essential hypertension through dopamine D₁ receptor-mediated kidney function; (2) the variant G_s α subunit (*GNAS* gene), which encodes G_s α , the ubiquitously expressed α -subunit; (3) the variant G _{β} subunit in essential hypertension, obesity, stroke, and myocardial infarction; and (4) the variants of G protein-coupled receptor kinase 4 GRK4 that alter dopamine D₁ receptor-mediated kidney function in essential hypertension. The role of accessory proteins in GPCR activation and inactivation is best discussed in the context of representative receptor systems.

6.1.1 The G Protein-Coupled Receptors

The largest subfamily by far is class A. It comprises almost 90% of all GPCRs (*1*). Various family members have been subjected to detailed study at both the molecular and the structural levels (*1*). The definition of the properties of these receptors has resulted in the isolation of putative receptors (*4–10*) that have become reagents for drug discovery (*10*).

These receptors share several common features, some of which are illustrated in our example, the cysteinyl leukotriene 2 (CysLT₂) receptor (see [Fig. 6.1](#)) (*11*). These features include: (1) insertion into the membrane and targeting to the plasma membrane of the cell; (2) the presence of seven conserved transmembrane domains; (3) three extracellular and three intracellular loops; (4) an extracellular amino terminus; and (5) an intracellular carboxyl terminus (*1,2*).

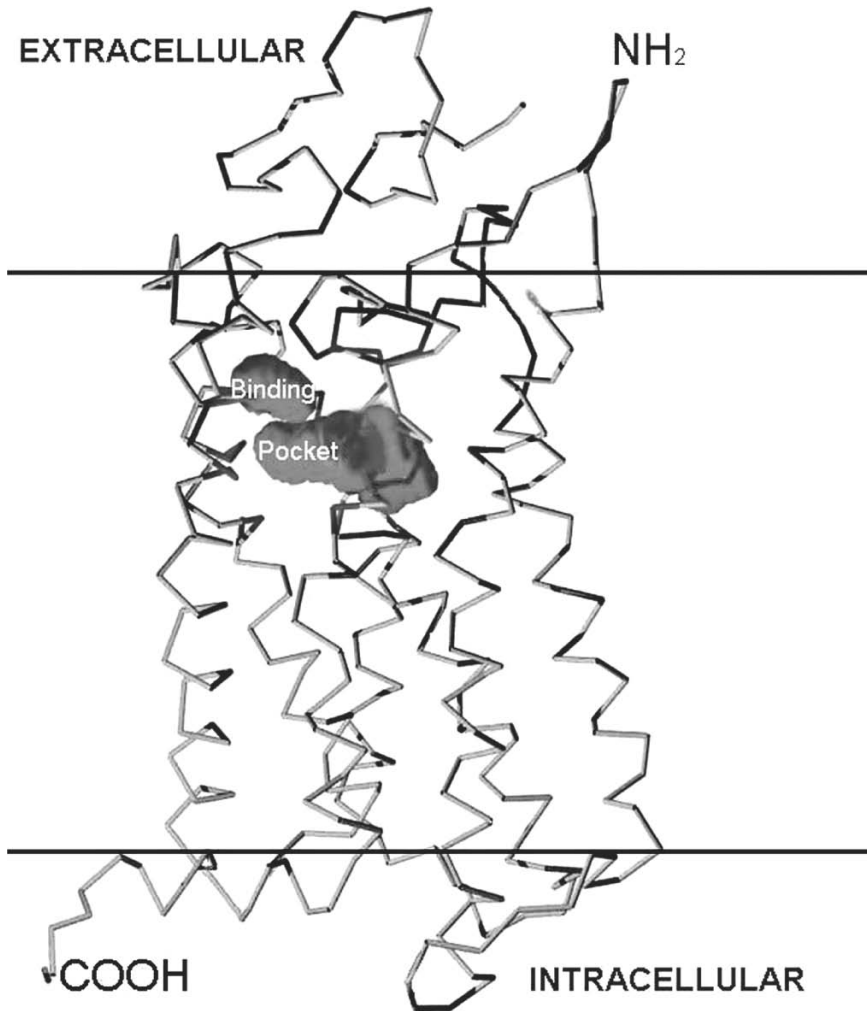


Fig. 6.1 Schematic representation of the cysteinyl leukotriene 2 (CysLT₂) receptor. Ribbon model of this family A G protein-coupled receptor (GPCR) is pictured in its heptahelical configuration. The extracellular amino terminus of the receptor, the transmembrane domains, and the intracellular carboxyl tail extend behind the intracellular palmitoylation site. The putative “binding pocket” for cysteinyl leukotriene ligands is derived from a rhodopsin model

All of the known class A receptors are subject to posttranslational modification at one or more *N*-linked glycosylation sequences, found either in the extracellular amino terminus or in the second extracellular loop. Glycosylation is required for the expression of some GPCRs at the plasma membrane (12,13). Furthermore, many receptors, such as rhodopsin and the dopamine D₁ receptors, are also subject to other posttranslational modifications, such as palmitoylation at the intracellular domains

(14,15). These palmitoylation sites may serve to anchor the intracellular carboxy tail to the plasma membrane (16,17). Indeed, X-ray crystallography studies have suggested that the prototypic family A receptor rhodopsin may effectively form an additional helical structure as a result of this membrane anchoring (18,19).

Activation has most often been studied by analyzing the effects in vitro mutated forms of the GPCRs or G protein subunits. Receptors targeted by bulky ligands, such as large peptides and hormones, tend to bind at the N-terminus extracellular loops as well as at the transmembrane domains. Ligands as diverse in structure as dopamine and the cysteinyl leukotriene (CysLT), however, bind to their cognate recognition sites within the hydrophobic core formed by the membrane-spanning α -helices (20,21). In the case of the CysLT₂ receptor (see Fig. 6.1), for example, naturally observed variants have been discovered that alter the region defining the putative binding pocket. Thus, multiple motifs define the ligand–receptor interaction (22).

Still other receptors have poorly defined binding pockets that accommodate ligands in many orientations and at alternative binding domains. In addition, many receptors have been found to assume different conformations with distinct signaling functions, especially as a result of receptor dimerization. As a result of these factors, single receptor types may impinge on multiple signaling pathways, while groups of receptors may all act on a single intracellular signaling cascade (3,23–25).

A special problem arises in assessing the therapeutic relevance of receptor families across the genome: the complex interaction of multiple closely related receptors that bind a single drug in somewhat different ways (3). For example, even though the CysLT₁ and CysLT₂ receptors have a unique rank order of ligand potency (26,27), their overlapping tissue distribution suggests that they may not always act as autonomous leukotriene-binding sites (28–30).

Like many GPCRs, the CysLT₁ and CysLT₂ receptors may contain a number of structures capable of facilitating functional interactions. As reported for other receptors, dimerization and oligomerization may occur as the result of posttranslational modification or the interaction between transmembrane domains (31,32). Dimers and oligomers of receptors such as the angiotensin II type I (33,34), the M₃ muscarinic (35), the dopamine D₁ (36,37), and the metabotropic glutamate (mGluR) (38) may form through a variety of mechanisms. These interactions may play a role in modifying the orientation of high-affinity ligand-binding sites (39–41). The effects of naturally occurring GPCR variants, such as the CysLT₁ and the CysLT₂ receptors, on functions relating to receptor dimerization and G protein coupling remain largely unknown (42).

6.2 GPCR Signaling

Significant advances in our understanding of the structure and function of GPCRs have resulted from the identification of particular residues critical to the cell signaling that results from ligand binding, receptor activation, and receptor inactivation (43). Each individual GPCR, when exposed to continuous stimulation by an agonist,

produces a self-limited activation signal. The process of activation, however, is reviewed (44) in the context of what is known about the genomics of G protein subunits and accessory proteins and the human disorders that result from disruption of these processes.

Several human disorders result from genetic G protein abnormalities. Several involve the imprinted *GNAS* gene, which encodes G_s α , the ubiquitously expressed α -subunit that couples receptors to adenylyl cyclase (AC), causing cyclic adenosine monophosphate (cAMP) generation. Loss-of-function, gain-of-function mutations and imprinting defects lead to many clinical phenotypes. Mutations of *GNAT1* and *GNAT2*, which encode the retinal G proteins (transducins), cause specific congenital visual defects. Common polymorphisms of the *GNAS* and *GNB3* (which encodes G β_3) genes have been associated with multigenic disorders (e.g., hypertension and metabolic syndrome). To date, only variants of the α - and β -subunits of the G protein have been implicated in human disease. No γ -subunit disruptions have been identified. A general overview of G protein coupling is necessary before a description of the G protein, accessory protein, and GPCR variants associated with disease is undertaken.

6.2.1 G Protein Coupling: Molecular Mechanism of GPCR Activation

The G protein-mediated signal transduction resulting from GPCR activation by an extracellular agonist causes a cascade of intracellular electrochemical signals. The release of second messengers is significant because it represents the way in which events subsequent to ligand binding are amplified within the cell, a process that accounts for the great sensitivity of GPCR signal transduction (2,25,45). The sensitivity of these pathways, however, can result in major disruptions in cell signaling when a receptor is subjected to natural or in vitro mutation (46).

Amplification of the signal is an elaborate process that depends on specific properties of the receptor, which G protein system is involved, and on the presence of auxiliary proteins that amplify or quench the signal (25). As a result, a single amino acid variation in one GPCR can cause a dramatic gain or loss of function. One species of GPCR interacts with many G protein molecules and many more effectors (47). Therefore, when the signal from a receptor with a gain-of-function mutation is amplified, pathophysiological dysregulation can result. Conversely, when the signal from a receptor with a loss-of-function mutation is amplified, it may abnormally reduce signaling activity below what would otherwise be considered basal (23,48).

6.2.2 G Protein Subunits

In classic models of G protein coupling, the process is often described to involve several steps. First, as ligand is bound to the GPCR, the GPCR assumes its “activated”

configuration. An activated GPCR then interacts with an inactive G protein complex, consisting of three subunits: the α -, β -, and γ -subunits. The inactive G proteins exist as heterotrimers with one guanosine 5'-diphosphate (GDP) bound to each α -subunit. It is the interaction of an activated GPCR with a heterotrimeric G protein that results in an activated, or high-affinity, receptor–G protein complex (2,25).

The complex subsequently releases GDP, and guanosine 5'-triphosphate (GTP) binds to the α -subunit in its place (47,49,50). While there is evidence supporting a model that allows for the dissociation of both the active $G\alpha$ -GTP and the noncovalently bound $\beta\gamma$ -heteromeric complex from the receptor–effector complex, other models can also account for these data (51). Auxiliary proteins may regulate the potentiation of the GPCR–G protein effector complexes that generate second messengers or specific transmembrane proteins such as ion channels (44,52). These processes are illustrated schematically in Fig. 6.2.

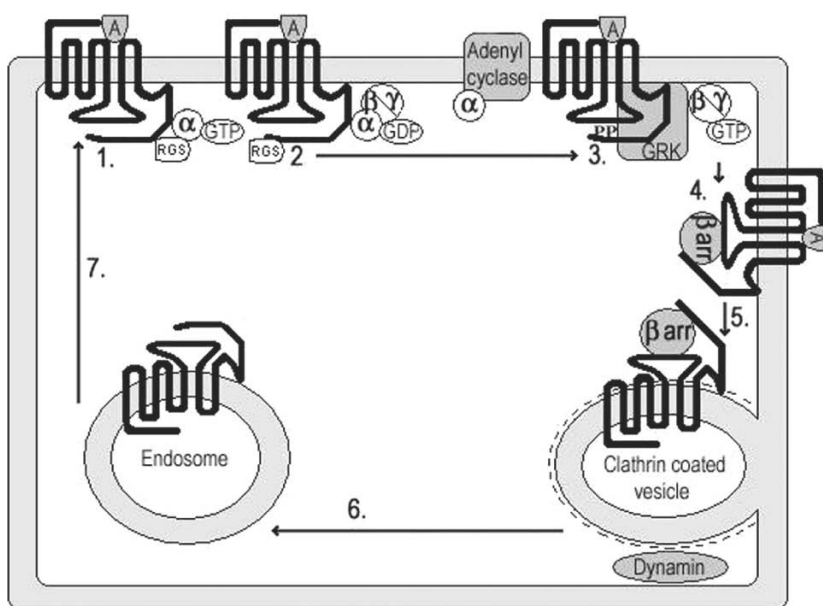


Fig. 6.2 Schematic of G protein-coupled receptor (GPCR) activation and inactivation. Following short-term exposure to agonist, common pathways of GPCR desensitization, internalization, and downregulation are initiated. The rapid effects, often described as resulting in homologous desensitization, are mostly associated with the G protein-coupled receptor kinase (GRK)-mediated phosphorylation of agonist-occupied receptor. They are summarized in this schematic as follows: (1) agonist (A) binds to GPCR, initiating conformational changes in the receptor, resulting in the recruitment of the regulator of G protein signaling (RGS); (2) G protein (α , β , and γ) couples, RGS facilitates guanosine triphosphatase (GTPase) activity, and the second-messenger cascade results after $G\alpha$ binds to adenylylase; (3) GRK is recruited, displacing enzyme and phosphorylating (PP) agonist-occupied receptor; (4) β -arrestin (β arr) forms a complex with the receptor; (5) the receptor is internalized at clathrin-coated pits; (6) internalization results in degradation of the endosome-internalized receptor; but (7) dephosphorylated receptor may be recycled to the plasma membrane (2,49,105,151). GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate

6.2.3 The G Protein β - and γ -Subunits

The β - and γ -subunits are less diverse compared with the $G\alpha$ -subunits; however, they have a role in both activation and inactivation of GPCR systems. In addition to G protein activation, the $\beta\gamma$ -subunits bind three classes of GRKs (GRK1, GRK2, and GRK3) and translocate them to the membrane. It is the membrane colocalization of GRKs and GPCRs that makes possible the GRK phosphorylation of GPCRs that is integral to the process of receptor inactivation (53).

The diversity in tissue expression of β - and γ -subunits also plays a role in regulating these processes. Ignoring splice variants, at least 5 β -subunits ($\beta 1$ to $\beta 4$) and 11 γ -subunits ($\gamma 1$ to $\gamma 11$) have been isolated (54). The considerable overlap in the distribution (55) of these subunits results in subtle phenotype penetrance.

6.2.4 β -Subunits Associated with Complex Phenotypes

While no variants of the β - and γ -subunits have been associated with monogenic disorders, polymorphisms have been associated with a variety of complex phenotypes. For example, a single-base substitution (c.825C>T) of the $G\beta_3$ gene (*GNB3*) has been associated with hypertension. The variant leads to alternative splicing that is predicted to generate shortened $G\beta_3$ proteins (56), which may cause enhanced G protein signaling (56–58). Biochemically, this may result from an abnormal stability of the functional interactions of the shortened $G\beta_3$ proteins (57).

While many reports find an association between the C825T allele of *GNB3* and other features of the metabolic syndrome, including obesity, insulin resistance, autonomic nervous changes, and dyslipidemia (58–61), the results are not unanimous (62–65). The polymorphism has also been implicated in Alzheimer's disease (66), sudden death (67), and tumor progression (68,69) and as a pharmacogenetic marker for drug response (57,70–73). The mechanisms linking the C825T polymorphism to clinical outcomes have not been identified. The *GNB3* polymorphisms, however, may become useful markers for disease risk and altered drug response.

6.2.5 The G Protein α -Subunits

The $G_s \alpha$ -subunit is critical to perpetuating the GPCR signal because it is the free α - and $\beta\gamma$ -subunits that activate effector proteins and ion channels, such as AC, guanylyl cyclase, phospholipases C and A_2 , Ca^{2+} and K^+ channels (74). For example, while the activated $G_s \alpha$ tends to activate AC (75,76), the $G_i \alpha$ tends to inhibit AC, and activated $G_q \alpha$ tends to activate phospholipase C- β (44,77). Variations in receptor structure can change the rate at which these G protein subunits are liberated. Enhanced or diminished receptor signaling can result from the disruption of these processes at any step.

6.2.6 *Tissue Variability of G Protein Subunits and GPCR Signaling*

Since there are more than 20 distinct G α subunit proteins, G α enzymatic activity can be a major determinant of the specificity and variability of GPCR signaling in health and disease. By definition, the characteristics of variant GPCR signaling will vary depending on the G protein subunits coexpressed in tissues or cells. The rate of GTP hydrolysis varies, depending on the type of G α subunit (78,79). The persistence of the signal depends on which G α subunit is involved since it is the guanosine triphosphatase (GTPase) activity inherent in the G α subunit that determines the rate of GTP hydrolysis. This process inactivates G protein signaling and restores the low-energy G α -GDP conformation, which can then respond to the binding of another ligand (78,79).

Four subfamilies that exert a physiological influence through their expression in different tissues have been identified by analysis of sequence homology. The subunit classifications include the following: The widely expressed G α_i subfamily, includes (1) the transducins; (2) G $\alpha_{i1,2,3}$, G α_o , G $\alpha_{t1,2}$ (expressed in rods and cones); (3) G α_{gust} , the gustatory G protein that transduces signals from the taste receptors on the tongue; and (4) G α_z , which stimulates cyclic guanosine monophosphate (cGMP) phosphodiesterase, inhibits AC and regulates the Ca²⁺ and K⁺ channels. Next is the G α_s family, which includes G α_s and G α_{olf} (the olfactory G proteins), which stimulate AC and regulate both Ca²⁺ and K⁺ channels. Third is the G α_q family, G α_q and G $\alpha_{11,14,15,16}$, which activates phospholipase C (PLC). Finally, there is the G α_{12} family, G α_{12} and G α_{13} , which stimulates Na⁺-H⁺ exchangers (47).

In addition to the classic signaling pathways, G α subunits, such as G α_{12} , activate other proteins, such as the small G protein Rho. This occurs in the presence of active accessory proteins, such as RhoGEF proteins. The RhoGEF proteins are part of a group of many proteins that are known to contain G α -GTPase-activating protein (GAP) domains (80). The RhoGEF proteins in particular promote nucleotide exchange on Rho in response to G $\alpha_{12/13}$ activation.

6.2.7 *G α Protein Subunit Disrupted in Disease*

The G α_s subunit, encoded by the *GNAS* gene on 20q13, is one multiple-gene product that results from alternative promoters and exon splicing. G α_s is the ubiquitously expressed G α subunit that is required for receptor-mediated cAMP production. A number of widely distributed activating variants, such as Arg201Leu, lead to McCune-Albright's syndrome (MAS) (81,82), in which patients can develop fibrous dysplasia (FD) of bone, cafe-au-lait skin lesions, gonadotropin-independent sexual precocity, or tumors (or nodular hyperplasia) of pituitary somatotrophs, thyroid, or adrenal cortex with associated hormonal oversecretion (83). Similar mutations have been identified in cases of adrenocorticotropin-independent macronodular adrenal hyperplasia (84) and premature breast development (85). The activating G α_s variants result in various phenotypes that are influenced by constitutive cAMP production (81).

Inactivating $G_s\alpha$ variants lead to Albright's hereditary osteodystrophy (AHO) in the heterozygote, suggesting that $G_s\alpha$ haploinsufficiency causes the disorder. AHO is characterized by short stature, obesity, brachydactyly (shortening of metacarpal and metatarsal bones), subcutaneous ossifications, and mental or developmental deficits (86,87). The severity of the phenotype is variable. Some patients with $G_s\alpha$ mutations have few or no symptoms.

The mechanism of $G_s\alpha$ disease may result from insufficient parathyroid hormone-related peptide signaling by the parathyroid hormone receptor 1 (PTHr1) in chondrocytes. This deficiency may inhibit the differentiation of chondrocytes within the endochondral growth plate (88,89). A variety of parathyroid hormone abnormalities can result.

The *GNAS1* gene imprinting causes patients who have inherited $G_s\alpha$ mutations from their fathers to develop only AHO or pseudopseudohypoparathyroidism (PPHP). On the other hand, patients who inherited mutations from their mothers develop both AHO and resistance to a variety of hormones, including parathyroid hormone (PTH), thyrotropin (TSH; formerly called thyroid-stimulating hormone), growth hormone-releasing hormone, and gonadotropins. This array of hormone resistance that results from $G_s\alpha$ insufficiency is known as pseudohypoparathyroidism (PHP) type 1A (87,90,91). Maternal-specific inheritance of hormone resistance results from $G_s\alpha$ expression from the maternal allele in hormonal target tissues such as the renal proximal tubule, thyroid, pituitary, and gonads (92–96). In many other tissues, $G_s\alpha$ is not imprinted; therefore, both mutations produce $G_s\alpha$ haploinsufficiency, which leads to the AHO phenotype.

$G_s\alpha$ loss-of-function mutations do not always result in pluripotent phenotypes, however. Those with pseudopseudohypoparathyroidism type 1B (PHP1B), for example, have renal PTH resistance without AHO or resistance to other hormones. In fact, G_s function is normal in some tissues of PHP1B patients. There is strong evidence that the imprinting status of the *GNAS1* exon 1A region determines the transcriptional status of the $G_s\alpha$ promoter in proximal tubules. Loss of this imprinting pattern (at XL α s) because of deletions in nearby genes, such as STX16 or NESP55, results in loss of the maternal imprinting pattern throughout the rest of *GNAS* (97–99). Since $G_s\alpha$ is usually expressed primarily from the maternal allele in renal proximal tubules (92), an abnormal paternal imprinting pattern would lead to $G_s\alpha$ deficiency and renal PTH resistance. It has been proposed that this would result because demethylation would activate a repressor, causing the $G_s\alpha$ promoter to cease activity. This leads to $G_s\alpha$ deficiency in affected tissues and PTH resistance (81).

The study of activating and inactivating *GNAS1* mutations, therefore, has served to elucidate the tissue-specific regulation of GPCR signaling. G protein subunits and accessory proteins have a great hold over the activity of a multitude of receptors. Disruptions to the $G_s\alpha$ subunit, on one extreme, can resemble phenotypes caused by numerous constitutively active receptor variants, while on the other extreme they can resemble complex phenotypic patterns of tissue-specific receptor inactivation.

6.3 Accessory Proteins

The complexity of the possible disruptions in GPCR signaling becomes more evident as other accessory proteins are discovered and subsequently studied in disease. In addition to the accessory proteins involved in regulating the duration of the GPCR signal, such as β -arrestin (reviewed in **Subheading 6.4.2**), there are classes of protein that facilitate GPCR signaling. These proteins include the RGS proteins and the AGSs (**100,101**). While RGS proteins act to enhance the GTPase activity of $G\alpha$ that follows G protein coupling (**102–105**), AGS proteins are receptor independent AGSs (**106,107**). In both cases, there are examples of accessory proteins implicated in human disease (*see* **Table 6.1**).

6.3.1 Activators of G Protein Signaling

The AGS proteins have been implicated in regulating functions as diverse as asymmetric cell division, addictive behavior, and circadian rhythm. They probably adjust signal processing when a cell meets a challenge in its microenvironment. As a result, AGS proteins may contribute to the pathological GPCR-mediated responses to environmental stressors that may result in disease. For example, AGS8 has been implicated in remodeling the G protein signaling networks of cardiomyocytes subjected to hypoxia (**108,109**).

AGS8 is hypoxia inducible and signals directly by interacting with $G\beta\gamma$. The upregulation of AGS8 in hypoxic cardiomyocyte cells probably represents a component of signal remodeling that occurs during ischemic heart disease. Thus, the kinase-dependent pathways involved in sustaining collateral growth may be engaged independent of GPCR activation. These studies suggest that AGS proteins represent a class of accessory proteins that are critical to refining GPCR signaling pathways.

6.3.2 Regulators of G Protein Signaling

The RGS proteins are GAPs and are involved in the inactivation of the signal resulting from the coupling of GPCRs to high-affinity heterotrimeric G protein. G protein coupling depends on the hydrolysis of $G\alpha$ -GTP to $G\alpha$ -GDP. As shown in **Fig. 6.2**, the RGS proteins bind directly to activated $G\alpha$ -GTP to serve as GAPs. These proteins limit the half-life of $G\alpha$ -GTP by activating the $G\alpha$ subunit's GTPase activity, thereby facilitating the termination of signaling (**102–105**).

The RGS proteins exemplify the significance of accessory proteins to receptor function. The contribution of genetic variants of RGS2 to essential hypertension serves as an example of how significant accessory proteins are to the signaling of GPCRs, such as the dopamine D_1 receptor (**110**). While some RGS protein subfamilies selectively bind and regulate a specific class of $G\alpha$, such as $G\alpha_{12/13}$, most RGS proteins are promiscuous regarding which $G\alpha$ they can bind (**103**).

Table 6.1 Examples of genes encoding accessory proteins for G protein-coupled receptors that are disrupted in human genetic disease

Gene	Variant/allele	Disease/ phenotype	Pharmacology	Reference
Gβ ₃ , guanine beta-3 (<i>GNB3</i>) 12p13	825C>T SNP alternative splice	Hypertension	Shortened Gβ ₃	(56–58)
		Metabolic syndrome, obesity, insulin resistance, dyslipi- demia	↑G protein signal Abnormal stability of the functional interactions of the shortened Gβ ₃ proteins	(58–61)
		Alzheimer’s disease, autonomic nervous system changes, sudden death		(58,66,67)
		Tumor progression Polymorphic drug response marker		(68,69) (57,70,73)
G _s , alpha <i>GNAS</i> 20q13.2	Arg201Leu	McCune–Albright’s syndrome; fibrous dysplasia of bone; café-au-lait skin lesions; sexual precocity; pituitary, thyroid, or adrenal tumors	Activating G _s α variants with constitutive cAMP production	(81–85)
		Albright’s hereditary osteodystrophy (AHO), short stature, obesity, brachydactyly, subcutaneous ossi- fications, develop- mental deficits,	Inactivating G _s α variants lead to variable phenotype related to insuf- ficient parathyroid hormone recep- tor (PTHRI) in chondrocytes	(86–89)
		Inheritance of paternally imprinted gene in exon 1A	Renal PTH (par- athyroid hormone) resistance without AHO	(88,89)
		Inheritance of maternally imprinted gene	AHO and resistance to multiple hormones	(87,90,91)
Regulator of G protein signaling 2 (<i>RGS2</i>) 1q31	1166A>C vari- ant located in the 3’UTR	Bartter’s/Gitelman’s (BS/GS) angi- otensin II-related vasomotor tones are blunted	RGS2 maximally stimulated: failure to regulate nitric oxide and cGMP	(115–118)
	Many SNPs, insertions/ deletions: 1891–1892 TC 2138–2139 AA	Haplotypes associated with hypertension in African Americans	RGS mRNA ↓ in fibroblasts and peripheral blood mononuclear cells	(110,119)

(continued)

Table 6.1 (continued)

Gene	Variant/allele	Disease/ phenotype	Pharmacology	Reference
G protein-coupled receptor kinase 1, rhodopsin kinase (<i>RHOK/GRK1</i>) 13q34	Exon 5 deletion	Oguchi disease, recessively inherited stationary night blindness	Impairment of GRK1-mediated desensitization of rhodopsin	(189–191)
G protein-coupled receptor kinase 4 (<i>GRK4</i>)	Arg65Leu, Ala142Val, and Ala486Vval	Hypertension, sodium sensitivity	GRK4 activity increased: ↑ Dopamine D ₁ receptor desensitization ↑ Angiotensin II type 1 receptor expression	(186–188)

cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; mRNA, messenger RNA; SNP, single-nucleotide polymorphism.

The process of RGS recruitment to the membrane-bound receptor, however, seems to be constitutive—it appears to be independent of the state of activation of the receptor or G protein. This recruitment may facilitate signal quenching because the combination of 30 RGS proteins and 20 Gα subunits allows for a diverse pattern of inactivation. RGS proteins, therefore, are recruited to the plasma membrane in cells expressing either Gα subunits (Gα) or linked GPCRs (e.g., D₁-dopamine receptor) in preparation for the GAP activity that quenches G protein signaling (104,105).

While RGS recruitment seems to be independent of the activation state of either receptor or G protein, there is evidence that RGS proteins can bind directly to GPCRs (111). It is possible that the receptors act as scaffolds serving to bring RGS proteins nearer to the G protein targets (112). Thus, RGS proteins may be selectively sorted at the plasma membrane by receptors to orient and optimize their GAP activity toward the linked Gα.

The recruitment of RGS proteins to the membrane therefore shadows their regulation of G protein function. Thus, insight into GPCR signal termination may suggest strategies for designing drugs that selectively optimize RGS activity (104,105) in a specific disease, such as essential hypertension. As with the other systems described, naturally occurring GPCR variants may alter receptor function by altering the interaction of RGS proteins with the receptor.

6.3.3 *Polymorphisms of the RGS2 Gene in Hypertensives*

RGS2 preferentially alters G α q-mediated signaling. It may be important for cardiovascular health because RGS2 knockout mice exhibit altered smooth muscle relaxation and hypertension (50,113,114). For example, RGS2 plays a role in the signaling of the angiotensin II type I receptor. The receptor itself has been independently implicated in hypertension because of the 1166A>C variant located in the 3' untranslated region (3'UTR) (115,116). In Bartter's/Gitelman's syndrome (BS/GS) patients, angiotensin II-related signaling and vasomotor tone are blunted. RGS2 expression is maximally stimulated in BS/GS, suggesting a link between BS/GS genetic abnormalities and abnormal vascular tone regulation (117). Pathogenic effects may result from the failure of RGS2 to regulate nitric oxide and cGMP through adequate phosphorylation of RGS2 by cGMP-dependent protein kinase 1 α (PKG) (117). Although BS/GS pathogenesis may not be directly attributed to RGS variants, understanding it does provides better insight into the regulation of RGS proteins by Rho inhibition of PKG (118).

The RGS2 gene does contain a number of variants, however, that are at various frequencies in different populations. Genetic variation in the human RGS2 gene consists of at least 14 single-nucleotide polymorphisms (SNPs) and 2 two-base insertion/deletions (in/del; 1891 to 1892 TC and 2138 to 2139 AA) (110,119). Most coding variants are reported at low allelic frequency; however, the C1114G polymorphism was associated with lower RGS2 expression in some populations (120).

The intronic 1891 to 1892 TC and 2138 to 2139 AA in/del variants, however, are more common. The variants have been reported to be in linkage disequilibrium and are associated with hypertension in African Americans. Significantly, two haplotypes are reported to have significantly different frequencies between hypertensives and normotensives—but only among African American groups—reflecting the unique epidemiology of essential hypertension in the African American population. The intronic in/del defined ethnicity-specific haplotypes may serve as ethnicity-specific genetic variants for essential hypertension (110,119). RGS2 messenger RNA (mRNA) expression was significantly lower in peripheral blood mononuclear cells (PBMC) and in fibroblasts from hypertensives in comparison to normotensives. C1114G polymorphism was associated with RGS2 expression, with the lowest values in GG hypertensives. The 1114G allele frequency was increased in hypertensives compared with normotensives.

6.4 Inactivation of GPCRs

Continuous exposure of a GPCR to an agonist produces a self-limited signal (44) that may be disrupted in disease states. Two examples worthy of discussion are Oguchi disease, caused by disruption of GRK1 inactivation, and essential hypertension associated with GRK4 variants. Disruption of GRK activity is discussed with

respect to Oguchi disease and to essential hypertension in separate discussions in **Subheading 6.4.3**

Inactivation, a process that reduces the cellular response to the agonist, is illustrated schematically in **Fig. 6.2**. It is often measured by quantitating the change in second-messenger production, such as cAMP production by AC, following prolonged exposure of one type of receptor to an agonist (*121*). For example, the study of natural and artificial mutations of GPCRs and the genes encoding proteins involved in inactivation, such as GRK1 and GRK4, has identified many protein motifs that are essential. These experiments measure the extent to which the signal is limited by the ability of wild-type and mutated GPCRs to inactivate in response to agonist (*122*).

6.4.1 Desensitization

The process known as desensitization, taking place within a time frame of seconds to minutes following agonist exposure, occurs when the receptor uncouples from its G protein. This results from conformational changes that result from agonist-dependent phosphorylation, often as a result of GRK activity. The desensitized receptors undergo plasma membrane clustering and endosome-mediated internalization and are finally targeted for degradation unless they are recycling back to the cell surface. If receptors are lost from the cell surface, downregulation is said to have taken place. This may be transient, in the case of intracellular sequestration, or longer term if protein synthesis is unable to keep pace with receptor loss (*44*). Two patterns of desensitization, homologous and heterologous, have been characterized (*123*). While phosphorylation of GPCRs is associated with both forms (*124,125*), it is the GRK enzymes that tend to be implicated in the homologous form that will be of interest in discussing the events relevant to Oguchi disease and various hypertension phenotypes.

Agonist-specific desensitization, generally termed homologous desensitization, is associated with agonist-dependent GRK phosphorylation. Originally characterized in the case of rhodopsin, it was later found to be common among GPCRs. Homologous desensitization occurs rapidly when GPCRs are exposed to high (micromolar) agonist concentrations (*126–128*). Nonactivated receptor systems are spared, however, and continue to function normally.

By contrast, heterologous desensitization is a slower response to an agonist (minutes rather than seconds) that occurs even when GPCRs are exposed to lower agonist concentrations. It involves the diminished response of many kinds of GPCRs, including receptors that have not been exposed to ligand. This appears to occur even if GPCRs share few, if any, common signaling pathways or effectors (*126–128*).

Second-messenger-dependent kinases, such as cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC), are most often implicated in heterologous desensitization (*123,129*); however, the systems involved may vary between cell types (*130*). These protein kinases are associated with GPCR desensitization that

occurs at slower rates than that reported for the GRKs ($t_{1/2}$ of 3 min compared to 15 s). This probably accounts for the slower time course of heterologous desensitization (131). For the most part, the following discussion centers on homologous desensitization.

6.4.1.1 Mechanisms of Homologous Desensitization

The desensitization of most GPCRs appears to be dependent on the carboxyl tail or third intracellular loop regions. For example, the α_{2A} -adrenergic (132), the α_{1B} -adrenergic (133), the N-formyl peptide (134), and the M2 muscarinic acetylcholine (135,136) receptors all contain clusters of residues in the third intracellular loop that are required for desensitization.

While GRK2, 3, and 5, phosphorylation has been associated with agonist activation of many receptors (44,137), only discrete regions of phosphorylation that are attributable to one specific enzyme appear to be essential for desensitization (122). With respect to the β_2 -adrenergic (138–141), the dopamine D_1 (122), the μ -opioid (142), the δ -opioid (143), the α_{1B} -adrenergic (133), the A_3 and A_{2a} adenosine (144–146), and the N-formyl peptide (134) receptors, the motifs may be located in the carboxyl tail.

The desensitization motifs in the dopamine D_1 receptor, as an example, may be at least partly located in the proximal carboxyl tail of the receptor (122). This region may interact with portions of the third intracellular loop to promote desensitization. These structures may also be involved in recycling and trafficking of inactivated receptors (147).

The carboxyl tail portion includes ^{360}Thr preceded by ^{359}Glu . The location of these is shown in Fig. 6.3. The cyclase method of assaying desensitization (121) may estimate the extent to which the receptor inactivates following agonist activation. Normal desensitization of the wild-type dopamine D_1 receptor, defined by an increase in K_m and decrease in $V_{\max}$ for agonist-pretreated compared with naïve cells (see Fig. 6.4A), was abolished when the Thr360 residue was substituted for Ala (see Fig. 6.4B). In these experiments, while desensitization appeared to remain intact when other carboxyl terminal serine and threonine residues were eliminated (see Fig. 6.3, distal carboxyl tail), it was eliminated when the acidic residue present at ^{359}Glu was mutated to alanine (data not shown). These data suggest that the acidic ^{359}Glu may be necessary to potentiate basal levels of phosphorylation of the critical ^{360}Thr residue (122). Taken together, ^{359}Glu – ^{360}Thr residues may represent a putative GRK desensitization motif of the dopamine D_1 receptor. In principle, these findings may be corroborated by evidence that the rhodopsin receptor requires critical acidic residues, such as ^{341}Glu , to maintain both basal phosphorylation and agonist-induced phosphorylation of ^{338}Ser (148).

There are probably other GRK-related mechanisms required for agonist-induced desensitization, however (149). There is evidence that phosphorylation of the serines and threonines located in the third intracellular loop may, in at least some cases, be a corequisite for desensitization (150). Third-loop mutations exhibited

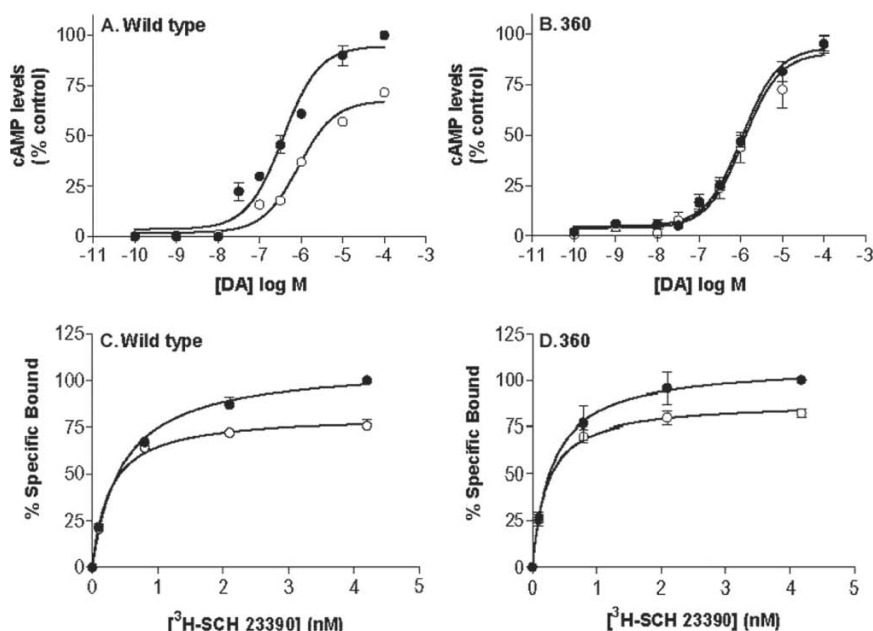


Fig. 6.4 In vitro effects of mutation on desensitization and internalization of the dopamine D₁ receptor. Shown here are effects of mutation on dose-dependent intracellular cyclic adenosine monophosphate (cAMP) accumulation (**A** and **B**) and binding curves (**C** and **D**) for artificial lig- and (SCH 23390) using three constructs: controls (wild type, **A** and **C**) and the Thr360Ala mutant (360, **B** and **D**). In the desensitization experiments, cells were preincubated with 10 μM dopamine (○) or vehicle (●) for 20 min, and increasing concentrations of dopamine (10⁻¹⁰ to 10⁻⁴ μM) were added to assess cAMP accumulation. Note that loss of efficacy and potency seen in wild-type cells (**A**) disappeared with the Thr360Ala mutation (**B**). Conversely, internalization, assessed by decrease in SCH23390 binding (**C**) after pretreatment with 10 μM dopamine (○, compared to vehicle ●), was essentially unchanged by the Thr360Ala mutation (**D**)

conformation that will allow its interaction with proteins integral to the desensitization process. One such group of proteins, indicated in **Fig. 6.2**, are the arrestins.

6.4.2 Internalization

Some forms of internalization are also arrestin-mediated. GRK-mediated phosphorylation of the receptor is required to promote the formation of the β-arrestin complex that can be internalized (**151,152**). The pathway of arrestin-mediated GPCR internalization that involves the transfer of ligand-activated receptors from the plasma membrane to an intracellular compartment (**153**) is shown in **Fig. 6.2**.

Although internalization is also often described to be a phosphorylation-dependent process, **Fig. 6.4C,D** shows that receptors do not always require phosphorylation of

the same residues to desensitize (**122**)—and for the recycling of inactivated receptors to the cell membrane (**147**)—as they do for receptor endocytosis (**122,147**). The integrated process of internalization, however, is integral to the processes underlying the membrane trafficking of GPCRs. The processes that are critical to the maintenance of the appropriate quantity of receptors expressed at the cell surface (**154**) can be teased apart, as exemplified by studying a number of different receptors.

The normal internalization of the wild-type receptor, defined as a loss of cell surface receptors (measured by decreased maximal binding or $B_{\max}$), was unaffected for the desensitization-deficient ³⁶⁰Thr mutant (see **Fig. 6.4C,D**) but may have been affected when distal carboxyl terminal residues were mutated (see **Fig. 6.3**). Therefore some, although not all, GPCRs show radical dissociation between desensitization and internalization. This is found not only in the dopamine D₁ receptor (**122**) but also in the N-formyl peptide (**134**), the CB1 cannabinoid (**17**), and the M2 muscarinic (**155**) receptors.

In case of the β_2 -adrenergic receptor, phosphorylation of serine and threonine residues in the carboxyl tail can be shown to be involved in desensitization and internalization (**141,156**). Other GPCRs—such as the μ - and δ -opioid receptors (**157,158**) and the A_{2b} adenosine receptor (**159**)—require analogous serine and threonine residues in the carboxyl tail for both desensitization and internalization (**157,158**).

While reproducible for many receptors, this phenomenon is not universal for GPCRs. For example, in the case of the M2 muscarinic receptor, while two-thirds of intracellular loop clusters of Ser/Thr residues (²⁸⁶Ser-²⁹⁰Ser and ³⁰⁷Thr-³¹¹Ser) mediate internalization, only the carboxyl terminal (³⁰⁷Thr-³¹¹Ser) cluster mediates desensitization (**136**). In conclusion, internalization may follow desensitization, or it may occur independently (**160**) with or without the influence of other regulatory processes (**161**).

Regardless of the GPCR residues involved, the involvement of β -arrestin in GPCR internalization has been particularly well elucidated. First, the binding of β -arrestin to the GPCR sterically inhibits interaction of the receptor with G proteins (**162**). The displaced receptor- β -arrestin complex is then free to bind with high affinity to clathrin chains (**163**). This recruitment of the complex to clathrin-coated pits allows the incorporation of the GPCRs into lipid vesicles. Internalization follows when the vesicles are pinched off the cell membrane by the GTPase dynamin (**164–166**). Subsequently, the internalized receptors are either recycled back to the plasma membrane or are targeted, within days or hours, for degradation in lysosomes (**167**).

In some cases, for example, in the case of the β_2 -adrenergic receptor, internalization has been found to be a precursor to resensitization of the receptor (**168,169**). This phenomenon may be common to many GPCRs. Internalization may afford the opportunity of receptor dephosphorylation through the action of an endosomal acid phosphatase (**170**), resulting in resensitization of the receptor (**171**).

While it is often convenient to model internalization as a process that follows desensitization, the evidence now suggests that, although often linked, these

processes can be distinct (*172*). For some receptors, such as the β_2 -adrenergic receptor (*138*), the forms of internalization that are distinct from desensitization may include those that are arrestin independent. Less is known, however, about the pathways of internalization that may not involve arrestin.

The residues required for internalization, like those implicated in desensitization motifs, do not always meet the requirements for putative sites of kinase-mediated phosphorylation. Among the numerous motifs that have been implicated, an NPXXY motif (*154,173*) may be required for agonist-induced activation and internalization of the β_2 -adrenergic receptor, and a dileucine motif in the carboxyl tail of many GPCRs (*154*) may be involved in internalization of receptors such as the β_2 -adrenergic (*174*) and the vasopressin V1a receptors (*175*).

While GPCR phosphorylation at serine and threonine residues is involved in the internalization pathways of many receptors (*134,176–178*), it is likely that for some GPCRs internalization pathways may be distinct (*134,176–178*). These apparently nonarrestin mechanisms of internalization, however, may vary more between receptors than those identified for GRK-dependent processes (*179,180*).

6.4.3 The Family of GRK Enzymes

The GRK family consists of seven well-characterized enzymes. These enzymes are distinguished by (1) the structural homology within the family; (2) the specific amino acid sequences that a given GRK can phosphorylate; (3) enzyme kinetics (*169,181*); and (4) GPCR disease phenotypes that are often manifested by dysregulation of GRK activity. Gain-of-function GPCR mutations are frequently found to be constitutively phosphorylated. Conversely, inadequate receptor desensitization and sequestration often result.

Much has been learned about GPCR biochemistry from contrasting the GRK1-like, GRK2-like, and GRK3-like subfamilies in health and disease (*169*). The role of the GRKs is indicated schematically in **Fig. 6.2**. Substrate specificity of the GRKs may be a factor in the degree to which specific tissues are affected by deleterious GPCR mutations (*182*). Of all the GRK family, the GRK2 amino acid sequence is most widely divergent from GRK1, which may also be a factor in defining which tissues are affected by ectopic GPCR phosphorylation (*148*). However, phosphorylation specificity is also defined by the amino acid sequence of GPCRs adjacent to serine/threonine residues. While GRKs 1 and 2 require adjacent acidic residues, respectively, on the carboxyl and amino terminal flanks of the phosphorylation site, GRK4 specifically phosphorylates at sites adjacent to basic amino acid residues. This evidence for GRK substrate specificity affords us significant insight into the molecular pathology of phenotypes that may involve GRK activity (*169*).

The GRK1 subfamily, consisting of GRK1 and GRK7, is known to be involved in the pathophysiology of deleterious rhodopsin mutations that underlie several inherited retinal disorders, including Oguchi disease. While GRK1 is the prototypic GRK enzyme rhodopsin kinase (*169*), both the GRK1 and GRK7 enzymes are

expressed in the retina and act to quench the rhodopsin signal transduction after light activation (183). The involvement of GRK7 in retinal disease has not been confirmed.

The GRK2 subfamily, consisting of GRK2 and GRK3, acts on a wide range of GPCRs that are expressed in many tissues. The GRK2 enzymes were first characterized in studies of the phosphorylation of agonist-occupied β_2 -adrenergic receptors (169). GRK2 enzymes contribute to disease. For example, GRK2 gain-of-function mutations affect the leuteinizing hormone (LH) receptors that are associated with Leydig cell hyperplasia (184).

The GRK4 subfamily is best understood in the context of the prototypical GRK1 and GRK2 subfamilies (169). The GRK4 subfamily consists of the GRK4, GRK5, and GRK6 enzymes (185). In contrast to GRK1 and GRK2 enzymes, GRK4 enzymes selectively phosphorylate residues with an amino terminal basic amino acid. GRK4 has been found to have potential significance in systems as well characterized as dopamine D₁ receptor desensitization (186). In the context of the role of the dopamine D₁ receptor in the kidney, GRK4 enzyme variants are in the sub-heading that deals with phenotypes associated with essential hypertension (187,188).

6.4.3.1 Oguchi Disease: Defective GRK1 Phosphorylation of Rhodopsin

Receptors that remain in the activated state even in the absence of ligand are often known as constitutively active mutants (CAMs). The resulting disruptions in rhodopsin signaling also often result in alterations in the phosphorylation of rhodopsin by rhodopsin kinase (GRK1), the specialized GRK enzyme expressed in the retina that is largely responsible for rapidly desensitizing the receptor when it is exposed to light.

In fact, a group of rhodopsin-related disorders *results* from mutations in the GRK1 gene itself. The result is Oguchi disease, a rare, recessively inherited retinopathy (189). The Oguchi mutations result in the impairment of GRK1-mediated desensitization of rhodopsin that is not compensated by normal expression of another GRK enzyme, such as GRK7 (183). The GRK1 mutations disrupt the pathway of light-dependent rhodopsin phosphorylation that is normally required for quenching light-induced signal transduction in photoreceptor cells.

In vitro experiments have demonstrated that a deletion of exon 5 of the *GRK1* gene is a null mutation that abolishes the enzymatic activity of GRK1 (189). Because both homozygous and heterozygous states for this mutation lead to disease (190), it is likely that GRK1 integrity is critical to retinal health. As a result of these observations, it is possible that a dominant negative effect or a GRK gene dose effect may be involved in retinal disease.

In vivo functional characterization of *GRK1* gene mutations has demonstrated that they prevent rhodopsin phosphorylation and subsequent arrestin binding. Interestingly, when studied ex vivo, rod cells expressing *GRK1* gene mutations also exhibited a greatly diminished attenuation of light sensitivity (191). Thus, the

function of GPCRs in healthy tissues may depend on the integrity of GRK-dependent processes.

6.4.3.2 Essential Hypertension: GRK4 Polymorphisms and Excessive Phosphorylation of the Dopamine D₁ Receptor

The GRKs have been implicated in genetic and acquired hypertension because they participate in the desensitization of GPCRs, including D₁ receptor and the angiotensin II type 1 receptor (*186,188*). For example, basal GRK-dependent phosphorylation of serine residues of the D₁ receptor is increased in the renal proximal tubules in animal models as well as in humans with essential hypertension. Of the α/β - and γ/δ -isoforms of *GRK4* expressed in the kidneys, the γ -isoform was found to be polymorphic, confirming the *GRK4* locus linkage with essential hypertension (*186,187*).

The *GRK4* SNPs include Arg65Leu, Ala142Val, and Ala486Val. Dopamine D₁ receptor-mediated cAMP production is reported to be markedly impaired by these variants. Expression of these SNPs is also associated with increased basal phosphorylation of the dopamine D₁ receptor. This suggests that increased basal phosphorylation of the dopamine D₁ receptor by GRK4 may be associated with the decreased responsiveness of the dopamine D₁ receptor in hypertension (*187,188*).

In vitro studies suggest that the *GRK4* SNPs impair the function of D₁ receptors, increase blood pressure, and impair the diuretic and natriuretic effects of dopamine D₁-like agonist stimulation. Inappropriate desensitization of the dopamine D₁ receptor in renal proximal tubules in hypertension may result in the decreased ability of the kidney to eliminate a sodium chloride load—a key risk factor in the development of hypertension.

The effect of GRK4 disruption is widespread in affected tissues. In addition to abnormal desensitization of the dopamine D₁ receptor, GRK4 polymorphisms are associated with increased expression of another regulator of sodium load, the angiotensin II type 1 receptor. The findings suggest that dysregulation of GPCR systems might be corrected by blocking the effects of GRK4 in patients who harbor GRK4 polymorphisms. The principle of targeting accessory proteins might be applied to other disorders that involve disruptions to normal GPCR signaling (*186–188*).

6.5 Conclusion

The identification and characterization of the processes of GPCR activation and inactivation have defined the genomics of the accessory proteins necessary to these processes. This has accelerated progress in understanding the fundamental mechanisms involved in GPCR synthesis, transport to the membrane, ligand binding, and activation and inactivation by GRK-mediated (and other) phosphorylation (*192*).

The catalog of G α and G β subunit polymorphisms that result in complex phenotypes has complemented this effort.

Significantly, the study of the genomics of GPCR accessory proteins has provided insight into pathways of disease, such as the contributions of RGS proteins to hypertension and AGS proteins to myocardial hypoxia. In the case of the GRKs, identified originally in the retina as integral to the pathways that involve rhodopsin, proteins such as GRK4 have been identified that have been subsequently associated with hypertension. These studies show how classical human genetics can become an *entree* into the genomics and pharmacogenetics of a given receptor system or systems.

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Chapter 7

G Protein-Coupled Receptors Disrupted in Human Genetic Disease

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7.1 Introduction.....	110
7.2 Receptor Genes and Disease.....	111
7.3 Conclusion	129
References.....	130

Summary Genetic variation in G protein-coupled receptors (GPCRs) results in the disruption of GPCR function in a wide variety of human genetic diseases. In vitro strategies have been used to elucidate the molecular pathologies that underlie naturally occurring GPCR mutations. Various degrees of inactive, overactive, or constitutively active receptors have been identified. These mutations often alter ligand binding, G protein coupling, receptor desensitization, and receptor recycling. The role of inactivating and activating calcium-sensing receptor (CASR) mutations is discussed with respect to familial hypocalciuric hypercalcemia (FHH) and autosomal dominant hypocalcemia (ADH). Among ADH mutations, those associated with tonic-clonic seizures are discussed. Other receptors discussed include rhodopsin, thyrotropin, parathyroid hormone, melanocortin, follicle-stimulating hormone, luteinizing hormone, gonadotropin-releasing hormone (GnRHR), adrenocorticotrophic hormone, vasopressin, endothelin- β , purinergic, and the G protein associated with asthma (GPRA). Diseases caused by mutations that disrupt GPCR function are significant because they might be selectively targeted by drugs that rescue altered receptors. Examples of drug development based on targeting GPCRs mutated in disease include the calcimimetics used to compensate for some CASR mutations, obesity therapeutics targeting melanocortin receptors, interventions that alter GnRHR loss from the cell surface in idiopathic hypogonadotropic hypogonadism and novel drugs that might rescue the P2RY12 receptor in a rare bleeding disorder. The discovery of GPRA suggests that drug screens against variant GPCRs may identify novel drugs. This review of the variety of GPCRs that are disrupted in monogenic disease provides the basis for examining the significance of common pharmacogenetic variants.

Keywords Follicle-stimulating hormone; gain of function; gonadotropin-releasing hormone (GnRHR); G protein-coupled receptor; loss of function; luteinizing hormone; melanocortin; monogenic disease; parathyroid hormone; rhodopsin; thyrotropin.

7.1 Introduction

In Chapter 6 the properties that define GPCRs, their utility in drug discovery and their signaling characteristics, were described. This chapter reviews the wide variety of GPCR variants and mutations that have been related to many human disorders. The receptors mutated in monogenic diseases are discussed in the context of signaling disruptions; many of these have been reviewed previously (1–3). Subsequent discussion in Chapter 8 extends to GPCR variants that are associated with a phenotype consisting of altered drug efficacy or altered susceptibility to disease.

Variation in genes encoding the G protein-coupled receptors (GPCRs) is associated with a spectrum of disease phenotypes and predispositions. GPCR sequence variability is significant because receptors are also the targets of therapeutic agents. As a result, each variant provides an opportunity to study receptor function in vivo that complements a plethora of available in vitro data on the pharmacology of the GPCRs. Refined knowledge of the genes that encode GPCRs is helping to define (1) the properties of the largest class of transmembrane (TM) receptors with respect to their genomic, protein, and signaling properties and the many putative drug targets available for drug discovery using “reverse pharmacology”; (2) the genetic predisposition to disease states that can result from sequence variation in the genes encoding these receptors; and (3) the basis of variability in drug response and toxicity (pharmacogenetics) and subsequent alterations in drug efficacy.

Estimates of receptor efficacy and potency are two of the common ways that pharmacologists use to determine whether a GPCR variant results in the radically disrupted signaling characteristic of disease or the more subtle alterations in signaling relevant to pharmacogenetics. *Drug efficacy* is a pharmacological term that describes the extent to which ligand activation of a receptor results in maximal stimulation $V_{\max}$ of a relevant signaling pathway (e.g., adenylyl cyclase generation of cyclic adenosine monophosphate [cAMP]). By contrast, *drug potency* denotes the concentration of ligand that results in half-maximal response EC_{50} of a signal such as cAMP stimulation. The plethora of recurrent genetic variants or polymorphisms includes coding and noncoding protein variants that sometimes alter efficacy and potency.

This chapter discusses mutant GPCR genes that are known to be disease causing through the expression of defective receptor proteins that have been shown in vitro to result in defective receptor proteins that are inactive or constitutively active receptors. Mutations that cause inactive receptor proteins are often referred to as loss-of-function (LOF) mutations. Among the LOF mutations, some result in a dominant negative phenotype, indicating that, among heterozygotes, expression of the LOF variant disrupts the function of the wild type. By contrast, constitutively active mutants (CAMs) result in autonomous signaling in the absence of agonist.

Although originally described for in vitro mutations, CAMs have now been described for many members of class A, B, and C families (3,4).

These two extreme receptor states are defined by changes in ligand binding, G protein coupling, receptor desensitization, and receptor recycling (3,4). The investigation of these mutations gives insight into the causes of human genetic disease and provides perspective on strategies for drug discovery that take into account the potential for the development of drugs targeted at mutated and wild-type GPCRs (3–7). Advances in our knowledge of both receptor structure and function also facilitate the discussion of GPCR pharmacogenetics outlined in Chapter 8.

Selected examples reviewed include those disorders resulting from mutations in rhodopsin, thyrotropin (formerly called thyroid-stimulating hormone, TSH), luteinizing hormone (LH), vasopressin, angiotensin receptors, and the GPCR associated with asthma (GPRA). By comparison, the recurrent pharmacogenetic variants may not result in monogenic disorders but are likely to result in an altered predisposition to developing a complex disease or drug response phenotype. In some cases, such as the calcium-sensing receptor (CASR), different classes of receptor variant may result in either monogenic disease or variable pharmacology. The pharmacological phenotypes are often reported to result from either a partial gain or a partial loss of receptor signaling. These phenomena, reviewed in Chapter 8, are often defined in terms of alterations of efficacy or potency of the variant receptor with respect to the wild-type receptor. As a result, some of the GPCRs reviewed in this chapter that cause disease are also discussed in Chapter 8 in relation to a different group of variants that are primarily pharmacogenetic variants.

7.2 Receptor Genes and Disease

The properties of some GPCR variants are reviewed with respect to what can be learned from prototypical receptors, beginning with rhodopsin. The examples selected are summarized in Table 7.1 with respect to the common single-nucleotide polymorphisms (SNPs) that cause the disorders.

Disease phenotypes have been associated with both LOF mutations leading to ligand resistance (or reduced binding) and gain-of-function mutations leading to constitutive activation of signaling pathways (or enhanced binding). The pharmacological phenotypes that have also been attributed to variant receptors because of either gain or loss of receptor efficacy or potency are reviewed in Chapter 8.

7.2.1 *Rhodopsin Variants in Retinal Disease*

Constitutively active mutants of GPCRs encode for receptors capable of enhanced signaling when they are activated without exposure to ligand. The majority of rhodopsin variants are CAMs. As a result, they have become useful tools in the study of conformational changes leading to receptor activation. Study of CAMs has also identified a class of ligands that acts as inverse agonists: agents causing conformational

Table 7.1 G protein-coupled receptor (GPCR) sequence variants associated with human genetic disease

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
Rhodopsin (RHO) 3q21-q24	G90D, A292E, T4K, N15S, T17M, P23H	Retinitis pigmen- tosa, congenital night blindness	Constitutively activate mutant (CAM) receptor	(10-17)
	L125R			(19)
	K296E, E113, and substitution of adjacent residues		Potentially ruptures the salt bridge by a competitive mechanism	(10,18)
	E134Q, E134D		↑ Activity; ↓ activ- ity: Substitution of E134 may disrupt structure	(33)
Luteinizing hormone/ chorionic gona- dotropin receptor (LHCGR) 2p21	Truncated TM5	Leydig's cell hyperplasia	Constitutively activated lutein- izing hormone (LH) receptor	(77-79)
	T398M, A568V, M571I, T577I, D578G	Association with familial male precocious puberty		
Follicle-stimulating hormone receptor (FSHR) 2p21-p16	A189V	Ovarian dysgenesis	↓ Affinity for ligand	(71)
	D567G	Semen production normal despite ↓ gonado- trophins	Constitutively active	(71-76)
	N680S	Pharmacogenetic variant	Population study	(72)
	D294H	Red hair/fair skin	↓ Affinity for ligand	
Melanocortin 1 receptor (MC1R) 16q24.3	D84E	Development of melanoma		
	V92M	Red hair/fair skin	Activating/ inactivating	(61-69)
Melanocortin 4 receptor (MC4R) 18q22	V103I, many SNPs	Morbid obesity, monogenic form of binge eating		(65-69)
Endothelin receptor, type B (EDNRB) 13q22	Many SNPs, W276C	Hirschsprung's disease (one of nine genes at four loci)	↓ G _q coupling in vitro	(109-114)
Adrenocorti- cotropin receptor (ACTHR/MC2R) 18p11.2	S120R, R201Stop, S74I, V254C, C360G	Isolated glucocorti- coid deficiency	Altered/loss of function	(95)
	Promoter polymorphism	Adrenocortical tumors	↓ Expression; loss of heterozygos- ity in tumors	(33,95)
Gonadotrophin- releasing hormone receptor (GNRHR) 4q21.2	N10K, N10R, E90K, R139H, S217R, T321I	Idiopathic hypogo- nadotropic hypogonadism (IHH)	Reduced or loss of function	(84-93)

(continued)

Table 7.1 (continued)

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
	H223R, T410P, I458R	Jansen's metaphyseal chondrodysplasia	Constitutively active receptor	(50)
Parathyroid hormone receptor (PTHr1) 3p22–p21.1	P132L, Delete. bp 1122 (frame shift), 1176 G/A	Blomstrand's chondrodysplasia	No accumulation of cAMP	(33,51)
	R150C	Endochondromatosis	Inactivating mutations	(33,50–52)
Thyroid-stimulating hormone receptor (TSHR)	P52T, G431S, V509A, C672Y	Autoimmune thyroid disease	Altered receptor function/conformation	(23–29,31)
14q31	D727E	Grave's disease	Population studies	(23,29,31)
	D619G, A623I (somatic)	Toxic multinodular goiter	Constitutive activation of adenylyl cyclase	(24,32)
		Hyperfunctioning thyroid adenomas		(23–29,31)
Arginine vasopressin receptor 2 (AVPR2) Xq28	W71 truncation and many SNPs	Nephrogenic diabetes insipidus	↓ Ligand binding/reduced expression of receptor	(98–105)
G protein-coupled receptor 154, associated with asthma (GPR154/GPRA) 7p15–p14	GPRA-B isoform overexpressed in bronchial epithelia of asthmatics	Asthma	Unidentified ligand suggests that GPRA is a potential drug target	(120,121)
Chemokine, cc motif, receptor 5 (CCR5) 3p21	Δccr5 (32-bp deletion) 59029 A/G	Partial resistance to HIV infection ↓ AIDS progression ↓ Non-Hodgkin's lymphoma	Altered binding affinity	(33) Chapter 8
	–homozygous –heterozygous			
Purinergic receptor, P2Y, G-protein coupled, 12 (P2RY12) 7p13	2-nt deletion	Bleeding disorder	Disrupted Gi/Go inhibition of cAMP accumulation	(117,118)
Calcium-sensing receptor (CASR) 3q13.3–q21	R185Q, E297K, R795W, Arg185Q, R220W	Familial hypocalcemic hypercalcemia (FHH)/neonatal severe hyperparathyroidism	Loss of function	(36–44)
	0.9-kb <i>alu</i> insertion in exon 7	Adenylyl cyclase		(43,44)
	A116T, N118K, etc.	Familial hypocalcemia	↑ IP ₃ response	(46,48)
	A986S, R990G.	Common polymorphisms	Predictive of serum Ca ²⁺	(38,48)

cAMP, cyclic adenosine monophosphate; SNP, single-nucleotide polymorphism.

changes in a receptor that restore basal levels of receptor signaling by uncoupling a constitutively activated receptor from the G protein. In the example of rhodopsin, it is the retinoic acid derivative 11-*cis*-retinal that acts as an inverse agonist (8,9).

These mutations not only constitutively activate transducin but also often result in constitutive phosphorylation of rhodopsin by rhodopsin kinase or GRK1 (G protein-coupled receptor kinase 4). As discussed in Chapter 6, GRK1 is a specialized enzyme expressed in the retina that is responsible for rapidly desensitizing the receptor when it is exposed to light. The phosphorylated rhodopsin in turn binds tightly to the inhibitory protein arrestin. This reaction quenches the activated receptor's interaction with the G protein transducin and inhibits further G protein signaling. A reciprocal relationship exists between GPCR activation during G protein coupling and rapid quenching, or desensitization, by one of the GRKs (10–12). The identification of aberrant rhodopsin phosphorylation and desensitization (13) for a wide variety of rhodopsin mutations suggests that the retinitis pigmentosa phenotype results partly from a pathology of GRK phosphorylation.

7.2.1.1 Night Blindness, Retinitis Pigmentosa, and Rhodopsin Phosphorylation

Rhodopsin CAMs are responsible for various ocular abnormalities, including night blindness and various retinal dystrophies, generically termed *retinitis pigmentosa*. The rhodopsin variants include Thr4Lys (14,15), Asn15Ser (16), Thr17Met, Pro23His (17,18), Pro23Leu, Gln28His, Gly90Asp, Glu113Gln, Ala292Glu, and Lys296Glu (10–12). In the case of each variant, both the disease phenotype and the effect of the mutation on receptor structure and function may vary. The mutations at positions Gly90Asp and Ala292Glu result in complete night blindness, while other mutations cause retinitis pigmentosa (12). In many cases, such as the variants at Gly90, different amino acid substitutions at the same position have been found to distinguish between phenotypes (19).

Study of another constitutively phosphorylated rhodopsin mutant, the Leu125Arg variant in TM domain 3, has resulted in an understanding of the specificity with which an amino acid substitution can determine whether a receptor is able to desensitize. When the amino acid at position 125 of rhodopsin was individually modified in vitro to each of the remaining 18 amino acids, it was found that receptors with smaller residues at position 125 were better able to activate transducin. In the case of the bulkier Leu125Tyr and Leu125Trp substitutions, very little G protein signaling was detected. This suggests that amino acid side chains exert a steric effect, leading to inhibition of G protein activation (20). In view of this, it seems likely that the Leu125 in TM helix III of rhodopsin, which is located near the ligand-binding pocket for 11-*cis*-retinal, may be important for the structure of the chromophore-binding pocket (20). This structural information provides new information about the structure of the ligand-binding site of the prototypical GPCR, rhodopsin (21).

7.2.1.2 Oguchi Disease and Defective GRK1 Phosphorylation of Rhodopsin

The group of rhodopsin-related disorders, resulting from mutations in the *GRK1* gene, was reviewed in Chapter 6 in relation to its significance to GPCR signaling. One example is autosomal recessive Oguchi disease. As reviewed in Chapter 6, Oguchi mutations result in the impairment of GRK1-mediated desensitization of rhodopsin (22,23). This disrupts the normal pathway of light-dependent rhodopsin phosphorylation and subsequent quenching of light-induced signal transduction in photoreceptor cells (24). Thus, regardless of the integrity of the receptor itself, disruption of GPCR accessory proteins can result in a disease phenotype attributable, biochemically, to receptor dysregulation.

7.2.2 *Thyroid Disease and Thyroid-Stimulating Hormone Receptor Mutations*

Similar to the rhodopsin receptor disorders, activating and inactivating mutations of the thyroid-stimulating hormone (TSH) and TSH receptor (TSHR) underlie many cases of thyroid disease. The TSHR mutations disrupt TSH signaling by blunting the G_s -mediated stimulation of adenyl cyclase. Disruption of TSHR may result in dysregulation of the TSH function and result in the abnormal growth of thyroid hormone-secreting cells.

Hyperthyroidism, for example, can result from activating germline mutations that are located in the TSHR TM domains. By contrast, thyroid adenomas and multinodal goiter (25–31) result from a variety of somatic mutations in other regions of the TSHR. For example, a rare constitutively active TSHR mutation in the first TM domain results from a Gly substitution at the conserved ⁴³¹Ser position (28).

Mutations with similar outcomes have been identified in nonautoimmune autosomal dominant hyperthyroidism (toxic thyroid hyperplasia) (25,26,28,32,33). These variants are located in the third TM (Val509Ala), the seventh TM (Cys672Tyr), and the carboxyl tail (Asp727Glu) regions (34). These variants result in a form of congenital hyperthyroidism that is the germline counterpart of a hyperfunctioning thyroid adenoma, with similar functional characteristics (25,33).

7.2.2.1 Toxic Multinodal Goiter and Activating TSHR Mutations

Although toxic multinodular goiter is pathogenetically heterogeneous, it also results in hyperthyroidism. The molecular pathology of this disorder is complicated by the discovery that activating mutations of both the $G_s\alpha$ subunit (reviewed in Chapter 6) and the TSHR have been identified in goiter. These variants result in autonomously hyperfunctioning thyroid adenomas (26) as well as the majority of nonadenomatous hyperfunctioning nodules scattered throughout the gland in patients with toxic or functionally autonomous multinodular goiter (35).

7.2.2.2 Variable Thyroid Phenotypes Result from Mutations

There is wide variability in phenotypic presentation of *TSHR* gene mutations even though they are tightly distributed within TM domains. As with the other receptors, single amino acid changes, such as Asp72Glu, have been associated with a broad phenotype. This variant is not, however, strictly considered to be a constitutive TSHR. It is another example of a pharmacogenetic variant that should be taken into account when evaluating congenital nonautoimmune hyperthyroidism of varying severity (36). On the continuum of receptor activity, it has enhanced sensitivity to agonist (37). To complicate matters, it may be of variable clinical significance depending on the genetic background (27,38) since many TSHRs also have defects in corepressor interaction that influence thyroid phenotype within kindreds (39). Discussion of GPCR variants that are associated with intermediate phenotypes is the focus of Chapter 8.

7.2.3 Calcium-Sensing Receptor Mutations and Hypercalcemia/Hypocalcemia

The CASR functions as an extracellular calcium sensor for the parathyroid gland and the kidney. CASR serves to maintain a stable calcium concentration, without which many aspects of homeostasis are adversely affected. For example, the effect of CASR variants on seizure threshold in the brain is reviewed in **Subheading 7.2.3.3** concerning autosomal dominant hypocalcemia (ADH). Because the CASR gene is highly polymorphic (40), the contribution of common polymorphisms to individual differences in calcium metabolism is under increasing scrutiny. These studies are reviewed in Chapter 8.

Unlike the majority of GPCRs discussed, the CASR belongs to family C, and as such, it shares considerable homology with the metabotropic receptors (family C), particularly the glutamate receptor. This distinction is associated with a significant difference in the ligand-binding domains. Unlike family A GPCRs, the ligand-binding domain of family C receptors often includes a large extracellular motif. Mutations of the CASR contribute to the altered set point for extracellular ionized calcium $[Ca^{2+}]$ required for parathyroid hormone (PTH) regulation that defines a variety of disorders characterized by hypercalcemia or hypocalcemia. These disorders include familial hypocalciuric hypercalcemia (FHH), secondary hyperparathyroidism and neonatal severe hyperparathyroidism (41–43).

7.2.3.1 Familial Hypocalciuric Hypercalcemia

The syndrome known as familial hypocalciuric hypercalcemia (FHH) was first called familial benign hypercalcemia to emphasize the asymptomatic nature of

lifelong hypercalcemia that results from inactivating *CASR* mutations (44, 45). The syndrome became known as FHH because of the abnormal renal calcium handling in affected family members (46). The prevalence of FHH is ill defined; however, it accounts for only a minority of cases of asymptomatic hypercalcemia. It is possible that the paucity of FHH families may reflect the fact that they are infrequently recognized rather than the rarity of the disorder itself.

Familial hypocalciuric hypercalcemia is inherited in an autosomal dominant manner with almost 100% penetrance but variable expressivity. The FHH locus was first mapped to 3q21–24 by linkage analysis (47). Most FHH families map to the long arm of chromosome 3, but one clearly maps to another locus, 19p13.3 (44).

Three different *CASR* gene missense mutations (Arg185Gln, Glu297Lys, Arg795Trp) were originally identified in three unrelated FHH families. Since then, more than 50 additional inactivating mutations have been identified (44). Many of these variants are shown in Fig. 7.1. The majority of mutations are missense, with a few nonsense, deletion/insertions, and splice-site mutations (48). In one case, an insertion of an 0.9-kb *Alu* sequence in exon 7 of the *CASR* gene was identified (49,50). At least three missense mutations are recurrent (Pro55Leu, Thr138Met, and Arg185Gln). Independent inactivating mutations that involve two different amino acid substitutions have been identified (Arg185X and Arg185Gln; Arg220Trp and Arg220Gln; Arg227Leu and Arg227Gln).

The *CASR* mutations are not evenly distributed but appear to be clustered in two regions: the NH₂ terminal 300 amino acids of the extracellular domain (ECD) and a 360-amino acid portion (residues 520–881) of the TM and intracellular domains. Few mutations are identified in the last 190 amino acid residues of the cytoplasmic tail or the proximal portion of the ECD (residues 300–520).

7.2.3.2 Hypocalcemia, Hypoparathyroidism, and Hypocalcemic Hypercalciuria

Families affected by ADH, autosomal dominant hypoparathyroidism, and hypocalcemic hypercalciuria have each been defined by gain-of-function mutations in the *CASR* gene (44). ADH is associated with the expression of constitutively activated *CASR*, which serves to suppress PTH secretion from the parathyroid gland. In the kidney it induces hypercalciuria, which further contributes to the hypocalcemia.

More than 20 activating *CASR* mutations (almost all missense) have been identified and appear almost equally divided between the amino-terminal third of the ECD and the TM domain (see Fig. 7.1). Of special interest is the cluster of six ECD mutations (Ala116Thr, Asn118Lys, Leu125Pro, Glu127Ala, Glu128Leu, and Cys129Phe) that cause an increase in receptor sensitivity to extracellular calcium, suggesting that this region is critical for receptor activation. This cluster overlaps the two cysteine residues ¹²⁹Cys and ¹³¹Cys, which are putatively involved in the formation of the mature *CASR* dimer (51). Although most cases of ADH are accompanied by a clear family history, de novo mutations are surprisingly common (43, 52).

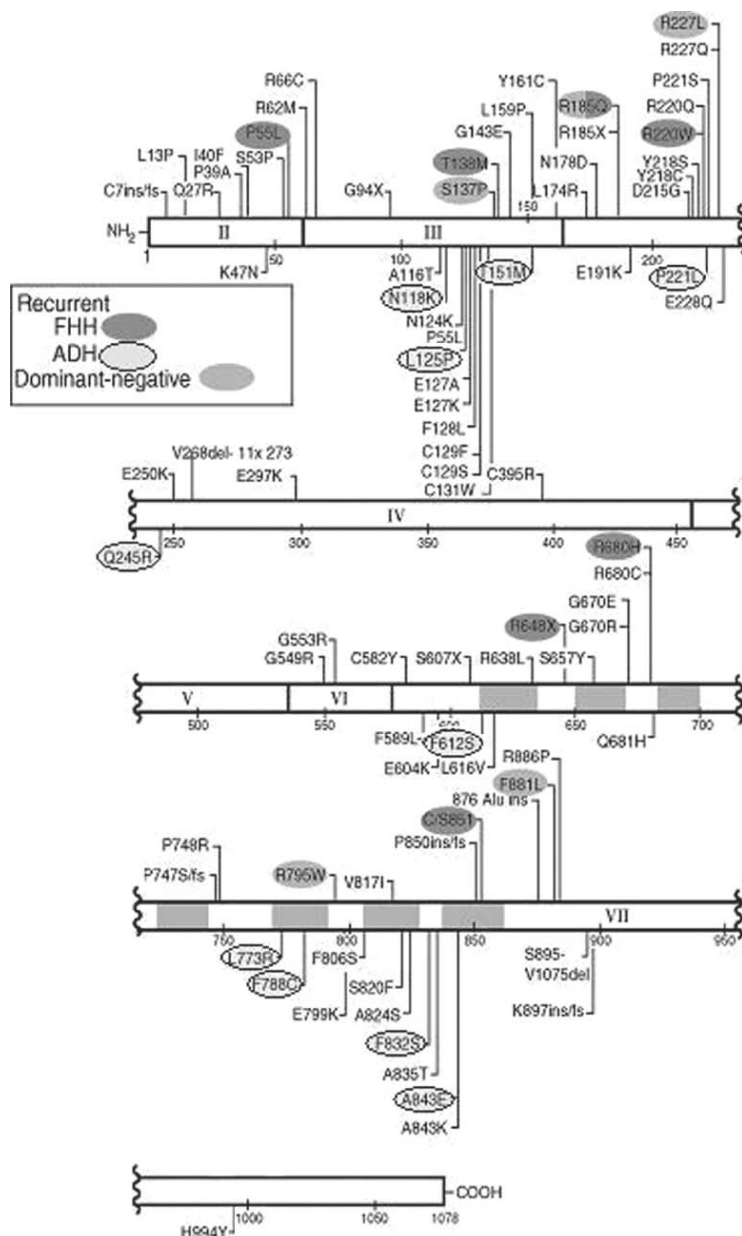


Fig. 7.1 Localization of some mutations and polymorphisms reported for the calcium-sensing receptor (CASR). The relationship between the CASR gene exons (II to VII) and the modular domains of the 1078-amino acid protein are indicated. The 610-amino acid extracellular domain (ECD) is encoded by exons II to VI. The beginning of exon VII encodes the ECD. The remainder of exon VII encodes the transmembrane domain (TMD) of approx. 250 amino acids that includes the membrane-spanning helices TM1–TM7 (indicated by the hatched boxes), the extracellular and intracellular

7.2.3.3 Neurological Phenotypes and Autosomal Dominant Hypercalciuria

In a subset of ADH families, the *CASR* gain-of-function mutations have been associated with the onset of tonic–clonic seizures. Although in general the molecular genetics of ADH have become better understood, the profound neurological implications of *CASR* mutations have not been widely explored (41–42). Expression of the *CASR* in brain regions, such as the hippocampus, suggests that many neurological functions relating to seizure threshold may be regulated by the *CASR*.

Up to a third of all cases of idiopathic hypoparathyroidism may be found to have activating *CASR* mutations. This suggests that the frequency of tonic clonic seizures caused by activating *CASR* mutations may be higher than expected (44,51–54). The neurological phenotypes may result from the dysregulation of *CASR* in central nervous system and peripheral tissues. Evidence of seizures in patients expressing activating *CASR* mutations may indicate that the *CASR* plays an important role in setting seizure threshold (53,54). The brain calcification that is seen in ADH patients—even those patients unaffected by seizures—suggests that the activating *CASR* mutations may profoundly alter calcium homeostasis in the brain (42).

The suppression of PTH secretion from the parathyroid gland that accompanies the constitutive activation of the *CASR* makes the disorder difficult to recognize and treat. In some cases, it has been reported that seizures can be intractable. The abnormal set point of calcium regulation complicates treatment with calcitriol and dietary calcium supplementation because the *CASR* expressed in the kidney controls calcium excretion. The constitutively activated *CASR* mutant induces hypercalciuria, which may compound the hypocalcemia (42).

Further work on ADH may identify the molecular mechanisms underlying the brain calcification and tonic–clonic seizures associated with the *CASR*-activating mutations. This information may refine therapy for ADH patients as well as hypoparathyroidism patients who harbor *CASR* mutations. Further details about ADH can be found in the *CASR* locus-specific database at [http://www.casrdb.mcgill.ca/\(41\)](http://www.casrdb.mcgill.ca/(41)).

7.2.4 Parathyroid Hormone Receptor Mutations and Skeletal Dysplasias

The parathyroid hormone receptor 1 (PTH1R) protein belongs to the GPCR family B. The PTH1R is a receptor for PTH and for parathyroid hormone-related peptide

Fig. 7.1 (continued) loops (ECL1 to ECL3, ICL1 to ICL3, respectively), as well as the intracellular domain (ICD) of approx. 200 amino acids. The locations of the inactivating mutations found in patients with FHH (familial hypocalciuric hypercalcemia) or neonatal severe hyperparathyroidism (NSHPT) are shown. Activating mutations found in patients with autosomal dominant hypocalcemia (ADH) are shown below. Those that are recurrent and dominant negative are highlighted (41). <http://www.casrdb.mcgill.ca/>

(PTHrP). The receptor signal is mediated by G proteins that activate adenylyl cyclase and the phosphatidylinositol–calcium second-messenger system. Mutations of *PTHrP* are associated with abnormalities of development related to altered PTHrP ligand binding. PTHrP is a key paracrine peptide responsible for osteochondrogenesis during fetal development (55,56).

Activating mutations cause Jansen's metaphyseal chondrodysplasia (JMC). This disorder is inherited in an autosomal dominant fashion, although most reported cases are caused by new mutations. The important features include short-limbed dwarfism secondary to severe growth plate abnormalities, asymptomatic hypercalcemia, and hypophosphatemia. Although the PTHR is found widely in fetal and adult tissues, it is most abundant in kidney, bone, and the metaphyseal growth plates. Molecular analysis showed that heterozygous gain-of-function mutations that give rise to constitutively active receptors (56,57) result in the altered mineral ion homeostasis and growth plate abnormalities of JMC.

By contrast, persons homozygous for inactivating mutations in the *PTHrP* gene manifest with Blomstrand's lethal chondrodysplasia, a recessive short-limbed dwarfism with craniofacial malformations, hydrops, hypoplastic lungs, and aortic coarctation (58). In keeping with the regulatory role that PTHR1 plays in bone formation in utero, the bones show accelerated endochondral ossification and deficient remodeling. For example, the Arg150Cys PTHR1 mutation was identified in two of six patients with enchondromatosis, a familial disorder with evidence of autosomal dominance characterized by multiple benign cartilage tumors and a predisposition to malignant osteosarcoma (59).

The phenotypic complexity noted for other GPCR diseases is true also for *PTHrP* mutations. Opposite clinical manifestations have been reported to result from distinct recessive mutations in the gene. These rare variants present as Eiken syndrome, a distinct entity from JMC and Blomstrand's chondrodysplasia and from enchondromatosis. The skeletal features are opposite those in Blomstrand's chondrodysplasia. The Eiken syndrome variant, resulting in a truncation at position 485, may result in a paradoxical phenotype caused by the consequences of disrupting the carboxyl tail of the receptor (60).

7.2.5 GPCR Mutations and Obesity

Specific brain regions, including parts of the hypothalamus, are known to be involved in the regulation of feeding, body adipose, and sensory integration of inputs—functions that are also discussed in Chapter 8 in relation to the orexin–hypocretin system. Candidates in obesity include melanin-concentrating hormone (MCH), a 19-amino acid hypothalamic neuropeptide that is important in the regulation of energy homeostasis (61–63) and melanocortin. Two MCH receptors have been identified: MCHR1, isolated from rodents and humans, and MCHR2, present only in humans. MCH signals via GPCRs coupled to $G_{i/o}$ downstream of the leptin pathway and is expressed on neurons known to regulate body weight (64).

The variants of MCHR1 and MCHR2 that are known, however, have little clinical correlation compared with melanocortin.

7.2.5.1 Melanocortin Receptor Mutations and Obesity

The melanocortin 4 (MCH₄) receptor (*MC4R*) gene may contribute substantially to the genetics of obesity that involve the hypothalamus (62,65–71). The natural ligand for this receptor, melanocyte-stimulating hormone (α MSH), is a neuropeptide derived from pro-opiomelanocortin (POMC). MC4R is also negatively regulated by endogenous inverse agonists, such as the agouti (Ag) and agouti-related proteins (AgRPs). Since the MC4R is constitutively active, it is the balance between the activity of AgRP-containing neurons and α MSH-containing neurons that determines the extent of melanocortin pathway activation (72).

The contribution of the MCH₄– α MSH pathway to obesity has been primarily identified from the study of *MC4R* knockout mice that are hyperphagic and severely overweight (73,74). More recently, however, large association studies in humans have identified polymorphisms, such as Val103Ile, as well as private mutations that account for a monogenic form of binge eating and obesity (75–77).

The discovery of a rare form of autosomal dominant obesity that results from an inactivating (frame-shift) *MC4R* mutation confirmed the role of the MCH₄ receptor in energy homeostasis. LOF *MC4R* mutations were identified as a result of the linkage studies in families with severe autosomal dominant obesity (67,68,78). The loss of constitutive activity in these receptors resulted in the identification of an important disruption to energy homeostasis. These observations suggest that the correct balance of agonists and inverse agonists may be achieved by pharmaceutical interventions which target the MC4R functions that maintain weight homeostasis. These considerations are being incorporated into MC4R drug design (79–82).

7.2.6 Follicle-Stimulating Hormone Receptor Mutations and Gametogenesis

The follicle-stimulating hormone (FSH) receptor (FSHR) is a key component of the endocrine axis governing gonadal function. FSH is essential for normal gametogenesis in both males and females. Inactivating FSHR mutations identified in female ovarian dysgenesis, however, appear to be benign in males, who instead occasionally harbor an asymptomatic constitutively active FSHR mutation. This difference reflects the developmental differences: In females, FSH is required for ovarian development and follicle maturation, whereas in males FSH determines Sertoli cell number and normal spermatogenesis. The prototypic inactivating (Ala189Val) and activating (Asp567Gly) FSHR mutations are reviewed next, respectively, in the discussion of ovarian dysgenesis and hypophysectomized males (83,84).

7.2.6.1 FSHR Mutants, Ovarian Dysgenesis, and Infertility

The Ala189Val mutation in the FSHR was first identified in a female patient with severely affected gametogenesis (83). The resultant female infertility phenotype was identified in a dominantly inherited pattern of ovarian dysgenesis. Identified in a homozygous form in affecteds, the mutation disrupts the large ECD of the FSHR implicated in ligand binding, while leaving intact the remaining TM-spanning domains and the carboxyl tail (83,84). In vitro studies suggested that the mutation probably affects FSH binding by disrupting the proper protein folding and thereby inactivating the receptor (84,85).

7.2.6.2 FSHR Mutations Unmasked in Hypophysectomized Males

Male patients hypophysectomized because of a pituitary tumor, who had normal semen counts despite undetectable serum gonadotropins after surgery, have been discovered to harbor constitutively active forms of the *FSHR* gene. Because the benign phenotype is only unmasked by the development of an unrelated tumor, the frequency of these mutations in the general population is difficult to evaluate (83,86).

The constitutive FSHR mutation Asp567Gly is encoded by a SNP located in exon 10 of the gene. As a result of its location, the substitution probably disrupts the third cytoplasmic loop. The constitutive mutation was found to result in an increase in basal cAMP production compared in vitro to the wild-type FSHR. The ligand-independent activation of the FSHR in the constitutive mutant explains why this heterozygote is capable of maintaining spermatogenesis in hypophysectomized patients (83,84,87,88).

Interestingly, although Ala189Val variants have been identified in both sexes, the Asp567Gly variant has only been identified in males. This suggests that this activating FSHR mutation may result in a lethal phenotype in females (83,84). In this context, it is intriguing that there is evidence for an association between homozygosity for the common Asn680Ser variant with increased FSH serum levels in normogonadotropic anovulatory infertile women (85).

Although inactivating FSH mutations are the only FSHR mutations known to cause monogenic disease (83), there are naturally occurring FSH variants, such as Asn680Ser, that affect a spectrum of phenotypes, such as the fertility of women from different genetic backgrounds (85). A contrasting example is provided by some cases of ovarian hyperstimulation syndrome (OHSS). This potentially life-threatening complication of ovarian stimulation treatments has been associated with an activating FSHR mutation (89,90). This is an example of how pharmacogenetics can focus attention on genetic predispositions that would not have otherwise undergone scrutiny. Pharmacogenetic topics are discussed with respect to other examples in Chapter 8.

7.2.7 *Luteinizing Hormone Receptor Mutations*

Luteinizing hormone is critical to male fertility because it stimulates testicular Leydig cells to produce the testosterone that maintains secondary male sex characteristics. The LH receptor mediates these functions by activating adenylyl cyclase via G_s (90).

There are a variety of constitutively active mutations in the gene encoding the LH receptor. These variants result in gonadotropin-independent disorders such as testotoxicosis and familial male precocious puberty (FMPP) (91). These disorders are inherited in an autosomal dominant, male-limited pattern (92,93).

7.2.7.1 Testotoxicosis

Testotoxicosis is a form of male precocious puberty. The disorder results from a constitutive activation of the $G_s\alpha$ protein (reviewed in Chapter 6). This results in LH receptor activation that is analogous to the LH receptor mutant phenotypes. The disorder often presents alongside paradoxical pseudohypoparathyroidism type Ia (PHP-Ia), a condition that is marked by resistance to hormones acting through cAMP (PTH and TSH) (91).

Molecular studies explained this apparent paradox when the temperature-sensitive $G_s\alpha$ Ala366Ser mutation of the $G_s\alpha$ protein was identified. At 32°C, the $G_s\alpha$ 366Ser mutation results in the constitutive cAMP accumulation that causes the testosterone secretion that is the hallmark of the testotoxicosis phenotype. At 37°C, however, the $G_s\alpha$ 366Ser mutation results in loss of adenylyl cyclase signaling, causing PHP-Ia. As a result, a single mutation that performs differently in different tissues causes precocious puberty and abnormalities of PTH and TSH (91).

7.2.7.2 Familial Male Precocious Puberty and Constitutive LH Receptor Mutants

Familial male precocious puberty is associated with Leydig cell hyperplasia, which may contribute to low sperm cell counts. Molecular studies have identified substitutions in the TM 6 domain of the LH receptor in affected males (94,95). The Asp567Gly mutation of the LH receptor, for example, was found to result in a constitutively active phenotype. The disorder was also found to result from a nearby Ala568Val mutation (95) and from Met571Ile and Thr577Ile mutations in the more cytoplasmic portion of helix 6. These mutations were found to result, in vitro, in receptors with constitutively active phenotypes characterized by significantly increased basal cAMP production in the absence of agonist. Although these variants have been reported in kindreds from various ethnic origins, including European (96) and Brazilian (94,95), it is unclear whether each variant constitutes a unique founder mutation.

7.2.7.3 LH Receptor Wild-Type vs LH Mutant Structure and Function

Constitutively active mutations, such as those reported in the LH receptor, provide insight into the dysregulated G protein coupling observed in a variety of disease states. Various important structural components of GPCRs are highlighted. For example, in vitro studies have shown that a constitutively active α_1 -adrenergic receptor can be generated by mutating the alanine residue homologous with the FSHR⁵⁶⁸alanine. Similar to the LH receptor variant, the resulting α_1 -adrenergic receptor variant is characterized by high basal adenylyl cyclase activation. These studies suggest that the alanine residue conserved in the TM 6 domain may be critical for downregulation of signal transduction (94,95).

7.2.8 Gonadotropin-Releasing Hormone Receptor Mutations and Idiopathic Hypogonadotropic Hypogonadism

Idiopathic hypogonadotropic hypogonadism (IHH) consists of those patients without commonly anosmia (a poor sense of smell) or adrenal insufficiency. This subset of IHH results in reproductive failure that is caused by mutations of the GnRH (gonadotropin-releasing hormone) receptor (*GNRHR*) gene. Like all IHH patients, those affected experience delayed sexual development and low or apulsatile gonadotropin levels. The impairment in sexual development, however, occurs in the absence of the anatomical abnormalities common to fertility disorders that affect the hypothalamic–pituitary axis (97,98).

The genetic defects for two of the more common X-linked subtypes of IHH, congenital IHH with anosmia (or Kallmann syndrome, KS), and IHH with adrenal insufficiency (adrenal hypoplasia congenita) are distinct from the forms of the disease caused by GnRH receptor (*GnRHR*) mutations. These forms of IHH are included for the sake of clarity.

The KS mutations were identified in the *KAL* gene and result in abnormal olfactory bulb development (99,100). The mutations responsible for the X-linked IHH with adrenal hypoplasia congenita were identified in the *DAX1* gene. *DAX1* encodes an orphan nuclear hormone receptor that regulates portions of reproductive development (101,102).

7.2.8.1 GnRHR Mutations that Result in Idiopathic IHH

Comparatively little is known about the molecular biology of the *GnRHR* mutations that result in idiopathic IHH. At least 15 mutations of the *GnRHR* have been described in IHH (98,103–105). Some of these mutations, such as Glu90Lys and Ser217Arg, have been found in vitro to be LOF mutations. Other *GnRHR* mutations, such as Asn10Lys, Thr32Ile, and Gln10Arg, have a somewhat reduced ability to elicit an inositol phosphate response in vitro (98).

Site-directed mutagenesis has been used to identify the significance of many GnRHR variants to receptor function. The Glu90Ala (**106**) and Arg139His (**107**) mutations were inactive *in vitro*, suggesting that these residues are probably critical to receptor activation. The ²¹⁷Ser variant of TM 5, however, illustrates how the effect of an amino acid substitution can be context sensitive. Although the variant identified in patients, Ser217Arg, is completely inactive; a substitution of Ser217Gln and Ser217Tyr using site-directed mutagenesis results in a GnRHR with partial function. Therefore, some residues may not always be critical to receptor function as long as the substitution does not disrupt receptor structure because of the steric hindrance (**98**). In this manner, portions of the GnRHR that are involved in specific molecular functions have been isolated.

7.2.8.2 GnRHR Pharmacogenomics

The advances made possible by isolating the GnRHR and its variants illustrate the potential applications of pharmacogenomics. The joining of clinical and structural biology has resulted in the identification of an antagonist that can selectively rescue most of the naturally occurring GnRHR mutants by increasing their cell surface expression (**108**). This is an example of a therapeutic strategy that would have been unimaginable before the pharmacogenomic paradigm of drug discovery.

This antagonist may act on GPCRs to stabilize misfolded proteins and prevent them from being targeted for degradation (**97,98,109**). The antagonist is *permeant*, named after its ability to recover the function of receptors before they are degraded or expressed incorrectly at the membrane. While still experimental, this example illustrates how an understanding of GPCR genomics and GPCR protein structure may facilitate the identification of drugs with novel mechanisms of action that may provide clinical intervention for complex developmental disorders.

7.2.9 Adrenocorticotrophic Hormone Receptor Mutations and Isolated Glucocorticoid Deficiency

Isolated glucocorticoid deficiency (IGD) is an autosomal recessive disorder characterized by progressive primary adrenal insufficiency but with normal mineralocorticoid metabolism. As a result of screening affected families, the gene encoding the human ACTH (corticotropin, formerly called adrenocorticotrophic hormone) receptor (MC2R) was found to be involved in the etiology of IGD (**110,111**). Several compound ACTH receptor (*ACTHR*) heterozygotes appear to be associated with IGD.

The genotype consists of two different *ACTH* receptor gene mutations in *trans*. For example, a germline nt.201C>T substitution results in the truncation of the entire carboxyl portion of the receptor because of the introduction of a premature

stop codon (TGA). A germline substitution at nt.360C>G, resulting in a Ser120Arg ACTHR mutation in TM 2, was found concurrently. In another family, the Ser120Arg mutation was found concurrently with a Tyr254Cys variant in the third extracellular loop of the receptor protein (*112*). Therefore, the growing number of variants identified may allow better assessment of the significance of a given compound heterozygote to IGD.

In this way, some variants of the ACTHR may be expressed on a background entirely lacking in functional ACTHR (*110*). Other examples have been identified. A truncation of the protein at Gly217 was found on the paternal chromosome concurrently with a substitution in the maternal chromosome located –2-bp positions from initiation of the transcription start site. Interestingly, although this substitution may be present in 6.5% of healthy individuals, its pathology only becomes evident when inherited concurrently with the truncation mutant (*111*). These studies exemplify how the study of inherited defects in a receptor gene may help to define not only the regulation of cell signaling but also the tissue levels for this class of receptors (*110*).

7.2.10 Vasopressin V₂ Receptor Mutations and Familial Nephrogenic Diabetes Insipidus

Nephrogenic diabetes insipidus (NDI) is characterized by renal tubular resistance to the antidiuretic effect of arginine vasopressin (AVP). NDI may be inherited as an autosomal dominant or X-linked recessive disorder. The autosomal dominant form of NDI results from mutations of the aquaporin 2 gene (AQP2). AQP2 encodes a water channel of the renal collecting duct. Its disruption causes autosomal dominant NDI (*113,114*) and occasionally recessive forms of the disease.

7.2.10.1 V₂ Vasopressin Receptor

The gene encoding the V₂ vasopressin receptor (AVPR2), located in the Xq28 region (*115*), is responsible for the X-linked nephrogenic diabetes insipidus. AVPR2 belongs to the cyclic nonapeptide-binding GPCR subfamily that also includes the V1a and V1b vasopressin receptors and the oxytocin receptor. AVPR2 is expressed predominantly in the distal convoluted tubule and collecting ducts of the nephron. Its primary role is to respond to the pituitary hormone AVP by stimulating mechanisms that concentrate the urine and maintain water homeostasis. More than 40 different single-nucleotide mutations, without any significant differences in phenotypic expression, have been reported in different families (*116*).

The variety of AVPR2 mutations that are known to cause X-linked NDI include SNPs, insertions, and deletions (*117*). For example, familial NDI may result from substitutions of Ser167Thr—a residue conserved across many GPCRs—and Leu44Pro. The Hopewell mutation, a Trp71 truncation, results in NDI in the largest

North American NDI pedigree. Most affecteds originate from Colchester County in Nova Scotia. In nearby Quebec, however, mutations were found in three families. These mutations include an Arg137His, a Arg113Trp, and an nt.804delG frame-shift mutation. A large kindred from Utah carries a Leu312 truncation mutation, and an Iranian family has been shown to harbor an Ala132Asp mutation. The geographical isolation of AVPR2-associated NDI mutations is consistent with evidence that suggests that de novo mutations are relatively common in X-linked diseases (118).

7.2.10.2 Loss-of-Function V₂ Vasopressin Receptor Mutations

Among those mutations that are more fully characterized in vitro are the missense mutations Cys112Arg, Asn317Lys, and Trp323Ser. These mutations, however, are associated with a range of phenotypes even among patients who share the same mutations (119). This suggests that some mutations of the AVPR2 gene may have varying degrees of penetrance depending on other genetic and environmental factors (115,120).

A variety of AVPR2 nonsense mutations causes the most severely affected NDI phenotypes (121). Although truncation frequently occurs within TM domain 3, severe phenotypes have also been reported as a consequence of the Arg137His mutation. The Arg137His mutation is representative of variant receptors that are unable to activate stimulatory G_s proteins (122). The receptor fails to respond to agonist through stimulated adenylyl cyclase activity. Many other AVPR2 mutations, such as frame-shift and small in-frame deletions, also result in AVPR2s that fail to couple to G_sα (123).

The Arg137His AVPR2 variant has been the subject of detailed study in heterologous expression systems (123). Vasopressin binds the variant with affinity similar to the wild type; however, it fails to stimulate G_sα. This evidence suggests that the conservation of an arginine at this position is necessary for receptor-coupled G protein activity (115,123,124).

In fact, Arg137 is part of the DRY motif at the boundary between the third TM region and the second intracellular loop that is found in the majority of this group of GPCRs (125). Data regarding the function of the Arg137His mutation of the AVPR2 (123) resulted in the identification of an homologous residue in the human β₂-adrenergic receptor, Arg131His, that has a similar function. Thus, the arginine in the DRY sequence may be essential for dissociation of the G protein following activation (124). In addition, some V₂ vasopressin mutations may act to induce constitutive arrestin-mediated desensitization in some patients who also carry the Arg137His mutation (126).

7.2.10.3 Downregulation of V₂ Receptor and Constitutively Phosphorylated Mutations

The Arg137His receptor, in contrast to the wild-type vasopressin receptor, is constitutively phosphorylated in vitro. This often leads to receptor sequestration in

arrestin-associated intracellular vesicles even in the absence of agonist. This may result in a disruption of the normal affinity of arrestins for phosphorylated GPCR in some NDI phenotypes. Should this be accompanied by inadequate dephosphorylation of the internalized receptor, significantly fewer receptors would be recycled back to the plasma membrane.

The discovery of a disruption to downregulation in some cases of NDI, however, may present a future NDI intervention. An intervention that targets V_2 vasopressin desensitization (126) may be analogous to the permeant antagonist that recovers the function of GnRHRs before they are degraded or expressed incorrectly at the membrane (97,98,109). Thus, it may be possible to treat NDI by pharmacological targeting of desensitization in patients who harbor certain *AVPR2* mutations.

7.2.11 Endothelin- β Mutations Associated with Hirschsprung's Disease

Hirschsprung's disease is a disorder that involves an enlargement of the colon that is defined by the absence of ganglion cells in the myenteric and submucosal plexuses of the gastrointestinal tract. Nine genes and four loci for susceptibility to Hirschsprung's disease are known (127). The disorder is characterized by incomplete penetrance and variable expressivity (128). Although the RET proto-oncogene accounts for the highest proportion of familial and sporadic cases (128), mutations in the endothelin 3 (EDN3) ligand and the endothelin- β (ETB) receptor gene (*EDNRB*) are important because of the extent to which they disrupt normal human development (129).

Although the endothelin system consists of two GPCRs, the ETB and endothelin- α (ETA) receptors, and three peptide ligands (129), Hirschsprung's disease is most frequently associated with ETB receptor variants such as the Trp276Cys mutation (130,131). Rare mutations in the EDN3 ligand gene (132) and the gene encoding the endothelin-converting enzyme 1 (ECE-1) (133), however, are also reported to be associated with Hirschsprung's disease (127).

Other ETB receptor mutations have been reported in sporadic cases of Hirschsprung's disease. These include the Gly57Ser, Arg319Trp, and Pro383Leu ETB receptor variants. In each case, the variants appear to inactivate the receptor (134).

The study of the ETB Trp276Cys receptor, however, has resulted in useful insight into the molecular pathology of Hirschsprung's disease. The high conservation between the endothelin receptor subtypes A and B has facilitated detailed molecular characterization (135). The homologous ²⁵⁷Trp and ²⁵⁸Trp mutations of the ETA and ETB receptors have been characterized with respect to their coupling properties with G_i , G_o , and G_q in vitro. The mutants have a similar affinity for endothelin 1, but the naturally occurring Trp276Cys ETB receptor mutation shows reduced G_q coupling in comparison to the engineered Trp276Ala ETB and Trp258Ala ETA receptor mutations.

7.2.12 Purinergic (P2RY12) Receptor Mutations and a Rare Bleeding Disorder

The purinergic receptors are a large family of GPCRs. Some subtypes have overlapping pharmacological selectivity for various adenosine and uridine nucleotides. The purinergic (P2RY12) receptor is involved in platelet aggregation and is a potential pharmacogenetic target for treatment of thromboembolism and other clotting disorders.

The P2RY12 receptor was identified as the result of linkage mapping of a pedigree exhibiting a severe bleeding disorder that was refractory to many treatments. This became evident because the wild-type P2RY12 receptor is the pharmacological target for the anticlotting agents ticlopine and elopidogrel (*136,137*). The P2RY12 receptor mutation, located in the TM 6 domain, is a two-nucleotide deletion that was found to have reduced efficacy and potency for these anticlotting agents. By expressing the mutation in vitro, it may become possible to identify novel pharmacological agents with efficacy in bleeding disorders, including those refractory to P2RY12 receptor agonists (*37*).

7.2.13 The G Protein-Coupled Receptor Associated with Asthma

The GPCR associated with asthma, GPRA (or GPR154), located on chromosome 7p13, was identified from linkage studies of asthma in a Finnish population and five other Western European populations (*138–140*). GPRA was identified as a candidate gene in the pathogenesis of asthma and other diseases mediated by immunoglobulin E (IgE). Like other GPCRs, GPRA may act as a receptor for unidentified ligands and is therefore a potential drug target. GPRA along with its two main isoforms GPRA-A and GPRA-B and its ligands define a distinct signaling pathway that is dysregulated in asthma (*141*). GPRA-B is more highly expressed in the bronchial epithelia and smooth muscle of asthmatics compared with healthy individuals: suggesting that the GPRA-B receptor is a promising reagent against which to screen asthma drugs (*141*).

7.3 Conclusion

As our understanding of the GPCR gene family grows, it becomes clear that many mutated forms of GPCRs are associated with a wide spectrum of disease phenotypes and predispositions. Monogenic disorders that result from a disruption of GPCR signaling provide a unique window on receptor function that complements the plethora of available in vitro data. In particular, an understanding of how mutant GPCR genes cause disease—especially through LOF or constitutively active

mutations—may suggest novel pharmacological interventions. Since disrupted receptors are also pharmacological targets, the identification of GPCRs mutated in disease provides the opportunity to identify drugs that specifically compensate for the disruption. These endeavors are intimately related to the field of GPCR pharmacogenetics reviewed in Chapter 8. Many receptors are known to have variants that, although not always directly resulting in a monogenic disease phenotype, may confer a phenotype that alters risk for a disease or altered reaction to a pharmaceutical (3,5–7).

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Chapter 8

G Protein-Coupled Receptor Pharmacogenetics

Miles D. Thompson, Katherine A. Siminoivitch, and David E. C. Cole

8.1	Introduction.....	140
8.2	GPCR Pharmacogenetics.....	141
8.3	Conclusion	168
	References.....	168

Summary Common G protein-coupled receptor (GPCR) gene variants that encode receptor proteins with a distinct sequence may alter drug efficacy without always resulting in a disease phenotype. GPCR genetic loci harbor numerous variants, such as DNA insertions or deletions and single-nucleotide polymorphisms that alter GPCR expression and function, thereby contributing to interindividual differences in disease susceptibility/progression and drug responses. In this chapter, these pharmacogenetic phenomena are reviewed with respect to a limited sampling of GPCR systems, including the β_2 -adrenergic receptors, the cysteinyl leukotriene receptors, and the calcium-sensing receptor. In each example, the nature of the disruption to receptor function that results from each variant is discussed with respect to the regulation of gene expression, expression on cell surface (affected by receptor trafficking, dimerization, desensitization/downregulation), or perturbation of receptor function (by altering ligand binding, G protein coupling, and receptor constitutive activity). Despite the breadth of pharmacogenetic knowledge available, assessment for genetic variants is only occasionally applied to drug development projects involving pharmacogenomics or to optimizing the clinical use of GPCR drugs. The continued effort by the basic science of pharmacogenetics may draw the attention of drug discovery projects and clinicians alike to the utility of personalized pharmacogenomics as a means to optimize novel GPCR drug targets.

Keywords Activation; agonist; antagonist; desensitization; efficacy; G protein-coupled receptor; pharmacogenetics; potency; single nucleotide polymorphism; variant.

8.1 Introduction

The genetic basis of drug response, pharmacogenetics, is important to both laboratory medicine and population health. As discussed in Chapter 6, genetic variation in G protein coupled receptors (GPCRs) is important because these receptors are the most abundant cell surface receptors that can be targeted clinically. The pharmacogenomic categorization of receptors and their accessory proteins facilitates the study of the numerous variants of different proteins that can result in similar phenotypes. Functional interactions between gene products are often suggested as a result of genomic information because some gene families encode proteins that commonly interact in vivo. In some cases, this insight is derived from genetic studies of complex human or animal phenotypes in which the disruption of converging pathways results in similar phenotypes. Biochemical characterization of common GPCR variants, however, is still greatly assisted by the kind of studies of mutated GPCRs and monogenic disease that were summarized in Chapter 7.

8.1.1 *Pharmacogenetics and Pharmacogenomics*

GPCR pharmacogenetics considers GPCR gene variants whether or not they cause disease. This definition originated with the early studies of GPCR variants in disease—focusing on mutations of prototypical receptors such as rhodopsin and the β -adrenergic receptor (1,2). These investigations generated the first insight into the locations and kinds of mutation that alter receptor function related to both pharmacogenetics and molecular pathology. At the same time, as new GPCR systems were identified, the concept of genomewide patterns of drug–host interactions began to emerge, and the field of pharmacogenomics came into its own.

Pharmacogenomics is a scientific endeavor that sets out to classify the structure and function of putative drug targets across the entire genome (3–5). Pharmacogenomics has enabled the identification of novel therapies by means of reverse pharmacology; that is, the use of a receptor class as “substrate” for novel compounds that might target them (6). These target receptors include those recognized to be of particular importance because of the disease state induced by a deleterious mutation. Pharmacogenomics also facilitates the identification of compounds that can compensate for pharmacogenetic variants (3), thereby eliminating a loss or gain of function associated with disease susceptibility or drug sensitivity (7). Pharmacogenomic studies, therefore, take pharmacogenetics into consideration when classifying and characterizing receptor systems.

This chapter traces developments in GPCR pharmacogenetics and pharmacogenomics that have resulted from the identification of GPCR variants in the general population that define individual drug responses (3,8,9). In some cases, early discoveries were contingent on the proof of principal that GPCR mutations could cause monogenic disease. Increasingly, however, GPCR pharmacogenetics has become an independent field that studies the genetic basis of drug response phenotypes

irrespective of disease state. The focus of this chapter is on variant GPCR genes that encode receptors that are important for clinical indications regardless of whether they are associated with disease predisposition.

8.1.2 Pharmacogenetics and Personalized Medicine

For example, rare variants of the human orexin-2/hypocretin-2 (OX2R) receptor, the Pro10Ser and Pro11Thr variants (**10**), have been associated with mild sleep disorders (**11**). Evidence that these single-nucleotide polymorphisms (SNPs) have lower efficacy for orexin ligands in sleep disorder patients who carry the variants suggests that the receptors might be useful as reagents in drug development (**11**). This example suggests the potential for “personalized medicine” that considers identifying the best pharmacological agent for a given variant, however rare it may be. The more moderate changes in signaling, while not always associated with disease, may have significant effects on drug efficacy in a clinical setting (**3**). Similar phenomena are reviewed with respect to many GPCR systems.

8.2 GPCR Pharmacogenetics

Although there has been an intensive effort to identify neurotransmitter GPCR variants associated with complex phenotypes, many of the phenotypes associated with these variants are pharmacogenetic. These studies are possibly confounded because recruiting patients with similar symptomatology is not a guarantee that they share the same underlying disorder (**3**).

The early work on monogenic diseases identified GPCR mutations in disorders such as stationary night blindness and rhodopsin (reviewed in Chapter 7 with respect to ligand binding, G protein signaling, and agonist-dependent desensitization and internalization). The framework for delineating genotype–phenotype relationships for GPCR pharmacogenetics, however, requires a distinct frame of reference (**1,12**). GPCR pharmacogenetics often deals with polygenic disorders with genetic and environmental risk factors that may not always coincide with those influencing drug responses.

8.2.1 The Calcium-Sensing Receptor: GPCR Variability in the Population

From the pharmacogenomic point of view, the calcium-sensing receptor (CASR) receptor differs from the majority GPCRs discussed in this chapter because it is not a family A receptor; it is a member of family C. However, study of the vast number of CASR variants provides useful insight into the pharmacogenetic principles that

apply to all GPCRs. Since the GPCRs share many characteristics of ligand binding, signaling, and downregulation, comparisons between members of GPCR families are valid and perhaps informative with respect to the biochemistry of receptor variants.

The *CASR* gene variants that give rise to monogenic disorders, reviewed in Chapter 7, are rare in the context of *CASR* variants found in the general population. By contrast, it is the more common variants that may be among the most clinically significant with respect to personalized medicine. This is because tissues that express variant forms of the *CASR* have altered $[Ca^{2+}]$ set points as a result of the altered sensitivity of the *CASR* receptor. Common polymorphisms in the *CASR* gene may account for significant population variation in calcium response and result in a variety of disease susceptibilities.

There are many polymorphisms scattered across the more than 100kb of genomic DNA that encodes the *CASR* protein; however, the common missense SNPs (Ala986Ser, Arg990Gly, and Gln1011Glu) are all clustered in the 3' cytoplasmic tail (*13,14*). The most common of these, the Ala986Ser variant, has proven to be predictive of the unbound, extracellular calcium fraction (*15,16*). Other *CASR* SNPs may also be important (*17*) in the general population.

If any of these variants are genetic determinants of the extracellular calcium concentration (*15*), they may also confer risk for disease states such as familial hypercalcemia. In some genetic backgrounds, the *CASR* variants may in turn be risk factors for a number of common disorders, such as hypertension and cancer. The Ala986Ser variant, for example, has been associated with bone mineral density (*18*), primary hyperparathyroidism (*19*), and Paget disease (*20*). The Ala986Ser variant is, however, a relatively mild inactivating variant that may predispose to hypercalcemia without being fully predictive of hypocalciuria. By contrast, the Arg990Gly variant appears to be better associated with activation of the renal *CASR*. This results in the increased calcium excretion that characterizes idiopathic hypercalciuria and is predictive of nephrolithiasis (*21*). Thus, the study of the different human phenotypes that result from the variable penetrance of polymorphisms of *CASR* (*22*) may result in a better understanding of the genetic basis of *CASR* pharmacological differences in the population.

8.2.2 Polymorphisms of the Angiotensin II Receptor in Hypertension

The complexity of GPCR pharmacogenetics is illustrated by studies of the genetic basis of hypertension and the efficacy of antihypertensives. Workers in this area are often concerned with the heterogeneity that underlies hypertension and the efficacy of antihypertensives.

Among antihypertensives, those targeting the renin–angiotensin system are among the best studied. The renin–angiotensin system consists of a two-enzyme cascade that is involved in the regulation of blood pressure and electrolyte homeostasis.

The renin enzyme cleaves the substrate, angiotensinogen, to angiotensin I, which is in turn cleaved by angiotensin-converting enzyme (ACE) (23) to generate angiotensin II (an octapeptide). Angiotensin II acts at the angiotensin II type 1 GPCR (AT1R) as a potent vasoconstrictor.

The cloning of the human genes encoding the AT1R (24) that recognizes the angiotensinogen ligand (25), produced by ACE (26), was quickly followed by the discovery of polymorphisms found to be significant risk factors for cardiovascular disease. Because antagonism of the AT1R is used to decrease blood pressure in hypertensive patients (27), the *AT1R* polymorphisms, such as *A1166C*, may be clinically significant. Located in the 3' untranslated region of the *AT1R* gene, the *1166 A>C* polymorphism is associated with hypertension (24), left ventricular hypertrophy (28), coronary heart disease, myocardial infarction (29), and progression of diabetic nephropathy (23,30). Pharmacological evidence suggests that the *A1166C* substitution is associated with altered receptor sensitivity. Some studies have suggested that the pharmacogenetics of *1166A>C* may be clinically important because it may be predictive of the success of antihypertensive drug treatment (31).

The fact remains, however, that *AT1R* gene *1166A>C* polymorphism may be a marker for cardiovascular disease as a result of linkage disequilibrium between this polymorphism and other variants of the *AT1R* gene or other genes in the region of chromosome 3q21–25 to which the *AT1R* gene maps. It is possible that *AT1R* gene expression in vivo is altered by the *1166A>C* polymorphism since, in vitro, homozygosity is associated with greater vasoconstriction (32). If this is the case, persons carrying the *C* allele may be at risk for the increased vasoreactivity (33) underlying higher blood pressure in some persons (24). The *C* allele may be of pharmacogenetic significance if it alters the outcome of AT1R antagonist treatment of high blood pressure.

The overall frequency of the *C* allele is approximately 25% in the Caucasian population (23). The *C* allele may well account for a variety of symptoms of heart disease, such as angina pectoris, in a large fraction of the population. Furthermore, it is possible that an epistatic interaction exists between the *AT1R* gene polymorphism, an ACE deletion/insertion variant, and a Met235Thr variant of the angiotensinogen gene (29) with respect to poor treatment outcome (23). In some study populations, the common *1166A>C* polymorphism of the *AT1R* gene appears to interact with the deletion (*D*) allele of the ACE gene in conferring risk for myocardial infarction (29). The heterogeneity of vascular disease risk factors (34,35), however, may explain why *AT1R* gene polymorphisms have not been consistently associated with disease (36) or pharmacology (27).

8.2.3 Neurotransmitter Pharmacogenetics

Antipsychotic drugs bind to many GPCRs and other targets, such as neurotransmitter transporters. These GPCRs include dopaminergic, serotonergic, and muscarinic receptors. The genomic structure and expression of several of these genes is probably relevant to understanding disease progression and therapeutic outcome (3).

8.2.3.1 Dopamine Receptor Pharmacology

Dopamine is a major catecholamine neurotransmitter in the central nervous system (37) that is involved in the neuroregulation of locomotor activity, emotion, and neuroendocrine secretion (38,39). Clinically, dopaminergic drugs are used to treat Parkinson's disease and schizophrenia by activating or blocking dopamine receptors, respectively (40).

Given that psychiatric disorders are probably the result of the complex interplay of genetic heterogeneity and environment, however, studies of GPCR gene sequence variants in a given population are not necessarily representative of all patient populations (41–43). Genomewide SNP association studies have met with some success in quantifying the additive contribution of GPCR genes, such as those encoding dopamine receptors, in some disorders (44,45). These studies have also been successful in identifying many non-receptor candidates.

Among the dopamine receptor genes, the dopamine D₁ receptor gene, for example, is essentially nonpolymorphic (41), at least in its exon–intron structure. While the 5' untranslated region (UTR) promoter SNP has been associated with a number of neuropsychiatric disorders and drug response phenotypes, these findings are not without controversy and may not always provide insight into receptor function in a disease state. It has been necessary to use site-directed mutagenesis *in vitro* to characterize receptor function with respect to the residues critical to desensitization, internalization (46), and downregulation (47,48)—subjects reviewed in Chapter 6. The fact that the dopamine D₁ receptor is so well conserved may reflect its importance to central nervous system function. It is the dopamine receptor with the widest expression in the brain and the highest affinity for dopamine (49).

Dopamine Receptor Variants

Caveats aside, the five dopamine receptors remain candidates in disease. The pharmacological properties that have been used to group them into dopamine D₁-like and dopamine D₂-like receptors may also be useful to consider when surveying the dopamine receptor gene association studies. For the most part, the D₁-like dopamine receptors D₁ (50–52) and D₅ (53) have not been as widely associated with disease as the D₂ dopamine receptors. The D₂-like receptors D₂ (54), D₃ (55), and D₄ (56) have similar dopamine sensitivities and are much more polymorphic than the D₁-like receptors (49,57).

The dopamine D₂-like receptor polymorphisms include SNPs, variable-number tandem repeats (VNTRs), and splice variants (58,59). The polymorphic forms of the dopamine D₄ receptor, for example, manifest as variable numbers of 48-bp repeat sequences (denoted D_{4,1} to D_{4,7}) (49).

The efficacy of antipsychotics, with respect to dopamine receptors, results mostly from blockade of D₂-like receptors. Binding of the classical antipsychotics (e.g., bromocriptine and raclopride), however, is about two orders of magnitude stronger at D₂ receptors compared with D₄ receptors. The atypical antipsychotics,

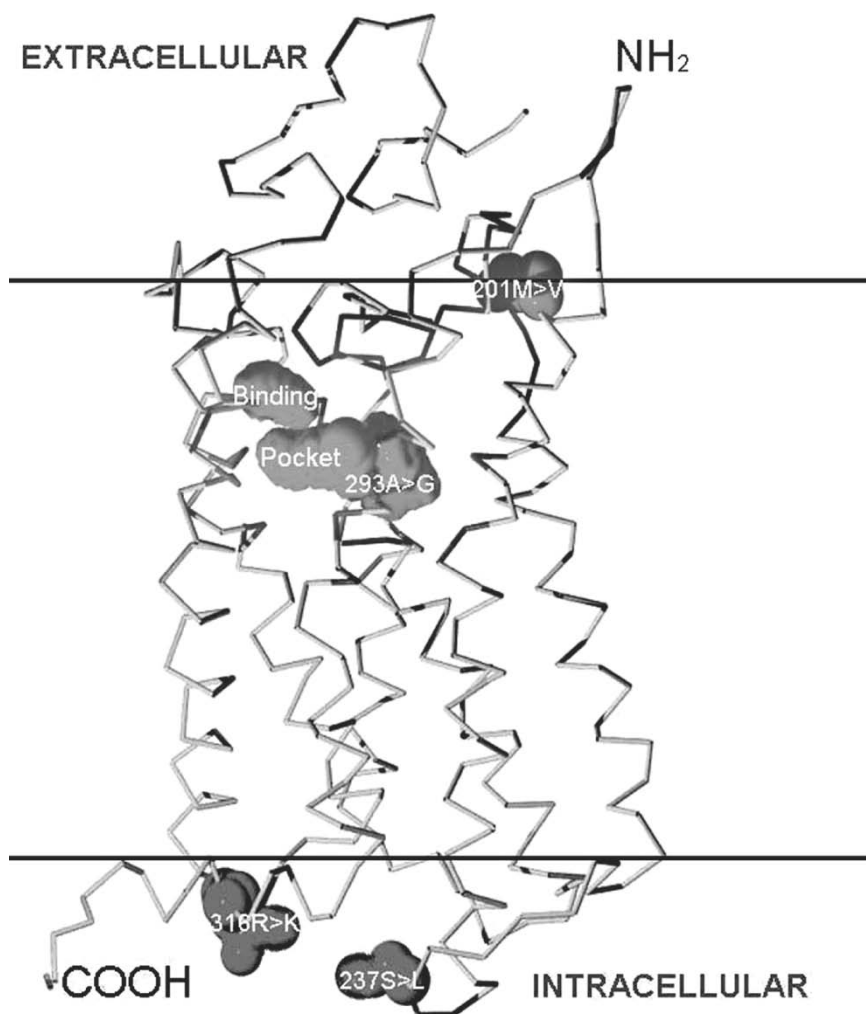


Fig. 8.1 Structure of the cysteinyl leukotriene 2 (CysLT₂) receptor and variants. The positions of the transmembrane (TM)-spanning domains of the CysLT₂ receptor, the putative binding pocket, and four naturally occurring amino acid substitutions are shown in relation to the cutaway plasma membrane. Of the four single amino acid variants discovered (Met201Val, Ser237Leu, Ala293Gly/Arg316Lys), only the partially inactivating Met201Val variant may be associated with the asthma or atopy phenotypes. The Ala293 variant, found in the context of a compound heterozygote, Ala293Gly/Arg316Lys, results in an activating variant that is predicted to disrupt the putative binding pocket that was predicted from rhodopsin

such as clozapine, however, are less potent at the dopamine D₂ and D₃ receptors compared with the D₄ receptor (49). Clinically, however, the potency of clozapine at dopamine D₄ receptor variants such as D_{4.2}, D_{4.4}, and D_{4.7} are probably similar under therapeutic conditions (49).

Dopamine Receptor Association Studies

Many variants of the dopamine receptors have been tested for association with psychiatric and drug response phenotypes (42). These studies have been based on the candidate gene hypothesis, which argues as follows: Because antipsychotic pharmaceutical agents target dopamine receptors, especially the D_2 -like receptors, disruptions in the receptors, and accessory proteins, may be the cause of disease (3).

With respect to the dopamine D_1 -like receptors, including the dopamine D_5 receptor, which is ten times more sensitive to dopamine and has a much more narrow tissue expression than the dopamine D_1 receptor, few studies have found evidence of coding variants associated with a disease state (41,42). Untranslated promoter SNPs, however, have been associated with various disease states (60). These studies, while equivocal, suggest association with bipolar disorder, alcoholism, and attention-deficit disorder to name a few (60–65).

By contrast, studies of the dopamine D_2 -like receptors have found evidence for the association of the receptor with disease (66); these studies have been replicated (41,42). From among the multitude of these studies, only selected examples are reviewed here. For example, evidence both for and against the association of the dopamine D_2 -like receptors with schizophrenia has been reported. Polymorphisms of the dopamine D_4 receptor, including the third intracellular loop VNTR, alter dopamine D_4 receptor expression. In addition to association with schizophrenia (3,67–70), the dopamine D_4 polymorphisms have been associated with the genetic basis of the variable efficacy of antipsychotics such as clozapine (or neuromuscular toxicity—tardive dyskinesia) (69,71,72). Similarly, promoter SNPs have been associated with altered clozapine efficacy (67,68,73).

The evidence indicating that dopamine D_4 polymorphisms contribute to schizophrenia remains under investigation even though the elevation of dopamine D_4 receptor expression in schizophrenia is reproducible (71,72). A V194G polymorphism has been associated with increased receptor protein expression (68,69). In addition to the major psychoses, disorders such as attention-deficit/hyperactivity disorder (ADHD) (74–77) and novelty-seeking behavior (78–80) have been also associated with D_4 receptor, although some negative findings have been reported.

Studies of the dopamine D_3 receptor in schizophrenia (81–83) have yielded variable results. Tardive dyskinesia in schizophrenic patients treated with clozapine, however, has been associated with D_3 receptor variants (84–86). These findings suggest that although GPCR gene variants may not always contribute to a disease phenotype, they may be associated with genetic variability in pharmacology or pharmacogenetics.

Similar associations have been reported between dopamine D_2 receptor variants with Tourette's syndrome, obesity (87–89), and alcohol dependence (90–93), although these findings are still the subject of debate in the literature. From the point of view of pharmacogenetics, the *Taq1A* polymorphism of the dopamine D_2 receptor is associated with the development of tardive dyskinesia (88,94,95). While the results of these association studies vary (3,12), these data clarify our under-

Many studies have shown associations of SNPs in such genes as the serotonin 5-HT_{2A} and 5-HT_{2C} receptors, genes and the histamine H₂ receptor gene, not to the phenotype of disease severity, but to phenotypes such as drug response or nonresponse (97). For example, the 5-HT_{2A} receptor gene variants and the variant that encodes the 5-HT_{2C} Cys22Ser receptor have been associated with altered responses to clozapine (97).

The 5-HT_{2A} variants such as H452Y have been associated with decreased calcium flux in response to clozapine that likely results from decreased Gq signaling (97,102–106). It is interesting to note that while depression has not been consistently associated with 5-HT_{2A} polymorphisms, such as –1348A/G in the promoter, no response to the antidepressant citalopram may be associated with 5-HT_{2A} in these patients (107,108).

There are data to confirm and reject the association of the Cys23Ser 5-HT_{2A} and the Gly22Ser 5-HT_{1A} receptor variants, characterized in vitro by reduced agonist potency, with phenotypes such as intractable suicidal ideation (98), ADHD (100), alcohol dependence, and schizophrenia (98,99,109–116). While the –1348 A/G polymorphism of the 5-HT_{2A} receptor has been associated with the negative symptoms of schizophrenia, other studies of eating disorders appear to be equivocal. A body of evidence is available, however, that 5-HT_{2A} variants may be associated with psychotic symptoms in Alzheimer's patients (94,100,117,118).

Studies of the 5-HT_{2C} receptor polymorphism are in many ways similar. While promoter polymorphisms have been associated with clozapine-induced weight gain (119–124), the C23S variant has been associated with increased clozapine response. Similar to that reported for the 5-HT_{2A} receptor, there is better evidence that the C23S variant may be associated with psychotic symptoms in Alzheimer's disease than other psychiatric symptoms such as suicide ideation (97,117,125,126). Further evidence of serotonin dysfunction in Alzheimer's disease can be found in the association of the 5-HT₆ gene C267T (267C allele) with increased risk for the disease (127–129).

Polymorphisms of major receptors for the triptan drugs used to treat migraine, the 5-HT_{1B} and 5-HT_{1D} receptors, have also been studied with respect to pharmacogenetics and disease. While a rare variant of the 5-HT_{1B} receptor, the F124C variant, has been reported, no coding SNPs for the 5-HT_{1D} receptor have been reported. While the F124C 5HT_{1B} variant has been shown to have a higher affinity for ligand, it has not been associated with any disease state (130–132). On the other hand, many SNP association studies have implicated the 5-HT_{1B} and 5-HT_{1D} receptors with, respectively, ADHD (132–134) and obsessive–compulsive disorder (135–139). With respect to both disorders, however, the evidence is far from conclusive.

In view of this, the key to providing better treatment to the surprisingly large number of poor responders both to antidepressants and antipsychotics may lie in genomewide association studies (140). Association studies have been reported with over 40 SNPs and ADHD symptoms (44). The pharmacogenomic approach to identifying candidate loci in depression depends on genomewide mapping of SNPs contributing to altered drug response (12). The pharmacogenomic strategy may also identify novel GPCR candidate genes and other genes that interact to create a polygenically determined responder/nonresponder phenotype (5).

8.2.3.3 Opioid Receptor Polymorphisms

Many studies of opioid receptor gene variants have been associated with altered pharmacology. Although there is evidence that addiction has a genetic component, studies of the μ -opioid receptor in relation to opioid addiction have general relevance to pharmacogenetics. This is not only because the μ -opioid receptor is an opioid drug target but also because opioid neurons have been implicated in other addictions, such as alcohol dependence (*12*).

In this context, there has been extensive study of the pharmacogenetics of the coding μ -opioid receptor variants, the Asn40Asp, Asn152Asp (*141,142*), His260Arg, His265Arg, and the Ser268Pro SNPs (*142,143*). While the Asn40Asp and Asn152Asp variants have thus far not been associated with addiction, however, they do have properties that are distinct from the wild type. These variant receptors bind the natural β -endorphin ligand with fourfold higher affinity and are trafficked to the cell membrane at reduced levels (*141*). These studies described the pharmacogenetics of the μ -opioid receptor.

Through disruption of the calmodulin kinase II site required to maintain a basal level of receptor signaling (*141,142*), the Ser268Pro variant results in diminished receptor desensitization. This effect may be attributable to elimination of the competition for the Ser268 residue that normally exists between calmodulin kinase and the G_i/G_o protein (*144*). As a result, the ²⁶⁸Pro receptor variant is more frequently found in the active conformation necessary for ligand binding. The variant is possibly involved in addiction because people expressing the receptor variant are predicted to have an altered tolerance for opioid ligands. The low frequency of the variant, even among addicted individuals, however, may limit its significance (*12*).

In view of the difficulty in identifying variants that are clearly associated with addiction, the pharmacogenomic approach may identify other variant genes disrupting portions of opioid signaling (*12*). A study of μ -opioid receptor identified haplotypes comprised of two coding SNPs, Ala6Val and Asp40Asn, that may be more frequent among opiate addicts of African American descent (*145,146*).

The difficulty of performing candidate gene studies on very rare SNPs suggests that studies of the complex genetics of psychiatric disease may move from a pharmacogenetic model (with candidate gene analyses) to a pharmacogenomic one (with genomewide searches). There is evidence suggesting that neurotransmitter GPCR variants are often associated with a drug response phenotype even when not associated with a neuropsychiatric disorder.

8.2.4 Pharmacogenetics of Adrenergic Receptors

An understanding of GPCR pharmacogenetic variants, however, may be important in populations who are at risk to unusual drug reactions. One of the best examples

of how pharmacogenetics has the potential to define personalized medicine has evolved from the study of adrenergic receptors. Mutated forms of GPCRs, such as adrenergic receptors, can elicit a wide spectrum of disease phenotypes or altered drug efficacies. Polymorphic adrenergic receptors have been reported to result in both gain and loss of receptor efficacy or potency phenotypes. Genetic variants do not always result in molecular defects so dramatic that they constitute a measurable risk for disease phenotype.

The adrenergic receptors are widely expressed. They serve as receptors for the catecholamines epinephrine and norepinephrine and are targets for therapeutic agonists or antagonists in asthma and heart failure treatment (*147,148*). Studies of adrenergic receptor genomics have revealed that allelic variants of these receptors are common. Although the adrenergic receptor variants were among the first GPCR polymorphisms to undergo extensive in vitro study (*149*), in many cases the clinical importance of these allelic variants is only now emerging (*147*).

Coding and promoter polymorphisms of adrenergic receptors that cause altered expression, ligand binding, coupling, or regulation phenotypes have been identified. For example, the Pro64Gly variant of the β_3 -adrenergic receptor, expressed in adipose tissues, is associated with some cases of obesity (*150*). A further example of adrenergic receptors with a phenotypic alteration in signaling properties has been found in studies of the β_1 -adrenergic receptor (*151*) and α_{2A} (*152,153*); however, neither inactivated nor constitutively activated receptors are the result. The Arg389Gly β_1 -adrenergic receptor variant and the Asn251Lys α_{2A} -adrenergic receptor variant result in a gain in second-messenger signaling (efficacy and potency). This results in a shift to the left in agonist-elicited second message that is similar to that shown in [Fig. 8.3A](#) with respect to variants of the cysteinyl leukotriene 1 (CysLT₁) receptor. While these variants are common in the population, and they are potentially significant with respect to drug efficacy, they are also potential risk factors for disease (*152–154*).

8.2.4.1 Downregulation Polymorphisms in the β_2 -Adrenergic Receptor

The gene encoding the β_2 -adrenergic receptor displays a fair degree of polymorphism in the human population. Like the dopamine receptors, the β_2 -adrenergic receptor variants are often relevant to pharmacogenetics. Constitutively active mutant (CAM) and loss-of-function (LOF) variants are in evidence.

Nonetheless, the pharmacogenetics of the β_2 -adrenergic receptor is complex. For example, the allele distributions of polymorphisms at amino acid positions 16, 27, and 164 are skewed in asthma, hypertension, obesity, and some immune disorders. Among these, the Arg16Gly receptor displays enhanced agonist-promoted downregulation, suggesting that this receptor may be rapidly lost from the cell surface and degraded in lysosomes. By contrast, the Gln27Glu polymorphism is actually resistant to downregulation (*148,155*).

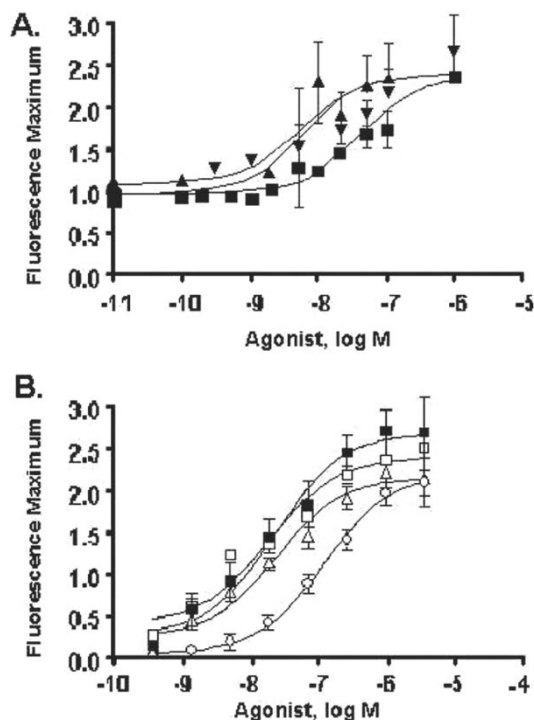


Fig. 8.3 In vitro effects of Gly300Ser cysteinyl leukotriene 1 (CysLT₁) receptor and Met201Val on CysLT₂ receptor signaling compared with wild type. **A** Cysteinyl leukotriene D₄ (LTD₄) concentration–response curve for CysLT₁ receptors in transfected cells. Inositol triphosphate (InsP) generation assay of the variants and wild-type forms of the CysLT₁ receptor. both 300S and 206S variants' EC₅₀ were significantly different from wild type. The concentrations of LTD₄ required to produce the InsP effect were much higher than those used in the [Ca²⁺]_i assay shown in **B**, in which calcium flux was assayed for the variants and wild-type and variant forms of the CysLT₂ receptor challenged with LTD₄. The resulting changes in intracellular calcium concentrations were measured as fluorescence maximum. For LTD₄, the Met201Val variant (o) had a significantly greater EC₅₀ compared to wildtype (■), while the Ser237Leu (Δ) and Ala293Gly/Arg316Lys (□) variants were not different. However, the Ala293Gly/Arg316Lys variant showed decreased efficacy (V_{max}). Interestingly, when the Ala293Gly/Arg316Lys receptor was challenged with the agonist Bay u9773 (data not shown), this rare variant was demonstrated to have a significantly smaller EC₅₀ compared to wild type, indicating that, under some circumstances, the variant is activating (167)

8.2.4.2 Heart Disease Associated with β₂-Adrenergic Receptor Polymorphisms

Variants of the β₂-adrenergic receptor, especially the Thr164Ile polymorphism, have been associated with increased severity of congestive heart failure. Heart failure subjects with the Thr164Ile mutation have a 1-year survival rate of 42%, as compared with 76% for a control group with the wild-type β₂-adrenergic receptor (154,156). Carriers of the 164Ile polymorphism therefore may be candidates for

more aggressive treatment of disease (*156*). By contrast, the Arg16Gly and Gln27Glu polymorphisms do not appear to influence the disease course (*157*).

8.2.4.3 Myasthenia Gravis and β_2 -Adrenergic Receptor Polymorphisms

Myasthenia gravis (MG) is an autoimmune-based failure of cholinergic transmission at the neuromuscular junction to which variant forms of the β_2 -adrenergic receptor have also been associated. The disorder is associated with decreased density of β_2 -adrenergic receptors on peripheral blood mononuclear cells, particularly in patients with the Arg16Gly variant. ^{16}Gly is also associated with antibodies to the variant β_2 -adrenergic receptor and the secretion of cytokines in response to β_2 -adrenergic receptor peptide fragments. In addition, acetylcholine receptor antibodies have been measured at higher levels in patients homozygous for β_2 -adrenergic receptor variants (*158,159*).

These and other findings suggest that the β_2 -adrenergic receptor is involved with the pathophysiology of MG. The role of the β_2 -adrenergic receptor in development of MG has been confirmed by evidence suggesting that increased ^{16}Gly homozygosity and lower prevalence of ^{16}Arg homozygosity is characteristic of MG patients (*158,159*). These data on the β_2 -adrenergic receptor suggest that GPCR pharmacogenetic variants are sometimes also associated with disease susceptibility.

8.2.5 Asthma GPCR Pharmacogenomics

Studies of GPCRs in asthma can be differentiated on the basis of whether they measure the contribution of candidate genes to atopy, bronchial hyperreactivity (BHR), drug response/nonresponse, or another phenotype. The contribution of selected GPCR variants to the risk for developing asthma or altered drug response is reviewed.

8.2.5.1 The β_2 -Adrenergic Receptor

The evidence suggests that the involvement of β_2 -adrenergic receptor (*ADRB2*) variants in the development of asthma and adrenergic drug pharmacogenetics (*160*). Although the β_2 -adrenergic receptor Arg16Gly variant (*p.R16G*) is associated with reduced lung function (*158*) and familial nocturnal asthma (*12,161*), it is also commonly resistant to some β_2 -adrenergic receptor agonists (*162*). This may result from receptor loss from the cell surface during defective downregulation. Not surprisingly, in the event that drug response phenotypes and disease phenotypes result from the same genetic variants, clinical management can become very difficult.

Given the difficulty of analyzing the contribution of the many β_2 -adrenergic receptor variants to various phenotypes, many studies refine the analysis by

constructing haplotypes consisting of two or more variants on the same chromosome (i.e., in *cis*). For example, the variants encoding the *R16G* and *Q27E* variants form a haplotype that has been used to predict treatment outcome to the β_2 -adrenergic receptor agonist albuterol (*12*). Carriers of these variants have a complicated phenotype because the downregulation-resistant *p.Q27E* receptor results in β_2 -adrenergic receptor hypersensitivity that potentially complicates the treatment (*163*). Because these phenotypes are complex, however, these findings may not explain all cases of albuterol hypersensitivity (*164*).

The β_2 -adrenergic receptor gene mutations therefore have been associated with a wide spectrum of respiratory phenotypes that include altered drug responses and bronchial hyperreactivity disease. The β_2 -adrenergic receptor polymorphisms probably represent only a few of the genetic variables involved in asthma pathophysiology (*157,165*). Their significance, however, may be great if SNP screening could be used more widely to personalize diagnosis and treatment options.

8.2.5.2 The Cysteinyl Leukotriene Receptor System

It is possible that genetic variability in the genes encoding proteins critical to the CysLT pathway (see [Fig. 8.1](#)) may contribute additively or synergistically to altered drug responses. *CysLT* gene variability has also been observed in mice, in which the *CysLT₁* receptor gene can undergo alternative splicing that has functional consequences (*166*). Studies of CysLT₁ and CysLT₂ variants focus on how they might alter the response of the receptor to agonists and on their possible contribution to the atopy phenotype (*167*).

CysLT₁ Receptor Pharmacogenetics

The CysLT₁ receptor has been associated with atopic asthma in at least one geographically isolated population resident on Tristan da Cunha. This is intriguing from the point of view of personalized medicine because drugs that act as high-affinity antagonist ligands of the CysLT₁ receptor (e.g., montelukast, pranlukast, zafirlukast) (*168–176*) or allergic rhinitis (*177*) have been reported to be ineffective in approx. 20% of patients (*178*). The discovery that there are at least four *CysLT₁* transcripts generated by alternative splicing contributed further to the heterogeneity and should be examined in conjunction with the SNP data (*179*).

Although it is possible that the *CysLT₁* receptor gene may harbor inactivating mutations in some populations, studies of the Tristan da Cunha population have only identified the unremarkable Ile206Ser (*p.I206S*) variant and an activating Gly300Ser (*p.G300S*) variant. Unfortunately, additional clinical correlations between *CysLT₁* receptor genotypes and drug response are not reported for the study population.

CysLT₂ Receptor Pharmacogenetics

The CysLT₂ receptor may also be important to the pharmacology of CysLT₁ pharmaceuticals if, like many GPCRs (*180*), these receptors form functional heterodimers with unique pharmacological properties. While specific CysLT₂ receptor antagonists have not been marketed, drug development based on targeting the CysLT₂ receptor may be important, given that approx. 20% of patients treated with CysLT₁ agents fail to respond. This problem may become particularly important in patients for whom both the CysLT₁ and CysLT₂ receptors are polymorphic.

A Met201Val variant has been associated with atopy in populations including Tristan da Cunha. Unlike the *p.G300S* CysLT₁ variant, however, the *p.M201V* variant is partially inactivating. The fact that CysLT₁ and CysLT₂ are both polymorphic in some individuals suggests that the coexpression of variant receptors may alter CysLT signaling.

Interaction of CysLT₁/CysLT₂

In the study of the isolated population of Tristan da Cunha, the activating CysLT₁ *p.G300S* variant and the inactivating CysLT₂ *p.M201V* variant receptor were both associated with atopic asthma. This raises the question: Could these variants interact in some populations to confer risk for atopy? This question, along with the question of whether the CysLT₁ and CysLT₂ receptor variants can form heterodimers, remains to be addressed. However, the fact that all persons on Tristan da Cunha reported to be heterozygous for both CysLT₁ and CysLT₂ receptor variants were atopic suggests that more work in this area should be done.

The relative location of each variant is shown in the alignment of each CysLT receptor with rhodopsin (*see Fig. 8.2*). This alignment was used to predict the transmembrane-spanning and the putative binding pocket of the CysLT₂ receptor. This suggests that variants of these receptors modify the putative CysLT binding site that is partially determined by the integrity of their respective transmembrane domains. The abnormal but opposite pharmacology of the variants of these receptors, causing increased potency of cysteinyl leukotriene D₄ (LTD₄) at the *p.G300S* receptor variant (located in the intracellular portion of in transmembrane domain 7) and decreased potency of LTD₄ at the *p.M201V* receptor variant (located in the extracellular portion of transmembrane domain 5) deserves further investigation.

8.2.5.3 Endothelin 1 Type A

Endothelin 1 (ET1) is a 21-amino acid peptide released from bronchial cells. It has potent vasoconstrictive agonist properties mediated by two receptor types (A and B). The involvement of the endothelin 1 type A (*EDNRA*) gene (*AfiII* SNP) in atopy, however, is marginal at best and as yet not widely replicated (*181*). For

example, the *Afil* SNP was associated with atopy concurrent with elevated antigen-specific immunoglobulin E (IgE) levels in a British population (182).

8.2.5.4 Prostanoid DP Receptor

The prostaglandin D_2 receptor (DP), a target for prostaglandin D_2 (PDG_2), is encoded by the *PTGDR* gene located on chromosome 14q22.1. The DP receptor SNPs associated with asthma are located in the gene's promoter. Determining the functional relevance of these variants is complicated by the fact that PDG_2 also acts on the chemoattractant on T-helper type 2 (T_H2) cells receptor (CRTH2). Although these receptors both bind the proinflammatory eicosanoid PGD_2 , they appear to have opposite signaling properties (183).

While DP receptor activation is associated with amelioration of asthma pathology, the activation of CRTH2 increases eosinophil recruitment at inflammatory sites—pathological changes characteristic of atopic dermatitis and allergic asthma (70). It is possible that maintaining a greater expression of prostanoid DP relative to CRTH2 may protect against the deleterious effects of PGD_2 . The DP receptor gene promoter polymorphism therefore appears to alter receptor expression to protect against BHR (184,185).

8.2.5.5 Chemoattractant Receptor

The *CRTH2* gene, which encodes the receptor for PGD_2 , is located within a linkage region for asthma on chromosome 11q (186–188). CRTH2 is expressed on basophiles and eosinophils (189–191) and is involved in the regulation of allergic inflammation (192). Two common SNPs, 1544G>C and 1651G>A, in the 3'-untranslated region of *CRTH2*, show evidence of linkage with asthma that was refined, by haplotype analysis, to the linkage disequilibrium of the 1544G + 1651G haplotype.

8.2.5.6 Thromboxane Receptors

Thromboxane A_2 ($TBXA_2$) binds to a specific receptor, the prostanoid thromboxane (TP) receptor ($TBXA_2R$), which in turn signals, through activation of the $G_{q/11}$ family of G proteins, the mitogen-activated protein kinase (MAPK) pathway and the protein kinase A pathway. $TBXA_2$ is the most potent of the prostanoids. The *TBXA2R* gene, located on chromosome 19p13.3, results in two receptor isoforms as a result of alternative splicing of the carboxyl terminus. It plays a vital role in inflammation, platelet aggregation, and the degree of vasoconstriction. Two *TBXA2R* gene isoforms result from alternative splicing of the carboxyl terminus. These isoforms, $TP\alpha$ and $TP\beta$, share the first 328 amino acids (193,194,311–313). *TP* receptor gene alternative splicing may represent a source of variability in BHR.

The TP β isoform, for example, undergoes agonist-induced internalization (195) that results in the loss of this isoform from the population of cell surface receptors.

Many aspects of BHR are potentially mediated by the TP receptor isoforms, making these variants candidates in the pathophysiology of asthma (196–198). In addition, a rare bleeding disorder that results from failure of platelet aggregation has been attributed to distinct variants of the TP receptor. The Arg60Leu variant of the TP α isoform has been associated with the failure of platelet aggregation (193) that probably results from a mechanism distinct from those resulting in BHR. Located in the first cytoplasmic loop of the receptor, the Arg60Leu variant impairs cyclic adenosine monophosphate (cAMP) accumulation and phospholipase C (PLC) activity, while leaving ligand binding intact. Interestingly, the homologous mutation of the TP β isoform was not deleterious, possibly because it acts through G $_i$ /G $_o$ systems (193), while the TP α isoform may act through G $_s$.

With respect to BHR phenotypes and asthma pharmacology (199), the relevance of the TBXA $_2$ system derives from the fact that alveolar macrophages, eosinophils, and platelets increase the production of TBXA $_2$ during lung inflammation. Blocking TBXA $_2$ action may prevent constriction of pulmonary vasculature and airway smooth muscle (ASM). Thus, TBXA $_2$ appears to be involved in microvascular leakage, mucus secretion, and ASM proliferation (199).

TP receptor signaling has been extensively documented in vascular smooth muscle and platelets, but its characterization in human ASM cells has been more limited until recently. ASM cells express messenger RNA (mRNA) for both TP receptor isoforms, and functional receptors respond to agonist with an increase in intracellular Ca $^{2+}$ concentration (200). As a consequence, besides potentiating the epidermal growth factor (EGF) mitogenic response independently from transactivation of the EGF receptor (EGFR) (200), TP receptor stimulation induces a concentration-dependent increase in DNA synthesis.

The TP receptor requires the G $_i$ /G $_o$ protein to activate the Src-Ras-ERK1/2 (extracellular signal-regulated kinase 1 and 2) cascade to induce the proliferative response, which in turn promotes the rapid nuclear translocation of activated ERK1/2 (201). Because TP receptor may be activated by many inflammatory mediators (202–204), these findings suggest new therapeutic strategies that alter the ASM hypertrophy or hyperplasia observed in the chronic airflow obstruction and airway inflammation that characterizes asthma, chronic bronchitis, bronchiolitis obliterans, and chronic obstructive pulmonary disease.

TBXA $_2$ R gene variability may also contribute to interindividual differences in the efficacy of pharmaceutical agents that act on this system. A positive association between a polymorphism in the TBXA $_2$ R gene and risk of asthma, atopy, and the aspirin-intolerant asthma (AIA) phenotype has been identified (205–208). These drugs include the synthase inhibitor ozagrel hydrochloride (OKY-046); the TP receptor antagonist seratrodast (AA-2414); and ramatroban (Bay u3405), a TP receptor antagonist that is undergoing clinical trial (199).

The TBXA $_2$ R gene splice variants result, therefore, in protein structures with distinct functions. An amino acid substitution that is deleterious in one splice isoform, however, may only be a polymorphic marker in another. This phenomenon

may have far-reaching pharmacogenetic consequences because one copy of the mutation is adequate to prevent TP α signaling and possibly disrupt receptor dimerization (193).

8.2.5.7 The Chemokine System

Chemokines are the largest family of cytokines. Four invariant cysteines define the chemokine proteins. They are grouped on the basis of the conservation of the domain containing the first two cysteines.

The involvement of the chemokine system in asthma has become evident since the genomics of chemokine receptors has been elucidated. Among these receptors, the CCR5 receptor binds natural ligands including the CC chemokines, such as RANTES (regulated on activation, normal T cell expressed and secreted), the macrophage inhibitory proteins MIP1 α and MIP1 β , and the monocyte chemoattractant protein 2 (MCP2). The gene is located at the chemokine receptor gene cluster region on 3p21.

The CCR5 Δ 32 polymorphism, a 32-bp deletion polymorphism of the promoter of this receptor, has been associated with protection against human immunodeficiency virus (HIV) infection and asthma (209,210). While the contribution of the CCR5 receptor to immune diseases is probably better understood in the case of HIV infection, however (210–212), our discussion opens with its role in asthma.

The CCR5 Δ 32 polymorphism diminishes CCR5 receptor expression in type 1 T-helper (Th1) cells, which may result, indirectly, in the greater Th1 cell activity that is associated with asthma. The variant causes a decrease in CCR5 binding to endogenous CC chemokine agonists such as RANTES MIP-1 α and MIP-1 β (213). This signal is associated with the greater Th1 cell activity that results in asthma.

This adds to the growing evidence that asthma is associated with a systemic increase in the production of the allergic Th2 cytokines (211,214) that was noted in the discussion of the prostenoid DP₁ receptor. Interestingly, the mechanism that preferentially maintains Th2 cells, TIM-1, has been implicated in pathways maintaining allergic responses (215).

The biochemistry of cytokine and chemokine involvement in asthma is complex (216,217). Future studies will distinguish the relative importance of these systems to asthma. The involvement of the cytokine pathway in asthma, however, has been confirmed by the linkage of a locus for an enzyme in this pathway, dipeptidyl peptidase (DPP10), located on chromosome 2q14–32.

8.2.6 Polymorphisms of the Chemokine Receptors in Infection and Immunity

Studies of coreceptors have suggested novel avenues for developing therapeutic and preventive strategies against HIV and acquired immunodeficiency syndrome

(AIDS). These strategies build on an understanding of the fundamental aspects of HIV-1 transmission and pathogenesis (218–221). This is briefly reviewed.

8.2.6.1 CCR5

CCR5 is known to be an important coreceptor for macrophage-tropic viruses, including HIV. Expression of CCR5 is detected in a promyeloblastic cell line, suggesting that this protein plays a role in granulocyte lineage proliferation and differentiation. The polymorphic 32-bp *CCR5* promoter deletion, resulting in promoter inactivation, confers strong resistance to HIV-1 infection.

8.2.6.2 Other Coreceptors

Other GPCRs that act as coreceptors for the HIV virus include the CCR2 and CCR4 receptors, which have been identified as receptors for T-cell line-tropic and macrophage-tropic HIV-1 isolates. The role of CCR2 and CCR3 was identified partly because another CCR5 variant, the Val64Ile variant, was found to be genetically associated with resistance to HIV infection and to result in abnormal heterodimerization with CCR2 and CCR4 in vitro (222–225). Thus, it is possible that aberrant CCR heterodimerization may be another contributor to the modulation of HIV resistance.

CCR2, CCR3, and other coreceptor proteins provide additional insight into resistance to infection and how some HIV-1 strains are selectively targeted to specific tissues. In contrast, it has been suggested that the expression of G protein-coupled receptor 1 (GPR1) in the kidney mesangial tissues results in increased susceptibility to variant HIV-1 infection. The GPR1 protein may also be involved in nephritis associated with AIDS progression (226). The transmission of macrophage-tropic variants and the subsequent appearance of T-cell line-tropic variants are two of the axes that can be tested with respect to coreceptor polymorphisms (220).

8.2.6.3 CCR2 and CCR3 Polymorphisms

The number and polymorphic variety of HIV coreceptors is still under investigation. The CCR2B and CCR3 receptors, however, appear to function as minor coreceptors. A common Val64Ile substitution of the CCR2 receptor is associated with the delayed progression of HIV infection to AIDS. Although the variant has been shown to delay disease progression, it does not reduce risk of infection (227).

CCR3 gene missense polymorphisms, including the Arg275Glu substitution in the third extracellular loop and the Leu302Pro substitution in the intracellular cytoplasmic tail, have been identified. As yet, however, no phenotype has been associated with these polymorphisms (228).

Thus, while polymorphisms in GPCR coreceptors are associated with altered viral infection, the phenotype corresponding to a given genetic variant may be difficult to identify. Genetic differences in amino acid sequences, however, might be useful in identifying persons with a specific disease-modifying phenotype that might be targeted by a specific drug response. The pharmacogenomic hypothesis anticipates that polymorphism-induced alterations in receptor–host interaction will be a valuable focus of future drug development efforts (229).

8.2.7 Polymorphisms of the Platelet-Activating Factor Receptor

The platelet-activating factor (PAF) receptor (PAFR) mediates the proinflammatory and vasoactive actions of PAF. Interindividual variation in PAF-related physiological response and anti-inflammatory drug responsiveness results from the substitution of Ala224Asp in the third intracellular loop of the PAFR (230). Since the ⁶³Asp residue is part of the DRY motif that is involved in regulating ligand affinity, it may be a structural requirement for G protein coupling to the receptor (231). In vitro studies suggested the Ala224Asp results in a significant reduction of the PAF-induced intracellular signals that include calcium mobilization, inositol phosphate production, and inhibition of adenylyl cyclase. The reduction in these signals is associated with a phenotype in vitro of reduced chemotaxis. These data suggest that this PAF variant may be selectively targeted in some patients. The pharmacological potential of targeting such variants by reverse pharmacology is suggested by the fact that the variant was present at an allele frequency of 7.8% in a sample from a population in Japan (230).

8.2.8 GPCR Mediation of Interactions Between Virus and Host

The study of the genomics of the GPCRs involved in infection, inflammation, and disease progression has identified novel classes of receptor genes that may become pharmaceutical targets. The potential for pharmaceutical intervention into viral infection has been established not only for HIV progression but also for the development of Kaposi sarcoma (KS), a common sequel resulting from the Kaposi herpes virus, KSHV. The potential of GPCR pharmacogenomics is suggested by experimental evidence supporting a key role for a particular *KSHV* gene, a constitutively active G protein-coupled receptor (vGPCR), in the development of KS. Although this receptor, like the cytomegalovirus (CMV)-encoded GPCR (232), originates in a nonhuman genome, it is able to function in human cells and thereby coopt many functions. In particular, it is able to function as a receptor for human ligands affecting immunomodulating cytokines such as interleukin 6. This GPCR may facilitate viral control of the host pathways that regulate angiogenesis needed to sustain tumor growth (233–236).

A complex interaction between human GPCR genes and those from nonhuman sources is emerging. The expression of some nonhuman GPCRs may be beneficial

in that they protect the host against the virus (providing constitutional resistance), while others may compound the difficulty of treating viral infections with new antiviral agents. In the case of CMV, for example, viral strains may encode four potential chemokine receptors (US27, US28, UL33, and UL78). Of these virally encoded chemokine receptors, US28 binds many endogenous human CC chemokines (232) *in vitro*. The expression of the foreign genomic material in human cells may therefore promote CMV infection (237).

Another example of the complex interaction between host and virus genomes has been demonstrated in the case of human Epstein–Barr virus infection. By contrast with herpes simplex virus (HSV) and CMV, the Epstein Barr virus is equipped to induce expression of the human GPCR genes encoding the EB1 and EB2 receptors during the course of infection, facilitating the spread of infection. The virus may promote infection (238) by interacting with human promoter elements—a common site for polymorphic mutation. The severity of infection therefore may hinge on whether a certain viral strain can coopt the regulation of a human GPCR gene that is critical to infection. GPCR pharmacogenomics thus provides insight into the contribution of viral and human GPCR to many human viral infections.

8.2.9 GPCR Mutations in Developmental Disorders and Cancer

In addition to their effects on metabolism, GPCRs and G proteins also play a role in the regulation of cell growth, differentiation, dysplasia, and neoplasia. Autonomous cell growth, resulting in neoplastic transformation (239), is associated with naturally occurring mutations both in GPCRs and in G protein α -subunits. These phenotypes suggest that the GPCR component of the genome is critical to normal differentiation and development.

Cell division can be induced by a number of mechanisms, including those transducing mitogenic signals from the cell membrane to the nucleus. Mitogenic signaling by GPCRs results from the convergence of signals emanating from many different classes of GPCRs expressed on the cell surface. The common pathway involves the ERK MAPK cascade, although receptor and nonreceptor tyrosine kinases also play central roles. The advent of pharmacogenomics has facilitated the understanding of how receptor, G protein, and tyrosine kinases contribute to the mitogenic signaling of normal and transformed cells. Reverse pharmacology may ultimately allow the rational design of pharmaceuticals to treat diseases involving uncontrolled cell proliferation (239,240).

8.2.9.1 CCK β /Gastrin Receptor Mutations and Gastrointestinal Carcinoma

The receptors for cholecystokinin (CCK) and gastrin, CCKR and CCK β /gastrin, respectively, have been implicated in the risk for a spectrum of human diseases that includes metabolic and neoplastic disorders (241–243). The dire consequences of

disrupting these receptors may reflect the role of the wild-type receptors in regulating food intake and pancreatic endocrine function. For example, the role of mature amidated gastrin, progastrin, and its intermediates has been identified in gastrointestinal neoplasia (244). Other disorders of gene regulation and development, including type 2 diabetes, have also been associated with activating variants of CCKRs (243). This insight into the disruption of gastrin signaling may allow development of pharmacological interventions at the gastrin receptor for affected patients.

Since the epidemiological evidence does not always confirm that elevated gastrin levels contribute to increased risk for colon cancer, it is worth reviewing the evidence of the molecular pathology of gastrin-related systems in colorectal cancer. This evidence is mostly derived from the study of colorectal cells cultured from biopsied tissue. It suggests that prolonged hypergastrinemia is associated with an increased risk for neoplastic changes.

Within this cohort, abnormal expression of CCK β /gastrin receptor has been associated with colon cancer since the receptor protein was expressed in 44% of colorectal cancers compared with 13% of controls (244,245). Mutation screening of tissues collected from colon cancer patients and controls discovered variants of the genes encoding the peptide G17 amide and the G protein-coupled CCK β /gastrin receptor (244,245). Several somatic mutations have been directly associated with disease. CCK β /gastrin receptor variants were associated with abnormal gastrin binding in vitro (246).

For example, the Val287Phe CCK β /gastrin receptor somatic mutation was found in some colon cancer patients. In vitro, the Val287Phe variant results in a loss of gastrin-induced MAPK p44/p42 signaling compared to wild type. It is associated with a 51% increase in clonal expansion. This structural alteration may be informative in the study of other GPCRs that are candidates in oncogenesis (244,245), particularly those with disruptions in the third intracellular loop. These studies suggest that it may be worth targeting the variant GPCRs that are expressed in tumors because they are known to be both pharmacogenetically distinct and associated with tumorigenesis.

8.2.10 *Thrombin, Inflammation, and Protease-Activated Inhibitor Receptors*

The protease-activated receptors (PARs), a subclass of GPCRs that function in the coagulation cascade, suggest that a comprehensive survey of the GPCR portion of the proteome provides information about the structure and function of this receptor class. The PAR factor II (thrombin) receptor-like 2 (F2RL2) is inactive in the cascade until proteolytic cleavage of its extracellular amino terminus. A Phe240Ser variant that is located in the second intracellular loop, found at a frequency of approx. 8%, disrupts receptor activation by proteolysis.

This illustrates how GPCR function can be influenced by structural changes that are genetically determined. The terminus created by proteolytic cleavage that, in

Table 8.1 Human G protein-coupled receptor (GPCR) sequence variants associated with altered risk for disease or altered pharmacology

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
α_2 -Adrenergic receptor (ADRA2A) 10q24–q26	N251K, third intracellular (IC3) loop		↑ Agonist-dependent G protein coupling—gain of function	(6,153)
	G389R (C-terminus)		↑ Basal and agonist-dependent G protein coupling—gain of function	(152,257)
β_1 -Adrenergic receptor (ADRB1) 10q24–q26	R16G	Nocturnal asthma/severity	Agonist-dependent down-regulation enhanced	(6,12,258)
β_2 -Adrenergic receptor (ADRB2) 5q32–q34		Hypertension risk ↑ ↓ Albuterol response		(259)
		Myasthenia gravis		(260)
	Q27E	Hypertension risk ↑ Heart failure/performance	Resistance to downregulation	(159,261)
		Drug hypersensitivity	Albuterol response ↑	(12,154,262) (163,165)
	R16G/Q27E “2/2” haplotype	Asthma, heart disease, and immune disorders	↓ β_2 -agonist affinity	(156–158,161,163–165)
μ_1 -Opioid receptor (OPRM1) 6q24–q25	A6V, N40D, N152D	Obesity; Heart failure/performance	Altered coupling to G _s /adenylyl cyclase system	(6,12,148)
	A6V, N40D	Haplotype associated with substance abuse	↑ Potency of β -endorphin; ↓ membrane trafficking ↓ Basal G protein coupling	(141–143) (145,146)
	H260R H265R	Idiopathic absence epilepsy Substance abuse/addiction		(263) (12)

	S268P, N273D			↓ CaM binding, ↓ desensitization	(141,142,144)
δ ₁ -Opioid receptor (OPRD1) 1p36.1–p34.3	N152D		↓ Receptor expression	↓ Desensitization and potency	(12)
Dopamine receptor D ₁ (DRD1)	<i>Dde</i> I (–48G, 5'UTR) SNP and haplotype association		Alcoholism; bipolar disorder, attention-deficit/hyperactivity disorder (ADHD)	Polymorphisms may be in linkage disequilibrium with regulators of D ₁ expression	(41,60–65)
5q35.1					
Dopamine receptor D ₂ (DRD2)	Short/long-/longer nt.414/443/TG splice A1 (<i>TaqI</i> RFLP)		↑ Expression of all dopamine D ₂ variants in schizophrenia Reward deficiency/addiction Obesity	Short is three times more sensitive to dopamine ↓ Receptor expression	(264) (91,96,265–267) (87–89) (91–93) (268,269)
11q23	A2, nt. C957T S311C, P310S, V96A, nt. A241G		Alcoholism Pathological gambling		
			Tardive dyskinesia	Altered drug affinity or clinical efficacy	(88,94,95) (270,271) (272,273)
Dopamine receptor D ₃ (DRD3)	<i>BclII</i>		Unipolar depression		
3q13.3	S9G (<i>MscI</i> RFLP) S9G + SNPs		Tardive dyskinesia SNPs and haplotypes associated with schizophrenia		(85,86) (81,82)
Dopamine receptor D ₄ (DRD4)	48-bp repeat in IC3		Effect on clozapine binding	Effect on G protein coupling	(56,67,69,74–77,274)
11p15.5			Novelty seeking; ADHD Elevated D ₄ -like sites in schizophrenic brains		(78–80) (68, 69)

(continued)

Table 8.1 (continued)

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
	V194G Promoter SNPs	Protein expression ↑		
Dopamine receptor D ₅ (DRD5)	L88F		↓ Sensitivity to dopamine and clozapine ↑ Affinity of agonist	(67,68,73)
4p16.1-p15.3	N351D			
5-Alphahydroxytryptamine receptor 1B (HTR1B)	F124C		↓ Affinity of agonist ↑ Affinity for ligand	(77,275) (130–132)
6q13	SNPs	ADHD uncertain		(132–134)
5-Alphahydroxytryptamine receptor 1D (HTR1D)	G861, SNPs	Obsessive-compulsive disorder		(135–139)
6q13	102 T/C (silent)	ADHD; Alzheimer's disease psychotic symptoms	↓ Response to clozapine	(94,100,117,118)
5-Alphahydroxytryptamine receptor 2A (HRT2A)		Schizophrenia marginal; Suicide association mar- ginal; Alcohol/behavioral possible		
13q14–q21		↓ Response to clozapine; ↓ G(q) and G(13) signaling		(98,99,109–116) (97,102–106)
	T25N, I197V, A447V, H452Y	Citalopram response; depression, negative symptoms of schizophrenia, eating disorders debatable	↓ Calcium mobilization	(107,108)
5-Alphahydroxytryptamine receptor 2C (HRT2C)	-1438 A/G promoter C23S	Alzheimer's disease psycho- sis: suicide ideation	May be associated with ↑ clozapine response	(276–278) (97,117,125,126)

Xq24	Promoter SNPs	May be associated with obesity, type 2 diabetes May be associated with an ↑ risk for Alzheimer's disease	May be associated with clozapine-induced obesity	(119–124) (127–129)
5-Alpha-hydroxytryptamine receptor 6 (HRT6)	C267T (267C allele)			
Histamine receptor H1 (HRH1)	E349D, L449S, Many SNPs	No association with atopic asthma	L449S; probably no effect on clozapine response	(97,279–281)
3p21-p14				
Histamine receptor H2 (HRH2)	R649G, SNPs	Some evidence for ↑ frequency in schizophrenia	No effect on clozapine response	(280,282,283)
	Promoter SNPs (1018 G/A)	No association with atopic asthma or schizophrenia		(97,280)
Histamine receptor H3 (HRH3)			↑ Constitutive activity of this receptor subtype	(284–286)
Cysteinyl leukotriene receptor 1 (CYSLTR1)	G300S	Atopy/asthma association: Tristan da Cunha population	Gain of function	(167,287–292)
Xq13–q21				
Cysteinyl leukotriene receptor 2 (CYSLTR2)	M201V, SNPs	Tristan da Cunha, Caucasian, and Japanese populations	Loss of function	(167,287–292)
13q14				
Angiotensin receptor 1 (AGTR1)	1166 A/C (3'UTR)	↑ Risk of hypertension Heart rate variability	↑ Angiotensin response	(27,293–295)
3q21–q25		↑ Risk of ischemic events Influences aortic stiffness		(24,32) (23,24,296)
	Many SNPs	Pharmacogenetic variants	Interact with ACE deletion	(32)
Endothelin receptor, type A (EDNRA)	Lys198Asn and –134delA, SNPs	Affect pulse pressure/baroreflex/heart failure association possible	↓ G _i coupling, in vitro	(31,34–36)
4q31.2				(297–307)

(continued)

Table 8.1 (continued)

Receptor	Variant/allele	Disease/phenotype	Pharmacology	Reference
	<i>Aβ</i> II	Rare reports of ↑ immunoglobulin E levels/atopy	Altered receptor expression	(181,182)
Prostaglandin D ₂ receptor (PTGDR)	Promoter SNPs undermine normal helper T anti-inflammatory response	Asthma	Altered receptor expression ↓ anti-immune response to PGD ₂	(184,185,308,309)
G protein-coupled receptor 44 (GPR44/CRTH2)	3' UTR SNPs: n.G1544C	Asthma	Altered receptor expression ↑ inflammatory response to PGD ₂ expressed on helper T, type 2	(186,189–191,310)
11q12–q13.3	n.G1651A n.G1544C/G1651A haplotype V64I	↓ AIDS progression	CCR5/CXCR4 heterodimer	(186,189–191,310) (227)
Chemokine, CC motif, receptor 2 (CCR2) 3p21	R275Q, L351P, L302P	↓ Sarcoidosis progression Susceptibility to insulin-dependent diabetes mellitus		(12,227) (12,224,314)
Chemokine, CC motif, receptor 2 (CCR3) 3p21.3	R275E, L302P	Population studies		(228,229)
Chemokine, cc motif, receptor 5 (CCR5) 3p21	Δccr5 (32-bp deletion) 59029 A/G -homozygous -heterozygous	Partial resistance to HIV infection; protection against hepatitis B infection possible ↓ AIDS progression ↓ Non-Hodgkin's lymphoma	Altered binding affinity	(220,221,315–317) (315–317) (315–317)

Chemokine CX3C motif, receptor 1 (CX3CR1) 3pter-p21	V249I, T280M	↑ AIDS progression	(318)
Calcium-sensing receptor (CASR) 3q13.2-q21	0.9kb <i>alu</i> insertion in exon 7	↓ Adenylyl cyclase	(319,320)
	A116T, N118K, etc. A986S, R990G	Familial hypocalcemia Common polymorphisms	(321-324) (14-21)
SNP, single-nucleotide polymorphism; Ip3, inositol triphosphate.			

the wild type, activates the receptor by creating a tethered ligand is absent in the variant. As a result, the variant causes the loss of the F2RL2 receptor as a cofactor in F2RL3 activation and subsequent thrombin-triggered phosphoinositide hydrolysis (247,248). In addition, any biological activity associated with the cleaved fragments is also absent. Although the relevance of GPCR fragments is largely unknown, the continued survey of the GPCR proteome will facilitate this understanding in relevant tissues (249–252).

8.3 Conclusion

With the increasing use of high-throughput screening, an ever-increasing number of genetic variants or polymorphisms are being identified in GPCR systems. The investigation of these mutations gives insight into pharmacogenetics—the study of the genetic risk factors that may predispose portions of the public to disease as a result of an altered drug response phenotype (*see Table 8.1*). At the same time, the discovery of these variants provides pharmacogenomic reagents that can be used to refine drug discovery (6,162,253,254).

With time, the relevance of in vivo mutations with respect to structural in vitro data will provide a detailed population model of the receptors of family A, which share structural similarity to rhodopsin. Comparison of these data with the family B GPCRs, the glucagon-like receptors (255,256), and the family C receptors, such as the CASR, may provide the detail necessary to model how GPCR structure and function are altered by common genetic variants.

These strategies should permit more widespread use of genetic screening to personalize the pharmacological interventions applied on an individual basis. This strategy, when applied to the entire class of GPCRs, will facilitate a pharmacogenomic understanding of the role of certain residues involved in receptor structure and function.

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Chapter 9

Epigenetic Alterations of the Dopaminergic System in Major Psychiatric Disorders

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9.1 Introduction.....	188
9.2 Materials	194
9.3 Methods	198
9.4 Summary and Future Scope.....	208
9.5 Notes	209
References.....	210

Summary Although there is evidence to link schizophrenia (SCZ) and bipolar disorder (BD) to genetic and environmental factors, specific individual or groups of genes/factors causative of the disease have been elusive to the research community. An understanding of the molecular aberrations that cause these mental illnesses requires comprehensive approaches that examine both genetic and epigenetic factors. Because of the overwhelming evidence for the role of environmental factors in the disease presentation, our initial approach involved deciphering how epigenetic changes resulting from promoter DNA methylation affect gene expression in SCZ and BD. Apparently, the central reversible but covalent epigenetic modification to DNA is derived from methylation of the cytosine residues that is potentially heritable and can affect gene expression and downstream activities. Environmental factors can influence DNA methylation patterns and hence alter gene expression. Such changes can be especially problematic in individuals with genetic susceptibilities to specific diseases. Recent reports from our laboratory provided compelling evidence that both hyper- and hypo-DNA methylation changes of the regulatory regions play critical roles in defining the altered functionality of genes in major psychiatric disorders such as SCZ and BD. In this chapter, we outline the technical details of the methods that could help to expand this line of research to assist with compiling the differential methylation-mediated epigenetic alterations that are responsible for the pathogenesis of SCZ, BD, and other mental diseases. We use the genes of the extended dopaminergic (DAergic) system such as membrane-bound catechol-*O*-methyltransferase (*MB-COMT*), monoamine oxidase A (*MAOA*), dopamine transporter 1 (*DAT1*), tyrosine hydroxylase (*TH*), dopamine (*DA*) receptors1 and 2 (*DRD1/2*), and related genes (e.g., reelin [*RELN*] and brain-derived neurotrophic factor [*BDNF*]) to illustrate the associations between differential promoter DNA

methylations and disease phenotype. It is our hope that comprehensive analyses of the DAergic system as the prototype could provide the impetus and molecular basis to uncover early markers for diagnosis, help in the understanding of differences in disease severity in individuals with similar or identical genetic makeup, and assist with the identification of novel targets for therapeutic applications.

Keywords Bipolar disorder; *COMT*; DNA methylation; *DRD1*; *DRD2*; epigenetic; *MAOA*; *RELN*; QM-MSP; schizophrenia.

9.1 Introduction

9.1.1 *Schizophrenia and Bipolar Disorder: Manifestation and Dilemma Faced by Genetic Studies in Psychiatry*

Schizophrenia (SCZ) and bipolar disorder (BD) are among the most severe forms of psychiatric disorders and elicit overlapping symptoms and cognitive deficits (1). SCZ is marked by hallucinations, delusions, illogical thinking, and disorganized behavior, and the main problem of patients with BD is circular mood changes that range from severe depression to severe mania with or without psychotic features. Approximately 2% of the population of any community suffers from SCZ and BD (2). Considering the early age of onset of these diseases, focus on the identification of genetic/epigenetic liabilities with the purpose of early intervention and prevention would address one of the major health issues of young adults. Although epidemiological and familial studies strongly suggest a genetic basis for the pathogenesis of these diseases, no specific genes that have a major or moderate role have been identified (3). This is likely to be because of the following reasons: polygenic inheritance, pleiotropy, and epigenetic aberrations.

9.1.1.1 Polygenic Inheritance

Genetic research in psychiatry provided strong evidence that the psychiatric disorders are multifactorial and polygenic in origin (2–5). Polygenic illnesses with overlapping symptoms may share several common disease-causing genes, and the coinheritance of a subset of them is apparently necessary for each of the diseases to reach the threshold to elicit the specific psychiatric phenotype (2–4). Therefore, the polygenic nature of the pathogenesis of psychiatric disorders is a legitimate reason for why genetic studies have tended to be inconclusive and could not be replicated. For example, several genes are involved in brain dopaminergic (DAergic) transmission, such as *TH* (tyrosine hydroxylase), *COMT* (catechol-*O*-methyltransferase), *MAOA* (monoamine oxidase A), *DAT1* (dopamine transporter 1), and dopamine receptor

genes *DRD1–5* (2,5). Since most of these genes interact and have various hypo-/hyperactive polymorphisms, tracking the influence of a single dysfunctional polymorphism in disease manifestations would be difficult in genetic association studies.

9.1.1.2 Pleiotropy

Pleiotropy occurs when one gene elicits different functions depending on the tissue types at the same time or during defined developmental periods (4,6). Thus, a dysfunctional pleiotropic gene may assume varying manifestations in different tissues at a time or other times when its expression is activated. Comorbidity of a psychiatric disorder with other psychiatric or somatic disorders indicates that a pleiotropic gene may be involved in the pathogenesis (3,4). However, the variability in presentations in the target phenotypes makes it difficult to find the responsible gene by most genetic studies, which focus on a single phenotype at a time. Most mental diseases have a high rate of comorbidity, warranting that an integrative approach considering both pleiotropy and polygenic inheritance is required to narrow down the effects of multipotent genes in psychiatric disorders (2–4).

9.1.1.3 Epigenetic Aberrations

Alterations in the level of DNA methylation are a known mechanism for fine-tuning of gene expression in response to a variable environment (7,8). There have been several studies suggesting an important role for environmental factors in early life development and in the pathogenesis of mental diseases (2,5). Thus, in some cases the epigenetic changes following deleterious environmental effects could substitute for the effects mediated by malfunctional polymorphisms. The environmental insults could alter the DNA methylation patterns, affecting the presentation of diseases, particularly in individuals with genetic susceptibilities and at the borderline threshold for predisposition to specific psychiatric diseases (3,5,9,10). Because of the importance of the role of DNA methylation as a cell memory for adaptive gene–environment interactions, in this chapter we primarily focus on *methylomics*, genomic methylation-mediated regulation of gene function, a concept that is widely known in cancer research and developmental biology and has only recently caught the attention of neuroscientists (10,11).

9.1.2 *Methylomics: The Science of DNA Methylation*

Methylomics is a novel science that studies the pathophysiology of DNA methylation and methylomic fingerprinting of the genome in various human tissues during developmental periods as well as during maintenance of a differentiated state (11).

In spite of the same genetic makeup of all cells of an organism, each tissue and even each cell has its own methylation pattern that determines its identity and functions as a result of dynamic interactions with other cells and the environment (7,8). Environmental stimuli, through the mediation of several elements/factors such as neurotransmitters, hormones, and transcription factors, modulate promoter methylation patterns and corresponding expression levels of various genes (11). For example, it is well known that dopamine (DA) and serotonin (5-HT), secondary to environmental stimulation, regulate defined sets of genes to produce the proteins/factors. The synapses that have already been conditioned by DA or 5-HT become susceptible to the influence of these proteins/factors that mediate growth and long-lasting structural changes (12,13).

BDNF (brain-derived neurotrophic factor) is among the proteins that are produced as a result of neuronal activation and that may play prominent roles in neuronal growth, connectivity, and functionality. This process is often mediated by cyclic adenosine monophosphate (cAMP) and cAMP response element binding protein (CREB), which as a consequence could also alter promoter/regulatory DNA methylation status and expression of the affected gene (13–16). As it has been shown for the glucocorticoid receptor in the hippocampus, even the style of maternal care could be associated with changes in methylation patterns of the genes in the offspring, which is likely to be maintained over a long period of time dependent on the persistence of the stimuli (17). Since successive bindings of transcription factors to a gene's regulatory regions are associated with a decrease in DNA methylation level and an increase in the capability of gene expression for a prolonged period of time in the future (18,19), DNA methylation is considered a mechanism for cell memory (7,8,19,20), which could also be the underlying mechanism for kindling.

After fertilization, there is a global demethylation of the genome, allowing multipotency of cells, which subsequently become differentiated into specific cell types depending on their fate, which is defined by an unknown mechanism (7). During the process of differentiation, there will be a resetting of methylation patterns in early life (7,8). There could be dynamic equilibrium in DNA methylation or demethylation according to critical periods of development and environmental conditions (21,22). The differential activities of methyltransferase enzymes and the presence of resources such as folic acid and S-adenosyl methionine, which determine the availability of methyl groups, could also play a role in the level of de novo methylation (21,22). For instance, it has been shown that methionine and other macro- and microenvironmental insults can induce pathologic DNA hypermethylation, which could lead to silencing of gene expression, as reviewed elsewhere (11). Several other nutritional or environmental factors, such as vitamin B₁₂, butyrate, tea polyphenols (23), alcohol (24–26), hypoxia, nitric oxide, and hormones (steroids, in particular) may also alter gene methylation patterns (11). Since the pattern of DNA methylation is generally maintained throughout cell division, any DNA methylation changes could either be global in the entire genome, affecting all progenies of the affected cell, or local, affecting only specific genes in specific cells (7,22).

9.1.3 Influence of Environment on the Methylome in Psychiatric Disorders: Alteration in the DAergic System in SCZ and BD

The DAergic neurons produce DA from tyrosine, a reaction that is catalyzed by tyrosine hydroxylase enzyme (2,4). DA is involved in several brain activities (e.g., attention, executive memory, desire, and natural rewards) mediated by an intricate network of cell signaling events. Most of these effects are mediated by binding of DA to DRD1- and DRD2-like receptors that activate downstream cell signaling pathways (2–5). COMT and MAOA are involved in dopamine degradation, while DAT1 reuptakes/recruits DA from the synaptic cleft (2,5). Genetic and pharmacological studies indicated that dysfunction of genes involved in the DAergic system (such as the D1- and D2-like receptors, membrane-bound [MB-COMT] and MAOA) contributed to the psychopathogenesis of SCZ and BD (3). Nevertheless, the genetic polymorphisms of these genes alone failed to provide a comprehensive molecular basis for the dysfunction of the DAergic system in major mental diseases (2–5). This along with the variability in phenotypic presentation in individuals with the same genetic makeup, such as the identical twins, and the lack of consistency of genetic associations in mental illnesses led to the current conclusion that environmental factors also influence the functionality of the DAergic system and presentation of disease symptoms (2,3,5,9,10).

This background prompted us to consider that the epigenetic changes brought about by altered DNA methylation patterns caused by environmental factors, by affecting the components of the DAergic system, are likely to play a major role in the pathogenesis of psychiatric diseases. To test this hypothesis, we analyzed 105 postmortem brain DNA and RNA samples from the frontal lobe donated by the Stanley Medical Research Institute and an additional 10 postmortem brain samples from the Harvard Brain Tissue Resources Center. To determine the DNA methylation status and corresponding levels of expression of *MB-COMT*, *RELN*, and *DRD2* genes, we used methylation-specific polymerase chain reaction (MSP)/bisulfite sequencing and reverse transcriptase polymerase chain reaction/quantitative real-time PCR (RT-PCR/qRT-PCR) (27,28).

Our samples were derived from the frontal lobe as it is the best-known dysfunctional region of the brain in SCZ and BD (29,30). We and others showed that hypermethylation-mediated silencing of the *RELN* gene correlated with SCZ pathogenesis (27,31). In subsequent studies (28), we found a higher frequency of hypomethylation of the *MB-COMT* promoter in SCZ and BD vs control subjects (methylation level = 26% and 29% vs 60%, $p = 0.005$ and 0.008 , respectively), particularly in the left frontal lobe (31% and 30% vs. 81%, respectively; $p = 0.004$). Furthermore, quantitative expression analyses (qRT-PCR) corroborated these observations as we found a significantly higher transcript level of *MB-COMT* in SCZ and BD vs the controls ($p = 0.02$) and an accompanying inverse correlation between *MB-COMT* and *DRD1* expression. In the same samples, there was a tendency for the enrichment of the Val allele of the *COMT* Val158Met polymorphism

with *MB-COMT* hypomethylation in the patients. Interestingly, the presence of a valine allele was associated with *RELN* promoter DNA hypermethylation ($p = 0.01$). In addition, *MB-COMT* promoter hypomethylation was correlated with *DRD2* promoter hypermethylation in SCZ and BD vs the controls ($p = 0.01$). Further, the *DRD2* and *RELN* promoters were almost always unmethylated in the controls with a hypomethylated *MB-COMT* but were significantly hypermethylated in SCZ and BD samples that exhibited a hypomethylated *COMT* promoter ($p = 0.01$).

These findings suggest that dopamine deficiencies arising from *MB-COMT* promoter hypomethylation (i.e., increased expression) may influence promoter methylation and expression of *RELN*, *DRD2*, *DRD1*, and potentially other genes via coordinated epigenetic events that may aggravate disease symptoms (see Fig. 9.1).

Despite our studies that have uncovered some epigenetic aberrations caused by promoter methylation changes in SCZ and BD, several other interacting genes

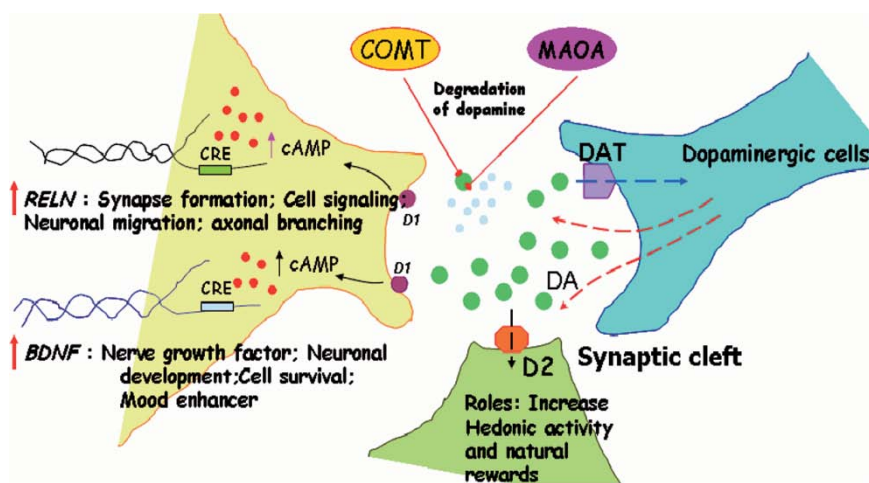


Fig. 9.1 Neuronal factors and receptors affected in schizophrenia (SCZ) and bipolar disorder (BD). Dopaminergic (DAergic) neurons release dopamine (DA), which is involved in several brain activities mediated by a network of cell signaling events, including attention, executive memory, desire, hedonic activities, and natural rewards. Most of these effects are likely to be downstream of D1- and D2-like receptors. Some of these effects could be mediated by cyclic adenosine monophosphate (cAMP) acting on the cAMP response element (CRE) regulatory region in the DA-responsive promoters of effector genes (e.g., brain-derived neurotrophic factor [*BDNF*] and reelin [*RELN*]). Our preliminary results support that catechol-*O*-methyltransferase (*COMT*) overactivity (caused by its promoter hypomethylation-associated overexpression or the hyperactive polymorphic valine allele at the Val158Met polymorphism) could lead to rapid DA degradation, leading to DA deficiency in the synaptic cleft. A similar scenario could be caused by rapid DA degradation by monoamine oxidase A (*MAOA*) or aberrant reuptake of DA by dopamine transporter 1 (*DAT1*). Subsequently, understimulation of the D1 and D2 receptors because of deficiency could impair the DA signaling events and end effects. These events may also influence primary or secondary promoter methylation of *BDNF* and *RELN*, leading to hypoeexpression of these genes, affecting neuronal outgrowth, positioning, and other functions

remain to be examined. Therefore, defining both the promoter methylation status and gene polymorphisms of these and other related genes will help in understanding the magnitude of frontal lobe DAergic dysfunction and the role of gene–gene interactions in SCZ and BD. Although several literature studies have focused on methods for genetic polymorphism analyses, the methods for screening of the epigenetic alteration caused by promoter DNA methylation in psychiatric studies is still in its infancy. Therefore, this chapter outlines the detailed methodology for analysis of the methylome for psychiatric disorders to promote a worldwide search for the epigenetically altered candidate genes in psychiatry.

9.1.4 Current Methods Used for DNA Methylation Analyses

We have extensively used bisulfite-treated genomic DNA sequencing and MSP (32) to map the differentially methylated CpG islands in the promoter regions of the *RELN*, *DRD2*, and *COMT* genes in psychiatric disorders (27,28). Similar methods have already been used for analyses of the promoter methylation status of *MB-COMT* (33) and other new methods have used successfully for the analyses of other genes (34). We recommend an initial evaluation of the candidate CpG islands using bisulfite sequencing of representative samples to assess the overall methylation profiles of the target CpG islands. Then, MSP analyses could be used to screen and to assess the methylation status of the selected group of specific CpGs. In brief, test genomic DNA is chemically modified with sodium bisulfite to convert the unmethylated cytosines to uracils; methylated cytosines remain unaffected. Then, primers are synthesized to amplify the target fragment for sequencing (35). In MSP, primers are synthesized to selectively amplify methylated and unmethylated DNA in separate PCR reactions (32). PCR reactions are resolved on nondenaturing polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet (UV) illumination. The bisulfite-modified placental DNA and in vitro methylated placental DNA are used as negative and positive controls for methylation, respectively. In addition, water is used as a negative PCR control to detect any contamination in each master mix solution.

MSP is used widely to assess the methylation status of a selected group of genes for high-throughput analyses of their methylation status at CpG sites (36,37). While the sensitivity of direct sequencing of the bisulfite-treated DNA or cloned DNA is about 10–20% (32,38), the sensitivity of MSP is known to be more than 1% (32). In fact, direct sequencing of the genomic DNA cannot detect the methylated signal from the noise background if the ratio of methylated DNA/total DNA is less than 10% (32,38). Although sequencing of the cloned DNA is a remedy to eliminate background noise, assuming that 10% of the cells of a specific tissue contain the methylated promoter, at least ten clones need to be provided to detect such a level of methylation in clinical samples, which is time consuming and labor intensive (32,36). Thus, MSP is considered a highly favored method for screening of methylated CpG sites within the CpG islands as it can detect the presence of less than 1%

of the methylated DNA in the pool of bisulfite-modified genomic DNA (32,36,37).

Although the standard MSP is an appropriate approach for promoter methylation status screening, our results clearly showed that subsequent evaluation of the differential promoter methylation level should be conducted by quantitative MSP (qMSP) to quantify the degree of these alterations to make clear correlations to the severity of the psychiatric disorders (H.M. Abdolmaleky and S. Thiagalingam, unpublished). In addition, it could be further validated using a complementary quantitative method such as immunoprecipitation (IP) of the methylated DNA to confirm the results of the qMSP (34).

9.1.5 Summary of Goals and Implications

Studies from our and other laboratories provided convincing data to support the notion that alterations caused by epigenetic DNA methylation and associated differential gene expression play a major role in the pathogenesis of SCZ and BD (27,28,31,39). These findings are consistent with (1) the lack of direct relationship between genotype and phenotype in major psychiatric disorders and variability in the manifestation of disease in individuals with identical or similar genetic makeup; (2) the existence of strong gene–environment interaction-mediated epigenetic modulation of gene functions derived from plasticity in DNA methylation. In addition, aberrant DNA methylation of the DAergic system in SCZ and BD and its association to *RELN* methylation provide compelling evidence for future validation of the initial observations as well as extension of these studies to additional candidate genes of the DA signaling/effector genes. We hope that providing detailed methods for these studies will help other researchers contribute to this novel branch of genomic research. In the long term, we are hopeful that these studies could become a prototype model system to aid in the development of strategies for better diagnosis, management, and therapy of SCZ, BD, and other mental diseases.

9.2 Materials

9.2.1 Bisulfite Treatment of Genomic DNA

9.2.1.1 Conventional Methods

Reagents required for the conventional method include sodium hydrogen sulfite (Sigma-Aldrich, cat. no. 243973), hydroquinone (Sigma), NaOH (3M), mineral oil, Wizard DNA Cleanup system (Promega), isopropanol, ammonium acetate (10M), glycogen, and ethyl alcohol.

9.2.1.2 Commercially Available Kit

A commercial bisulfite treatment kit (EpiTect Bisulfite Kit) is available from Qiagen (cat. no. 59104).

9.2.2 *Polymerase Chain Reaction and Methylation-Specific Polymerase Chain Reaction*

1. PCR reaction mix: Standard PCR buffer 10X (2.5 μ L), 10 μ M deoxynucleotide 5'-triphosphate (dNTP) (0.4 μ L), dimethyl sulfoxide (DMSO) (1.5 μ L), platinum *Taq* DNA polymerase (2.5 U, Invitrogen) in a total volume of 25 μ L in ultrapure deoxyribonuclease/ribonuclease (DNase/RNase)-free water for each PCR reaction.
2. Primers are listed in [Table 9.1](#).

9.2.3 *Polyacrylamide Gel Electrophoresis*

1. Running buffer/TBE: (9.3 g ethylenediaminetetraacetic acid [EDTA].Na. $2H_2O$, 55 g boric acid, 108 g Tris-HCl): Dissolve in water in a total volume of 1 L for a 10X buffer and keep at room temperature.
2. Acryl/bis 37.5:1 solution (Amresco or other available similar solutions).
3. Ammonium peroxodisulfate (APS) (Fisher Scientific): Prepare 10% solution in water and store at 4°C.
4. TEMED (tetramethylethylenediamine). Store at 4°C.

9.2.4 *qMSP and Quantitative Multiplex MSP (QM-MSP)*

1. SYBR green master mix (light sensitive; store at -20°C for long storage and 4°C after opening).
2. Plates (96 or 384 wells).
3. Adhesive cover sheet for the plates
4. Primers (*see* [Table 9.1](#)).

9.2.5 *Bisulfite Sequencing*

1. PCR purification kit (Qiagen or other manufacturer).
2. Primers (*see* [Table 9.1](#)).

Table 9.1 Primers for methylation analyses of the target genes

Genes and primer type	Forward (5'–3')	Reverse (5'–3')	Annealing temperature, °C (fragment size)
β -Actin promoter amplification	TTGGGAGGGTAGTTTAGTTGTGGT	CAAAACAAAACACACCTTTTACCCTAA	60 (197)
β -Actin nested primers for second-round amplification in QM-MSP ^a	GGTGGGTTTAGATTAGGTTGTGTA	CTACCTACTTTTAAAAATAACAATCAC	60 (125)
<i>MB-COMT</i> promoter amplification	GGATTTTGTAGTAAGATTAGATT	CAATATTCCACCCCTAAATCTAAAA	56 (431)
<i>MB-COMT</i> MSP M ^a (33)	TATTTTGGTTATCGTCGCGC	AACGAACGCAAAACCGTAACG	56 ^b (142)
<i>MB-COMT</i> MSP U ^a (33)	TATTTTGGTTATTTGTTGTGT	AACAAACACAAAACCATAAACA	56 (142)
<i>MB-COMT</i> nested primers for second-round amplification in QM-MSP ^a	TATTTTACGAGGATATTT	AACAACCTAACTACCCCAAAAACC	56 (201)
<i>MB-COMT</i> nested primer for sequencing	GATATTTTAC(T)GAGGATATT or reverse of <i>MB-COMT</i> promoter amplification		56
<i>MAOA</i> promoter amplification	TAATGTGTTGGTTTTTTAAAGTGA	CTATCTAAACAAAAATAAACTTAAAA	56 (333)
<i>MAOA</i> MSP M ^a	CGTAGATTTTCGACGGTTTATATGAC	GACTAAACCAACAAAAATCGCAAAATCG	60 (92)
<i>MAOA</i> MSP U ^a	TGTAGATTTTGTATGGGTTTATATGAT	AACATAACCAACAAAAATCACAATACTCA	60 (92)
<i>TH</i> promoter amplification	GGATTTGGTTGTTTAGTTT	ACAACCTCTTATAAACTAAACTAAC	56
<i>TH</i> MSP M1	CGAGTTTGGGTTTTCGTAAAGTTC	ACAACCTCTTATAAACTAAACTAACG	60 (224)
<i>TH</i> MSP U1	TGAGTTTGGGTTTGTAAAGTTT	ACAACCTCTTATAAACTAAACTAACG	60 (224)
<i>TH</i> MSP M2 ^a	TTACGGACGAGTTTTTTTTTGC	ACAACCTCTTATAAACTAAACTAACG	58 (150)
<i>TH</i> MSP U2 ^a	TTATGGATGAGTTTTTTTTTTGT	ACAACCTCTTATAAACTAAACTAACG	58 (150)
<i>DAT1</i> promoter amplification	TAAATCGTTAGGGTTTTTTAGGT	CAAAACTAACGACTCCCAACTAA	56 (405)
<i>DAT1</i> MSP M ^a	TAGGCGTATTAGATGTCGGTAAGGC	GCTCTAACCAACGAACTCGAACCCCG	60 (163)
<i>DAT1</i> MSP U ^a	TAGGTGTAATTAGATGTGGTAAGGT	ACTCTAACCAACAAACTCAAAACCCCA	60 (163)
<i>DRD1</i> promoter amplification	ATTAGTTTTGGGAGTGTTTTTTTT	TCTCTCAAAAACCCCTAAAACTTA	56 (206)

<i>DRD1</i> MSP M ^a	GATTTT TTTT TAAACGTA TTTCCGC	AAACTTCGCTAAAAACAACGACTCCG	60 (125)
<i>DRD1</i> MSP U ^a	GATTTT TTTT TTTAAATGTA TTTGGT	AAACTTCACATAAAAAACAACACTCCA	60 (125)
<i>DRD2</i> promoter amplification	GGTTT TTTGAG TTTT TAAAGGAGAAGAT	ACACTAAAAC TAAACAAC TCTTA	56 (304)
<i>DRD2</i> MSP M ^a	CGTTAGGTCGGGGATCGTCG	GACGCCGAAACGCGAAAAACGCG	67 (118)
<i>DRD2</i> MSP U ^a	TGTTAGGTTGGGGATTGTTG	AACACCCAAACACAAAAAACACA	56 (118)
<i>DRD2</i> nested primer for sequencing	GGATTAGTTTGTAA TTA TAGT		60
<i>RELN</i> promoter amplification	GTATTT TTTTAGGAAAAATAGGGT	CTCCCAAAAATTACTTTAAA	56 (506)
<i>RELN</i> MSP M1 ^a	CGGGGTTT TGACGTTTTC	CGCCCTCACGAACTCGACG	60 (184)
<i>RELN</i> MSP U1 ^a	TATTTGGTTATTGTTGTG	CACCTCACAAACTCAACA	60 (184)
<i>RELN</i> MSP M2 ^a	CGGGAGGTGTTT TTTGCGGGT TTTTGAC	CCGAAAAACAAAAAAAACGCCCC	60 (115)
<i>RELN</i> MSP U2 ^a	TGGGAGGTGTTT TTTGTGGGGT TTTTGAT	CCCAAAAAACAAAAAAAACACCCA	60 (115)
<i>RELN</i> nested primer for sequencing	GTTAAAGGGGTGGTT or reverse of RELN promoter amplification		57
<i>BDNF</i> MSP M	ATGATAGCGTACGTTAAGGTATCGT	AACGAAAAACTCCATTTAATCTCG	60 (154)
<i>BDNF</i> MSP U	TATGATAGTGTATGTTAAGGTATTGT	CAACAAAAAACTCCATTTAATCTCA	60 (155)

M, methylated; U, unmethylated specific primers.

BDNF, brain-derived neurotrophic factor; *DAT*, dopamine transporter; *DRD*, dopamine receptor; *MAOA*, monoamine oxidase A; *MB-COMT*, membrane-bound catechol-*O*-methyltransferase; QM-MSP, quantitative multiplex methylation-specific polymerase chain reaction; *RELN*, reelin; *TH*, tyrosine hydroxylase.

^aThese primers are suitable for QM-MSP.

^bAnnealing temperature for QM-MSP at 63°C.

9.2.6 Immunoprecipitation of Methylated DNA

1. IP buffer (10mM sodium phosphate pH 7.0, 140mM NaCl, 0.05% Triton X-100).
2. Mouse antimethylcytidine antibody (Eurogentec or EMD Biosciences).
3. Magnetic Dynabeads with M280 sheep antibody to mouse immunoglobulin G (IgG; Dynal Biotech/Invitrogen).
4. Proteinase K, phenol chloroform, isopropanol, sodium acetate, glycogen, and ethyl alcohol.

9.3 Methods

9.3.1 Bisulfite Treatment

9.3.1.1 Conventional Methods

Traditionally, 1–2µg genomic DNA is diluted in 20µL water and denatured by treatment with 2M NaOH (5.5µL) and then treated with 10mM hydroquinone (31µL of a freshly prepared solution consisting of 55 mg hydroquinone dissolved in 50mL water) and 3M sodium hydrogen sulfite–NaOH, pH 5.0 (520µL freshly prepared solution consisting of 3.76 g sodium hydrogen sulfite dissolved in water in a final volume of 10mL) at 50°C for 16–20h, overlaid with 0.2mL mineral oil and under aluminum foil to avoid evaporation and light exposure (35). Next, the bisulfite-treated DNA is purified using the Wizard DNA Cleanup system according to the manufacturer's instructions. Subsequently, NaOH treatment (5.5µL 3M NaOH for 50µL of eluted DNA/5 min at room temperature) changes uracil to thymine (35). DNA is ethanol precipitated (650µL 100% ethanol %, 34µL 10M ammonium acetate, 2µL glycogen), kept at –80°C for 2h, and centrifuged for 30min at 16,000rpm at 4°C), washed with 70% ethanol (700µL), resuspended in 20µL water, and stored at –20°C or –80°C until ready for use in further experiments.

Bisulfite treatment kits have been developed and are commercially available (e.g., Qiagen bisulfite treatment kit). Kits work with the same efficacy and have helped streamline the procedure, which will help expedite the process because of the elimination of setup time. Beginners are encouraged to use a kit, especially when there is not enough DNA to calibrate the conditions for bisulfite treatment.

9.3.1.2 Bisulfite Modification of Genomic DNA Using a Bisulfite Treatment Kit

1. Quantify your DNA and dilute 100–2000 ng (based on the availability or number of the required experiments) in 20µL water for bisulfite treatment according to the manufacturer's instructions (e.g., EpiTect Bisulfite).

2. As the bisulfite-treated DNA is a single-strand nucleotide and subject to faster degradation compared to the double-strand DNA, aliquot the elution in two or three tubes and keep at -20°C or -80°C for further experiments.

9.3.2 Methylation-Specific PCR

To perform an accurate high-throughput MSP analysis and design highly stringent primer pairs, optimization of PCR cycles and annealing temperature are important for ensuring that the unmethylated and methylated template-specific primers can only generate PCR products that represent the unmethylated (placental DNA) or methylated (in vitro methylated DNA) samples, respectively. The presence of PCR products corresponding to the methylated template in unmethylated control template sample (placental DNA) and vice versa would suggest additional optimization steps, including adjusting the PCR conditions (e.g., increasing the annealing temperature or reducing the PCR cycles) or designing new primer pairs. This process has to be repeated until you have only an unmethylated product with placental DNA and only the methylated product using in vitro methylated DNA as the template (see [Note 1](#) for more information).

Furthermore, MSP analysis should not yield any products using the same amount of unmodified genomic DNA as the template. The presence of products would indicate that the bisulfite treatment is incomplete, which more likely will generate a false-positive signal for methylation. This may also indicate that your primers are not specific for the modified DNA (see clues for designing primers for MSP, 9.3.4).

9.3.3 Scanning for the CpG-Rich Islands in the Regulatory Promoter Regions of Genes and Designing Primers for MSP and Bisulfite Sequencing

Approximately 50% of the genes contain CpG islands in their promoter region, making them promising candidates for epigenetic regulation and experimentally for analysis of alteration in DNA methylation (7,8,40). Our evaluations revealed that, among the genes of the DAergic system, only the DRD3 gene lacked a CpG-rich promoter. To search for CpG among the genes promoter region, perform the following steps:

1. Go to University of California at Santa Cruz (UCSC) genome bioinformatics Web site (<http://genome.ucsc.edu/bestlinks.html>) or a similar Web site.
2. Find the gene of interest and copy the sequence of the promoter region if known. If not known, copy the sequence of 2,500 bases of the gene before the coding region.

3. Then, go to the DNA methylation database Web site (<http://www.methdb.de/>).
4. Paste the gene promoter sequence in the designated location and submit your sequence for MSP primers or sequencing primers.

The methylation database Web site will convert the submitted sequence to a bisulfite-modified sequence, and a graphic view of the CpG islands will be presented along with the suggested MSP or bisulfite sequencing primers (41). In addition, all Cs followed by G that are a candidate for methylation will be marked with + along the sequences. The original sequence is also placed on the top of the bisulfite-modified sequence for reference by this program. Although this Web site will suggest several alternative MSP primers, you may also design your own primers appropriate for sequencing or MSP.

Note that, although the UCSC Web site indicates the 5' of the genes' transcription start site as the genes' promoter region by default, it may be not the exact promoter region. However, in general, this region consists of the promoter region of the gene of interest (42).

9.3.4 Clues for Designing Primers for MSP

1. Design the MSP primers from those sites that contain at least four scattered Cs that are not followed by G in each of your primer sequences to prevent amplification of the unmodified DNA.
2. Select a DNA sequence that contains three to five scattered CGs in your primer sequence. However, the presence of even one CG at the 3' end of each primer site could be acceptable for methylation analyses of these CGs.
3. Typically, the last base of the original DNA sequence selected for designing the primers should be C (that is followed by G). For example, if the original sequence of the desired location for a forward prime is 5'-CCGTATCTACCGAATCTGCACGTGACG-3', after bisulfite treatment if the DNA is methylated it will be converted to TCGTATTTATCGAATTTGTACGTGACG-3' and if unmethylated to 5'-TTGTATTTATTGAATTTGTATGTGATG-3'. Thus, your methylation-specific primer sequence would be 5'-TCGTATTTATCGAATTTGTACGTGAC-3' and the unmethylated DNA-specific primer would be 5'-TTGTATTTATTGAATTTGTATGTGAT-3'. If this location is selected for designing the reverse primer, the methylated primer sequence would be 5'-CGTCACGTACAAATTCGATAAATACG-3', and the unmethylated primer sequence would be 5'-CATCACATACAAATTCAATAAATACA-3'. If you suspect that the last C in your targeted sequence is partially methylated (or unmethylated), to emphasize the outcome of the PCR for evaluation of the overall methylation status of other Cs located in your primer site, you could include one or two additional bases in your primer sequences after the last C for PCR amplification. In this case, the last base of the forward primer would be G, but for the reverse primer it could be any of the bases. As the C before CG could be partially methylated, for the

reverse primer it would be reasonable to include two bases in the primer sequence after the last C, if indicated.

4. In general, the annealing temperature of the unmethylated primers is less than for the methylated primers. To have the U and M primers with a closer annealing temperature, include a few more bases from the 5' end to the U primers.
5. If you find a regulatory binding site that contain CGs in the promoter region (e.g., TGACGTCA for CRE and GGGCGG for SP1 binding site), it would be a good candidate for the C residue of the regulatory element to be considered as the last base of the primer.
6. The ideal size for the amplified fragment in MSP is between 100 and 200 bp (*see Note 2*).

9.3.5 PCR Conditions for MSP

Master mixes are prepared and used in the PCR amplifications. A typical reaction consists of 2.5 μ L of 10X standard PCR buffer, 0.4 μ L 10 μ M dNTP, 1.5 μ L DMSO, 2.5 U platinum *Taq* DNA polymerase (Invitrogen), 25 pmol of each methylated or unmethylated DNA-specific primers, and ultrapure DNase-/RNase-free water in a total volume of 25 μ L. Approximately 30 ng bisulfite-modified genomic DNA are used as the template. PCR amplification is carried out with an initial incubation at 94°C for 2 min, followed by 30 cycles (35 cycles for gene promoter amplification) at 94°C for 30 s, 56°C–67°C for 40 s (for each primer, *see Table 1*), 72°C for 30 s, followed by a final extension for 5 min at 72°C. Separately analyze 20 μ L of each of the MSP products on a 6% polyacrylamide gel by electrophoresis, staining with ethidium bromide, and visualization under UV light.

9.3.6 Polyacrylamide Gel Electrophoresis of MSP Products

1. Prepare the apparatus for vertical gel electrophoresis by cleaning the glasses and assembling the parts.
2. Use a 10-mL pipet and load 10 mL polyacrylamide gel (made according to [Table 9.2](#)) between the glasses, wait a few seconds, and let the bubbles disappear; insert the forks of a 1.5-mm comb into the gel at the top (between the glasses) to create wells 8–10 mm deep for loading the PCR reactions (be careful to prevent the formation of any bubbles and remove the bubbles that arise).
3. Wait for 5–10 min and let the gel solidify. Then, remove the combs slowly and assemble plates containing the gel in the designed place in the vertical electrophoresis apparatus.
4. Add 800–900 mL 1X TBE buffer into the space in the electrophoresis apparatus up to 5 mm above the upper edge of the gel.

Table 9.2 Materials and instruction for making 6% polyacrylamide gel

Material	Amount	Manufacturer/composition
Water	15 mL	Distilled water
Acryl/bis 37.5:1 solution	3 mL	(Amresco)
TBE 10X buffer	2 mL	1. EDTA.Na.2H ₂ O, 9.3 g 2. Boric acid, 55 g 3. Tris-HCl, 108 g Solve in water in a final volume of 1 L for a 10X buffer (ph 8.3) and store at room temperature
APS 10%	0.3 mL	Ammonium peroxidisulfate: prepare 10% w/v solution in water (e.g., 5 g per 50 mL water) and store at 4°C
TEMED	30 μL (add at the end)	Tetramethylethylenediamine (Invitrogen, cat. no. 15524-0100); store at 4°C
	Mix by inverting or vortex briefly and use immediately	

5. Mix 20 μL of the PCR product with 4 μL of loading dye and load the mixture in the wells (usually the U and M product of each sample are loaded side by side for an easy comparison).
6. In one of the wells, load 1.5 μL of 100-bp DNA ladder for tracking your target fragments and for the estimation of the molecular weight of the bands.
7. Electrophoresis is conducted at 100–120 V for 30–40 min.
8. Remove the upper glass carefully and cut an angle in the gel for orientation, then transfer the gel to a dish containing 100 mL of the running buffer (1X TBE).
9. Add appropriate amount of ethidium bromide solution in the corner of the dish (do not add on the gel).
10. Use a shaker for 5 min to help distribute the ethidium bromide equally and to penetrate into the gel.
11. Transfer the gel in a UV chamber carefully (as ethidium bromide is carcinogen, use two pairs of nitrile rubber gloves and be careful to prevent any contamination; deposit the buffer containing ethidium bromide in a special waste bottle).
12. Focus the camera and adjust the exposure time appropriately; print or save the picture.

You are expected to see a picture similar to [Fig. 9.2](#).

9.3.7 Bisulfite Sequencing

In general, bisulfite sequencing is a more complicated procedure than the other types of sequencing because of the incomplete modification of Cs to Us and differences in the frequency of CGs in the promoter region of genes. Furthermore, the

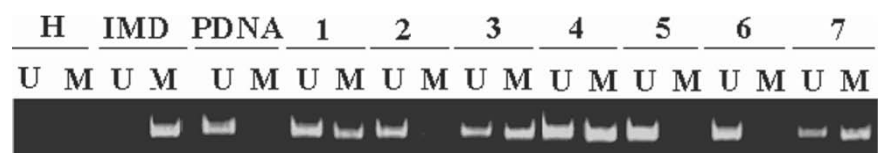


Fig. 9.2 Representative examples of the methylation-specific polymerase chain reaction (MSP) analyses for gene promoter regions. Lanes U and M indicate the presence of unmethylated and methylated template, respectively. Placental DNA (PDNA) and in vitro methylated DNA (IMD) served as negative and positive controls, respectively. Water (H) was used to detect contamination. Samples 1, 3, 4, and 7 indicate the presence of a methylated promoter DNA with various degrees of methylation, and samples 2, 5, and 6 represent an unmethylated promoter

remnants of the primers from the first PCR may also interfere with the sequencing reaction. To overcome these problems, do the following:

1. Use 50–100 mM of each primer for the amplification of the promoter region in 25 µL of PCR reaction.
2. Run 10 µL of the reaction in polyacrylamide gel (6%) to be sure that you have a single product.
3. Purify the rest of the PCR reaction using PCR purification kits (e.g., Qiagen cat. no. 28004) according to the manufacturer’s instructions.
4. Measure the purified DNA and use 10 ng DNA with 5 pg of the nested primer for sequencing (follow the special instructions for each sequencer).

You are expected to have a sequence profile such as that of [Fig. 9.3](#).

9.3.8 Clues for Designing the Bisulfite Sequencing Primers

1. Always design the forward and reverse primers from a CG-free region to amplify the promoter region. In rare cases, one CG could be included in the primer sequence. However, that C could not be the last few bases of the primer and avoid it as the last base.
2. Design the primers from those sites that contain at least four scattered Cs (that are not followed by G) in your primer sequence to prevent amplification of the unmodified DNA.
3. In addition to these primers, a nested primer from a CG-free region should also be designed for the sequencing. However, the forward or reverse primer could also be used for the sequencing.
4. In general, the ideal fragment size of the amplified promoter region for bisulfite sequencing is 200–300 bp.

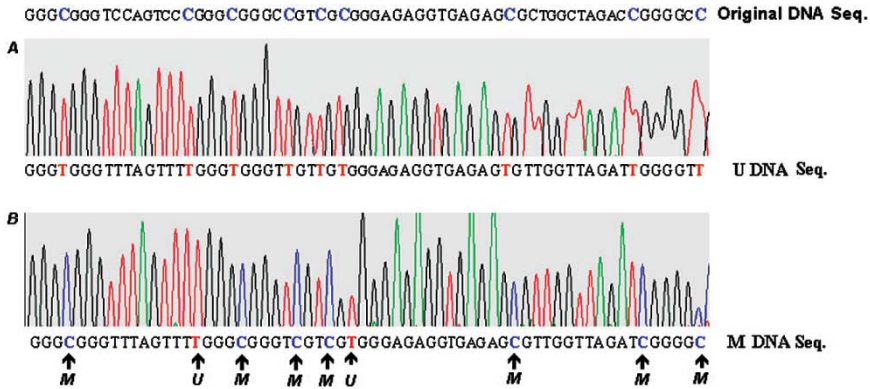


Fig. 9.3 Representative examples of the DNA methylation analyses of *MB-COMT* (membrane-bound catechol-*O*-methyltransferase) by bisulfite sequencing. Bisulfite treatment converts the unmethylated cytosines to uracil, while methylated cytosines remain unchanged. **A** Sequencing of the first exon of *MB-COMT* in a sample with unmethylated template correlates with conversion of cytosines (C) to thymine (T) in the DNA sequence corresponding to CpGs. **B** The cytosines exhibiting methylation (M arrows) contained sequence traces for Cs. This sequence trace exhibited cytosine methylation at several CpGs (which include two SP1 binding sites), indicating that the template is mostly methylated. However, two cytosines are totally unmethylated (U) in this fragment, and a small T signal in addition to C is seen in the last C, indicating that the tissue contains some cells with an unmethylated cytosine in that location (partially unmethylated). The original DNA sequence is indicated at the top. The bold Cs indicated in the CpG sequences are targets for methylation. Then, an unmethylated and a predominantly methylated sequence are indicated in the middle and bottom, respectively

9.3.9 SYBR Green-Based qMSP and QM-MSP

The high level of sensitivity of MSP allows efficient high-throughput examination of the DNA methylation status of the test samples. Although we have been successful in using the standard MSP procedure, our results clearly showed that it could improved by increasing its sensitivity to be able to quantify these alterations in a minute number of cells located in specific brain cortical layers to make clear correlations to the severity of the psychiatric disorders. Along these lines, we have established a real-time PCR-based qMSP using SYBR green in our laboratory as described in this chapter (H.M. Abdolmaleky and S. Thiagalingam, unpublished; see Fig. 9.4).

In this approach, bisulfite-treated DNA is used as the template, and 50–100 nM unmethylated or methylated DNA-specific primers are used for PCR amplification in separate reactions. For quantification, a $\Delta\Delta C_T$ method is used and normalized with the C_T (cycle threshold) for the β -actin gene (43). Alternatively, a fragment of the target gene promoter will be amplified using primers designed from a CpG-free area as an internal control (see Note 3). To eliminate any primer dimer that will compromise the accuracy of the results, an additional step in the PCR cycles above

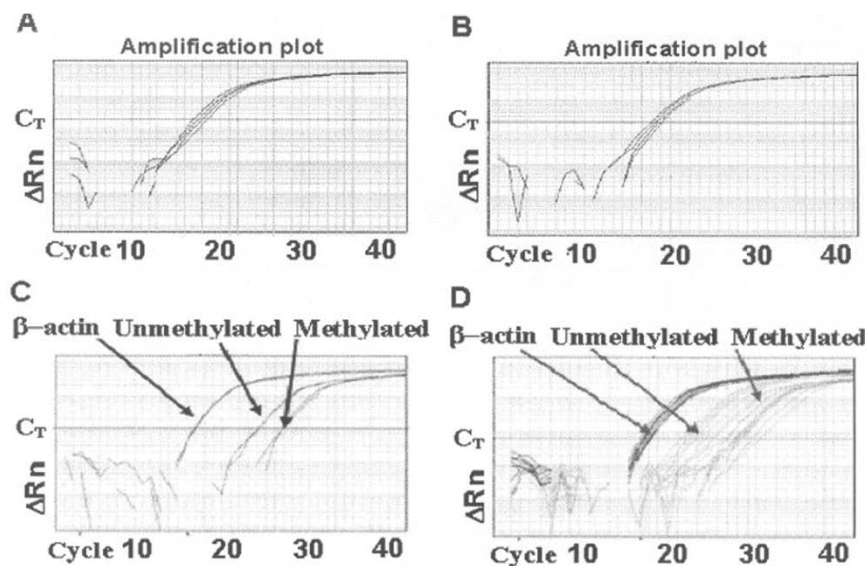


Fig. 9.4 Establishment of the quantitative methylation-specific polymerase chain reaction (MSP) analytical procedure. The ABI PRISM 7900 HT Sequence Detector system was used to perform real-time polymerase chain reaction (PCR) using MSP primers and bisulfite-modified template DNA. Upper panels: Setting up the conditions to obtain the standard curves with 50% (A) or 25% (B) sequential dilution of the template. Lower panels: The amplification curves on the left represent β -actin, unmethylated, and methylated MSP products, respectively, for reelin (RELN) (C). Amplification curves were compared at the set threshold before 40 cycles. Amplification curves from various samples are shown in the lower panel right (D)

the melting temperature of the primer dimer or nonspecific products could be used (44). We have also succeeded in adapting the SYBR green-based qMSP for multiplexing. By employing this method, we have successfully determined the methylation status of *MB-COMT*, *MAOA*, *DAT1*, *DRD1*, *DRD2*, *NRG1*, *RELN*, and other relevant genes.

For QM-MSP, first the promoter regions of several genes will be amplified using primers that correspond to the CG-free regions (45). Then, 1 μ L of the diluted PCR product (30–50 times) is used as the template in real-time QM-MSP to quantify the methylated and unmethylated template in separate reactions using methylation- or nonmethylation-specific primers. The benefits of this method are that (1) the instability of bisulfite-modified DNA is remedied using an initial round of PCR; (2) nonspecific products that compromise the accuracy of SYBR green-based real-time PCR are eliminated in the second-round PCR; (3) the likelihood of the development of primer dimers is minimized as the first-round PCR product is diluted 50 times and a minimal amount of MSP primers is used in the second-round PCR; (4) the amount of the precious DNA used in this approach is substantially less than in the other methods.

9.3.10 Experimental Procedures for Performing QM-MSP

1. Make a PCR master mix as described containing excess amount of dNTP and Taq polymerase enzyme. For instance, we found that if multiplexing for amplification of promoters of five genes has to be performed at the same time, 0.2 μ L 10 μ M dNTP and 1.25 U Taq DNA polymerase (Invitrogen) for each gene in the reaction are required. For each gene, consider 10 μ L of this PCR master mix.
2. Make 50 μ M stock solutions of each of the primers (assuming that 25 nM of the primers are diluted in 500 μ L of ultrapure DNase-/RNase-free water, concentration of the stock primer would be 50 μ M).
3. Mix equal amounts of promoter amplification primers for each of the genes (*see Table 1*) and for the β -actin gene. Use 2/10 μ L of the mixed primers for each gene in the reaction (for example, if you are mixing five primer pair sets, use 1 μ L of the mixed primers for each reaction, $\sim$ 200 nM of each primer in the PCR reaction).
4. Set the PCR cycles for 2 min at 94°C, 30 s at 94°C, 1.5 min at 56°C, and 1.5 min at 72°C. Repeat the last three cycles 25 times (it is expected that you will see a smear if you run 10 μ L of PCR product in the gel).
5. Dilute this PCR product 30–50 times (*see Note 3*).
6. Use 1–2 μ L of the diluted PCR product as the template for QM-MSP in a 10- or 20- μ L reaction.
7. Normalize the C_T of unmethylated or methylated product with the C_T of the β -actin gene, amplified in separate reactions for quantitation (*see also Note 4*).

9.3.11 Optimizing the Primer Concentration and Other Conditions for qMSP and QM-MSP

To obtain accurate results in qMSP/QM-MSP analyses, the best conditions have to be worked out to achieve reliable standard curves during the test trials. This could be achieved with the use of unmethylated and methylated templates such as placental DNA and in vitro methylated DNA, respectively, and by performing bisulfite treatment as described. To find the best condition for each gene, purify the DNA, calculate the concentration and copy numbers, and dilute the DNA sequentially (e.g., 1, 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64) and perform real-time PCR with several dilutions of the primers (e.g., 25, 50, 75 ng each in various combinations). For example, you should see the amplification plots as indicated in **Fig. 9.4** with 50% (**Fig. 9.4A**) or 25% (**Fig. 9.4B**) sequential dilution. These test trials and any other quantitative PCR (qPCR)/QM-MSP experiments need to be done in duplicate or triplicate to ensure that the required skills and instruments for equal pipeting are in place, or the impacts could be minimized by averaging the results of the triplicate experiments. Similar to MSP, for each qMSP or QM-MSP trials use placental and

in vitro methylated DNA as standards and reference templates for unmethylated and methylated DNA, respectively (see Refs. **43**, **45**, and **46** for details of quantitation procedures in qMSP and QM-MSP).

9.3.12 CpG Methylation-Specific Immunoprecipitation (CMIP)

We have also successfully adopted the use of antimethylated cytosine antibody to precipitate methylated DNA to examine suspected candidate genes that are likely to be regulated by altered DNA methylation to obtain preliminary results in a relatively short time frame before resorting to labor-intensive methods such as bisulfite-modified DNA sequencing and MSP for a number of genes, as indicated in **Fig. 9.5**. This method could also be used as a screening tool to determine whether several likely candidate genes are subjected to regulation caused by differential DNA methylation changes (**34**).

The IP of methylated DNA fragments is achieved using mouse antimethylcytidine antibody (Eurogentec or EMD Biosciences). In brief, 4 μ g of isolated genomic DNA from the test tissue are sonicated and evaluated by 2% agarose gel electrophoresis to verify that random genomic DNA fragments range from 300 to 1000 bp. Next, DNA is denatured at 95°C for 10 min and suspended in 500 μ L IP buffer (10 mM sodium phosphate pH 7.0, 140 mM NaCl, 0.05% Triton X-100) in ice. Ten μ L mouse antimethylcytidine antibody are added and mixed for 2 h at 4°C to IP the methylated DNA fragments. The mixture is incubated in 30 μ L magnetic Dynabeads with M280 sheep antibody to mouse IgG (DynaL Biotech/Invitrogen) overnight at 4°C in a shaker. It is washed three times with 700 μ L IP buffer using the magnet; the beads are treated with proteinase K for 3 h at 50°C, and the DNA is recovered with phenol chloroform extraction followed by isopropanol/sodium acetate plus

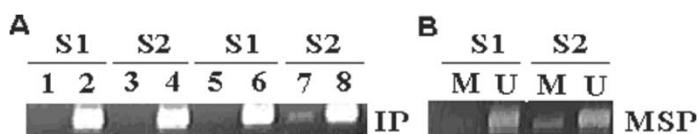


Fig. 9.5 CpG methylation-specific immunoprecipitation (CMIP) of DNA fragments. We used anti-5-methylcytidine monoclonal antibody (EMD Biosciences) or control mouse immunoglobulin G (IgG) for CMIP. The validation of the immunoprecipitation (IP) technique was carried out by MSP methylation-specific polymerase chain reaction analysis of the predicted methylated monoamine oxidase A (MAOA) gene as shown in **A**. We captured methylated promoter regions using CMIP and evaluated the presence of MAOA in the immunoprecipitated DNA using primers specific for polymerase chain reaction (PCR) amplification of the promoter region of the gene. In lanes 1 and 3, the control mouse IgG showed that the DNA could not be precipitated. Lane 5 indicates that unmethylated MAOA promoter DNA (revealed by MSP) is not precipitated by antibody against methylated DNA. However, methylated DNA is precipitated by the same antibody as indicated in lane 7. Lanes 2, 4, 6, and 8 show the presence of input DNA before IP, as control. **B**. The original DNA used for CIMP was analyzed by conventional MSP. As could be predicted, only the DNA template that contained methylated MAOA gave a positive signal for promoter methylation of MAOA (sample S2) and not the sample that lacked methylated MAOA (S1)

glycogen precipitation. An equal amount of sonicated DNA not subjected to IP should be kept (as input DNA) for comparing with the amount of the precipitated DNA. A neutral antibody is also simultaneously used for precipitation to confirm that the technique (and the antimethylcytidine antibody) is specific for methylated cytosine-containing DNA precipitation. Primers are designed from the original DNA sequence to amplify the desired target gene promoter region by qRT-PCR. The C_T value of the immunoprecipitated DNA (methylated DNA) is compared against the C_T of the input DNA for relative quantification of the methylated DNA (34).

9.3.13 Experimental Procedures for CMIP

1. Isolate genomic DNA from tissue/cells using DNeasy tissue kit (Qiagen) or phenol chloroform extraction.
2. Sonicate 4 μ g genomic DNA (one or two pulses, adjusted to 3.5 power).
3. Run 10 μ L sonicated DNA (from 100 μ L total) in 2% agarose gel to verify that random genomic DNA fragments range from 300 to 1000 bp. If indicated, repeat the sonication.
4. Store half of the remaining sample as input DNA for comparing the degree of the precipitation of DNA, achieved in the following steps.
5. Denature the other half of the DNA at 95°C for 10 min (or by using boiling water).
6. Suspend it immediately in 500 μ L IP buffer in ice (10 mM sodium phosphate pH 7.0, 140 mM NaCl, 0.05% Triton X-100).
7. Add 10 μ L mouse antimethylcytidine antibody and rotate for 2 h at 4°C to bind the methylated DNA fragments to the antibody.
8. Incubate the mixture with 30 μ L magnetic Dynabeads with M280 sheep antibody to mouse IgG at 4°C, rotating overnight in a shaker.
9. Wash three times with 700 μ L IP buffer using the magnet.
10. Treat the beads with proteinase K for 3 h at 50°C.
11. Recover the DNA by phenol chloroform extraction followed by isopropanol/sodium acetate plus glycogen precipitation.
12. Dilute the DNA in 45 μ L water (equal to the volume of the input DNA) and measure the concentration of IP DNA.
13. Use 1 μ L of this DNA for PCR using primers designed to amplify the promoter region of the candidate genes and compare to the PCR product obtained from the input DNA.

9.4 Summary and Future Scope

In summary, this chapter introduced methods to the wider scientific community to facilitate the extension of the compelling preliminary observations that we and others have made toward deciphering the role of DNA methylome in the pathogenesis of

SCZ and BD. It is now becoming increasingly clear that the major psychiatric disorders are caused by both genetic variations and epigenetic alterations in response to the influence of gene–gene and gene–environment interactions. Environmental insults that cause epigenetic alterations could be more problematic in individuals who already possess genetic susceptibility to a specific disease. Indeed, if one already carries a compromised allele, it may not elicit any illness until it reaches the diagnostic threshold. On the other hand, epigenetic insults, through alteration in the promoter DNA methylation changes of critical genes, could potentially worsen the background hypo-/hyperactivity, causing the individual to reach the disease threshold. Such genes as *COMT*, *MAOA*, *TH*, *DAT1*, *DRD1–2*, *BDNF*, and *RELN* were initially selected for our studies because of their involvement in major mental disorder psychopathologies as indicated by meta-analyses or based on more frequently reported association studies as discussed elsewhere (3,28). These studies should be extended by the selection of additional genes based on pathway analyses and construction of an interconnecting network model for the functionality of the DAergic system. The epigenetic data generated from these studies should be pooled to establish an integrated model for pathogenesis resulting from the dysregulation of DA signaling and the functional status of effector genes of the complex major mental disorders SCZ and BD.

We predict that the consideration of the interactions between epigenetic risk factors and genetic susceptibility genes could provide a comprehensive view of the pathogenesis of these diseases in an integrated model. We hope the data and knowledge provided from such studies could provide the basis for further research to establish a more extensive interconnecting comprehensive network model that will incorporate several other pathways (e.g., *N*-methyl-D-aspartate [NMDA], serotonin [5-HT], etc.) in addition to the DAergic system.

9.5 Notes

1. In some locations, the CpGs of placental DNA may be methylated, particularly when the target CpGs are located in the coding regions. If you could not eliminate the methylated product by increasing the annealing temperature and other evidence (e.g., bisulfite sequencing) indicates that the placental DNA is methylated in the target region, the DNA of 5-azacytidine-treated cells in culture could be used as the negative controls for methylation.
2. In general, the size of the amplified target fragment in qPCR needs to be less than 150bp. Therefore, when designing the primers for MSP, this issue should be considered if planning to use the same primers for qMSP or QM-MSP. However, it is likely that positioning and designing of the MSP primers may not conform to this limit. If the product size is larger than 150bp (e.g., RELM M1/U1 or DAT1 in our experiments), the accuracy of the qMSP/QM-MSP results should also be examined using serial dilutions of a known DNA template in primary test trials.
3. We designed the primers for the promoter amplification of genes with a low annealing temperature and the MSP primers with a higher annealing temperature so that the remaining promoter amplification primers in the reaction would not anneal during QM-MSP and mediate amplification of nonspecific products. In addition, when a minimum amount of promoter amplification primers is used and PCR products are diluted 30–50 times, the remnant of these primers

will be minimal in the second reaction. This also avoided compromising the specificity of the PCR product in QM-MSP in our experiments. Nevertheless, if you have evidence that promoter amplification primers have interfering effects, you may dilute the PCR product 100 times (or more if necessary) and use less promoter amplification primers in the first-round PCR or purify the first-round PCR products using appropriate PCR purification kits to get rid of the remaining unused primers.

4. There could be a competition for the PCR amplification of specific genes based on the affinity of the corresponding primer pairs and availability of the template. We found that the most accurate results could be obtained when, for each gene, a set of nested primers from a CG-free region is designed, and the C_T of the amplified fragment in real-time PCR is used for normalization (instead of β -actin). Alternatively, if the annealing temperature of methylated and unmethylated DNA-specific primers is the same, you may mix both unmethylated and methylated primers in the third reaction and use the C_T of this reaction for normalization of the unmethylated or methylated product. We had the best results when we mixed 25 nM of the unmethylated primers with 12.5 nM of the M primers. In this primer concentration, unmethylated DNA will be amplified as it is amplified in a separate reaction; concurrently, the methylated DNA is also amplified efficiently with no formation of the primer dimers. If you have evidence that the level of methylated DNA of your target gene is higher than the level of unmethylated DNA (e.g., DAT1 in our experiment), then use 25 nM of the methylated primers and 12.5 nM of the unmethylated primers in the reaction. Note that test trials should be done to quantify the best primer concentrations for each gene.

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Chapter 10

Pharmacogenomics in Alzheimer's Disease

Ramón Cacabelos

10.1	Introduction	214
10.2	Genomics	217
10.3	Alzheimer's Disease Therapeutics	260
10.4	Pharmacogenomics.....	271
10.5	Conclusions	324
	References.....	326

Summary Pharmacological treatment in Alzheimer's disease (AD) accounts for 10–20% of direct costs, and fewer than 20% of AD patients are moderate responders to conventional drugs (donepezil, rivastigmine, galantamine, memantine), with doubtful cost-effectiveness. Both AD pathogenesis and drug metabolism are genetically regulated complex traits in which hundreds of genes cooperatively participate. Structural genomics studies demonstrated that more than 200 genes might be involved in AD pathogenesis regulating dysfunctional genetic networks leading to premature neuronal death. The AD population exhibits a higher genetic variation rate than the control population, with absolute and relative genetic variations of 40–60% and 0.85–1.89%, respectively. AD patients also differ in their genomic architecture from patients with other forms of dementia. Functional genomics studies in AD revealed that age of onset, brain atrophy, cerebrovascular hemodynamics, brain bioelectrical activity, cognitive decline, apoptosis, immune function, lipid metabolism dyshomeostasis, and amyloid deposition are associated with AD-related genes. Pioneering pharmacogenomics studies also demonstrated that the therapeutic response in AD is genotype-specific, with apolipoprotein E (*APOE*) 4/4 carriers the worst responders to conventional treatments. About 10–20% of Caucasians are carriers of defective cytochrome P450 (*CYP*) 2D6 polymorphic variants that alter the metabolism and effects of AD drugs and many psychotropic agents currently administered to patients with dementia. There is a moderate accumulation of AD-related genetic variants of risk in *CYP2D6* poor metabolizers (PMs) and ultrarapid metabolizers (UMs), who are the worst responders to conventional drugs. The association of the *APOE-4* allele with specific genetic variants of other genes (e.g., *CYP2D6*, angiotensin-converting enzyme [*ACE*]) negatively modulates the therapeutic response to multifactorial treatments

affecting cognition, mood, and behavior. Pharmacogenetic and pharmacogenomic factors may account for 60–90% of drug variability in drug disposition and pharmacodynamics. The incorporation of pharmacogenetic/pharmacogenomic protocols to AD research and clinical practice can foster therapeutics optimization by helping to develop cost-effective pharmaceuticals and improving drug efficacy and safety.

Keywords *ACE*; Alzheimer's disease; anxiety; *APOE*; cholinesterase inhibitors; cognition; combination therapy; *CYP2D6*; genetics; genomics; pharmacogenetics; pharmacogenomics.

10.1 Introduction

Alzheimer's disease (AD) is the most frequent cause of dementia (50–70%), followed by vascular dementia (30–40%) and mixed dementia (15–20%). These prevalent forms of age-related neurodegeneration represent a major problem of health in developed countries, with more than 25 million people affected and probably more than 75 million people at risk during the next 20–25 years worldwide. The prevalence of dementia increases exponentially, from approx. 1% at 60–65 yr to more than 30–35% in people older than 80 yr. It is very likely that in those patients older than 75–80 yr most cases of dementia are mixed in nature (degenerative plus vascular), whereas pure AD cases are very rare after 80 yr (1–3).

The average annual cost per person with dementia ranges from U.S.\$15,000 to U.S.\$50,000 depending on disease stage and country, with a lifetime cost per patient of more than U.S.\$175,000 (4). In some countries, approx. 80% of the global costs of dementia (direct plus indirect costs) are assumed by the patients or their families. It has been postulated that dementia appears to be the most costly disease for society in many countries in the population segment older than 60 yr (5). About 10–20% of the costs in dementia are attributed to pharmacological treatment, including antidementia drugs, psychotropics (antidepressants, neuroleptics, anxiolytics), and other drugs currently prescribed in the elderly (antiparkinsonians, anticonvulsants, vasoactive compounds, anti-inflammatory drugs, etc.). In addition, since 1990 more than 300 drugs have been partially or totally developed for AD (6), with the subsequent costs for the pharmaceutical industry, and only 5 drugs with moderate-to-poor efficacy and questionable cost-effectiveness have been approved in developed countries (7–9).

With the advent of recent knowledge on the human genome (10,11) and the identification and characterization of AD-related genes (12,13) as well as novel data regarding cytochrome P450 (*CYP*) family genes and other genes with enzymatic products that are responsible for drug metabolism in the liver (e.g., *N*-acetyltransferases [*NATs*], ATP-Binding Cassette, subfamily B/Multidrug Resistance (*ABCBs/MDRs*), thiopurine methyltransferase [*TPMT*]), it has been convincingly postulated that the incorporation of pharmacogenetic and pharmacogenomic procedures (see Figs. 10.1 and 10.2) in drug development might

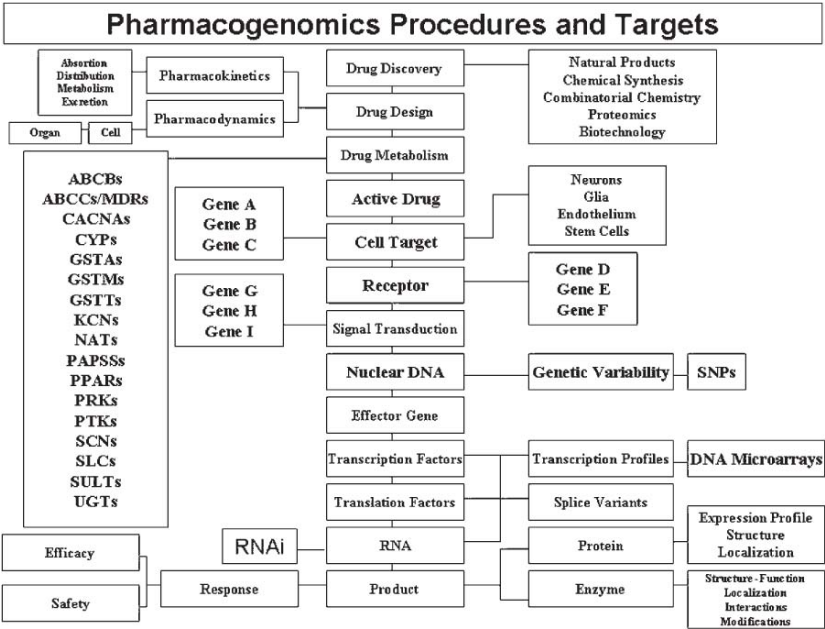


Fig. 10.1 Sequential processing in drug development and pharmacogenomics. (Adapted from refs. 18–20.) (For abbreviations see Table 10.3)

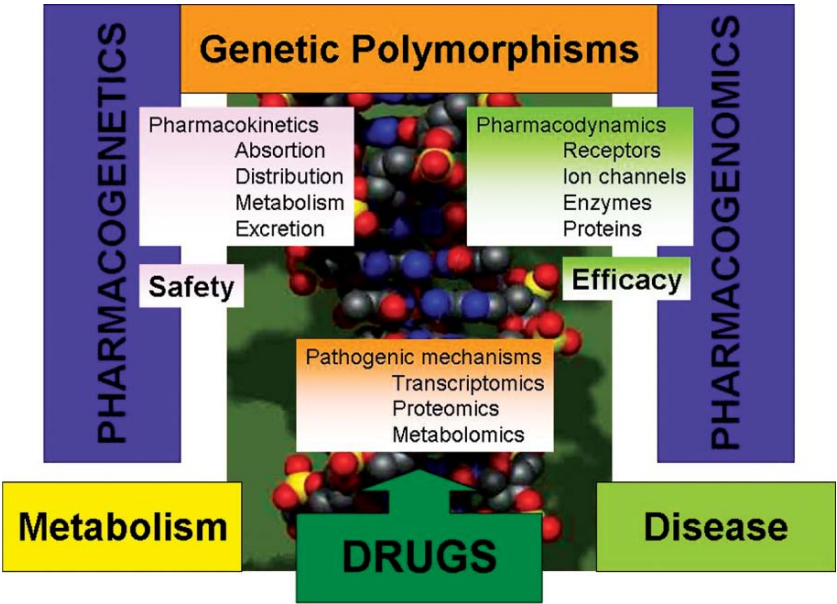


Fig. 10.2 Efficacy and safety issues associated with pharmacogenetics and pharmacogenomics. (Adapted from ref. 20.)

bring about substantial benefits in terms of therapeutics optimization in dementia (13–22) and in many other complex disorders (12,17,21), assuming that genetic factors are determinant for both premature neuronal death in AD (12,23–25) and drug metabolism (26–28).

However, this field is still in its infancy, and the incorporation of pharmacogenomic strategies in drug development and pharmacological screening in dementia and other central nervous system (CNS) disorders is not an easy task. The natural course of technical events to achieve efficient goals in pharmacogenetics and pharmacogenomics include the following steps: (1) genetic testing of mutant genes or polymorphic variants of risk; (2) genomic screening and understanding of transcriptomic, proteomic, and metabolomic networks; (3) functional genomics studies and genotype–phenotype correlation analysis; and (4) pharmacogenetics and pharmacogenomics developments addressing drug safety and efficacy, respectively (13–16,18–20).

With pharmacogenetics, we can understand how genomic factors associated with genes encoding enzymes responsible for drug metabolism regulate pharmacokinetics and pharmacodynamics (mostly safety issues) (18–20,29–31). With pharmacogenomics, we can differentiate the specific disease-modifying effects of drugs (efficacy issues) acting on pathogenic mechanisms directly linked to genes with mutations that determine alterations in protein synthesis or subsequent protein misfolding and aggregation (15–22). The capacity of drugs to reverse the effects of the activation of pathogenic cascades (phenotype expression) regulated by networking genes basically deals with efficacy issues. At present, the terms *pharmacogenetics* and *pharmacogenomics* are often used interchangeably to refer to studies of the contribution of inheritance to variation in the drug response phenotype (28); however, for historical and didactic reasons (until a more suitable and universal definition be established), it would be preferable to maintain the term of *pharmacogenetics* for the discipline dealing with genetic factors associated with drug metabolism and safety issues, whereas *pharmacogenomics* would refer to the reciprocal influence of drugs and genomic factors on pathogenetic cascades and disease-associated gene expression (efficacy issues) (18–20). Finally, with nutrigenetics/nutrigenomics, we can evaluate the effects of the most influential environmental factor (nutrition) on genomic function, disease induction, and drug metabolism as well as the influence of structural and functional genomics on the appropriate metabolism of nutritional factors (19,20,32–36).

The application of these procedures to dementia is a very difficult task because dementia is a complex disorder in which more than 200 genes might be involved (12,13,19,20) (see Table 10.1). In addition, it is very unlikely that a single drug would be able to reverse the multifactorial mechanisms associated with premature neuronal death in most dementing processes with a complex phenotype represented by memory decline, behavioral changes, and progressive functional deterioration (37). This clinical picture usually requires the utilization of different drugs administered simultaneously, including memory enhancers such as the conventional anti-dementia drugs (tacrine, donepezil, rivastigmine, galantamine, memantine) approved by the Food and Drug Administration (FDA); psychotropics (antidepressants, neuroleptics, anxiolytics); anticonvulsants; antiparkinsonians; and other

types of drugs of current use in the elderly because of the presence of concomitant ailments (e.g., hypertension, cardiovascular disorders, diabetes, hypercholesterolemia, etc.).

In fact, the average number of drugs taken by patients with dementia ranges from six to more than ten per day depending on their physical and mental conditions. Nursing home residents receive, on average, seven to eight medications each month, and more than 30% of residents have monthly drug regimes of nine or more medications, including (in descending order) analgesics, antipyretics, gastrointestinal agents, electrolytic and caloric preparations, CNS agents, anti-infective agents, and cardiovascular agents (38). In population-based studies, more than 35% of patients older than 85 yr are moderate or chronic antidepressant users (39). Polypharmacy, drug–drug interactions, adverse reactions, and noncompliance are substantial therapeutic problems in the pharmacological management of elderly patients (40), adding further complications and costs to the patients and their caregivers. In 2000–2001, in ten U.S. health maintenance organizations (HMOs) 23.0–36.5% of elderly individuals (N = 157,517) received at least 1 of 33 potentially inappropriate medications (41). Although drug effect is a complex phenotype that depends on many factors, it is estimated that genetics accounts for 20–95% of variability in drug disposition and pharmacodynamics (30). Under these circumstances, therapeutics optimization is a major goal in the elderly population, and novel pharmacogenetic and pharmacogenomic procedures may help in this endeavor (1,6,13–21).

In the present chapter, a brief summary of recent findings and data of interest related to the pharmacogenomics of AD is introduced, including (1) the involvement of polygenic factors in the pathogenesis of AD; (2) the influence of particular genotypes on the phenotypic expression of AD-related traits and biological markers; (3) the genetic variation observed in AD compared with the normal population and with other forms of dementia, such as vascular dementia; (4) the potential influence of pharmacogenetic factors (e.g., *CYP2D6* genotypes) in AD therapeutics; (5) the effect of pharmacogenomic factors (AD-related genes, apolipoprotein E [*APOE*], angiotensin-converting enzyme [*ACE*]) on the therapeutic response to conventional drugs in dementia; and (6) original data derived from studies using bigenic clusters (*APOE/ACE*, *APOE/CYP2D6*) to investigate the influence of genetic associations on AD therapeutics (18–20,42).

10.2 Genomics

10.2.1 Genetics

A few years after the pioneering studies of Alois Alzheimer (1864–1915) with August D., Johann F., and other patients, reported between 1907 and 1911 (43–46), it soon became clear that AD was a clinical entity that accumulated in some families,

suggesting that an important genetic component might be influencing the pathogenesis of this neurodegenerative disorder. Familial predisposition to dementia has been documented in the second patient of Alzheimer, who died in October 1910 at age 57 years, representing the index case of a family with clear predisposition to presenile dementia (46).

Our present knowledge of AD genetics derives from population, family, twin, adoption, and molecular biology studies carried out during the past 50 years (47–50). After the pioneering work of Schotky, Lowenberg, Waggoner, and MacManemey in the 1930s, Sjögren in the 1950s, and Heston and associates in the 1960s and 1970s (47), complex segregation analysis in the early 1990s led to the conclusion that AD is determined, in part, by a major autosomal dominant allele with an additional multifactorial component (51,52). The frequency of the AD susceptibility allele was estimated to be 0.038, with a potential major locus accounting for 24% of the transmission variance, indicating an important role for several genetic loci and other nongenetic mechanisms (51). Autosomal recessive forms of AD cannot be ruled out in specific populations (53). Epidemiological studies also suggested that most cases of AD (>80%) are familial (12,47,54,55). For some authors, the familial incidence of AD was about 43% (55), and for others the cumulative incidence of AD among relatives was 49% by age 87 (54). Advances in molecular genetics during the past two decades allowed the identification of several genetic loci associated with AD (see Table 10.1) and the genetic classification of AD (AD1 to ADn) as depicted in the Online Mendelian Inheritance in Man (OMIM) database (12,56).

The genetic defects identified in AD since the early 1980s can be classified into three main categories. First is Mendelian or mutational defects in genes directly linked to AD, including (1) 32 mutations in the amyloid β -protein (β APP) precursor protein (*APP*) gene (21q21); (2) 165 mutations in the presenilin 1 (*PS1*) gene (14q24.3); and (3) 12 mutations in the presenilin 2 (*PS2*) gene (1q31–q42) (12,13,19,20,23–25,57,58) (see Table 10.1). Second, multiple polymorphic variants of risk characterized in more than 200 different genes distributed across the human genome can increase neuronal vulnerability to premature death (12,13,19,20) (see Table 10.1). Among these genes of susceptibility, the *APOE* gene (19q13.2) is the most prevalent as a risk factor for AD, especially in those subjects harboring the *APOE-4* allele, whereas carriers of the *APOE-2* allele might be protected against dementia (12,20). *APOE*-related pathogenic mechanisms are also associated with brain aging and with the neuropathological hallmarks of AD (1,12,19,20,25,59,60). Third, diverse mutations located in mitochondrial DNA (mtDNA) through heteroplasmic transmission can influence aging and oxidative stress conditions, conferring phenotypic heterogeneity (12,61–63). It is also likely that defective functions of genes associated with longevity may influence premature neuronal survival because neurons are potential pacemakers defining life span in mammals (12,20).

All these genetic factors may interact in still-unknown genetic networks, leading to a cascade of pathogenic events characterized by abnormal protein processing and misfolding with subsequent accumulation of abnormal proteins (conformational changes), ubiquitin–proteasome system dysfunction, excitotoxic reactions, oxidative

Table 10.1 Selected human genes investigated as potential candidate genes associated with dementia and age-related neurodegenerative disorders

Locus	Symbol	Title/Gene	MIM
1p21.3–p131.1	<i>SORT1</i>	Sortilin	602458
1p31	<i>BBP</i>	β -Amyloid-binding protein precursor	
1p32	<i>ZFYVE9</i>	Zinc finger, FYVE domain containing 9	
	<i>SARA</i>	SMAD anchor for receptor activation	
	<i>MADHIP</i>	MADH-interacting protein	
1p34	<i>LRP8</i>	Low-density lipoprotein receptor-related protein 8	602600
	<i>APOE receptor 2</i>		
1p36	<i>AD7CNTP</i>	Alzheimer disease neuronal thread protein (ADNTP)	607413
1p36.3	<i>MTHFR</i>	Methylenetetrahydrofolate reductase	236253
			104300
1q21	<i>S100A</i>	S100 calcium-binding protein A1	176940
1q21–q23	<i>APCS</i>	Serum amyloid P component	104770
1q23	<i>NCSTN</i>	Nicastrin	605254
	<i>APH2</i>		
1q25	<i>SOAT1</i>	Acyl-CoA:cholesterol acyltransferase	102642
	<i>STAT</i>	Sterol O-acyltransferase 1	
	<i>ACAT</i>		
1q31–q42	<i>AD4</i>	Presenilin 2	600759
	<i>PSEN2</i>		104300
	<i>STM2</i>		
Chr. 1	<i>APH1A</i>	<i>Caenorhabditis elegans</i> anterior pharynx defective homolog	607629
2p14–p13	<i>RTN4</i>	Neurite outgrowth inhibitor (reticulon 4)	604475
	<i>NOGO</i>		
2p25	<i>ADAM17</i>	A desintegrin and metalloproteinase domain 17	603639
	<i>TACE</i>	Tumor necrosis factor- α converting enzyme	
2q14	<i>IL-1A</i>	Interleukin 1 α	147760
2q21.1	<i>CSEN</i>	Calsenilin	604662
	<i>DREAM</i>		
	<i>KCNIP3</i>		
2q21.2	<i>LRP1B</i>	Low-density lipoprotein receptor-related protein 1B	608766
3q26.1–q26.2	<i>BCHE</i>	Butyrylcholinesterase	177400
3q32.3–q34	<i>CREB1</i>	Cyclic adenosine monophosphate (cAMP) response element-binding protein	123810
Chr. 4	<i>APBB2</i>	Amyloid β A4 precursor protein-binding, family B, member 2	602710
	<i>FE65L1</i>		
5q15–q21	<i>CAST</i>	Calpastatin	114090
5q31	<i>APBB3</i>	Amyloid β A4 precursor protein-binding, family B, member 3	602711
	<i>FE65L2</i>		
5q35.3	<i>DBN1</i>	Drebrin E	12660
6p21.3	<i>AGER</i>	Advance glycosylation end product-specific receptor	600214
	<i>RAGE</i>		

(continued)

Table 10.1 (continued)

Locus	Symbol	Title/Gene	MIM
6p21.3	<i>TNFA</i>	Tumor necrosis factor- α	191160
		Cachectin	
7p21	<i>IL-6</i>	Interleukin 6	147620
	<i>IFNB2</i>	β 2-Interferon	
7q36	<i>NOS3</i>	Nitric oxide synthase 3	163729
8p22	<i>CTSB</i>	Cathepsin B	116810
	<i>CPSB</i>	Amyloid precursor protein secretase	
9q13	<i>APBA1</i>	Amyloid β A4 precursor protein-binding, family A, member 1	602414
	<i>X11</i>		
	<i>MINT1</i>		
	<i>LIN10</i>		
10p13	<i>AD7</i>	Alzheimer disease 7	606187
10q23-q25	<i>IDE</i>	Insulin-degrading enzyme	146680
10q24	<i>AD6</i>	Alzheimer disease 6	605526
			104300
10q24	<i>PLAU</i>	Plasminogen activator, urokinase	191840
	<i>URK</i>		
11p15	<i>APBB1</i>	Amyloid β A4 precursor protein-binding, family B, member 1	602709
	<i>F65</i>		
11p15.1	<i>SAA1</i>	Serum amyloid A1	104750
11q23.2-q24.2	<i>SORL1</i>	Sortilin-related receptors	602005
11q23.3	<i>BACE1</i>	β -Site amyloid β A4 precursor protein-cleaving enzyme	604252
	<i>BACE</i>	β -Secretase	
		Memapsin 2	
11q24	<i>APLP2</i>	Amyloid β A4 precursor-like protein 2	104776
12p11.23-q13.12	<i>AD5</i>	Familial Alzheimer disease 5	602096
12p12.3-p12.1	<i>IAPP</i>	Islet amyloid polypeptide	147940
	<i>IAP</i>	Amylin	
	<i>DAP</i>	Diabetes-associated peptide	
12p13.3-p12.3	<i>A2M</i>	α_2 -Macroglobulin	103950
12q13.1-q13.3	<i>LRP1</i>	Low-density lipoprotein-related protein 1	107770
	<i>A2MR</i>	α_2 -Macroglobulin receptor	
14q24.3	<i>FOS</i>	FBJ murine osteosarcoma viral (v-fos) oncogene homolog	164810
		Oncogene Fos	
14q24.3	<i>AD3</i>	Presenilin 1	104311
	<i>PSEN1</i>		
14q32.1	<i>SERPINA3</i>	α_1 -Antichymotrypsin	107280
	<i>AACT</i>		
	<i>ACT</i>		
14q32.1	<i>CYP46</i>	Cytochrome P450	604087
	<i>CYP46A1</i>	Family 46, subfamily A	
		Polypeptide 1	
		Cholesterol 24-hydrolase	
Chr. 15	<i>APH1B</i>	Homolog of <i>C. elegans</i> anterior pharynx defective 1B	607630

(continued)

Table 10.1 (continued)

Locus	Symbol	Title/Gene	MIM
15q11–q12	<i>APBA2</i> <i>X11L</i>	Amyloid β A4 precursor protein-binding, family A, member 2	602712
16q22	<i>APPBP1</i>	Amyloid β precursor protein-binding protein 1	603385
17q11.2	<i>BLMH</i> <i>BMH</i>	Bleomycin hydrolase	602403
17q21	<i>STH</i>	Saitohin	607067
17q21.1	<i>MAPT</i> <i>MTBT1</i> <i>DDPAC</i> <i>MST</i>	Macro tubule-associated protein tau	157140 600274 168610 172700 601104
17q21–q22	<i>GPSC</i>	Familial progressive subcortical gliosis	221820
17q22–q23	<i>APPBP2</i> <i>PAT1</i>	Amyloid β precursor protein-binding protein 2	605324
17q23	<i>ACE</i> <i>ACE1</i> <i>DCP1</i>	Angiotensin I-converting enzyme Dipeptidyl carboxypeptidase 1	106180 104300
17q23.1	<i>MPO</i>	Myeloperoxidase	254600
17q24	<i>FALZ</i> <i>FAC1</i>	Fetal Alzheimer antigen	601819
18q11.2–q12.2	<i>TTR</i> <i>PALB</i>	Transthyretin Prealbumin	176300
19p13.2	<i>NOTCH3</i> <i>CADASIL</i> <i>CASIL</i>	<i>Drosophila</i> Notch 3 homolog	600276
19p13.2	<i>AD8</i>	Alzheimer disease 9	608907
19p13.3–p13.2	<i>ICAM</i> <i>CD54</i> <i>BB2</i>	Intercellular adhesion molecule 1	147840
19p13.3	<i>APBA3</i> <i>X11L2</i>	Amyloid β A4 precursor protein binding, family A, member 3	604262
19q13.12	<i>PEN2</i>	Presenilin enhancer 2	607632
19q13.2	<i>APOE</i>	Apolipoprotein E	107741
19q13.2	<i>APOC1</i>	Apolipoprotein C-I	107710
19cen-q13.2	<i>AD2</i>	Alzheimer disease 2	104310
19cen-q13.2	<i>APLP1</i>	Amyloid β A4 precursor-like protein 1	104775
19q31-qter	<i>APPL1</i>	Amyloid β A4 precursor protein-like 1	104740
20p	<i>AD8</i>	Alzheimer disease 8	607116 104300
20p11.2	<i>CST3</i>	Cystatin 3	604312
20p11.2	<i>CST3</i>	Cystatin C	604312
21q21	<i>AD1</i> <i>APP</i> <i>AAA</i> <i>CVAP</i>	Amyloid β (A4) precursor protein Amyloid of aging and Alzheimer disease Cerebrovascular amyloid peptide Protease nexin II	104760

(continued)

Table 10.1 (continued)

Locus	Symbol	Title/Gene	MIM
21q22.3	<i>BACE2</i>	β-Site amyloid βA4 precursor protein-cleaving enzyme 2	605668
	<i>ALP56</i>		
	<i>DRAP</i>	Down syndrome-region aspartic protease	
22q11	<i>RTN4R</i>	NOGO receptor (reticulon 4 receptor)	605566
	<i>HN^{NOGOR}</i>	Humanin	606120

Source: Adapted from refs. 12 and 20.

and nitrosative stress, mitochondrial injury, synaptic failure, altered metal homeostasis, dysfunction of axonal and dendritic transport, and chaperone misoperation (12,19,20) (see Fig. 10.3). These pathogenic events may exert an additive effect, converging in final pathways that lead to premature neuronal death. Some of these mechanisms are common to several neurodegenerative disorders that differ depending on the gene affected and the involvement of specific genetic networks, together with cerebrovascular factors, epigenetic factors (DNA methylation), and environmental conditions (nutrition, toxicity, social factors, etc.) (1,12,19,20,64–69). The higher the number of genes involved in AD pathogenesis, the earliest the onset of the disease, the faster its clinical course, and the poorer its therapeutic outcome (12,18–20).

10.2.2 Approaches to Structural and Functional Genomics

Structural genomics has as its goal the provision of structural information for all possible open reading frame sequences through a combination of experimental and computational approaches (70). According to their mutational profile, there are more than 200 different AD types; however, the integration of genomic profiles into polygenic clusters associated with AD can yield more than 5000 AD variants in the population (12,19,20). Full-length genomic screening in AD is a technically difficult task, and most studies use proteomic analysis for the identification of abnormal proteins in AD tissues (71–75) or in transgenic animals with AD-linked mutations (76–79). Association studies of diverse genes in different populations show contradictory results of difficult validation (80–83).

It is likely that about 80% of the human genes can be expressed in the CNS. More than 1000 full-open reading frames of transcripts expressed in the CNS, many of which encode yet-uncharacterized proteins, have been identified (84). In neurodegenerative processes in general, and in dementia in particular, thousands of networking genes are altered, leading to defective transcriptomic, proteomic, and metabolomic expression (12,19,20). Genomewide transcription profiling is a powerful technique

for studying cell function, and data obtained from high-density microarrays are complex, posing important challenges in data mining. Dealing with microarray data, novel genes as well as uncharacterized expressed sequence tags (ESTs) can be identified in AD (85,86). Proteome-scale studies of protein three-dimensional structures can provide valuable information for investigating basic biology and pathogenic mechanisms and developing therapeutics (87).

Although the amyloid hypothesis is recognized as the *primum movens* of AD pathogenesis (12,23,24), mutational genetics associated with *APP* and *PS* genes alone (<10% of AD cases) does not explain in full the neuropathological findings present in AD, represented by amyloid deposition in senile plaques and vessels (amyloid angiopathy), neurofibrillary tangle (NFT) formation caused by hyperphosphorylation of tau protein, synaptic and dendritic desarborization, and neuronal loss, accompanied by neuroinflammatory reactions, oxidative stress, and free-radical formation probably associated with mitochondrial dysfunction, excitotoxic reactions, alterations in cholesterol metabolism and lipid rafts, deficiencies in neurotransmitter and neurotrophic factor function, defective activity of the ubiquitin–proteasome and chaperone systems, and cerebrovascular dysregulation (12,19,20,37). All these neurochemical events are potential targets for treatment (18–20) (see Table 10.2 and Fig. 10.3).

10.2.2.1 Genetic Variation

Approximately 5% of the human genome is structurally variant in the normal population, involving more than 800 genes (87). The spectrum of variation in the human genome includes (1) single changes (single-nucleotide polymorphisms [SNPs], point mutations) (1 bp); (2) small insertions/deletions (I/Ds) (binary I/D events of short sequences) (1–50 bp); (3) short tandem repeats (microsatellites) (1–500 bp); (4) fine-scale structural variation (deletions, duplications, tandem repeats, inversions) (50 bp to 5 kb); (5) retroelement insertions (SINEs, LINEs, LTRs, ERVs) (300 bp to 10 kb); (6) intermediate-scale structural variations (deletions, duplications, tandem repeats, inversions) (5–50 kb); (7) large-scale structural variation (deletions, duplications, large tandem repeats) (50 kb to 5 Mb); and (8) chromosomal variations (euchromatic variations, cytogenetic deletions, duplications, translocations, inversions, and aneuploidy) (>5 Mb) (87). Segmental duplications of low copy repeats are blocks of DNA ranging from 1 to 400 kb in length that occur at multiple sites within the genome and typically share a high level (>95%) of sequence identity (87). Segmental duplications frequently mediate polymorphic rearrangements of intervening sequences via nonallelic homologous recombination (NAHR) with major implications for human disease. SNPs and I/D events are the most frequent types of structural variation. I/D polymorphisms of several genes with functions in enzymatic pathways or in drug-metabolizing enzymes (e.g., *CYP2D6*) may drastically influence a variety of common phenotypes with pathogenic or pharmacogenetic relevance.

Table 10.2 Potential therapeutic strategies in Alzheimer’s disease and dementia

Pathogenic mechanism	Therapeutic strategy	Drug	Company
Genetic factors Single-gene related	Gene therapy	AAV-NGF (CERE-110)	Ceregene
	NGF gene therapy RNAi	OM-99-2 KMI-008 Fs(OMOO-3)dR9 Hisidin WO 0399202 WO 0302122 WO 0345913 WO 0402483 US 6562783 BMS LY-411575 WEP-III-31C WO 0393264 WO 0314095 WO 0359335 WO 366592	Universities of Illinois and Oklahoma Kyoto Oklahoma/Tokyo/Zapag Kyungpook National University Merck Sharp and Dohme Elan/Pfizer GlaxoSmithKline Actelion NeuroLogic Bristol-Myers Squibb Lilly/Athena Brigham/Harvard Medical School Merck Sharp and Dohme Sanofi-Synthélabo Bayer Pharmacoceia/Schring-Plough
Polygenic related β-Amyloid deposition	β-Secretase inhibitors		
	γ-Secretase inhibitors		
	α-Secretase activators	PTI-00703	ProteoTech
	Aβ-fibrillization and aggregation inhibitors	PPI-1019/APAN NC-531 β-Sheet breaker protein RS-0406 Oxigon HF-0420	Praecis Neurochem Serono BTG/Sankyo Mindset BioPharmaceuticals Hunter-Fleming

Table 10.2 (continued)

Pathogenic mechanism	Therapeutic strategy	Drug	Company
Tau pathology	Amyloid immunotherapy	AN-1792	Elan/Wyeth
		AAB-001 (hMAb)	Elan/Wyeth
		AAC-001	Elan/Wyeth
		Aβ Synthetic homologs	Mindset BioPharmaceuticals
		Aβ Immunoconjugates	Neurochem/Praecis
	Copper chelating agents Solubilizers of Aβ aggregates	Aβ/High-ordered config	Cytos/Novartis
		AAV-CB-Aβ42	Peking Union Med Col
		MAbs	Merck and Co./Acumen
		Clioquinol/PBT-1	Prana
		DP-109	D-Pharm
Apoptosis	APP production inhibitors	PBT-2	Prana
		Phenserine tartrate	Axonyx
		Posiphen	Axonyx/National Institutes of Health
		Reticulons	
		Chaperones/SLF-CR	Howard Hughes Medical Institute
	Aβ-Selective regulators		
Neurotransmission deficits Acetylcholine	Phosphatase activators GSK-3 inhibitors Cdk5 inhibitors P38 inhibitors JNK inhibitors Caspase inhibitors Neurotrophic agents		
Acetylcholine	Acetylcholine-release stimulant	Montirelin	Nippon Seiyaku
		T-588	Toyama
		Dexefaroxan/RX-821037	bioMerieux-Pierre Fabre

(continued)

Table 10.2 (continued)

Pathogenic mechanism	Therapeutic strategy	Drug	Company
Enzymes	Acetylcholine reuptake inhibitor	MKC-231	Mitsubishi
		Tacrine	First Horizon/OTL Pharma
	Cholinesterase inhibitors	Donepezil	Eisai/Pfizer
		Rivastigmine	Novartis
		Galantamine	Johnson and Johnson/Shire
		Metrifonate	Bayer
		7-Methoxytacrine	Research Institute for Pharmacy and Biochemistry
		Zanapezil/TAK-147	Takeda
		Ensaculin/KA-672	Schwabe
		Phenserine	Axonyx
Muscarinic receptors	Choline-acetyl-transferase stimulant	Ganstigmine/CHF-2819	Chiesi
		P-11149	Aventis
		NIK-247	Nikken
		Methanosulfonyl fluoride	Texas University
		T-82	SSP/Arena/Seiyaku
		Teserstigmine/CHF-2060	Chiesi
		ZT-1	Jiangsu Yangtse
		Nefiracetam	Dai-ichi
		Cevimeline/AF-102A	Dai-ichi/Nippon Kayaku
		PD-151832	Pfizer
Nicotinic receptors	Muscarinic agonists	Sch-211803	Schering-Plough
		SIB-1553A	Merck
		Fasoracetam	Nippon Seiyaku
		NDG-97-1	Neurogen/Pfizer
GABA	Muscarinic antagonists		Shionogi/GlaxoSmithKline
	Nicotinic agonists	S-8510	

Table 10.2 (continued)

Pathogenic mechanism	Therapeutic strategy	Drug	Company
Glutamate NMDA AMPA	GABA modulators	S-189861	Servier
	Inverse GABA receptor agonist	Memantine CX-516	Merz/Lundbeck/Forest Cortex/Servier
		Ampalex/CX-516 CX-691	Cortex/Servier
Dopamine	Glutamate agonists	NS-2330	Neurosearch/Boehringer
Noradrenaline	NMDA antagonists	Nicergoline	Pharmacia
Histamine	Ampakines	Cipralisant T82	Gliatec/Merck SSP/Arena
Serotonin	Dopamine reuptake inhibitors	Xaliproden/SR-57746A	Sanofi-Synthelabo
Neurotrophic deficit	Adrenoreceptor modulators	742457	GlaxoSmithKline
	Histamine H3 antagonists	FK-960	Fujisawa
	5-HT3 receptor agonist		
	5-HT1A receptor agonist		
	5-HT6 antagonist		
	Serotonin stimulant		
	Neurotrophic agents		
Neuronal loss	NGF agonists	Cerebrolysin	Ebewe/Abbot
	Growth factors	Xaliproden/SR-57746A	Sanofi-Synthelabo
	Synthetic neuropeptides	AAV-NGF (CERE-110)	Ceregene
	Neuronal stem cells		
	Growth factors		
	Neurite outgrowth activators		
	Synaptogenesis activators		
	Nogo inhibitors		
	MOP inhibitors		
	GSK-3 inhibitors		
	JNK inhibitors		

(continued)

Table 10.2 (continued)

Pathogenic mechanism	Therapeutic strategy	Drug	Company
Neuroinflammation	P38 inhibitors		
	Cyclooxygenase-1 inhibitors	Naproxen	NIA/Johns Hopkins
	Cyclooxygenase-2 inhibitors	Celecoxib	Pfizer
		Rofecoxib	Merck
	Complement activation inhibitors		
Oxidative stress	P38 inhibitors		eNOS Pharmaceuticals
	Caspase-1 inhibitors	Argisat	
	eNOS inhibitors		
	PPAR α agonists		
	PPAR γ agonists		
	Novel NSAIDs		
	Cytokine inhibitors	Flurizan/R- Flurbiprofen	Myriad
	Antioxidants	Interferon- α_{2A}	NCRR
	Caspase inhibitors	Vitamin E	
	Antioxidating enzyme enhancers		National Institute on Aging (NIA)/ National Cancer Institute (NCI)
Excitotoxic reactions	NMDA antagonists		
Calcium dysmetabolism	Ampakines		
	Modulators of glutamate transporters		
	Calcium channel blockers		
	PPAR γ agonists		
	GSK-3 inhibitors		
Lipid dysfunction	HMG-CoA reductase inhibitors	Atorvastatin Lovastatin/ADX-159	Pfizer/Yamanouchi Andrx
	PPAR γ agonists		
	Novel biomarine lipoproteins	E-SAR-94010 E-JUR-94013 E-CAB-94011	Ebiotec Ebiotec Ebiotec
Cerebrovascular dysfunction	Vasoactive substances	Nicergoline	

Table 10.2 (continued)

Pathogenic mechanism	Therapeutic strategy	Drug	Company
Neuronal dysfunction associated with nutritional deficiency	NO inhibitors		
	HIF inhibitors		
	Dandrolene-related agents		
	Novel lipoproteins	E-SAR-94010	Ebiotec
	Liver X receptor agonists		
Neuronal dysfunction associated with nutritional deficiency	Nutrigenomics	E-SAR-94010	Ebiotec
	Nutraceuticals	E-JUR	Ebiotec
	Brain metabolic enhancers	E-CAB	Ebiotec
		Nutritional BME	NIA/Burke Medical Research Institute
		ABPI-124	Mitokor/AHP
Other pathogenic mechanisms	Estrogen agonists	Estrogen	NIA/National Center for Research Resources
	Estrogen replacement	Estrogen	
	Brain targeted	Vasopressin modulator	Ivax
	Dabelotine/S-12024	Rasagiline	Servier
	MAO-B inhibitors	SL-251188	Lundbeck/TEVA
		FK-960	Sanofi-Synthelabo
	Somatostatin stimulant	Avandia/rosiglitazone	Fujisawa
	Insulin sensitizer	Cyclophosphamide	VA Medical Research Services
	Anti-inflammatory agents		National Institute of Mental Health (NIMH)
	Immunostimulants	Colostrinin	
	MAP kinase inhibitors	CEP-1347	ReGen Therapeutics
		CPI-1189	Cephalon/Lundbeck
			Centaur

(continued)

Table 10.2 (continued)

Pathogenic mechanism	Therapeutic strategy	Drug	Company
	Muscarinic M1-receptor density	P-58	Phytopharm
	Choline uptake enhancer	MKC-231	Mitsubishi
	Prolyl-endopeptidase inhibitors	Z-321	Zeriaaw
		SR-57667	Sanofi-Synthelabo
	Antineurodegenerative agents	Anapso	Alacan/ASAC Pharma
	Immunotrophins	CDP-Choline	Takeda, Ferrer
	Endogenous nucleotides		
Antibiotics		β -Lactam antibiotics (penicillin, ampicillin, ceftriaxone)	Johns Hopkins/Columbia Univ., Palm Harbor ALS Assoc.
		737552	GlaxoSmithKline/Shionogi
Benzodiazepine partial inverse agonist		MEM 1003	Memory Pharmaceuticals
		MEM 1414	Memory Pharmaceuticals
Others		MPC-7869	Myriad Genetics
		Rosiglitazone	GlaxoSmithKline
		Ladostigil hemitartrate	TEVA
		NS-2330	Boehringer Ingelheim
		SGS111	Saegis Pharmaceuticals
		SGS518	Saegis Pharmaceuticals
		SGS742	Saegis Pharmaceuticals
		SRA-333	Wyeth Pharmaceuticals

Source: Adapted from ref. 20.

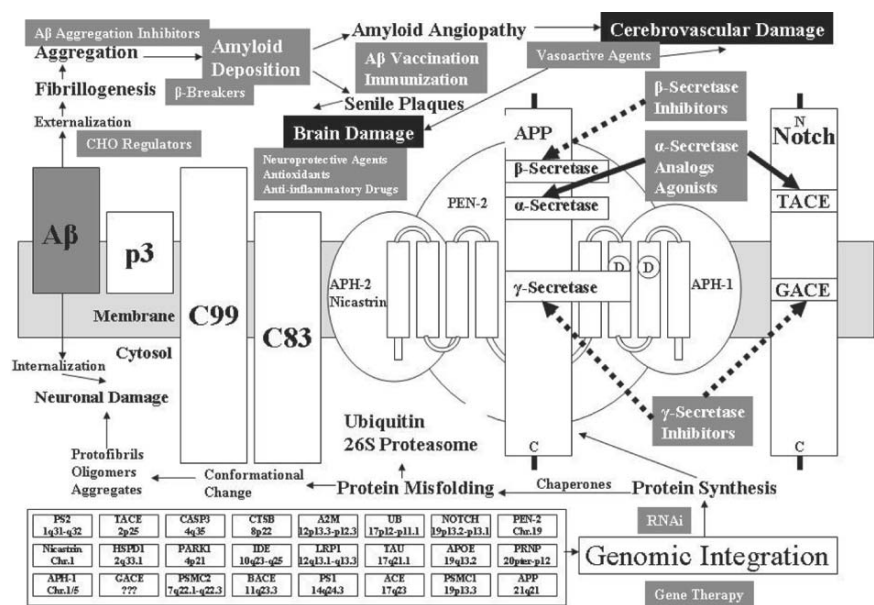


Fig. 10.3 Brain amyloidogenesis and potential therapeutic interventions in Alzheimer’s disease. (Adapted from refs. 18–20.)

The differential expression of common variants is a major source of genetic variation with important repercussion in human diversity and disease heterogeneity. Prior to the completion of the Human Genome Project and the emergence of dense genetic maps, scientists used linkage studies and positional cloning to identify DNA mutations in rare diseases, but in the past two decades association study designs became more powerful compared with linkage study designs in identifying susceptibility loci and SNP variation (88,89). Currently, more than 10 million DNA sequence variations have been uncovered in the human genome (88).

It has been observed that the genetic variation rate (GVR) is higher in AD patients than in the general population (12,13,20,90). The variability of bigenic, trigenic, tetragenic, and polygenic genotypes of AD-related genes is currently higher in AD patients than in controls, with an absolute genetic variation (AGV) of 40–60% and a relative genetic variation (RGV) of 0.85–1.89% depending on the number of genes included in the haplotype-like cluster. Approximately, 40% of AD cases exhibit a GVR higher than 1% compared to controls when a trigenic cluster integrated by combinations of *APOE* plus *PS1* plus *PS2* polymorphic variants is examined. Increased GVR in AD might indicate that the overrepresentation of a series of genes involved in brain maturation and in the maintenance of higher activities of the CNS has surpassed a natural selection threshold (excessive genome complexity, genomic overdiversification), constituting a Darwinian disadvantage that shortens life span in humans (12,20,90).

Recent observations support the contention that serial segmental duplication events might have orchestrated primate evolution by the generation of novel fusion/fission genes as well as potentially by genomic inversions associated with decreased recombination rates facilitating gene divergence (91). *Alu* repeats, the most abundant family of repeats in the human genome, with over 1 million copies comprising 10% of the genome, can also contribute to increase genetic variability and genomic disorders (92). Inversions, deletions, and insertions are important mediators of genomic variability and disease susceptibility. About 297 sites of structural variation have already been identified in the human genome, including 139 insertions, 102 deletions, and 56 inversion breakpoints, some of which are of biomedical relevance (93).

Evolutionary horizontal gene transfer might also contribute to increase genetic variation in AD. Horizontal transfer events can be classified into distinct categories of acquisition of new genes, acquisition of paralogs of existing genes, and xenologous gene displacement by which a gene is displaced by a horizontally transferred ortholog from another xenolog, as currently seen in prokaryotes (94). The fixation and long-term persistence of horizontally transferred genes might confer a selective advantage or disadvantage on the recipient organism, contributing to the phenotypic manifestation of a novel trait.

There are roughly 7–10 million positions in the human genome that can show variability among individuals, and differences in DNA sequence are the genetic basis of human variability and complex traits. Hinds et al. (95) characterized whole-genome patterns of common human DNA variation by genotyping 1,586,383 SNPs in 71 Americans of European, African, and Asian ancestry. Approximately 7 million SNPs show a minor allele frequency (MAF) of at least 5% across the human population, and 4 million SNPs show a MAF of 1–5%. More than 95% of the genome is in inter-SNP intervals of less than 50kb, and two-thirds of the genome is covered by inter-SNP intervals of less than 10kb. There are 735,094 SNPs (46%) in genic regions of the genome defined as within 10kb of the transcribed intervals for 22,904 protein-coding genes, and 20,165 SNPs (1.3%) are present in amino acid coding sequences (95). According to Hinds and coworkers (95), 94% of SNPs have two alleles in the African American population, 81% in the European American sample, and 74% in the Han Chinese sample, with a MAF greater than 10% in 68% of African Americans and 57% of Chinese and a MAF of less than 10% in 17% (263,029 SNPs) of the 1,586,383 SNPs of the three populations. These findings show that most common DNA variation is shared across human populations, with differences in allele frequencies between populations, and are consistent with the conclusion that most functional human genetic variation is not population specific (95). In general terms, this study predicts 73% of common variation in the European American population and 54% of common variation in the African American population (95).

Because trigenic and tetragenic clusters of AD-related genes exhibit a higher AGV with respect to the control population with no family history of dementia (12,13,20,90), AD markers with large between-population variance might be useful for admixture mapping studies to identify genetic variants potentially associated

with phenotypic differences between AD and controls or among different types of dementia. It is also important to take into account that non-African populations generally have higher levels of linkage disequilibrium than African populations, probably because of the generation of linkage disequilibrium by bottlenecks in the history of non-African populations (96).

Furthermore, nonsynonymous SNPs (nsSNPs) are coding variants that introduce amino acid changes in their corresponding proteins. The human population is estimated to have 67,000–200,000 common nsSNPs, and each person is thought to be heterozygous for 24,000–40,000 nsSNPs. These coding variants that can affect protein function are believed to have the largest impact on human health compared with SNPs in other regions of the genome (97).

AD is a perfect paradigm to potentially explain how the stochastic process of aging interacts with genetic factors, leading to reduced neuronal survival and consequently human longevity because neurons are major sensors of longevity, and longevity depends on genome stability, metabolic factors, and environmental factors (98). In this regard, increased genetic variation in AD might represent an evolutionary disadvantage capable of reducing human longevity because of the induction of premature neuronal death (12,20,90). At the base of this deleterious mechanism, epigenetic factors and chaperone dysfunction-related protein misfolding might be present. The accumulation of misfolded proteins, with loss of function or toxic gain of function, can have cellular consequences such as stress response, proteasome inhibition, chaperone sequestration, transcription/cell cycle factor sequestration, fibril pore formation, calcium overload, oxidative stress, glutamate overload, mitochondrial dysfunction, and cell death (99), all present in AD brains (12). In this complex process, with the final common pathway of premature neuronal death and reduced life span, specific forms of chronic apoptosis (100) or accelerated telomere shortening (101) cannot be excluded.

10.2.3 Alzheimer's Disease Pathogenesis

Alzheimer's disease is a complex disorder in which multiple pathogenic mechanisms may be involved to give rise to a common phenotype. From a didactic point of view, it has been established that primary pathogenic events in AD are represented by genetic factors (mutations, susceptibility SNPs) and programmed neuronal death because neurons start to die 30–40 yr before the onset of the disease (12,20). Secondary pathogenic events are associated with the phenotypic expression of senile plaques (amyloid deposition) and NFT, together with synaptic loss, dendritic desarborization, and neuronal death, as the major hallmarks of AD pathology. Tertiary and quaternary pathogenic events are reflected by neurotransmitter deficits, neuroinflammatory reactions, oxidative stress phenomena and free-radical formation, excitotoxic reactions, alterations in calcium homeostasis, deficit of neurotrophic factors, and cerebrovascular perturbations, among many other neurochemical phenotypes (12).

The molecular mechanisms underlying β -amyloid deposition in brain tissue and blood vessels, as well as abnormalities in tau protein leading to NFT formation, have been elegantly elucidated by many groups all over the world (*12,23,24,102*) (see [Fig. 10.3](#)), defining the fundamentals for promising therapeutic strategies oriented to inhibit the formation of amyloid deposits or to reduce senile plaque burden (*6,23,24,103–107*). Notwithstanding, the complexity of the pathogenic cascade in AD invites prediction that many other genetic factors and pathogenic mechanisms may be involved in the etiology of AD, together with epigenetic phenomena, cerebrovascular dysfunction, and environmental events (*12*).

10.2.3.1 Amyloid Precursor Protein Gene

The *APP* gene (21q21.2–q21) encodes the amyloid precursor protein (*APP*), a type I integral membrane glycoprotein containing the ABP region (4 kDa) extending to 28 amino acids of the ectodomain and 11–14 amino acids of the adjacent transmembrane (TM) domain (*108,109*). *APP* has at least ten isoforms generated by alternative splicing of a 19-exon gene with three predominant transcripts (*APP695*, *APP751*, *APP770*), of which *APP695* is preferentially expressed in neurons (*108*). Exons 16 and 17 encode the ABP domain of *APP* (*109*). The cytoplasmic tail of *APP* forms a multimeric complex with the nuclear adaptor protein Fe65 and the histone acetyltransferase TIP60 to stimulate transcription via heterologous Gal4 or LexA DNA binding domains (*110*).

APP is processed by several different proteases called secretases. β -Secretase generates the NH_2 -terminus of ABP, producing a soluble fragment of *APP* (β -*APPs*) and a 99-residue COOH-terminal fragment (C99) bound to the membrane (see [Fig. 10.3](#)). α -Secretase cleaves *APP* at the ABP region to produce α -*APPs* and an 83-residue COOH-terminal fragment (C83) (see [Fig. 10.3](#)). γ -Secretase acts on the C99 and C83 substrates at the TM domain to produce C99-derived 4-kDa ABP and C83-derived 3-kDa p3 peptide (see [Fig. 10.3](#)). γ -Secretase-related proteolysis is heterogeneous, yielding an abundant 40-residue peptide (ABP40) and small amounts of a 42-residue COOH-terminal variant (ABP42), which has hydrophobic properties that facilitate amyloidogenic fibril formation (*111*) (see [Fig. 10.3](#)).

Several missense mutations have been identified in *APP* that potentially result in early-onset AD (EOAD), including separate mutations in codon 717 of the *APP* transcript found in familial AD (fAD) (V717I, V717F, V717G) (*112–116*), referred to as the London *APP717* mutation; the Swedish *APP670/671* double mutation (Lys670Asn/Met671Leu) (*117*); and the Florida *APP716* mutation (Ile716Val) (*118*). These mutations involve codons near the β -secretase and γ -secretase cleavage sites, while the Flemish *APP692* mutation (C692G transversion, A692G), the Dutch *APP693* mutation (Glu22Gln), the Arctic *APP693* mutation (Glu22Gly), and the Italian *APP693* mutation (Glu22Lys, E22K) in the *APP* gene are located within ABP near the α -secretase cleavage site (*119,120*). The E22Q peptide exhibits the highest content of β -sheet conformation and the fastest aggregation/fibrillization properties. The Dutch mutation induces apoptosis of cerebral endothelial cells,

whereas the wild-type (wt) ABP and the E22K mutant have no effect, indicating that different amino acids at position 22 confer distinct structural properties to the peptides that appear to influence the onset and aggressiveness of the disease rather than the phenotype (*I20*). All these mutations are grouped into the genetic classification of type 1 fAD (*I2*).

Other genes potentially influencing *APP* metabolism and ABP formation include the following: (1) *BACE1* (β -site amyloid β A4 precursor protein-cleaving enzyme; β -site *APP*-cleaving enzyme, β -secretase, memapsin 2) (11q23.3); (2) *BACE2* (β -site amyloid β A4 precursor protein-cleaving enzyme 2; β -site *APP*-cleaving enzyme 2; 56-kDa aspartic-like protease, ALP56; Down syndrome region aspartic protease, DRAP) (21q22.3); (3) *APPBP2* (amyloid β -precursor protein-binding protein 2; protein interacting with *APP* tail 1, PAT1) (17q22–q23); (4) *APBA1* (amyloid β A4 precursor protein-binding, family A, member 1; X11; D9S411E; MUNC18-1-interacting protein 1, MINT1; vertebrate LIN19 homolog, LIN10) (9q13); (5) *APBB2* (amyloid β A4 precursor protein-binding, family B, member 2; FE65-like1, FE65L1) (Chr. 4); (6) *APPBP1* (amyloid β -precursor protein-binding protein 1) (16q22); (7) *CASP3* (caspase 3, apoptosis-related cysteine protease; PARP cleavage protease; apopain, CPP32) (4q35); (8) *FE65-like 2* (*FE65L2*) (Chr. 5); (9) ubiquitin B (*UBB*) (17p12–p11.1); (10) *APBA2* (amyloid β A4 precursor protein-binding, family A, member 2; X11-like, X11L) (15q); (11) *APBB1* (amyloid β A4 precursor protein-binding, family B, member 1; *FE65*) (11p15); (12) *PS1* (14q24.3); and (13) *PS2* (1q31–q42) (*I2*).

β -Amyloid formation in senile plaques and brain vessels (amyloid angiopathy) is a major neuropathological hallmark in AD because of mutations in the *APP* gene, alterations in *APP* metabolism or processing, secretase-related dysfunction, *APOE*-associated misregulation, and probably other unknown mechanisms linked to genomic dysfunction and cerebrovascular alterations. ABP formation as a proteolytic by-product of a degradation process that leads to brain amyloidogenesis can result from different mechanisms, including (1) point mutations in the *APP* gene, (2) excess amounts of *APP*, (3) expression of aberrant *APP* isoforms, (4) structural misfolding, and (5) abnormalities in posttranslational modifications (*I21*). Disease-linked mutations in the *APP* and presenilin (*PS1*, *PS2*) genes result in increased production of the ABP42 form, predominant in AD senile plaques. ABP occurs in the above-mentioned two predominant forms (ABP40 and ABP42), and overproduction of ABP42 was suggested as a common cause of fAD.

ABP generation depends on proteolytic cleavage of the *APP* by the proteases α -, β -, and γ -secretases (see Fig. 10.3). Normal *APP* cleavage is produced by α -secretase precluding BAP formation into the amyloidogenic pathway (*I22*, *I23*) (see Fig. 10.3). Practically all *APP* mutations in the proximity of the γ -secretase site (see Fig. 10.3) can induce ABP formation and an increase in the ABP42-to-ABP40 ratio, inversely correlating with the age of onset in different families (*I24*). ABP accumulation is toxic for neurons and can induce apoptosis by a mechanism that requires c-Jun N-terminal kinase (JNK) activation (*I25*).

One potential target of neurotoxic ABP may be a novel ABP-binding protein (BBP) containing a G protein-coupling module that regulates caspase-dependent

vulnerability to ABP toxicity (*126*). Many studies suggested that neuronal death in AD is the result of an apoptotic mechanism (*127*), but the stereotypical profile of the terminal phases of apoptosis (chromatin condensation, apoptotic bodies, blebbing) are not seen in AD. Caspase 6, the protease that cleaves *APP* and presenilin, is localized in senile plaques. Some authors have reported that in AD there is a lack of effective apoptotic signal propagation to downstream caspase effectors (*128*). This novel phenomenon of apoptotic avoidance, termed *abortive apoptosis* or *abortosis*, may represent an exit from the caspase-induced apoptotic program leading to neuronal survival in AD (*128*). However, AD lymphocytes show a clear apoptotic behavior, which might reflect the peripheral expression of similar mechanisms occurring at the central level in neurons and microglia (*20,129,130*).

Nevertheless, *APP* is directly and efficiently cleaved by caspases during apoptosis, resulting in elevated ABP formation (*131*). The caspase-mediated *APP* proteolysis occurs within the cytoplasmic tail of *APP*. Caspase 3 is the predominant caspase involved in *APP* cleavage. Caspase 3 is markedly elevated in AD neurons and colocalizes with ABP in senile plaques (*131*). For some authors, caspases might play a dual role in AD influencing proteolytic processing of *APP* and increasing ABP formation and regulating the ultimate apoptotic death of neurons as well (*131*).

Activated microglia are found to be intimately associated with senile plaques and may play a pivotal role in mediated chronic inflammatory conditions in AD (*132*). Activation of microglia by ABP may result in the secretion of neurotoxic factors that kill neurons. To understand molecular pathways underlying ABP-induced microglia activation, Gan and coworkers (*133*) analyzed the expression levels of transcripts isolated from ABP42-activated murine microglial BV2 cells using high-density filter arrays and identified 554 genes that are transcriptionally upregulated by ABP42. Small interfering RNA-mediated silencing of the cathepsin B gene in ABP42-activated BV2 cells diminished the microglial activation-mediated neurotoxicity, and the specific cathepsin B inhibitor CA-074 also abolished the neurotoxic effects induced by ABP42-activated BV2 cells, suggesting that cathepsin B plays an essential role in neuronal death mediated by ABP-activated inflammatory responses (*133*).

Other mediators of inflammation may also affect *APP* processing. IL-1 β causes nuclear export of a specific NCOR corepressor complex, resulting in derepression of a specific subset of nuclear factor-kappa B (NF κ B)-regulated genes. One of these genes (tetraspanin *KAI1*) regulates membrane receptor function. It has been demonstrated that the nuclear export of the NCOR/TAB2/HDAC3 complex by IL-1 β is linked to selective recruitment of a TIP60 coactivator complex, and that *KAI1* is directly activated by a ternary complex, dependent on the acetyltransferase activity of TIP60, consisting of a presenilin-dependent C-terminal cleavage product of *APP* (Fe65) and TIP60. This is a specific *in vivo* gene target of an *APP*-dependent transcription complex in the brain (*134*).

Alterations in *APP* processing associated with dysfunctions in the ubiquitin-proteasome system may be responsible in part for ABP accumulation (*12*). The protein deposits in NFTs, neuritic plaques, and neurofibrillary tangles in AD cortex contain forms of *APP* and ubiquitin-B that are aberrant in the C-terminus (*135–137*).

Anomalous ubiquitin-B molecules are also present in the brains of control subjects, probably indicating a preneurodegenerative stage. The aberrant proteins show a cellular colocalization compatible with alterations at the transcriptional level or dysfunctional posttranscriptional RNA editing (*138*).

The conversion of ABP to a fibrillar form markedly increases binding to specific neuronal membrane proteins, including *APP*, suggesting that *APP* may be one of the major cell surface mediators of ABP toxicity (*139*). Alpha-2-macroglobulin (A2M) via its receptor, the low-density lipoprotein receptor-related protein 1 (LRP1), mediates the clearance and degradation of ABP. LRP1 is required for the A2M-mediated clearance of ABP40 and ABP42 via a receptor-mediated cellular uptake mechanism (*140*). Glycogen synthase kinase-3- α (GSK3A) is also required for maximum production of ABP40 and ABP42 generated from *APP* by presenilin-dependent γ -secretase cleavage (*141*). The secretion of ABP40 and ABP42 is also influenced by the phosphotyrosine interaction domain of JIP1B, but not the JNK-binding domain, indicating that the modulation of *APP* metabolism is independent of the JNK signaling pathway (*142*). The GYENPTY motif of the cytoplasmic domain of *APP* interacts with the C-terminal phosphotyrosine interaction domain JIP1 (MAPK8IP1). A specific splice variant of JIP1 (JIP1B) modulates the processing of *APP* in an interaction-dependent manner, stabilizing immature *APP* and suppressing the secretion of the large extracellular N-terminal domain of *APP*, the release of the intracellular C-terminal fragment, and the secretion of ABP40 and ABP42 (*142*). *APP* processing may be also regulated by the Rho-Rock pathway, on which some anti-inflammatory drugs exert an inhibitory effect (*143*).

The E693Q mutation in the *APP* leads to cerebral amyloid angiopathy (CAA), with recurrent cerebral hemorrhagic strokes and dementia. In contrast to AD, the brains of those affected by hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) show few parenchymal amyloid plaques. Neuronal overexpression of human E693Q *APP* in mice (*APP*^{Dutch} mice) causes extensive CAA, smooth muscle cell degeneration, hemorrhages, and neuroinflammation. In contrast, overexpression of human wild-type *APP* (*APP*^{wt} mice) results in predominantly parenchymal amyloidosis, similar to that seen in AD (*144*).

10.2.3.2 Presenilins

Familial AD3 and fAD4 are caused by more than 100 different mutations in the *PS1* and *PS2* genes located on chromosomes 14 (14q24.3) and 1 (1q31–q42) (see [Table 10.1](#)), respectively (*12,20*). *PS1* mutations are associated with early-onset familial AD and fAD with spastic paraparesis and usual plaques. In these AD cases, dementia coexists with spastic–ataxic paresis, white matter abnormalities reflecting ischemic leukoencephalopathy, and amyloid angiopathy (*145–147*). *PS1* (463 amino acids) and *PS2* (448 amino acids) are 46- to 49-kDa proteins that share 67–80% amino acid identity. PSs are serpentine integral membrane proteins with eight or nine TM domains localized in the endoplasmic reticulum (ER) and the Golgi subcellular compartments of neurons and other cells throughout the

animal kingdom (**148**). PSs are rapidly cleaved by proteolysis to yield 30-kDa N-terminal and 20-kDa C-terminal fragments and can accumulate in the aggregates, a cytoplasmic structure reflecting cell stress and overloading of the proteasome compartment (**149**). The subcellular localization of PSs in the ER and early Golgi overlaps with the intracellular sites of amyloidogenic ABP42 with which they coprecipitate. PSs function within macromolecular complexes and are necessary for the regulated intramembranous proteolysis of certain type 1 TM proteins (*APP*, Notch, p75).

There may be several distinct PS complexes. Gu et al. (**150**) proposed the existence of at least three complexes: (1) an approx. 150-kDa nicastrin-APH1 (anterior pharynx defective 1 protein) complex (which is likely to be a precursor complex); (2) a stable and abundant intermediate complex of 440kDa that contains APH1, PEN2 (presenilin enhancer 2), nicastrin, and *PS1*; and (3) a high mass (>670-kDa) heteromeric complex (*PS1*-APH1-NCT-PEN2) associated with the highest γ -secretase-specific activity (**150**) (see **Fig. 10.3**).

PS1 mRNA expression is primarily in cortical and hippocampal neurons, with less expression in subcortical structures. The main biological functions of PSs may include (1) *APP* processing, (2) protein sorting/trafficking, (3) Notch signaling, (4) chromosome organization and segregation, (5) neuronal differentiation, and (6) apoptosis (**151–154**). Proteins interacting with PSs include *APP*, nicastrin, β -catenin, calsenilin, filamin/Fh1, and Sel-10 (**153,154**). Nicastrin is a type 1 TM glycoprotein coded on chromosome 1 that interacts with both *PS1* and *PS2* regulating PS-mediated *APP* processing. Nicastrin also binds C-terminal derivatives of B-*APP* and modulates ABP production from these derivatives. Calsenilin is a substrate for caspase-3 that interacts with the fAD-associated C-terminal fragment of *PS2*. Calsenilin increases ABP42 production in cells expressing the *APP* Swedish mutation; this effect is potentiated by *PS2*, suggesting a role for apoptosis-associated ABP42 production of calsenilin/DREAM/KChIP3 (**155**). The expression of mutant proteins (V717L, V717F, V717G), but not of normal *APP695*, induces nucleosomal DNA fragmentation in cultured neuronal cells. The induction of DNA fragmentation requires the cytoplasmic domain of the mutants and appears to be mediated by heterotrimeric guanosine triphosphate-binding proteins (G proteins) (**156**).

PS1 forms a complex with β -catenin (CTNNB1), increasing β -catenin stability, and β -catenin levels are markedly reduced in the brains of AD patients with *PS1* mutations (**157**). One pathological characteristic of AD is extensive synapse loss. *PS1* is localized at the synapse, where it binds N-cadherin and modulates its adhesive activity. *PS1* is essential for efficient trafficking of N-cadherin from the ER to the plasma membrane. *PS1*-mediated delivery of N-cadherin to the plasma membrane is important for N-cadherin to exert its physiological function, and it may control the state of cell–cell contact (**158**).

The axonal transport of *APP* in neurons is mediated by the direct binding of *APP* to the kinesin light chain subunit of kinesin I. An axonal membrane compartment contains *APP*, β -secretase, and *PS1*, and the fast anterograde axonal transport of this compartment is mediated by *APP* and kinesin I. *APP* proteolysis in this

compartment generates ABP and a carboxy-terminal fragment of *APP* and liberates kinesin I from the membrane, suggesting that *APP* functions as a kinesin I membrane receptor, mediating the axonal transport of β -secretase and *PS1* (**159**).

Presenilin mutations apparently do not affect the intrinsic physiological functions of presenilins or cause lethal effects but (1) increase their toxic functions; (2) alter *APP* processing, leading to ABP deposition and accumulation by inducing functional changes in γ -secretase proteolytic activity; (3) increase neuronal sensitivity to apoptosis; (4) perturb calcium homeostasis, activating excitotoxic phenomena; (5) promote mitochondrial dysfunction; and (6) disrupt cholinergic signaling and responses to NGF (**152**). In some circumstances, *PS1* mutation may result in a gain of function or a partial loss of function. Coexpression of mutant *PS1* in mice transgenic for *APP^{swe}* dramatically accelerates the rate of amyloid deposition in the brain. Mice harboring only one functional *PS1* allele and coexpressing Mo/Hu*APP^{swe}* do not develop amyloid deposits at ages comparable to mice expressing mutant *PS1*. Studies reported by Jankowsky et al. (**160**) using different variants of transgenic mice demonstrated that the accelerated amyloid pathology, caused by so many different mutations in *PS1*, is clearly not a result of haploinsufficiency that might result from inactivating mutations; instead, the results obtained by Jankowsky et al. are consistent with a gain-of-property mechanism.

PSs affect *APP* processing, acting on γ -secretase, and are involved in the cleavage of the Notch receptor regulating γ -secretase activity or serving as protease enzymes (**149,154**). γ -Secretase activity is associated with PS-related complexes integrated by PS–PEN2–NCT–APH1 (**161**) (see **Fig. 10.3**). PSs probably contribute the catalytic activity to the protease complex, although some authors reported normal levels of ABP generation in cells expressing PSs mutated at the putative catalytic site residue Asp257.

Nyabi et al. (**162**) demonstrated that PSs with mutated Asp residues are catalytically inactive. Unexpectedly, these mutated PSs are still partially processed into amino- and carboxy-terminal fragments by a presenilinase-like activity and are able to rescue PEN2 expression and nicastrin glycosylation, and then they become incorporated into large 440-kDa complexes, demonstrating that the catalytic activity of PS and its other functions in the generation, stabilization, and transport of the γ -secretase complex can be separated and extends the concept that PSs are multifunctional proteins (**162**).

Novel PS-related families of proteins (IMPAS/PSH/signal peptide peptidases [SPSs]) have been identified (**163**). Intramembrane protease-associated or intramembrane protease aspartic protein Impas 1 (IMP1)/SPS induces intramembraneous cleavage of *PS1* holoprotein in cultured cells coexpressing these proteins. Mutations in evolutionary invariant sites in hIMP1 or specific γ -secretase inhibitors abolish the hIMP1-mediated endoproteolysis of *PS1*. In contrast, AD-like mutations in neither hIMP1 nor *PS1* substrate abridge the *PS1* cleavage (**163**).

Several proteins appear to regulate accumulation of PS proteins in cells. One such protein is ubiquilin 1 (UBQLN1), which increases levels of coexpressed *PS2* protein in a dose-dependent manner. Overexpression of ubiquilin 2, which is 80% identical to ubiquilin-1, also increases the levels of coexpressed *PS1* and *PS2* proteins in cells.

UBQLN1 overexpression decreases ubiquitination of coexpressed *PS2* proteins, suggesting that binding of ubiquilin might block ubiquitin chain elongation. Ubiquilin proteins are present within the inner core of aggresomes, which are structures associated with accumulation of misfolded proteins in cells (164).

PS1 mutations disturb the unfolded protein response (UPR), which is activated in response to ER stress caused by the accumulation of misfolded proteins in the lumen of the ER. *PS1* mutants inhibit activation of ER stress transducers Ire1- α , ATF6, and PERK, leading to attenuation of the induction of the ER chaperone GRP78/BiP and inhibition of the translation-suppressing molecules eIF2- α and PRK. This complex perturbation of the UPR leads to further accumulation of proteins in the ER, increasing vulnerability to ER stress (165).

Amyloid production and deposition are increased in AD patients with *PS* mutations, in transgenic mice with *PS* mutations, and in mutant *APP* and *PS1* yeast artificial chromosome transgenic mice (166–169). Transgenic mice with AD-related *PS1* mutations show accelerated neurodegeneration with intracellular ABP deposition and without amyloid plaque formation, suggesting that *PS1* mutation is upstream of the amyloid cascade in AD (170).

The expression of wild-type (wt)-*PS2* in human HEK293 cells increases the production of α -secretase-derived product *APP*- α , and *APP*- α production is drastically reduced in cells expressing the N141I-*PS2* mutation. The PS-associated *APP*- α -secretase nonamyloidogenic pathway is under the catalytic control of proteasome enzymes (171). Mutations in either of two conserved TM aspartate residues in *PS1*, Asp257 in TM6 and Asp385 in TM7, reduce ABP production and increase the amount of the carboxy-terminal fragments of *APP* that are the substrates of γ -secretase (172). In the soluble fraction of AD brains, ABP electrophoretically resolves into three bands of relative molecular mass of 4.5, 4.2, and 3.5 kDa. The 4.5 kDa species contains ABP(1–40/1–42), the 4.2 kDa species is ABP(py3–42), and the 3.5 kDa species is ABP(4–42) and ABP(py11–42) (173). The smallest band is more prominent in AD patients harboring *PS1* mutations than in those with sporadic AD or *APP* mutation-related AD, indicating that amino-terminally truncated forms are increased in *PS1* mutants, and that overexpression of amino-terminally truncated ABP species is the consequence of *PS1* mutation-related γ -secretase and β -secretase alterations (173).

PSs also regulate the Notch signaling pathway, involved in axon pathfinding, neurite outgrowth, and neuronal stem cell differentiation and maturation (174). *PS1* is indirectly implicated in Notch1 cleavage (175). Activation of mammalian Notch receptor by its ligands induces TNFA-converting enzyme-dependent ectodomain shedding, followed by intramembrane proteolysis caused by PS-dependent γ -secretase activity. Monoubiquitination as well as clathrin-dependent endocytosis are required for γ -secretase processing of a constitutively active Notch derivative, DeltaE, which mimics the TNFA-converting enzyme-processing product (176).

Missense mutations in the *Notch3* gene cause CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). Notch is a type I integral membrane protein proteolytically processed in its extracellular domain by furin and the metalloproteinase kuzbanian. The signal transduction cascade

is activated by Notch receptor binding to members of the DSL ligand family (Delta, Serrate, Lag-2), then the Notch cytoplasmic domain is cleaved, released, and translocated to the nucleus. The Notch receptor acts in a core pathway as a membrane-bound transcription factor (TF) that is released in response to ligand binding by two proteases, and the intracellular domain enters the nucleus to switch a DNA-binding corepressor complex, the activation of which is responsible for the initiation of biological activity in selected targets (177).

The core Notch signaling pathway involves a complex proteolytic processing with the participation of the Notch receptors 1–4, DSL transcriptional factors (Delta and Serrate/Jagged), CSL DNA-binding proteins (CBF1/RBPjk), and target genes (HES family of basic helix-loop-helix transcriptional regulators) (178). Deletion of the *PS1* gene in transgenic mice induced developmental abnormalities (failed somite formation, altered neurogenesis), suggesting a loss of Notch function. The neurons of these transgenic mice showed a decrease in γ -secretase *APP* processing and about 50–70% decline in ABP production (179). It has also been demonstrated that Notch1 releases ABP-like peptides via a PS/ γ -secretase-mediated cleavage in the middle of its TM domain, confirming the similar proteolysis of Notch1 and ABP (180). The bulk of the studies about the Notch–PS–*APP* link seem to indicate that PS itself is a protease with γ -secretase activity acting on type 1 and type 2 membrane proteins (178).

In this regard, β -secretases and γ -secretases associated with PS function might be potential targets for AD treatment (103–105,181,182) (see Table10.2; Fig. 10.3). Although *PS1* and *PS2* appear to be γ -secretases, it is not clear if the two enzymes normally have similar or different substrates because they reside in different complexes. PS heterodimers associate with APH1A and APH1B to form the γ -secretase complex that is required for the intramembrane proteolysis of membrane proteins, including *APP* and *Notch* (183). RNA interference (RNAi) assays that inactivate *APH1*, *PEN2*, or *nicastrin* in *Drosophila* cells reduce the levels of PS and γ -secretase cleavage of b*APP* and Notch substrates, suggesting that *APH1*, *PEN2*, and *nicastrin* are required for the activity and accumulation of γ -secretase (184). *PEN2* is a critical component of *PEN1*/ γ -secretase and *PEN2*/ γ -secretase complexes. Downregulation of *PEN2* by RNAi is associated with reduced PS levels, impaired nicastrin maturation, and deficient γ -secretase complex formation (185).

γ -Secretase inhibitors designed to function as transition-state analog inhibitors directed to the active site of an aspartyl protease show photoaffinity labeling to *PS1* and *PS2* (186). However, presenilin-like γ -secretase inhibitors might have deleterious effects acting on the physiological functions linked to Notch receptors in neurons. Notch signaling regulates the capacity of neurons to extend and elaborate neurites, and upregulation of Notch activity is concomitant with an increase in the number of interneuronal contacts and cessation of neurite outgrowth. The formation of neuronal contacts results in activation of Notch receptors, leading to restriction of neuronal growth and arrest in adult brains (187). PS is required for the normal proteolytic production of the carboxy-terminal Notch fragments necessary for maturation and signaling (188,189). Loss of PS function leads to Notch/*lin-12*-like

mutant phenotypes in *Caenorhabditis elegans* and to reduced Notch expression of the paraaxial mesoderm in mice. The PS-regulated Notch signaling might also be associated with ABP production in AD (188). Because *ws-PS2*, *N141I-PS2*, and the *PS2* C-terminal maturation fragment are degraded by proteasome multicatalytic complex, selective proteasome inhibitors [Z-IE(Ot-Bu)A-leucinal, lactacystin] potentiate *APP- α* secretion and decrease *APP- α* production in *N141I-PS2* mutation carriers. However, secretase inhibitors acting on Notch and *APP*-ABP production might have unwanted effects on interneuronal communication, neurite outgrowth, hematopoietic systems, and immune function (190,191). Because the *PS1* mutation-related pathogenic events seem to be upstream of the amyloid cascade (170), a preventive therapeutic intervention on PS function might preclude ABP formation and further neuronal degeneration. However, deleterious effects with γ -secretase inhibitors may arise in the treatment of AD patients (192).

During normal development Notch receptor signaling is important in regulating numerous cell fate decisions. Mutations that truncate the extracellular domain of Notch receptors can cause aberrant signaling and promote unregulated cell growth. Das et al. (192) examined two types of truncated Notch oncoproteins that arise from proviral insertion into the *Notch4* gene (Notch4/int-3) or a chromosomal translocation involving the *Notch1* gene (*TAN-1*). Both Notch4/int-3 and TAN-1 oncoproteins lack most or all of their ectodomain. Normal Notch signaling requires γ -secretase/PS-mediated proteolytic processing, but whether Notch oncoproteins are also dependent on γ -secretase/PS activity is not known. Notch4/int-3-induced activation of the downstream TF CSL is abrogated in cells deficient in PSs or treated with an inhibitor of γ -secretase/PSs. Both Notch4/int-3 and TAN-1 accumulate at the cell surface, where PS-dependent cleavage occurs, when γ -secretase/PS activity is inhibited. γ -Secretase/PS inhibition effectively blocks cellular responses to Notch4/int-3, but not TAN-1, apparently because some TAN-1 polypeptides lack TM domains and do not require γ -secretase/PS activity for nuclear access. These studies of Das and coworkers highlight potential uses and limitations of γ -secretase/PS inhibitors in targeted therapy of Notch-related neoplasms and constitute an elegant advice regarding caution for AD treatment.

Fraering et al. (193) purified and characterized the human γ -secretase complex. γ -Secretase is a member of an unusual class of proteases with intramembrane catalytic sites. This enzyme cleaves many type I membrane proteins, including the *APP* and the Notch receptor. Biochemical and genetic studies have identified four membrane proteins as components of γ -secretases: heterodimeric PS composed of its N- and C-terminal fragments (PS-NTF/CTF), a mature glycosylated form of nicastrin (NCT), Aph1, and Pen-2 (see Fig. 10.3). Studies in *Drosophila*, mammalian, and yeast cells suggest that PS, NCT, Aph-1, and Pen-2 are necessary and sufficient to reconstitute γ -secretase activity (178,193). Using the purified γ -secretase, Fraering et al. described factors that modulate the production of specific ABP species: (1) Phosphatidylcholine and sphingomyelin dramatically improve activity without changing cleavage specificity within an *APP* substrate; (2) increasing CHAPSO concentrations from 0.1% to 0.25% yields an approx. 100% increase in ABP1-42 production; (3) exposure of an *APP*-based recombinant

substrate to 0.5% sodium dodecyl sulfate (SDS) modulates cleavage specifically from a disease-mimicking pattern (high ABP42/43) to a physiological pattern (high ABP40); and (4) sulindac sulfide directly and preferentially decreases ABP1-42 cleavage within the purified complex (**193**). Affinity precipitations suggested that only a discrete fraction of cellular PS is present in the active γ -secretase complex, and that both γ -secretase-40 and γ -secretase-42 activities are mediated by the same molecular entity (**194**).

Maturation of γ -secretase requires an endoproteolytic cleavage in *PS1* within a peptide loop encoded by exon 9 of the corresponding gene. Deletion of the loop has been demonstrated to cause familial AD. The peptide derived from exon 9 can adopt a variable conformation, one that is compact enough to occupy the putative substrate-binding site without necessarily interfering with binding of small molecule inhibitors at other sites of γ -secretase. Knappenberger et al. (**195**) hypothesized that γ -secretase cleavage activation may be a result of a cleavage-induced conformational change that relieves the inhibitory effect of the intact exon 9 loop occupying the substrate-binding site of the immature enzyme.

APP is endoproteolytically processed by *BACE1* and γ -secretase to release ABP1-40 and ABP1-42, which aggregate to form senile plaques in AD brains. The C-terminus of ABP, C99, is generated by γ -secretase, which has activity that is dependent on *PS1* or *PS2*. It has been suggested that PS proteins are the catalytic core of the proteolytic activity of the complex, but a number of other proteins mandatory for γ -secretase cleavage have also been discovered. The exact role of PS in the γ -secretase activity remains a matter of debate because cells devoid of PS still produce some forms of ABP.

Pitsi and Octave (**196**) demonstrated that the expression of *PS1*, which binds C99, not only increases the production of ABP but also increases the intracellular levels of C99 to the same extent. A functional inhibitor of γ -secretase does not alter the ability of *PS1* to increase the intracellular levels of C99, suggesting that the binding of *PS1* to C99 does not necessarily lead to its immediate cleavage by γ -secretase, which could be a spatiotemporally regulated or an induced event (**196**). Missense mutations in *PS1* and *PS2* shift the ratio of ABP1-40/ABP1-42 to favor ABP1-42. A possible explanation of this outcome is that mutant *PS* alters the specificity of γ -secretase to favor production of ABP1-42 at the expense of ABP1-40.

In transgenic animals that coexpress the Swedish mutation of *APP* (*APP^{sw}*) with two *fAD-PS1* variants that differentially accelerate amyloid pathology in the brain, Jankowski et al. (**169**) demonstrated a direct correlation between the concentration of ABP1-42 and the rate of amyloid deposition. The shift in ABP42:ABP40 ratios associated with the expression of *fAD-PS1* variants is caused by a specific elevation in the steady-state levels of ABP1-42, while maintaining a constant level of ABP1-40. These data suggest that *PS1* variants do not simply alter the preferred cleavage site for γ -secretase, but rather that they have more complex effects on the regulation of γ -secretase and its access to substrates (**169**).

PS1 plays a role in β -catenin signaling and in the regulation of apoptosis. Phosphorylation of *PS1* is regulated by two independent signaling pathways

involving protein kinase (PK) A and PKC. Both kinases can phosphorylate the large hydrophilic domain of *PS1*. A phosphorylating site at Ser346 is specifically phosphorylated by PKC but not by PKA. This site is localized within a recognition motif for caspases, and phosphorylation strongly inhibits proteolytic processing of *PS1* by caspase activity during apoptosis. Furthermore, *PS1* phosphorylation reduces the progression of apoptosis, indicating that phosphorylation/dephosphorylation at the caspase recognition site provides a mechanism to reversibly regulate properties of *PS1* in apoptosis (197); however, the overexpression of *PS2* does not cause proapoptotic effects in transfected cells, according to some authors (198); in contrast, others have reported that overexpression of presenilinase-derived maturation product of *PS2* (CTF-*PS2*) increases ABP recovery, the production of which is almost abolished by a caspase-3 inhibitor and increased by staurosporine. CTF-*PS2* degradation clearly links CTF-*PS2* to apoptotic cascade effectors. CTF-*PS2* overexpression decreases cell viability and augments caspase-3 activity, accompanied by lowered bcl2-like activity and increased poly(ADP-ribose) polymerase cleavage and cytochrome c translocation into the cytosol. CTF-*PS2*-induced caspase activation is prevented by pifithrin- α , a selective blocker of p53 transcriptional activity. These data, reported by Alves da Costa et al. (199), indicate that the C-terminal fragment of *PS2* triggers p53-mediated staurosporine-induced apoptosis, a function independent of the presenilinase-derived N-terminal counterpart. Others have shown that N141I mutant *PS2* enhances neuronal cell death and decreases bcl2 expression (200).

The *PS2* gene is estrogen responsive in HepG2 cells in the presence of estrogen receptor- α (ERA). The estrogenic activity is mediated through an estrogen response element (ERE) in the 5'-flanking region of the *PS2* gene. Estrogen-induced synergistic activity by the p160 coactivator steroid receptor coactivator 1 (SRC-1) is mediated via the ERE and the AP1 response element in the *PS2* promoter. Another p160 coactivator, the transcriptional intermediary factor 2, is a more potent activator of *PS2* gene expression.

A common single adenine (A) nucleotide deletion polymorphism in the 5'-upstream promoter region of the *PS2* gene for regulatory elements was detected by Riazanskaia et al. (201). Examination of cohorts of AD patients and age-matched control individuals revealed no statistically significant differences in the frequency of this polymorphism in different populations (201,202). However, subgroup and regression analysis suggested that the relatively rare -A/-A genotype increases the risk of AD among subjects lacking *APOE-4* and among persons aged 65 yr and younger (201). DNA sequence and DNA-protein binding analysis demonstrated that this mutation negates binding with putative repressor TF interferon regulatory factor 2 (IRF2) in nuclear extracts prepared from the aged human brain neocortex. However, this mutation creates a potential regulatory element, C/EBP β , that is responsive to proinflammatory induction. The mutant *PS2* regulatory region exhibits a 1.8-fold higher level of basal expression and is sensitive to IL-1B and ABP1-42, and it is synergistically induced 3.2-fold over the wild-type *PS2* by IL-1B plus ABP. These results suggest that under proinflammatory and oxygen stress conditions relatively minor variations in *PS2* promoter DNA sequence structure can

enhance *PS2* gene expression, and that consequently these may play a role in the induction or proliferation of a proinflammatory response in AD brain (201).

10.2.3.3 Microtubule-Associated Protein Tau Gene

The microtubule-associated protein tau (*MAPT*) gene maps to 17q21.1 and encodes a 352-amino acid protein that contains a characteristic amino acid repeat in its carboxyl-terminal half (12). The microtubule-associated proteins (MAPs) coassemble with tubulin into microtubules, and the *MAPT* protein is enriched in axons. In the adult human brain, six tau isoforms produced by alternative splicing have been identified. The proteins are composed of 352–441 amino acids, differing from each other by the presence or absence of 29-amino acid or 58-amino acid inserts located in the N terminus and a 31-amino acid repeat located in the C-terminus. The C-terminus 31-amino acid repeat encoded by exon 10 of the tau gene gives rise to the three tau isoforms with four repeats each; the other three isoforms contain three repeats each. The repeats and some adjoining sequences constitute the microtubule-binding domains of tau, which in normal brains show similar levels of the four-repeat (4R) and three-repeat (3R) isoforms. The tau isoforms have specific core microtubule-binding domains that lead to complex intramolecular folding interactions. Flanking regions are also found to contribute to the binding activity in the 3R isoform, but less so in the 4R isoform.

It is very likely that the two types of isoform form distinct structures with different functional capabilities (203). The abnormally high ratio of 4R to 3R tau in the *MAPT* gene might lead to neuronal cell death by altering normal tau functions in adult neurons. Both isoforms promote microtubule polymerization and decrease the tubulin critical subunit concentration to approximately similar extents, but 4R tau stabilizes microtubules significantly more strongly than 3R tau, suggesting a dosage effect or haploinsufficiency model in which both tau alleles must be active and properly regulated to produce appropriate amounts of each tau isoform to maintain microtubule dynamics within a correct level of activity. Elevated levels of tau inhibit intracellular transport in neurons, particularly the plus-end-directed transport of kinesin motors from the center of the cell body to the neuronal process. This inhibition is significant because critical organelles, such as peroxisomes, mitochondria, and transport vesicles carrying supplies for the growth cone, are unable to penetrate the neurites, leading to stunted growth, increased susceptibility to oxidative stress, and likely pathologic aggregation of proteins such as ABP. The tau:tubulin ratio is normally low, and increased levels of tau become detrimental for the cell (204).

Tau protein interacts with many other proteins that can contribute to abnormal fibrillogenesis. One example is α -synuclein, which induces fibrillization of tau. Coincubation of α -synuclein and tau synergistically promotes fibrillization of both proteins in vitro. Mice with α -synuclein mutation or a tau mutation exhibit filamentous inclusions of both proteins, which are abundant neuronal proteins that normally adopt an unfolded conformation but polymerize into amyloid fibrils in

disease (205). Another example of interaction is oncogene DJ1 protein, encoded in a gene that maps to 1p36, which colocalizes within a subset of pathologic tau inclusions in tauopathies and that alters its solubility when it aggregates in these inclusions (206).

It has been convincingly demonstrated that tau protein mutations and tau protein pathology can cause neurodegeneration and are associated with a diverse group of diseases currently called *tauopathies* (102,207–211). Diseases with abundant tau-positive filamentous lesions include the following: Alzheimer's disease, corticobasal degeneration, dementia pugilistica, dementia with tangles only, dementia with tangles and calcification, Down syndrome, frontotemporal dementias, and parkinsonism linked to chromosome 17 mutations, myotonic dystrophy, Niemann–Pick disease type C, parkinsonism–dementia complex of Guam, Pick disease, postencephalitic parkinsonism, prion diseases with tangles, progressive supranuclear palsy (PSP), and subacute sclerosing panencephalitis (210). Tau-positive neurofibrillary lesions, representing cytoskeletal changes in AD neurons, constitute a well-recognized neuropathological feature of AD.

Intracellular neurofibrillary lesions appear in the neocortex, hippocampus, and some subcortical nuclei of AD, correlating with the presence of dementia. These lesions are found in nerve cell bodies and apical dendrites as NFTs, in distal dendrites as neuropil threads and in the abnormal neurites associated with senile plaques (102,210,211). These neurofibrillary lesions are integrated by paired helical filaments (PHFs) and straight filaments made of MAPT in a hyperphosphorylated state. The six isoforms of tau in brain are involved in microtubule assembly and stabilization. The tau isoforms are produced by alternative splicing of mRNA from a single gene located on the long arm of chromosome 17 (17q21.1), which has missense mutations and splicing defects that can lead to different forms of tauopathies (12).

Multiple exonic and intronic mutations in the tau gene have been identified in frontotemporal dementia and in other neurodegenerative disorders (12). Different *FTDP-17* (frontotemporal dementia with parkinsonism, associated with chromosome 17) missense mutations might be responsible for disease pathogenesis by reducing the ability of tau to bind microtubules and promote microtubule assembly. Tau mutations are divided into three groups according to their locations in the intron after exon 10, in exon 10, or in the remaining tau-coding region, causing the different phenotypic expression of heterogeneous, atypical dementias (102,211). Some *FTDP-17* mutations alter the MT-binding properties of tau, and others alter the ratio of 4R/3R tau isoforms. The missense mutations P301L, V337M, and R406W alter the biochemical properties of tau. Hyperphosphorylation and abnormal phosphorylation are major biochemical abnormalities of PHF-tau and early events in NFT formation due to the incapacity of tau to bind microtubules.

Tau pathology in AD is circumscribed to neurons, while in other tauopathies, such as corticobasal degeneration, PSP and familial multiple system tauopathy with presenile dementia, both nerve cells and glial cells are affected (210,211). PIN1 binds hyperphosphorylated tau, resulting in depletion of soluble PIN1 in AD brains.

The peptidyl prolyl *cis/trans*-isomerase *PIN1* recognizes the phospho-Thr212-Pro213 site on tau (212). *PIN1* depletion induces mitotic arrest and apoptotic cell death, and sequestration of *PIN1* into PHF may contribute to neuronal death (213). *PIN1* is able to restore the ability of phosphorylated tau to bind microtubules and promotes microtubule assembly in vitro. The level of soluble *PIN1* in AD brains is greatly reduced compared to that in age-matched control brains (213). Abnormal tau phosphorylation is reduced in transgenic *APP^{sw}* mice deficient in CD40 ligand, suggesting that the CD40–CD40 ligand interaction is an early event in AD pathogenesis (214).

Senile plaques and NFT, the two hallmark lesions of AD, are the result of the pathologic deposition of proteins normally present in the brain. Senile plaques are extracellular deposits of fibrillar ABP, and NFTs represent intracellular bundles of self-assembled hyperphosphorylated tau protein (215). It has been demonstrated that these two phenotypic expressions of brain degeneration are interrelated. Tau plays a key role in fibrillar ABP-induced neurite degeneration in the CNS (216), and there is a synergistic effect of amyloid aggregation in the propagation of tau pathology (217). In primary tauopathies (PSP, corticobasal degeneration, Pick disease, frontotemporal dementia), tau fibrillar pathology predominates, while in secondary tauopathies (Alzheimer's disease, familial British dementia, prion disease) extracellular ABP deposition coexists with tau pathology. *FTDP-17* mutations are very rare in AD, with a mutational rate in the 17q21.1 exon 10 lower than 1:1000 (12,20). Conrad et al. (218) demonstrated an association between PSP and a dinucleotide TG repeat polymorphism in intron 9 of the *MAPT* gene. An overrepresentation of the most common allele (a0) and genotype a0/a0 was associated with SPS (218).

A series of polymorphisms scattered throughout of the *MAPT* gene and two extended haplotypes, designated H1 and H2, that cover the entire gene have been identified by Baker et al. (219). The dinucleotide TG polymorphism alleles a0 (11 repeats), a1 (12 repeats), and a2 (13 repeats) are inherited with the H1 haplotypes, whereas the a3 (14 repeats) and a4 (15 repeats) alleles are inherited with the H2 haplotype (219). An association between the H1 haplotype or H1H1 genotype in the *FTDP-17 MAPT* gene with frontotemporal dementia and other tauopathies has been confirmed (211,220–222). The primary haplotype H1 of the *MAPT* gene is associated with Parkinson disease (223).

However, *FTDP-17* caused by mutations in the tau gene shows a wide range of phenotypic characteristics, and it is very unlikely that a single-gene mutation can be responsible for this type of dementia. Not all families with *FTDP-17* have mutations and deposition of hyperphosphorylated tau in the brain but show ubiquitin-positive, tau-negative inclusions, and neuropathological assessment also shows heterogeneity (224). Therefore, as proposed by van Swieten et al. (225), future research should focus on the role of other genetic and environmental factors in this form of *FTDP-17*, whereas the responsible defective genes still have to be identified for hereditary FTD without tau mutations (225–227). FTD is also linked to chromosome 3 (*FTD-3*) (228). Soluble ABP is increased in *FTDP-17* patients. The aggregation of tau protein might produce an accumulation of ABP, which does not reach the critical concentration needed for amyloid plaque formation (229).

A novel polymorphism (IVS11 + 90G-A) in the *MAPT* gene has been associated with EOAD, with no apparent relation to the *Saithoin* gene (230). Cerebral spinal fluid (CSF) tau levels are increased and ABP1-42 levels are decreased in AD (231). Some authors proposed a novel mechanism to link together tau and BAP in AD. According to Gambelin et al. (232), ABPs promote pathological tau filament assembly in neurons by triggering caspase cleavage of tau and generating a proteolytic product with enhanced polymerization kinetics. Despite international effort to genetically link tau pathology to AD, no relevant *MAPT* mutations have been clearly associated with AD worldwide. In contrast, tau pathology (NFT formation) is an almost omnipresent pathological feature in AD. This fact suggests that NFT formation is not necessarily a consequence of *MAPT* mutations, or that *MAPT* mutations are not essential for AD-related tau pathology (12).

MAPT is abnormally hyperphosphorylated and aggregated into NFTs in brains of patients with AD and other tauopathies with or without *MAPT* mutations as well as in animal and cell models of mutational tauopathies (208–211,222–233). Tau pathology seems to be critical to AD pathogenesis and correlates with the severity of dementia, but the mechanisms leading to abnormal hyperphosphorylation still remain elusive. New insights into tau-related pathology have been revealed. MAPT is phosphorylated in vitro by Akt, an important kinase in antiapoptotic signaling regulated by insulin and growth factors. Akt phosphorylates tau separately at T212 and S214, two sites previously shown to be phosphorylated by glycogen synthase kinase 3 β (GSK3B) and protein kinase A (PKA), respectively. Hyperphosphorylation of tau at most sites appears to precede filament assembly. Many of the hyperphosphorylated sites are serine/threonine-proline sequences. C-Jun N-terminal kinases JNK1, JNK2, and JNK3 phosphorylate tau at many serine/threonine-prolines (234). Kyong Pyo et al. (235) have demonstrated that Akt selectively phosphorylates tau at S214 rather than T212 and raised the possibility that tau S214 may participate in Akt-mediated antiapoptotic signaling.

Iqbal and coworkers (236) reported that abnormal hyperphosphorylation of tau may result from decreased tau O-GlcNAcylation, which probably is induced by deficient brain glucose uptake/metabolism in AD. Human brain tau was modified by O-GlcNAcylation, a type of protein O-glycosylation by which the monosaccharide β -N-acetylglucosamine (GlcNAc) attaches to serine/threonine residues via an O-linked glycosidic bond. At most of the phosphorylation sites, O-GlcNAcylation negatively regulates tau phosphorylation. In an animal model of starved mice, low glucose metabolism produced a decrease in O-GlcNAcylation and consequent hyperphosphorylation of tau. The O-GlcNAcylation level in AD brain extracts was decreased compared to controls (236).

Human tau Tyr18 can also be phosphorylated by the src family tyrosine kinase fyn, which might participate in neurodegeneration (237). Casein kinase I delta phosphorylates tau and disrupts its binding to microtubules (238). Cdk5 (cyclin-dependent kinase 5) is a key factor in tau aggregation and NFT formation (239). Nitration of tau protein has been linked to neurodegeneration and tauopathies (240). The heat shock protein 27 (Hsp27) preferentially binds pathological hyperphosphorylated tau and PHFs directly, but not nonphosphorylated tau. The formation of this complex alters the

conformation of pathological hyperphosphorylated tau and reduces its concentration by facilitating its degradation and dephosphorylation. The CHIP–Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival (241). The chaperone effect of Hsp27 is likely to provide a neuroprotective effect in AD and other tauopathies (242), and the effect of a defective ubiquitin–proteasome–chaperone system in tau pathology cannot be ruled out (243).

Tau PHF from AD brains and assembled in vitro are based on β -structure in the core domain, suggesting that the repeat domain of tau within the core of PHFs adopts an increasing level of β -structure during aggregation, whereas the N- and C-terminal domains projecting away from the PFH are mostly random coil (244,245). Reduction of detyrosinated microtubules and Golgi fragmentation are linked to tau-induced degeneration in astrocytes (246). Interactions between α -synuclein and tau can promote their fibrillization and drive the formation of pathological inclusions in neurodegenerative diseases (205). In normal conditions, however, tau might show the ability to protect DNA from hydroxyl radical attack, implying that tau may function as a DNA-protecting molecule to the radical (247).

Studies with exon 6 of the *MAPT* gene established that tau shows a unique expression pattern and splicing regulation profile, and that it utilizes alternative splice sites in several human tissues. The mRNAs from these splicing events, if translated, would result in truncated tau variants that lack the microtubule-binding domain. At least one of these tau variants is present as a stable protein in several tissues (248). Luo et al. (248) identified a novel isoform in the hippocampus in both normal individuals and in those with AD, particularly in dentate gyrus granular cells and CA1/CA3 pyramidal cells. However, this variant does not colocalize with canonical tau but rather partly colocalizes with MAP2 (248). Sze et al. (249) also found that downregulation of WW domain-containing oxidoreductase (WOX1) induces tau phosphorylation in vitro. WOX1 binds tau via its COOH-terminal short-chain alcohol dehydrogenase (ADH)/reductase domain, probably to regulate tau hyperphosphorylation and NFT formation (249).

Mutations that stimulate exon 10 inclusion into the human tau mRNA, which is regulated by an intricate interplay of *cis* elements and *trans* factors, cause FTDP-17 and other tauopathies. This suggests that the ratio of exon 10 inclusion to exclusion in the adult brain is one of the factors to determine biological functions of the tau protein.

Using minigene constructs with intron deletions from the full length of tau exons 9–11 minigene construct, Yu et al. (250) demonstrated that there is a minimum distance requirement between exon 10 and 11 for correct splicing of the exon 10. In addition, SRp20, a member of the serine-arginine (SR) protein family of splicing factors was found to facilitate exclusion of exon 10 in a dosage-dependent manner. Significantly, SRp20 also induced exon 10 skipping from pre-mRNAs containing mutations identified in FTDP-17 patients (250). In transgenic mice engineered to express the longest human tau isoform (T40) with or without the R406W mutation that is pathogenic for some FTDP-17 cases, Zhang et al. (251) found that altered tau function led to increased accumulation and reduced solubility of RW tau in an age-dependent manner, culminating in the formation of filamentous intraneuronal tau aggregates similar to that observed in tauopathy patients (251).

All these data together clearly indicate that tau pathology is important in neurodegeneration; however, the real role played by NFT in AD and other dementias still needs further elucidation.

10.2.3.4 Alternative or Complementary Pathogenic Events

The functional genomics of AD-related genes is still in a very primitive stage. Most genetic studies are the result of linkage analysis and genetic association analysis. Although *APP* and *PS* mutations are considered causative factors for AD, the total number of mutations identified in the *APP*, *PS1*, and *PS2* genes accounts for less than 10% of the cases with AD, clearly indicating that neurodegeneration associated with AD pathogenesis cannot be exclusively attributed to *APP*-/*PS*-related cascades (amyloid hypothesis). Additional evidence questioning the amyloid hypothesis as the primary pathogenic event in AD is the presence of abundant senile plaques in centenarians with an apparently normal cognitive function, suggesting that amyloid formation in brain tissue and vessels not necessarily has to be pathogenic under any circumstance. The same applies for *MAPT* mutations associated with tauopathies. Alterations in the ubiquitin–proteasome system and biochemical disarray in the chaperone machinery are alternative or complementary pathogenic events potentially leading to defects in protein synthesis, folding, and degradation with subsequent conformational changes, aggregation, and accumulation in cytotoxic deposits (12).

Typical examples of conformational disorders associated with fibrils and aggregates of specific proteins include the following: (1) hemoglobin in sickle cell anemia, unstable hemoglobin inclusion body hemolysis, and drug-induced inclusion body hemolysis; (2) prion proteins in Creutzfeldt–Jakob disease (CJD), the new variant CJD, Gerstmann–Straussler–Scheinker syndrome, fatal familial insomnia, and kuru; (3) serpins in α 1-antitrypsin deficiency-emphysema and cirrhosis, antithrombin deficiency thromboembolic disease, and C1-inhibitor deficiency angioedema; (4) α -synuclein in Parkinson disease and Lewy body dementia; (5) glutamine repeats in Huntington disease, spinocerebellar ataxia, dentate-rubro-pallido-Luysian atrophy, and Machado–Joseph disease; (6) ABP in AD and Down syndrome; (7) ubiquitin and superoxide dismutase (SOD) in amyotrophic lateral sclerosis; (8) Abri peptide in familial British dementia; and (9) other proteins in the systemic amyloidoses. These other proteins include immunoglobulin (Ig) light chain in systemic AL amyloidosis and nodular AL amyloidosis, serum amyloid A protein in reactive systemic AA amyloidosis and chronic inflammatory disease, transthyretin in senile systemic amyloidosis, familial amyloid neuropathy and familial cardiac amyloid, β 2-microglobulin in hemodialysis amyloidosis and prostatic amyloid, APOA-I in familial amyloid angiopathy and familial visceral amyloid, cystatin C in hereditary (Icelandic) cerebral angiopathy, and lysozyme in familial visceral amyloidosis (12,252–254).

Phenotypically, different neurodegenerative disorders, including AD, Parkinson disease, and prion diseases (transmissible spongiform encephalopathies), are

characterized by progressive neuronal loss and fibrillar protein aggregates, suggesting that protein aggregation is neurotoxic rather than a by-product of neuronal death. Modeling studies indicated that protofibrils or prefibrillar oligomers may be inducers of neuronal death, and that the fibrillar forms currently present in autopsy tissues might be reactive proteins with a potential neuroprotective effect (254).

Increasing evidence indicates that accumulation of aberrant or misfolded proteins, protofibril formation, ubiquitin–proteasome system dysfunction, and the direct or indirect consequences of abnormal protein aggregation and accumulation represent deleterious events linked to neurodegeneration (255,256). Ubiquitination is an essential cellular process affected by a multienzyme cascade involving E1s (ubiquitin-activating enzymes), E2s (ubiquitin-conjugation enzymes or UBCs), and E3s (ubiquitin–protein ligases) (12,257) (see Fig. 10.4).

Protein aggregation is a common feature of all of the chronic human neurodegenerative disorders. The intraneuronal inclusions in many of these diseases contain deposits of ubiquitylated proteins, indicating that perturbations of ubiquitin-dependent proteolysis may occur. The neuropathological hallmarks of AD are intraneuronal NFTs composed of hyperphosphorylated protein tau and extracellular amyloid plaques (12,23,24,191,207). Most of the ubiquitylated, hyperphosphorylated tau protein in NFTs is monoubiquitylated, with the remainder polyubiquitylated, as the substrate of the 26S proteasome (258). The protein deposits in NFT, neuritic plaques, and neuropil threads in the cerebral cortex of AD patients and those with

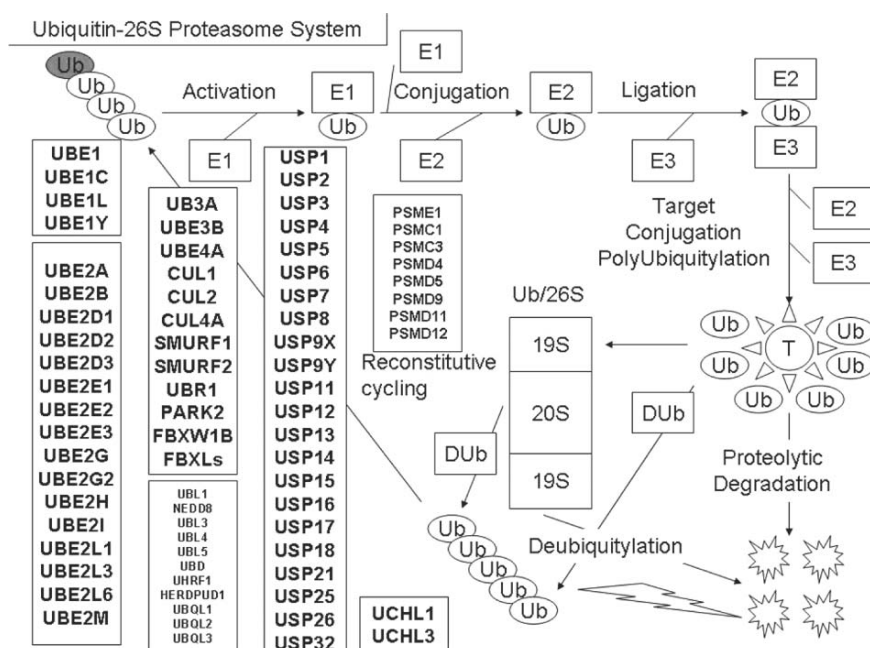


Fig. 10.4 The ubiquitin–26S proteasome system. (Adapted from ref. 12.)

Down syndrome contain forms of *APP* and ubiquitin-B that are aberrant in the C-terminus. These proteins do not appear in the brains of young control subjects; however, the presence of anomalous UBB in elderly control subjects might represent early stages of neurodegeneration. The two species of aberrant proteins display cellular colocalization, suggesting a common origin, operating at the transcriptional levels or by posttranscriptional editing of RNA (*135–138*). This type of transcript mutation is probably an important factor in the occurrence of nonfamilial cases of EOAD and late-onset AD (LOAD).

The mechanism of dinucleotide deletion at the transcript level might preclude the ubiquitin–proteasome system from proper operation, leading to accumulation of aberrant proteins, causing neurodegeneration (*12,135–138*). Proteasomes seem to play a crucial role upstream of the proteolytic cascade involving calpains and caspases by contributing to tau- and *APP*-altered breakdown and consequent tendency to aggregation of their degradation fragments (*259*).

Genetic variants in the *UBQLN1* gene (9q21.2–q21.3), which potentially link the ubiquitination machinery to the proteasome, substantially increase the risk of AD (*260,261*). Studies implicated ubiquitin as an important factor in regulating presenilin biogenesis and metabolism (*164,262*). Inhibition of the proteasome results in decreased presenilin fragment production, and reversal of proteasome inhibition restores presenilin fragment production, suggesting that the proteasome may be involved in presenilin endoproteolysis (*164,262*).

In conjunction with studies performed by van Leeuwen et al. (*135–138*), Layfield et al. (*263*) proposed a novel mechanism that could account for an inhibition of 26S proteasome activity in cases of nonfamilial AD. Mutant forms of ubiquitin may inhibit proteolysis within neurons, predisposing these cells to inclusion formation. Molecular misreading of the *UBB* gene results in a dinucleotide deletion in UBB mRNA (*135–138,264*). In AD, an age-related posttranscriptional defect in primary transcript RNA processing may occur, leading to dinucleotide deletions within open reading frames that result in frameshifts and produce abnormal extension proteins, as demonstrated by van Leeuwen and coworkers (*138*).

The nucleotide deletion of the ubiquitin primary transcript causes loss of the C-terminal Gly residue of ubiquitin (Gly76), producing residues of 1–75 of ubiquitin linked to a 20-amino acid nonsense extension, termed ubiquitin+1 (UBB+1). Unlike the normal ubiquitin transcript, this extended ubiquitin cannot be processed by deubiquitylating enzymes because the essential Gly76 is lost and therefore cannot be conjugated to target proteins. The UBB+1 can be polyubiquitylated by wild-type ubiquitin and incorporated into the growing proximal end of an unanchored polyubiquitin chain by the E2-25K enzyme (*265*). This unanchored polyubiquitin inhibits the 26S proteasome and its proteasomal activity. The isopeptidase-T deubiquitylating enzymes normally disassemble these unanchored chains, thus restoring proteasome activity; however, isopeptidase-T requires the presence of a free C-terminal Gly residue in the proximal ubiquitin moiety to operate efficiently, but chains with ubiquitin+1 are poor substrates for isopeptidase-T (*265*) and may therefore accumulate in AD neurons (*263*). In addition, proteasomal inhibition might directly contribute to protein aggregate formation (*266,267*). UBB+1 inhibits

proteasomal proteolysis and is also an ubiquitin fusion degradation substrate for the proteasome.

UBB+1 transcripts are present in tauopathies and synucleopathies and even in some control brains. In contrast to UBB+1 transcripts, UBB+1 protein accumulation in the ubiquitin-containing neuropathological hallmarks is restricted to the tauopathies such as Pick disease, frontotemporal dementia, PSP, and argyrophilic grain disease, but UBB+1 protein is not detected in the major forms of synucleopathies (Lewy body disease, multiple system atrophy). The intact brain can cope with UBB+1 as lentivirally delivered UBB+1 protein is rapidly degraded in rat hippocampus, whereas the K29,48R mutant of UBB+1, which is not ubiquitinated, is abundantly expressed. The finding reported by Fischer et al. (264) that UBB+1 protein only accumulates in tauopathies implies that the ubiquitin–proteasome system is impaired specifically in this group of neurodegenerative diseases, and that the presence of UBB+1 protein represents a form of proteasomal dysfunction in the brain (264).

UBB+1 also induces accumulation of a green fluorescent protein reporter carrying a constitutively active degradation signal in ubiquitin/proteasome transgenic mice (268). Proteasome inhibition by drug action induces a heat shock response and renders protection against stress. Expression of UBB+1 also induces expression of heat shock proteins. This priming of the chaperone system in these cells promotes a subsequent resistance to tert-butyl hydroperoxide-mediated oxidative stress, indicating that UBB+1-expressing cells have a compromised ubiquitin–proteasome system and are protected against oxidative stress conditions (269). However, overexpression of UBB+1 can cause nuclear fragmentation and cell death (270). In neuronal cell culture models, inhibition of the proteasome leads to cell death and formation of fibrillar ubiquitin and α -synuclein positive inclusions, thus modeling some aspects of Lewy body disease (271).

The intracellular deposition of ABP as the first pathogenic event prior to the appearance of tau pathology in AD (272) is an indirect evidence of ABP aggregation and incapacity of the ubiquitin–proteasome–chaperone system to repair the distorted metabolism of APP–ABP leading to neurodegeneration. A similar phenomenon with aggregation of conformation-abnormal peptides probably plays a key role in many other neurodegenerative disorders.

An example that alterations in the ubiquitin–proteasome system may be a primary event in AD, after ABP-induced toxicity or accumulation, was provided by Konishi et al. (273), who found that frameshift ubiquitin-B was present in subjects with AD pathology prior to development of dementia, probably accumulating in the initial steps of AD pathogenesis, whereas complement proteins were detected in AD patients but not in subjects with AD pathology and no symptoms of dementia, indicating the involvement of complement proteins in the later stage of dementia (273).

Metsaars et al. (274) elegantly studied the chronology of AD-related lesions in the primary visual cortex (Brodmann's area 17) of AD and identified four grades. At grade 1, only deposits of ABP were noticed. At grade 2, Congo red-positive deposits and processes containing ubiquitin and cathepsin D immunoreactivity around plaque cores could also be found. At grade 3, neuritic plaques and neuropil

threads were present, and at grade 4, NFTs appeared. The density of all the lesions dramatically increased at grade 4. The sequence of isocortical lesions from grade 1 to grade 4 is compatible with a cascade of events beginning with deposition of ABP and ending with the formation of NFTs (274). The presence of ubiquitin in grade 2 follows the initial expression of ABP, probably reflecting the inability of the ubiquitin–proteasome system to destroy the pathologic accumulation of ABP (275).

Ubiquitin interacts avidly but not covalently with ABP and such complexes can be isolated from AD brain extracts (276). Ubiquitin and *APP* colocalize to endosome-lysosomes implicated in *APP* proteolysis (277). A clear link between defective *APP* processing and the ubiquitin–proteasome system was demonstrated by Chen et al. (278). *APP*-BP1, first identified as an *APP*-binding protein, is the regulatory subunit of the activated enzyme for the small ubiquitin-like protein NEDD8. *APP*-BP1 drives the S- to M-phase transition in dividing cells and causes apoptosis in neurons (279). *APP*-BP1 binds to the COOH-terminal 31 amino acids of *APP* (C31) and colocalizes with *APP* in lipid rafts. Coexpression of a peptide representing the domain of *APP*-BP1 that binds to *APP*, abolishes the ability of overexpressed *APP* or the V642I mutant of *APP* to cause neuronal apoptosis and DNA synthesis. A dominant negative mutant of the NEDD8-conjugating enzyme hUbc12, which participates in the ubiquitin-like pathway initiated by *APP*-BP1, blocks neuronal apoptosis caused by *APP*-V642I, C31, or overexpression of *APP*-BP1. Neurons overexpressing *APP* or *APP*-V642I show increased *APP*-BP1 protein levels in lipid rafts. A similar increase in *APP*-BP1 in lipid rafts is observed in the AD hippocampus but not in less-affected areas of the AD brain. This translocation of *APP*-BP1 to lipid rafts is accompanied by a change in the subcellular distribution of the ubiquitin-like protein NEDD8, which is activated by *APP*-BP1 (278).

NEDD8 is covalently ligated to cullin family proteins, which are components of certain ubiquitin E3 ligases, by a pathway analogous to that of ubiquitin. NEDD8 protein expression is widely observed in most types of tissues. Accumulation of the NEDD8 protein is commonly observed in ubiquitinated inclusion bodies, including Lewy bodies in PD, Mallory bodies in alcoholic liver disease, and Rosenthal fibers in astrocytoma. About 20% of NFTs and senile plaques of AD show intense staining for NEDD8 as well as for ubiquitin. The NEDD8 system might be involved in the metabolism of these inclusion bodies via the ubiquitin–proteasome system (280).

Additional information provides support for a direct link between ABP-related neurotoxicity and the ubiquitin–proteasome system. Song et al. (281) have isolated an unusual ubiquitin-conjugating enzyme, E2-25K/Hip-2, as a mediator of ABP toxicity. The expression of E2-25K/Hip-2 was upregulated in neurons exposed to ABP1-42 in vivo and in culture. Enzymatic activity of E2-25K/Hip-2 was required for both ABP1-42 neurotoxicity and inhibition of proteasome activity. E2-25K/Hip-2 functioned upstream of apoptosis signal-regulating kinase 1 (ASK1) and JNK in ABP1-42 toxicity. Further, the ubiquitin mutant UBB+1 was colocalized and functionally interacted with E2-25K/Hip-2 in mediating neurotoxicity, suggesting that E2-25K/Hip-2 is a crucial factor in regulating ABP neurotoxicity in AD pathogenesis (281).

Some reports have shown reexpression of cell-cycle-related proteins in vulnerable neurons in AD. Ogawa et al. (282) have hypothesized that this attempt by neurons

to reenter mitosis is a response to external growth stimuli that leads to an abortive reentry into the cell cycle and, ultimately, neuronal degeneration. They have investigated p27, a cyclin-dependent kinase inhibitor that plays a negative regulatory role in cell cycle progression that, once phosphorylated at Thr187, is degraded via an ubiquitin–proteasome pathway. Both p27 and phosphorylated p27 (Thr187) show increases in the cytoplasm of vulnerable neurons in AD. Phosphorylated p27 shows considerable overlap with tau-positive neurofibrillary pathology, including NFTs, dystrophic neurites, and neuropil threads. The ribosomal S6 PK p70 S6 kinase is known for its role in modulating cell cycle progression, cell size, and cell survival. In response to mitogen stimulation, p70 S6 kinase activation upregulates ribosomal biosynthesis and enhances the translational capacity of the cell. The levels of phosphorylated p70 S6 kinase (at Thr389 or at Thr421/Ser424) are increased in accordance with the progressive sequence of neurofibrillary changes according to Braak’s criteria and correlated with hyperphosphorylated tau and ubiquitin, suggesting that the activated proteolytic system might not be sufficient to degrade the overproduced and overphosphorylated tau protein. A p70 S6 kinase modulated upregulation of tau translation might contribute to PHF-tau accumulation in neurons with neurofibrillary changes (283).

To better understand the whole pathogenic process in AD—from DNA duplication, transcription, and translation to protein conformation and degradation—to design novel pharmacogenomic strategies oriented to prevent the onset of the disease or to halt its progression, additional studies of functional genomics are urgently needed, including studies of the mechanisms of methylation and acetylation in neurons; transcription-related factors (284); transcriptional regulatory elements (core promoters, proximal promoters, distal enhancers, silencers, insulators/boundary elements, locus control regions); and the molecular machinery (general TFs, activators, coactivators) that interacts with the regulatory elements to mediate controlled patterns of gene expression (285). In addition, proteomics (286) and metabolomics studies (287) are fundamental issues awaiting elucidation to elaborate an integral, unitary hypothesis on primary and accessory causes of neurodegeneration in AD. All this information requires powerful bioinformatics tools and a “connectivity map” to define functional connections among disease process, genetic alterations, and drug actions (288).

10.2.4 Genotype–Phenotype Correlations

Functional genomics studies have demonstrated the influence of many genes on AD pathogenesis and phenotype expression (see Table 10.1). Mutations in the *APP*, *PS1*, *PS2*, and *MAPT* genes give rise to well-characterized differential neuropathological and clinical phenotypes of dementia (12,19,20). The analysis of genotype–phenotype correlations has also revealed that the presence of the *APOE-4* allele in AD, in conjunction with other genes, influences disease onset, brain atrophy, cerebrovascular perfusion, blood pressure, β -amyloid deposition, *APOE* secretion, lipid metabolism, brain bioelectrical activity, cognition, apoptosis, and treatment outcome (1,3,12–16,18,59,289–291).

The characterization of phenotypic profiles according to age, cognitive performance (MMSE and ADAS-Cog score); serum *APOE* levels; serum lipid levels, including cholesterol (CHO), high-density lipoprotein (HDL)-CHO, low-density lipoprotein (LDL)-CHO, very low-density lipoprotein (VLDL)-CHO, and triglyceride (TG) levels; as well as serum nitric oxide (NO), β -amyloid, and histamine levels (20), reveals sex-related differences in 25% of the biological parameters and almost no differences (0.24%) when patients are classified as *APOE*-4(-) and *APOE*-4(+) carriers. This probably indicates that gender-related factors may influence these parametric variables more powerfully than the presence or absence of the *APOE*-4 allele. In contrast, when patients are classified according to their *APOE* genotype, dramatic differences emerge among *APOE* genotypes (>45%), with a clear biological disadvantage in *APOE*-4/4 carriers who exhibit (1) earlier age of onset; (2) low *APOE* levels; (3) high CHO and LDL-CHO levels; and (4) low NO, β -amyloid, and histamine levels in blood (12,13,19,20,25,289,290).

These phenotypic differences are less pronounced when AD patients are classified according to their *PSI* (15.6%) or *ACE* genotypes (23.52%), reflecting a weak impact of *PSI*- and *ACE*-related genotypes on the phenotypic expression of biological markers in AD. *PSI*-related genotypes appear to influence age of onset, blood histamine levels, and cerebrovascular hemodynamics, as reflected by significant changes in systolic (Sv), diastolic (Dv), and mean velocities (Mv) in the left middle cerebral arteries (MCAs) (20). *ACE*-related phenotypes seem to be more influential than *PSI* genotypes in defining biological phenotypes, such as age of onset, cognitive performance, HDL-CHO levels, *ACE* and NO levels, and brain blood flow Mv in MCA. However, when *APOE* and *PSI* genotypes are integrated in bigenic clusters and the resulting bigenic genotypes are differentiated according to their corresponding phenotypes, an almost logarithmic increased expression of differential phenotypes is observed (61.46% variation), indicating the existence of a synergistic effect of the bigenic (*APOE* + *PSI*) cluster on the expression of biological markers, apparently unrelated to *APP/PSI* mutations because none of the patients included in the sample were carriers of either *APP* or *PSI* mutations (20,289).

These examples illustrate the potential additive effects of AD-related genes on the phenotypic expression of biological markers. Furthermore, the analysis of genotype-phenotype correlations with a monogenic or bigenic approach documents a modest genotype-related variation in serum amyloid- β (ABP) levels, suggesting that peripheral levels of ABP are of relative value as predictors of disease stage or as markers of disease progression or treatment-related disease-modifying effects (20,289). The peripheral levels of ABP in serum exhibit an *APOE*-dependent pattern according to which both *APOE*-4(+) and *APOE*-2(+) carriers tend to show higher ABP levels than *APOE*-4(-) or *APOE*-3 carriers (20,59,289,290) (see Fig. 10.5).

This trend is even clearer when *APOE*, *PSI*, and *PS2* genotypes are integrated in bigenic or trigenic clusters in which the 3322, 3212, and 4412 genotypes show the highest ABP levels compared with other genotypes (20,59,289,290) (see Fig. 10.6). The incorporation of genotype assessment in biochemical studies (e.g., phenotype expression profile) in AD (12,20,25,290) would avoid inconsistencies and unnecessary controversies, such as those reflected in recent articles concerning

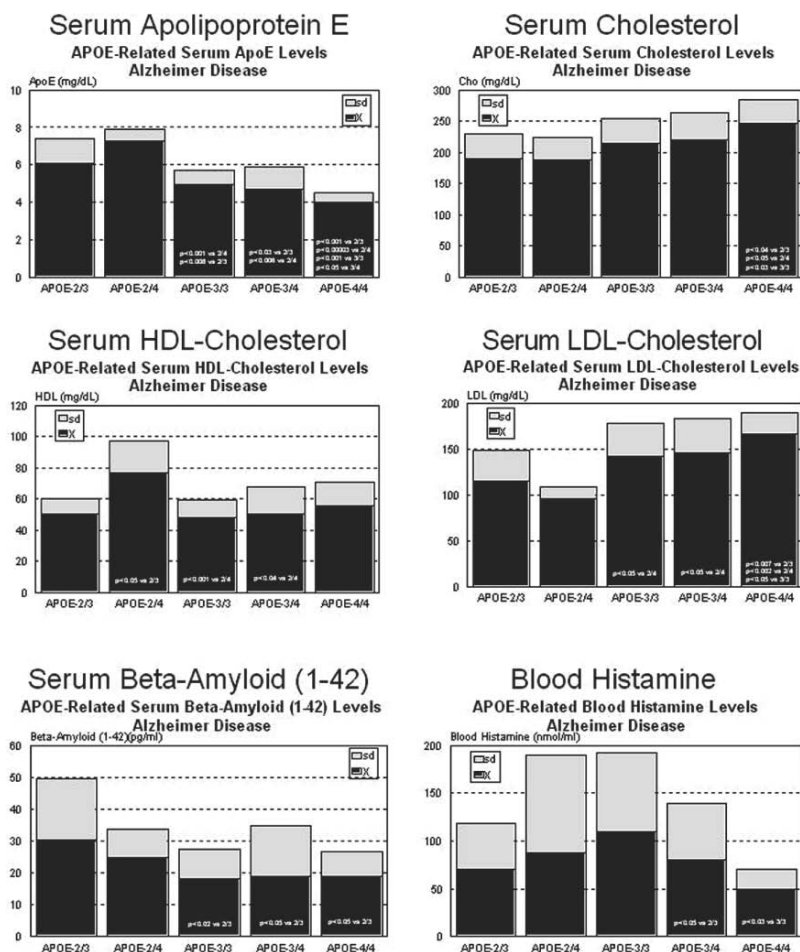


Fig. 10.5 Apolipoprotein E (*APOE*)-related serum levels of *APOE*, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, amyloid- β peptide (1-42), and histamine in Alzheimer's disease. (Adapted from refs. 12,59, and 289.)

variability in ABP levels in AD (292,293). Likewise, in drug clinical trials with β -breakers or amyloid scavengers, as well as in cases of vaccination against ABP deposits or treatment with β -secretase inhibitors, *APOE* genotyping should be included to discriminate specific genotype-related responses.

In contrast to the inconsistent variability in ABP levels, genotype-related serum histamine changes exhibit an outstanding variation that can be modified by therapeutic intervention (294-296) (see Figs. 10.5 and 10.6). *APOE*-related serum histamine levels exhibit an opposite pattern to that observed in ABP levels (see Figs. 10.5 and 10.6). The lowest concentration of serum histamine is systematically present in *APOE*-2(+) and *APOE*-4(+) carriers, and the highest levels of histamine are seen in *APOE*-3(+) carriers (see Figs. 10.5 and 10.6). Central and

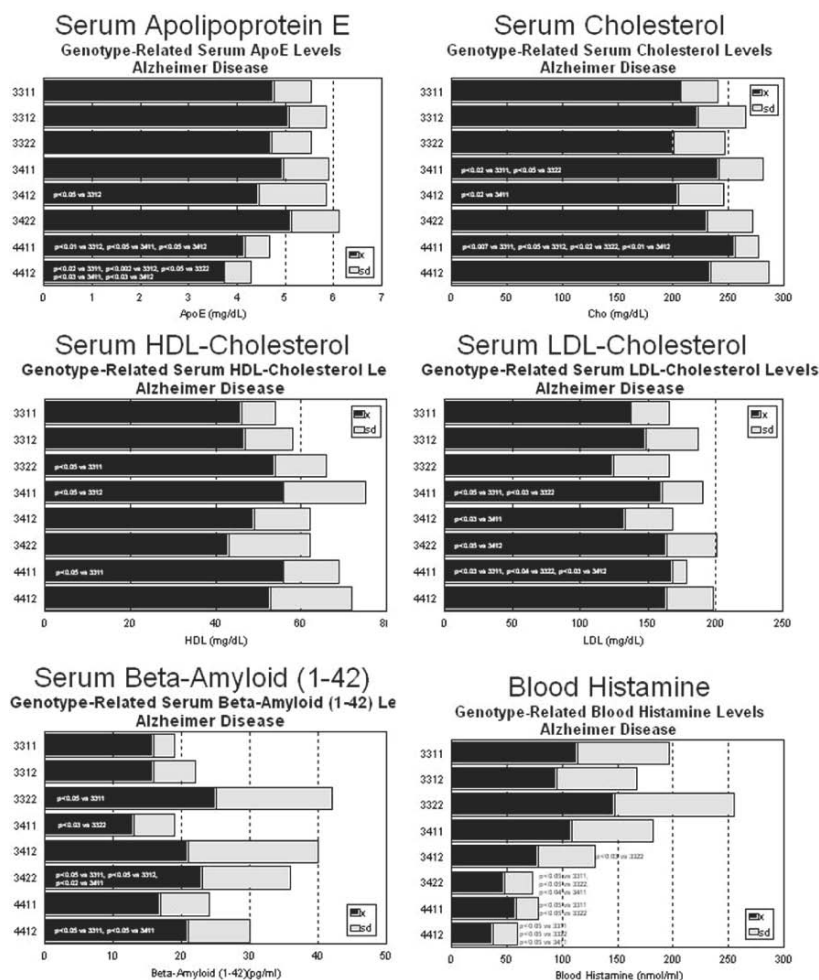


Fig. 10.6 Bigenic cluster (apolipoprotein E [*APOE*] + presenilin 1 [*PSI*])-related serum levels of apolipoprotein E, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, amyloid- β peptide (1–42), and histamine in Alzheimer's disease. (Adapted from refs. 12,59, and 289.)

peripheral histaminergic mechanisms may regulate cerebrovascular function in AD (12,20,294–296), which is significantly altered in *APOE-4/4* carriers (1,3,12,13,20,290). These observations can lead to the conclusion that the simple quantification of biochemical markers in fluids or tissues of AD patients with the aim of identifying pathogenic mechanisms or monitoring therapeutic effects, when they are not accompanied by differential genotyping for sample homogenization, is of very poor value.

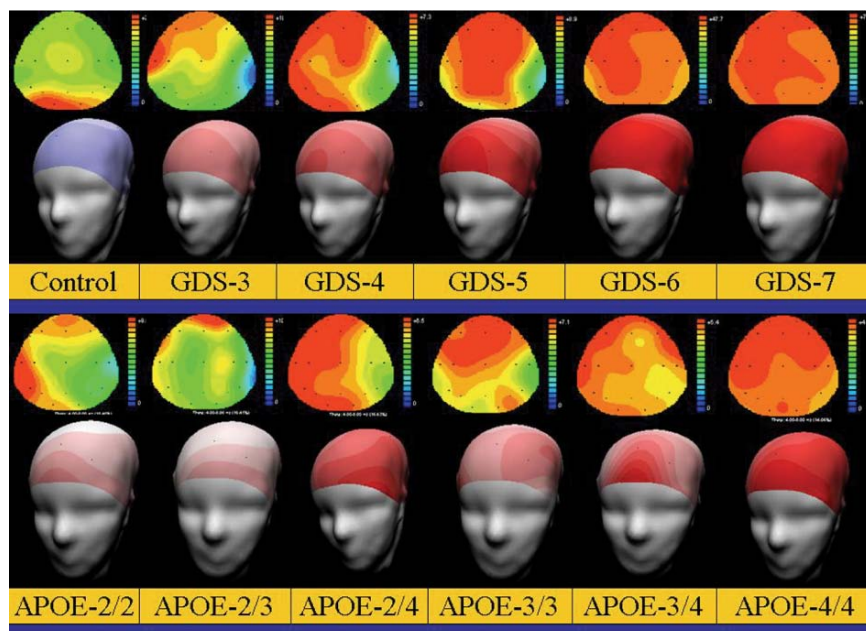


Fig. 10.7 Brain mapping activity (theta band) according to Global Deterioration Stage (GDS) staging (cognitive deterioration) and apolipoprotein E (*APOE*) genotype in Alzheimer's disease. (From refs. 19 and 20.)

Differential patterns of *APOE*-, *PS1*-, *PS2*-, and trigenic (*APOE* + *PS1* + *PS2*) cluster-related lymphocyte apoptosis have been detected in AD (129,130,297). Fas receptor expression is significantly increased in AD, especially in *APOE*-4 carriers, for whom lymphocyte apoptosis is more relevant (20).

It has been demonstrated that brain activity slowing correlates with progressive GDS staging in dementia (12,19,20) (see Fig. 10.7). In the general population, subjects harboring the *APOE*-4/4 genotype exhibit premature slowing in brain mapping activity represented by increased slow delta and theta activities as compared with other *APOE* genotypes (12,13,19,20). In patients with AD, slow activity predominates in *APOE*-4 carriers with similar GDS stage (12,13,19,20) (see Fig. 10.7).

All these examples of genotype–phenotype correlations, as a gross approach to functional genomics, illustrate the importance of genotype-related differences in AD and their impact on phenotype expression (12,13,19,20,289,290). Most biological parameters, potentially modifiable by monogenic genotypes or polygenic cluster profiles, can be used in clinical trials for monitoring efficacy outcomes. These parametric variables also show a genotype-dependent profile in different types of dementia (e.g., AD vs vascular dementia). For instance, striking differences have been found between AD and vascular dementia in structural and functional genomics studies (3,12,19,20,59,289,290) (see Fig. 10.8).

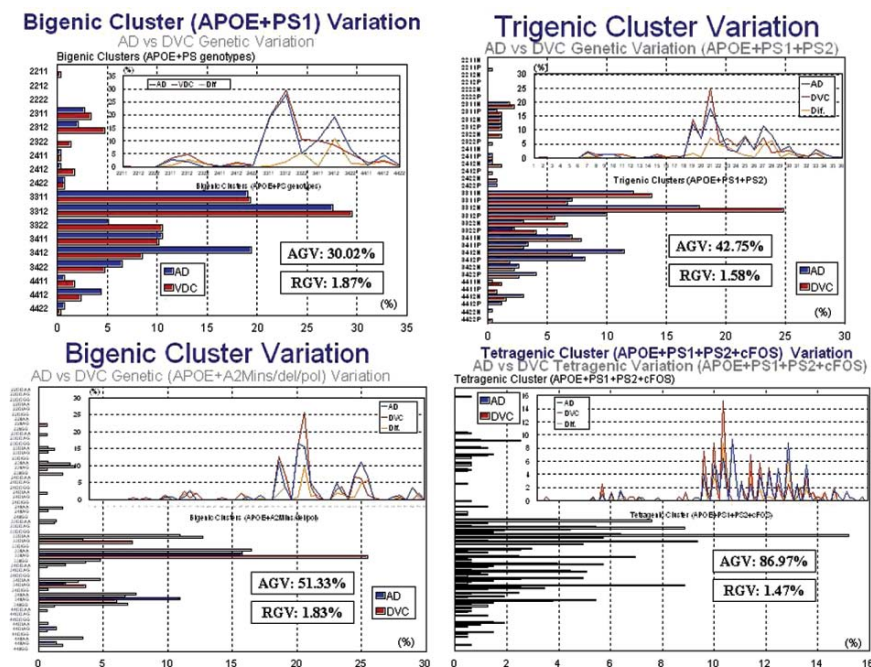


Fig. 10.8 Absolute genetic variation (AGV) and relative genetic variation (RGV) between Alzheimer's disease and vascular dementia associated with bigenic, trigenic, and tetragenic clusters of Alzheimer's disease (AD)-related genes. *APOE*, apolipoprotein E; PS, presenilin. (Adapted from refs. 12,19,20, and 59.)

10.3 Alzheimer's Disease Therapeutics

Drugs approved by the FDA and regulatory authorities in Europe and Japan include the cholinesterase inhibitors (ChEIs) tacrine, donepezil, rivastigmine, and galantamine (6,7,298–301) and the *N*-methyl-D-aspartate (NMDA) receptor partial antagonist memantine (6,303–305). Some studies assessing the cost-effectiveness of ChEIs suggested that ChEI therapy provides a benefit at every stage of disease, with better outcomes resulting from persistent, uninterrupted treatment (306–308), whereas other studies indicated that ChEIs are not cost-effective, with benefits below minimally relevant thresholds (7,9,309) or cost-neutral (4,9,310,311). Memantine shows benefits on cognitive and global function on the same order of magnitude as seen for ChEIs (306,312,313). Methodological limitations in some studies reduced the confidence of independent evaluators in the validity of the conclusions drawn in published reports (302). Although the therapeutic value and cost-effectiveness of current antidementia treatment is questionable (9), these drugs are of common use in AD (7,298) and still require further evaluation from a pharmacogenetic/pharmacogenomic perspective to avoid side effects and unnecessary costs (20).

10.3.1 *Potential Therapeutic Strategies*

Modern therapeutic strategies in AD are addressed to interfere with the main pathogenic mechanisms potentially involved in AD (see [Table 10.2](#)). Major pathogenic events (drug targets) and their respective therapeutic alternatives include the following: (1) genetic defects: gene therapy and RNAi; (2) β -amyloid deposition: β -secretase inhibitors, γ -secretase inhibitors, α -secretase activators, $A\beta$ -fibrillation and aggregation inhibitors, amyloid immunotherapy (active and passive vaccination), copper chelating agents, solubilizers of $A\beta$ aggregates, *APP* production inhibitors, and $A\beta$ selective regulators (reticulons, chaperones); (3) tau-related pathology: phosphatase activators, GSK-3 inhibitors, Cdk5 inhibitors, p38 inhibitors, JNK inhibitors; (4) apoptosis: caspase inhibitors; (5) neurotransmitter deficits: acetylcholine enhancers (acetylcholine-release stimulants, acetylcholine reuptake inhibitors, ChEIs, choline-acetyl-transferase stimulants, muscarinic antagonists, nicotinic agonists), γ -aminobutyric acid (GABA) modulators (inverse GABA-receptor agonists), glutamate modulators (NMDA antagonists, ampakines), dopamine reuptake inhibitors, adrenoreceptor modulators, histamine H3 antagonists, and serotonin (5-HT) modulators (5-HT3 and 5-HT1A receptor agonists, 5-HT6 receptor antagonists, 5-HT stimulants); (6) neurotrophic deficits: neurotrophic factors, growth factors, synthetic neuropeptides, and natural compounds with neurotrophic activity; (7) neuronal loss: neuronal stem cells, growth factors, neurite outgrowth activators, NOGO inhibitors, MOP inhibitors, GSK3 inhibitors, JNK inhibitors, and p38 inhibitors; (8) neuroinflammation: cyclooxygenase 1 and 2 (COX1 and COX2) inhibitors, complement activation inhibitors, p38 inhibitors, eNOS inhibitors, PPAR α agonists, PPAR γ agonists, novel nonsteroidal anti-inflammatory drugs (NSAIDs), and cytokine inhibitors; (9) oxidative stress: antioxidants, caspase inhibitors, and antioxidating enzyme enhancers; (10) calcium dysmetabolism: calcium channel blockers; (11) neuronal hypometabolism: PPAR γ agonists and GSK3 inhibitors; (12) lipid metabolism dysfunction: HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors, PPAR γ agonists, and novel biomarine lipoproteins; (13) cerebrovascular dysfunction: vasoactive substances, NO inhibitors, hypoxia-inducible factor (HIF) inhibitors, dandelene-related agents, novel lipoproteins with antiatherosclerotic activity, and liver X receptor (LXR) agonists; (14) neuronal dysfunction associated with nutritional deficits: brain metabolism enhancers, nutrigenomic agents, and nutraceuticals; and (15) a miscellany of pathogenic mechanisms potentially manageable with diverse classes of chemicals or biopharmaceuticals ([6,15,16,18–20,24,103–107,314](#)) (see [Table 10.2](#)).

10.3.2 *Molecular Pharmacology of Alzheimer's Disease with Novel Therapeutic Strategies*

It is very likely that a therapeutic approach to slow ABP formation or inhibit amyloidogenesis, as well as ABP scavenging, would help to partly neutralize or reduce

neuronal damage and neurodegeneration in AD (see Fig. 10.3). *APP* proteases are prime therapeutic targets to control *APP* metabolism, and future development of BACE and γ -secretase inhibitors may be beneficial for AD (104,111,112,181,182,315,316). It has also been demonstrated that ABP immunization of PDAPP transgenic mice overexpressing mutant human *APP* (P717V) prevented the development of ABP plaque formation, neuritic dystrophy, and astrogliosis, suggesting that immunization with ABP may be effective in preventing and treating AD (317,318); however, it is unlikely that similar mechanisms are fully effective in humans because ABP deposition is but a factor among many other pathogenic events underlying AD neuropathology (6,18–20,104,319,320).

Furthermore, studies in familial Danish dementia (Strömberg's hereditary ophthalmoto-encephalica), an early-onset autosomal dominant neurodegenerative disorder linked to a genetic defect in the *ABRI2* gene, in which Danish and Alzheimer amyloid subunits coexist, demonstrated that compact plaques (fundamental lesions for the diagnosis of AD) are not essential for the mechanism of dementia (321). In fact, to date, there is no reasonable explanation regarding why plaques and tangles simultaneously accumulate in AD.

McGeer and coworkers (322) demonstrated that a stable complex can form between tau and ABP. This complex enhances tau phosphorylation by GSK3 β , but the phosphorylation then promotes dissociation of the complex. ABP binds to multiple tau peptides, especially those in exons 7 and 9, and this binding is sharply reduced or abolished by phosphorylation of specific Ser or Thr residues. Conversely, tau binds to multiple ABPs in the mid- to C-terminal regions of ABP, and this binding is also significantly decreased by GSK3 β phosphorylation of tau. These studies led the authors to hypothesize that an initial step in the pathogenesis of AD may be intracellular binding of soluble ABP to soluble nonphosphorylated tau, thus promoting tau phosphorylation and ABP nucleation, and that blocking the sites where ABP initially binds to tau might arrest the simultaneous formation of plaques and tangles in AD.

At present, the majority of the novel strategies postulated by the industry and the academy to effectively fight AD are addressed to halt or reduce ABP accumulation in plaques and vessels (103–107,314), including α -secretase modulators (batimastat, marimastat); β -secretase inhibitors (OM991, OM992); γ -secretase inhibitors (difluoroketone peptidomimetics, dipeptide aldehydes, fenchylamine sulfonamides, L-685,458, calpain inhibitor MDL28170, serine protease inhibitor AEBSF); HMG-CoA reductase inhibitors (statins); compounds affecting fibril formation; inhibitors of ABP aggregation (nicotine, melatonin, antioxidants, antibiotics, IMAOs, laminin, entactin); accelerators of ABP disaggregation (2,4-dinitrophenol, 3-nitrophenol, N-methylated congeners of hydrophobic core domain ABP16–22); regulators of ABP catabolism and removal (neprilysin [NEP] inhibitors, endothelin-converting enzyme [ECE]); modulators of ABP neurotoxicity (ABP antibodies); *APP* gene-knockdown agents; and RNAi of pathogenic genes (314).

The cerebrovascular component of dementia (3,25,59) has never been therapeutically addressed in AD. Novel interventions with HIF prolyl-4-hydroxylase

inhibitors may be beneficial for cerebrovascular disorders and dementia as well as other diseases associated with oxidative stress (323). HIF prolyl-4-hydroxylases are a family of iron- and 2-oxoglutarate-dependent dioxygenases that negatively regulate the stability of several proteins involved in adaptation to hypoxic or oxidative stress (323).

Another important therapeutic issue in AD is the control of microglial activation and consequent neuroinflammation phenomena (143,324). It remains to be determined whether microglial activation plays a role in the initial steps of the pathogenic process or occurs merely as a response to neuronal damage, aggravating neurodegeneration by activation of proinflammatory and neurotoxic pathways (325). To date, poor results have been obtained with steroids, glucocorticoids, NSAIDs, and COX2 inhibitors in AD despite initial noisy expectations (143,326,327).

Interesting studies indicate that NF- κ B signaling in microglia is critically involved in neuronal death induced by ABP. Constitutive inhibition of NF- κ B signaling in microglia by expression of nondegradable I κ B α uperrepresor blocked neurotoxicity. Stimulation of microglia with ABP increased acetylation of RelA/p65 at lysine 310, which regulates the NF- κ B pathway. Overexpression of SIRT1 deacetylase and the addition of the SIRT1 agonist resveratrol markedly reduced NF- κ B signaling stimulated by ABP and had a strong neuroprotective effect. These studies highlight the therapeutic potential of resveratrol and other sirtuin-activating compounds in AD (328).

Although the number of potential targets and candidate drugs is apparently large (see Table 10.2), during the past decade most efforts have been addressed to identify and characterize compounds to inhibit ABP formation and reduce ABP load by clearing amyloid deposits. Several groups have also tried to test chemicals with the capacity to reduce NFT formation by inhibiting tau hyperphosphorylation (20,327).

10.3.2.1 Secretase Inhibitors and Modulators

The most prevalent hypothesis of AD pathogenesis considers the amyloid plaque as the central star of the Alzheimer's constellation. According to this, the elimination of the plaque or the blockade of its generation should be enough to halt disease progression. Although this simple reasoning is not accepted by many, what is true is that the amyloid hypothesis represents the most appealing theory of AD-related neurodegeneration to date. Drug discovery efforts aimed at preventing the accumulation of ABP have been directed toward inhibition of ABP production and prevention of ABP aggregation as well as enhancement of ABP clearance.

The molecular understanding of the proteolytic processing of *APP* by α -, β -, γ -, NO-, ϵ -, and ζ -cleavages (329,330), and the structural and functional characterization of α -, β -, and γ -secretases led to the conclusion that α -secretase modulators (either inhibitors or agonists), β -secretase inhibitors, and γ -secretase inhibitors might be potential candidate drugs to inhibit ABP formation, thus avoiding further ABP multimerization into neurotoxic oligomers that coalesce into fibrils, ultimately

forming amyloid plaques. Consequently, prevention of ABP production has focused on targeting the enzymes involved in *APP* processing.

Transition state-based inhibitors of *BACE1* have been described (331), and many γ -secretase inhibitors [L-658,485, L-852,646, DAPT (WO 9822494), LY411,575 (WO 9828268), (Z-LL)2-ketone, TBL4K, NVP-AHW700-NX, MG132, L685458] have been shown to block ABP processing (332–336); however, γ -secretase plays an essential role in the processing of other, disparate targets, and mechanism-based toxicity is a major concern (337). To date, more than 25 γ -secretase substrates have been identified. γ -Secretase plays a key role in mediating signaling via the Notch receptors. Nectin-1 α , an immunoglobulin-like receptor involved in the formation of synapses, is a substrate for PS/ γ -secretase-like cleavage (338), and Sortilin, SorCS1b, and SorLA Vps10p sorting receptors are also γ -secretase substrates (339).

The mammalian Vps10p sorting receptor family is a group of five TM homologs (Sortilin, SorLA, and SorCS1–3) that bind various cargo proteins via their luminal Vps10p domains, mediating a variety of intracellular sorting and trafficking functions. SorLA is downregulated in AD brains, interacts with *APOE*, and modulates ABP production. Sortilin has been shown to be part of proNGF-mediated death signaling that results from a complex of sortilin, p75^{NTR}, and proNGF (339).

Inhibition of γ -secretase has the expected benefit of reducing ABP in animal models of AD but has potentially undesirable biological effects as well, most likely because of the inhibition of Notch processing (337). Notch1 competes with *APP* for γ -secretase and downregulates *PSI* gene expression (340). Molecules known as γ -secretase modulators selectively inhibit γ -secretase processing of *APP* at the 42-cleavage site and may have a reduced incidence in processing other substrates. Espeseth et al. (341) described a novel series of benzofuran-containing compounds that inhibit *APP* processing by a previously undescribed mechanism of action. Although the compounds were initially identified in a search for *BACE1* inhibitors, they inhibit *BACE1*, *BACE2*, and γ -secretase-mediated cleavage of *APP* by binding *APP* within the ABP domain of the protein and differ in their mechanism of action from γ -secretase modulators (341).

Despite the promising effects of secretase inhibitors and modulators, some other players have to be taken into consideration in *APP* processing and secretase activity. High levels of *BACE1* activity are sufficient to elicit neurodegeneration, and this pathogenic pathway involves the accumulation of *APP* C-terminal fragments but does not depend on increased production of ABP. Thus, inhibiting *BACE1* may block not only ABP-dependent but also ABP-independent pathogenic mechanisms (342). Studies also indicated that there is a close interaction between BACE, *PSI*, and LRP on the cell surface, and that LRP is a novel BACE substrate (343) and a competitive substrate of *APP* for γ -secretase as well (344). γ -Secretase is a functional component of phagosomes, and γ -secretase activity may be involved in the phagocytic response of macrophages to inflammatory cytokines (345). PAR-4 (prostate apoptosis response 4) is a leucine zipper protein that was initially identified as associated with neuronal degeneration and aberrant ABP production. The C-terminal domain of PAR-4 is necessary for forming a complex with the

cytosolic tail of *BACE1*. Overexpression of PAR-4 significantly increases, whereas silencing of PAR-4 expression by RNAi significantly decreases, β -secretase cleavage of *APP*, suggesting that PAR-4 may be directly involved in regulating the *APP* cleavage activity of *BACE1* (346).

The regulation of β -secretase and BACE access to *APP* is lipid dependent and involves lipid rafts (347–349). These enigmatic plasmalemmal organelles, with virtually ubiquitous tissue distribution, have been implicated in a wide range of cellular functions. The caveolin proteins (caveolin 1, 2, and 3) are the structural component of caveolae in lipid rafts, and they function as scaffolding proteins capable of recruiting numerous signaling molecules to caveolae (350). Neutral glycosphingolipids (cerebrosides), anionic glycerophospholipids, and sterols (cholesterol) stimulate proteolytic activity of BACE- (351) modulating *APP* cleavage (352). The sphingolipid ceramide is almost universally generated during cell stress and apoptosis (353). This lipid second messenger is elevated in AD brains, increases the half-life of *BACE1*, and thereby promotes ABP biogenesis (354). Low cellular cholesterol levels favor the α -secretase pathway and decrease ABP secretion, presumably within the endocytic pathway; in contrast, low isoprenoid levels result in the accumulation of *APP*, amyloidogenic fragments, and ABP likely within biosynthetic compartments, suggesting that isoprenylation is involved in determining levels of intracellular ABP (355). Both *APP* and ABP can oxidize cholesterol to form 7 β -hydroxycholesterol, a proapoptotic oxysterol that is neurotoxic at nanomolar concentrations, and the oxidation of cholesterol is accompanied by stoichiometric production of hydrogen peroxide in the presence of divalent copper (356).

The syndecan family of heparin sulfate proteoglycans (HSPGs) plays critical roles in several signal transduction pathways, and syndecan 3 intramembrane proteolysis is presenilin/ γ -secretase dependent (357). COX2 and COX1 potentiate ABP formation through mechanisms that involve γ -secretase activity. Sulindac sulfide and other NSAIDs (ibuprofen, indomethacin, R-flurbiprofen) selectively decrease the secretion of ABP independently of COX activity, probably via γ -secretase inhibition (358–360). Pepstatin A methylester, sulfonamides, and benzodiazepines can also act as potent, noncompetitive, γ -secretase inhibitors (335). These are but a few examples of the potential repercussions and biochemical consequences that the pharmacological manipulation of secretases in AD may bring about.

10.3.2.2 Amyloid Scavengers

An alternative strategy to reduce ABP levels in the brain or clean up amyloid deposits is (1) altering catabolism and (2) disintegrating oligomers of abnormally aggregated fibrils. Many proteases/peptidases have been reported with the capability of cleaving ABP either in vivo or in vitro. Some of these enzymes include NEP (neutral endopeptidase-24.11, EC 3.4.24.11, enkephalinase, neutrophil cluster-differentiation antigen 10 [CD10], common acute lymphoblastic leukemia antigen [CALLA]) (3q21–q27); endothelin-converting enzyme (ECE) 1 (EC 3.4.24.71) (1p36.1); insulin-degrading enzyme (IDE) (EC 3.4.24.56) (10q23–q25); *ACE*

(EC 3.4.15.1) (17q23); uPA/tPA-plasmin system (EC 3.4.21.31) (10q24; 8p12); cathepsin D (11p15.5); gelatinase A (matrix metalloproteinase 2, MMP2) (16q13); gelatinase B (matrix metalloproteinase 9, MMP9) (20q11.2–q13.1); coagulation factor XIa (4q35); antibody light chain c23.5 and hk14; and α 2-macroglobulin complexes (12p13.3–p12.3) (**12,361**). Many of these proteases are capable of removing extracellular ABP, and some of them also remove the cytoplasmic product of γ -secretase-cleaved *APP* (**362,363**).

In conformational disorders with ABP aggregates, novel therapeutic strategies to disintegrate ABP deposits include (1) active vaccination (**328**), (2) passive immunization with antibodies against ABP, and (3) use of disruptors of ABP aggregation, including several low molecular weight substances such as the anthracycline 4'-iodo-4'-deoxydoxorubicin; Congo red dye; antibiotics rifampicin and iodochlorhydroxyquin; sulfonated molecule NC-758 (Alzhmed), which interferes with the binding of glycosaminoglycans to the ABP; and a series of dimeric derivatives of the amino acid proline (R-1-[6-[R-2-carboxy-pyrrolidine-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid; Ro 63-8695 or CPHPC) that link two pentamers of the serum amyloid P (SAP) protein, present in AD and peripheral amyloid deposits, preventing the binding of SAP to amyloid deposits for the drug-SAP complex to be rapidly removed from blood and then transformed by hepatic degradation. Other inhibitors of SAP binding of this series include Ro 15-3479, Ro 63-3300, Ro 15-3743, and Ro 63-2346 with IC_{50} ranging from $5\mu M$ to more than $100\mu M$ (Ro 63-8695 $IC_{50} = 0.9\mu M$) (**20,364,365**).

ABP neurotoxicity can be inhibited in part by tachykinins, some calcium channel blockers, neurotrophic factors, NMDA receptor blockers, some NSAIDs, inhibitors of free-radical formation and lipid peroxidation, estrogen replacement therapy, and β -sheet breaker peptide fragments (iA β 11/LPFFD) analogous to the ABP sequence (**366–369**). Disregulations in MMPs might also account for alterations in *APP* metabolism and ABP accumulation (**370**). Novel compounds able to help proteolytic enzymes involved in the remodeling of the extracellular matrix might also be useful to preserve brain microstructural changes caused by abnormal accumulation of protein aggregates, including ABP deposition and other degradation products (**6,20,104,314**).

Assuming that ABP is a metalloprotein with high affinity for Cu^{2+} , Fe^{3+} , and Zn^{2+} in vitro, there is evidence that highly specific chelators of metal ions that bind ABP (e.g., 5-chloro-7-iodo-8-hydroxyquinoline, iodochlorhydroxyquin, clioquinol) might reduce amyloid load in AD transgenic models by inhibiting oxidative stress (**371**). Preliminary clinical data also support a positive role for clioquinol as a candidate drug for AD (**372**), although conflicting results concerning the benefits and risks of metal chelation with clioquinol recommend caution with this potential treatment (**373,374**). Other strategies have been proposed for chelating agents in AD (**375,376**).

A series of natural and synthetic products have also shown antiamyloid properties as amyloid scavengers or β -breakers. One example is the phenolic yellow curry pigment curcumin, which has potent anti-inflammatory and antioxidant activities and can suppress oxidative damage, inflammation, cognitive deficits, and amyloid

accumulation, effectively disaggregating ABP and preventing fibril and oligomer formation (377). The omega-3 (n-3) polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) can also modulate *APP* processing by degrading both α - and β -*APP* C-terminal fragment products and full-length *APP*, not affecting *BACE1*, *APOE*, and transthyretin gene expression, suggesting that dietary DHA could be protective against ABP production, accumulation, and potential downstream toxicity (378).

Some studies have reported that a five-amino acid β -sheet breaker peptide (iA β 5p), end-protected to minimize exopeptidase cleavage, has the ability to induce a dramatic reduction in amyloid deposition in AD transgenic animals (368,369), and a series of improved iA β 5p derivatives has been developed (379). A new concept was introduced for the rational design of β -sheet ligands, which prevent protein aggregation. Oligomeric acylated aminopyrazoles with a donor–acceptor–donor hydrogen bond pattern complementary to that of a β -sheet efficiently block the solvent-exposed β -sheet portions in ABP1-40 and thereby prevent formation of insoluble protein aggregates (380). Some compounds based on the chemical structure of apomorphine were found to interfere with ABP1-40 fibrillization. The ability of these small molecules to inhibit amyloid fibril formation appears to be linked to their tendency to undergo rapid autooxidation, suggesting that autooxidation products act directly or indirectly on ABP and inhibit its fibrillization (381).

ABP oligomerization can also be potentially controlled by the use of naphthalene sulfonates. Sulfonated hydrophobic molecules such as AMNS (1-amino-5-naphthalene sulfonate), 1,8-ANS (1-anilinonaphthalene-8-sulfonate), and bis-ANS (4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonate) were able to stabilize small ABP oligomers (382). Residues 16–20 of the ABP function as a self-recognition element during ABP assembly into fibers. Peptides containing this motif retain the ability to interact with ABP and potentially inhibit its assembly. D-Enantiomers of five peptides (KLVFFA, KKLVFFA, KLVFFA, KIVFFA, and KVVFFA) were active as inhibitors of amyloid fibrillogenesis (383).

10.3.2.3 Amyloid Vaccination/Immunization

Since 1999, when Schenk et al. (316) demonstrated that monthly immunization with injections of ABP42 was able to prevent the development of amyloid plaque formation, neuritic dystrophy, and astrogliosis in mice transfected with the V717F mutant human *APP* gene, more than 100 articles have been published reporting on the benefits and drawbacks of amyloid immunization in animals and humans (384). The pioneering clinical trials with this novel strategy were very promising (385,386), but unfortunately 6% of immunized patients developed autoimmune meningoencephalitis (387), and the trials had to be suspended. Since then, an intense program to improve ABP immunization procedures, avoiding autoimmune reactions, has been developed, and renewed hopes regarding active and passive immunization have emerged during the past few years in the United States, Europe, and Japan (318,388–391).

10.3.3 *Regulators of Tau Phosphorylation*

The hyperphosphorylation of tau appears to be responsible for the loss of its biological function, gain of its toxicity, and aggregation into PHF (392). A series of microtubule-stabilizing agents (393), and antiphosphorylating or dephosphorylating agents might also be of some utility in inhibiting tau phosphorylation and tangle formation (394). Some conventional drugs have proven to be effective in inhibiting herapin-induced assembly of tau into filaments in vitro, including several phenothiazines (methylene blue, azure A, azure B, quinacrine mustard); polyphenols (myricetin, epicatechin 5-gallate, gossypetin, 2,3,4,2',4'-pentahydroxybenzophenone); and the porphyrin ferric dehydroporphyrin IX (395).

Compounds that inhibit tau filament assembly were also found to inhibit the formation of ABP fibrils without effect on the ability of tau to interact with microtubules (395). Several anthraquinones (emodin, daunorubicin, adriamycin) can also inhibit tau aggregation and dissolve PHF (396). Enhancers of protein phosphatase 5 (PP5) might also be useful for preventing tau hyperphosphorylation because tau may be a physiological substrate of PP5, and the abnormal hyperphosphorylation of tau in AD might result in part from the decreased PP5 activity in AD brains (397). Protein-tyrosine phosphatases (PTPs) are important signaling enzymes, encoded in more than 100 human genes, that have emerged as a new class of drug targets. PTPs function to remove the phosphoryl group from tyrosine-phosphorylated proteins. Suramin itself and several suramin derivatives act as powerful inhibitors and activators of PTPs (398).

Among the kinases capable of phosphorylating tau in vitro are both proline-directed kinases and non-proline-directed kinases. Some of these enzymes include glycogen synthase kinase-3 β (GSK3 β), extracellular signal-regulated kinase, stress-activated PK, Cdk5, CDC2-cyclin A kinase, MARK kinase, Ca²⁺/calmodulin-dependent PK, cyclic AMP-dependent PK, PKC, casein kinase I and II, double-stranded DNA-dependent PK, microtubule-associated protein/microtubule affinity-regulating kinase, and tau-tubulin kinase (241,242). GSK3 β , also called tau phosphorylating kinase I, is a serine/threonine kinase that abounds in the brain and is localized primarily in neurons. GSK3 β has been implicated in AD pathogenesis because of its links with PHF and ABP. GSK3 β and tau are parts of an approx. 400- to 500-kDa microtubule-associated tau phosphorylation complex in the brain, where the 14-3-3 ζ dimer, a member of a family of conserved acidic proteins with seven isoforms, simultaneously binds and bridges tau and GSK3 β and stimulates GSK3 β -catalyzed tau phosphorylation (399). GSK3 β may also act downstream of ABP. Exposure of neurons to ABP induces GSK3 β activation and cell death. Blockade of GSK3 β expression by antisense oligonucleotides or its activity by lithium inhibits ABP-induced neurodegeneration. Many groups have been searching for GSK3 β inhibitors (AR-A014418, indirubins) as potential candidate drugs for AD (400,401), and insights into the pathogenic role of the Wnt signaling pathway have generated a growing interest in the development of drugs that inhibit GSK3 with therapeutic potential in AD, diabetes, and stroke (402).

Dickey et al. (403) reported a list of tau-reducing compounds identified from an initial in-cell Western screening assay. Drugs resulting in more than 25% reductions in tau levels with less than 10% reductions in GAPDH include aggregation inhibitors (diaziquone, methylene blue); antibiotics (alexidine HCl); antiproliferatives (colchicine, albendazole, chelidonine, rotenone); and steroids (norethindrone) (403).

10.3.4 Chaperone and Ubiquitin–Proteasome System Modulators

Additional therapeutic targets to regulate aggregation and deposition of ABP and other abnormal proteins in conformational disorders (i.e., Alzheimer's disease, Parkinson disease, prion disease, polyglutamine disease, tauopathies, familial amyotrophic lateral sclerosis) are the molecular chaperones and the ubiquitin–proteasome system regulated by the ubiquitin ligase system and the ubiquitin–C-terminal hydrolase (404). Enhancement of components of the cellular quality control machinery, specifically the levels and activities of molecular chaperones, suppress aggregation and toxicity phenotypes to allow cellular function to be restored (405). Heat shock transcription factor 1 (HSF1), the master stress-inducible regulator, is fundamental in the regulation of molecular chaperones and components of protein homeostasis (405). Several activators, coinducers, and inhibitors of HSF1 have been identified. Activators of HSF1 include protein synthesis inhibitors (puromycin, azetidine); proteasome inhibitors (MG132, lactacystin); serine protease inhibitors (DCIC, TPCK, TLCK); Hsp90 inhibitors (radicicol, geldanamycin, 17-AAG); inflammatory mediators (cyclopentenone prostaglandins, arachidonate, phospholipase A2); and triterpenoids (celastrol). NSAIDS (sodium salicylate, indomethacin) and hydroxylamine derivatives (bimoclomol, arimoclomol) are major HSF1 coinducers, and flavonoids (quercetin) and the benzylidene lactam compound KNK437 act as HSF1 inhibitors (405).

Proteasome inhibition by lactacystin and Bz-LLL-COCHO (benzyl-Leu-Leu-glyoxal) causes a significant increase of ABP and cell death by altering APP processing at the γ -secretase site (406). Resveratrol does not inhibit ABP production because it has no effect on β -, or γ -secretases, but promotes instead intracellular degradation of ABP via a mechanism that involves the proteasome. The resveratrol-induced decrease of ABP can be effectively prevented by several selective proteasome inhibitors and by small interfering RNA-directed silencing on the proteasome subunit $\beta 5$ (407).

The presence of ER stress and impaired ubiquitin–proteasome system activity may activate defective reverberating circuits, leading to conformational changes in proteins that induce cell toxicity (408). Phosphorylated tau isoforms, especially those phosphorylated by GSK3 β and Cdk5, are neurotoxic, and the Hsp70/CHIP chaperone system plays an important role in the regulation of tau turnover and the selective elimination of abnormal tau species (256,409). AD tau binds to Hsc70,

and its phosphorylation is a recognition requirement for the addition of ubiquitin by the E3 ubiquitin ligase CHIP (carboxyl terminus of the Hsc70-interacting protein) and the E2-conjugating enzyme UbcH5B. CHIP can rescue phosphorylated tau-induced cell death, and therefore the CHIP–Hsc70 complex may provide a new therapeutic target for the tauopathies (241). Hyperphosphorylated tau and PHF tau also bind to Hsp27. The formation of this complex alters the conformation of hyperphosphorylated tau and reduces its concentration by facilitating its degradation and dephosphorylation. Similarly to the CHIP–Hsc70 complex, Hsp27 rescues pathological hyperphosphorylated tau-mediated cell death, suggesting that Hsp27 and related Hsps may provide some neuroprotection in AD (241). Genetic and pharmacological chaperone manipulation may arise in the future as a powerful therapeutic approach in neurodegenerative disorders (410).

10.3.5 RNA Interference and Gene Silencing

RNAi has led in recent years to powerful approaches to silence targeted genes in a sequence-specific manner with potential therapeutic applications in neurodegenerative diseases (411–413). MicroRNAs (miRNAs) are small noncoding RNA products 22bp long that provide a regulatory role by binding to target mRNAs and either repress transcription or induce cleavage of the target mRNA. It was initially thought that approx. 0.5–1% of all mammalian genes would be miRNAs. At present, more than 300 human miRNA genes have already been described in the miRNA database, miRBase, and it has been estimated that the total number may be at least 800 (414).

RNAi procedures for gene selective inhibition include (1) cytoplasmic delivery of short sdRNA oligonucleotides (small interfering RNA [siRNA]), which mimics an active intermediate of an endogenous RNAi mechanisms, and (2) nuclear delivery of gene expression cassettes that express a short hairpin RNA (shRNA), which mimics the microinterfering RNA (miRNA) active intermediate of a different endogenous RNAi mechanism. These technologies are complemented by nonviral gene delivery systems and ligand-targeted plasmid-based nanoparticles for RNAi agents (415,416).

RNAi has had an important impact on the development of novel disease models in animals, and it is likely that siRNAs, the trigger molecules for RNA silencing, will become an invaluable tool for the treatment of genetic disorders. The rational design of siRNAs, the introduction of chemical modifications into siRNAs to improve their pharmacokinetic and pharmacodynamic properties for in vivo application with high specificity, and the development of efficient delivery system will foster the therapeutic application of RNAi in AD and other neurodegenerative disorders (413,417).

Several authors have successfully demonstrated the feasibility of targeting AD genes (e.g., *APP*-London mutation, *APP*-Swedish mutation, *PS1*, *APLP1*, *APLP2*, *PEN-2*, *APH-1a*, *Nicastrin*, *BACE*, *MAPT*-V337M) with RNAi (418–423). Some authors have speculated about the possibility that ABP overproduction might be caused by the loss of epigenetic control in the expression of the genes involved in

APP processing. Two of the genes responsible for ABP production (*BACE*, *PS1*) appear to be controlled by the methylation of their promoters via S-adenosylmethionine metabolism (424). If this becomes true, genomic strategies to regulate gene silencing through methylation might be explored to regulate the expression of genes abnormally expressed in AD.

A better understanding of the genes and their enzymatic products involved in ABP deposition and tangle formation and their importance or not in AD pathogenesis will help in defining novel therapeutic targets for AD. However, the potential physiological functions of ABP (synaptic function, memory consolidation, neuronal growth and survival, protection against oxidative stress, surveillance against neuroactive compounds, toxins, and pathogens) and tau should be taken into account to preclude deleterious effects derived from anti-ABP or tau dephosphorylating strategies in AD (20).

In dealing with drug development in AD, it is important that decision makers in the pharmaceutical industry, governmental offices and regulatory agencies, neuroscientists, and clinicians keep in mind that senile plaques and tangles are but the phenotypic expression of a severe problem upstream of amyloid deposition and tau hyperphosphorylation. The final goal in AD therapeutics is not to eliminate the phenotype but to restore to normality the abnormal genomic dysfunction underlying premature neuronal death. Cleaning plaques and tangles from AD brains is not enough to protect neurons because AD pathology and neuronal death starts 20–30yr before the onset of the disease when memory dysfunction appears; when a drug shows some benefit in reducing AD neuropathology, it is fundamental that the candidate compound also be able to restore memory, precluding further neuronal death. Thousands of drugs have successfully passed primary screening, and more than 1000 compounds have entered into primary phases of drug development, but only a few have demonstrated a minimum neuroprotective effect, and none of them reached the category of curative treatment. In the end, what AD therapeutics requires is to target the primary cause of AD at the genomic level (either structural or functional), and then, when several groups of pharmaceutical products are able to demonstrate some potential curative effects, structural and functional genomics features will be the rate-limiting factors responsible for drug efficacy and safety on an individual basis. This is the patrimony of pharmacogenomics.

10.4 Pharmacogenomics

10.4.1 General Concepts

Pharmacogenomics is a novel science that refers to the genomic conditions by which different genes determine the behavior and sensitivity of drugs in relation to a specific organism or genotype. Another definition of pharmacogenomics is the practice of designing drugs according to individual genotypes to enhance

safety or efficacy. From a “postgenomic” perspective, pharmacogenomics would be defined as the determination and analysis of the genome and its products (RNA and proteins) as they relate to drug response. In contrast, *pharmacogenetics* is currently used to define the spectrum of inherited differences in drug metabolism and disposition, the pharmacological responses, and their modification by hereditary influences or the study of variability in drug responses attributed to hereditary factors in different populations (29). In its broadest meaning, pharmacogenetics relates heritable variation to interindividual variation in drug response (15–21). Pharmacogenomics relates to the application of genomic technologies, such as genotyping, gene sequencing, gene expression, genetic epidemiology, transcriptomics, proteomics, metabolomics, and bioinformatics, to drugs in clinical development and on the market, applying the large-scale systematic approaches of genomics to speed the discovery of drug response markers, whether they act at the level of drug target, drug metabolism, or disease pathways (20,28,425,426). The potential implications of pharmacogenomics in clinical trials and molecular therapeutics is that a particular disease could be treated according to genomic and biological markers, selecting medications and diseases that are optimized for individual patients or clusters of patients with a similar genomic profile (28,425). For many medications, interindividual differences are mainly due to SNPs in genes encoding drug-metabolizing enzymes, drug transporters, or drug targets (e.g., genome-related defective enzymes, receptors, and proteins that alter metabolic pathways leading to disease phenotype expression) (28).

The therapeutic lessons obtained from pharmacogenetics in the past, as pointed out by Meyer (427), can be the following: First, all drug effects vary from person to person, and all drug effects are influenced by genes. Next, most drug responses are multifactorial. Third, genetic polymorphisms of single genes, including mutations in coding sequences, gene duplications, gene deletions, and regulatory mutations, affect numerous drug-metabolizing enzymes, including several *CYP* enzymes (*CYP*-related genes), *NATs* (*NAT* genes), *TPMT*, and uridine diphosphate–glucuronosyltransferases (*UDP-GT*); individuals who possess these polymorphisms are at risk of experiencing documented adverse reactions or inefficacy of drugs at usual doses. Fourth, genetic polymorphisms of drug targets and drug transporters are increasingly recognized (receptors, ion channels, growth factors) as causing variation in drug responses. Fifth, several targets respond to treatment only in subgroups of patients who carry sensitizing mutations of these targets. Sixth, the frequency of variation of drug effects, whether multifactorial or genetic, varies considerably in ethnically defined populations. Finally, application of response-predictive genetic profiles to clinical outcomes has so far been done mostly in academic centers and has not yet reached clinical practice (427).

To gain expertise in the pharmacogenomics of a particular disease, it is necessary to understand the principles of three basic steps: (1) the genetics of the disorder to be studied in all its modalities (mendelian genetics, susceptibility genetics, mitochondrial genetics, epigenetic phenomena, genome–environment interactions); (2) structural and functional genomics; and (3) pharmacogenomic development in its two main facets, pharmacogenetics (associated with drug safety) and pharmacogenomics (associated

with drug efficacy). The development of new compounds or retesting of old drugs using pharmacogenetic strategies encompasses the following steps in a multidisciplinary fashion: (1) genetic screening (genotyping) of single genes to identify major gene targets; (2) analysis of genetic variation to differentiate populations; (3) structural and functional genomic analyses, including genetic clusters and haplotypes; (4) analysis of genotype–phenotype correlations to characterize major phenotypes as therapeutic targets associated with a particular gene or a cluster of genes involved in a metabolic pathway; and (5) implementation of basic and clinical pharmacogenomics procedures for drug development (20) (see Figs. 10.1, 10.2, and 10.9).

Basic pharmacogenomics strategies include the identification of drugs and cell targets together with the characterization of specific pharmacological responses in *in vitro* models, *in vivo* models, or transgenic animals incorporating high-throughput screening methods, cell biochips, and DNA microarrays in drug development systems that should be validated (13–21,428). Once a drug fulfills pharmacogenomic criteria of efficacy and safety at the preclinical level, then the target drug enters clinical evaluation. In drug clinical trials with a pharmacogenomics approach, despite conventional pharmacokinetic and pharmacodynamic analyses, the candidate drug should be effective in modifying genotype- and phenotype-related responses of therapeutic value (13–21,290,291,428).

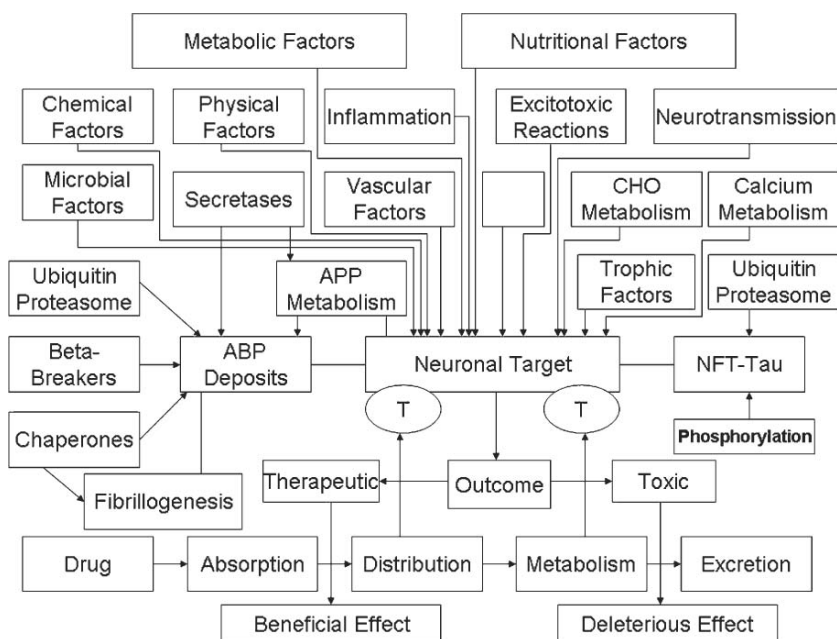


Fig. 10.9 Pathogenic factors acting on neuronal targets in Alzheimer's disease and the process of pharmacological treatment. ABP, amyloid β -protein; APP, ABP precursor protein; CHO, cholesterol; NFT-Tau, neurofibrillary tangle tau

The heterogeneity of AD and how apparently identical phenotypes assessed with international clinical criteria (NINCDS-ADRDA, DSM-IV, ICD-10) do not always respond to the same drugs has been well known (19,20). This response may be caused by different factors, including pharmacokinetic and pharmacodynamic properties of drugs, nutrition, liver function, concomitant medications, and individual genetic factors. In fact, the therapeutic response of AD patients to conventional ChEIs is partially effective in only 10–20% of cases, with side effects, intolerance, and noncompliance in more than 60% of patients because of different reasons (e.g., efficacy, safety) (6,7,20,298,303). Therefore, the individualization of therapy or pharmacological tailoring in AD and other CNS disorders is just a step forward to the long-standing goal of molecular pharmacology (28,31,429–431) taking advantage of the information and procedures provided by the sequencing of the entire human genome (10).

10.4.2 Pharmacogenetics of Drug Metabolism

Although drug effect is a complex phenotype that depends on many factors, it is estimated that genetics accounts for 20–95% of variability in drug disposition and pharmacodynamics (30). ChEIs of current use in AD, such as donepezil and galantamine (and tacrine) are metabolized via *CYP*-related enzymes (see Table 10.3). These drugs can interact with many other drugs that are substrates, inhibitors, or inducers of the *CYP* system, with this interaction eliciting liver toxicity and other adverse drug reactions (ADRs) (18–20) (see Table 10.4).

AD patients are currently treated with ChEIs, neuroprotective drugs, antidepressants, anxiolytics, antiparkinsonian drugs, anticonvulsants, and neuroleptics at a given time of the disease clinical course to palliate memory dysfunction, behavioral changes, sleep disorders, agitation, depression, parkinsonism, myoclonus and seizures, or psychotic symptoms (6,432). Many of these substances are metabolized by enzymes known to be genetically variable, including (1) esterases (butyrylcholinesterase [BuChE], paraoxonase/arylesterase); (2) transferases (*N*-acetyltransferase, sulfotransferase [ST], thiol methyltransferase, thiopurine methyltransferase, catechol-*O*-methyltransferase (COMT), glutathione-*S*-transferases [GSTs], UDP-GTs, glucosyltransferase, histamine methyltransferase [HMT]); (3) reductases (NADPH [nicotinamide adenine dinucleotide phosphate]:quinine oxidoreductase, glucose-6-phosphate dehydrogenase); (4) oxidases (ADH, aldehydehydrogenase, monoamine oxidase B, catalase, SOD, trimethylamine *N*-oxidase, dihydropyrimidine dehydrogenase [DPD]); and (5) *CYP* enzymes, such as *CYP1A1*, *CYP2A6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP2E1*, *CYP3A5* (see Table 10.3), and many others (20). Polymorphic variants in these genes can induce alterations in drug metabolism that modify the efficacy and safety of the prescribed drugs (433).

Drug metabolism includes phase I reactions (i.e., oxidation, reduction, hydrolysis) and phase II conjugation reactions (i.e., acetylation, glucuronidation, sulfation, methylation) (31). The principal enzymes with polymorphic variants involved in phase I reactions are the following: *CYP3A4/5/7*, *CYP2E1*, *CYP2D6*, *CYP2C19*,

Table 10.3 Cytochrome P450 (*CYP*) genes encoding *CYP*-related enzymes involved in human pharmacogenetic activities

Gene	Locus	Name	Alternate names	Related drugs	Related diseases	OMIM phenotype	Alternate symbols
<i>CYP1A2</i>	15q22-qtter	Cytochrome P450, P450 form 4; aryl hydrocarbon subfamily (aromatic compound-inducible), polypeptide 2	hydroxylase; cytochrome P450, subfamily 1 (aromatic compound-inducible), polypeptide 2; dioxin-inducible P3-450; flavoprotein-linked monooxygenase; microsomal monooxygenase; xenobiotic monooxygenase	Amiodarone, caffeine, citalopram, clozapine, cyclobenzaprine, dex-amethasone, echinacea, estradiol, etoposide, fluvoxamine, haloperidol, imipramine, interferon- α , lidocaine, mifepradil, midazolam, modafinil, naproxen, ondansetron, propranolol, ribavirin, riluzole, ropivacaine, tacrine, teniposide, theophylline, thiotepa, ticlopidine, verapamil, zolmitriptan, zoxazolamine	Chronic hepatitis C, schizophrenia, psychosis		CP12; P3-450; P450(PA)
<i>CYP1B1</i>	2p21	Cytochrome P450, Aryl hydrocarbon hydroxylase; cytochrome P450, subfamily 1 (dioxin-inducible), polypeptide 1 (glaucoma 3, primary infantile); flavoprotein-linked monooxygenase; microsomal monooxygenase; xenobiotic monooxygenase		Estrogens	Breast neoplasms	Primary congenital glaucoma 3A; early-onset digenic glaucoma; Peters anomaly	CPIB; GLC3A
<i>CYP2A6</i>	19q13.2	Cytochrome P450, family 2, subfamily A, polypeptide 6	Coumarin 7-hydroxylase; cytochrome P450, subfamily IIA (phenobarbital-inducible), polypeptide 3; cytochrome P450, subfamily IIA (phenobarbital-inducible), polypeptide 6; flavoprotein-linked monooxygenase; xenobiotic monooxygenase	5-Fluorouracil, dexamethasone, etoposide, fadrozole, fluorouracil, midazolam, nicotine, rifampin, teniposide	Neoplasms	Coumarin resistance, protection from nicotine addiction	CPA6; CYP2A3

(continued)

Table 10.3 (continued)

Gene	Locus	Name	Alternate names	Related drugs	Related diseases	OMIM phenotype	Alternate symbols
<i>CYP2B6</i>	19q13.2	Cytochrome P450, family 2, subfamily B, polypeptide 6	Cytochrome P450, subfamily IIB (phenobarbital-inducible), polypeptide 6	Aflatoxin B1, bupropion, cyclophosphamide, dexamethasone, etoposide, ifosfamide, midazolam, phenobarbital, propofol, rifampin, teniposide, thiotepa, vitamin D, xenobiotics	Nicotine addiction		CPB6; <i>CYP</i> IIB6; P450
<i>CYP2C19</i>	10q24.1–q24.3	Cytochrome P450, family 2, subfamily C, polypeptide 19	Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 19; flavo-protein-linked monooxygenase; mephenytoin 4-hydroxylase; microsomal monooxygenase; xenobiotic monooxygenase	Amitriptyline, carisoprodol, citalopram, cyclophosphamide, diazepam, fluoxetine, fluvoxamine, glucocorticoids, hexobarbital, lansoprazole, mephenytoin, modafinil, nelfinavir, nilutamide, omeprazole, pantoprazole, proguanil, rifampin, thiotepa, ticlopidine	Lupus nephritis, gastroesophageal reflux disease, peptic ulcer disease, visual disorders	Mephenytoin poor metabolizer	CPCL; <i>CYP</i> 2C; P450C2C; P450IIC19
<i>CYP2C9</i>	10q24	Cytochrome P450, family 2, subfamily C, polypeptide 9	Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 10; cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 9; flavoprotein-linked monooxygenase; mephenytoin 4-hydroxylase; microsomal monooxygenase; xenobiotic monooxygenase	Acenocoumarol, amiodarone, celecoxib, coumadin, dexamethasone, diclofenac, etoposide, fluconazole, fluoxetine, fluvastatin, fluvoxamine, glimepiride, glipizide, glyburide, ibuprofen, irbesartan, isoniazid, losartan, midazolam, phenylbutazone, phenytoin, rifampin, teniposide, tenoxicam, thiotepa, tolbutamide, toremsemide, vitamin D, warfarin	Arthritis, blood coagulation disorders, diabetes mellitus, epilepsy, hypertension, thrombolytic disease	Tolbutamide poor metabolizer, warfarin sensitivity	CPC9; <i>CYP</i> 2C10; P450 MP-4; P450 PB-1; P450IIC9

Table 10.3 (continued)

Gene	Locus	Name	Alternate names	Related drugs	Related diseases	OMIM phenotype	Alternate symbols
<i>CYP2D6</i>	22q13.1	Cytochrome P450, family 2, subfamily D, polypeptide 6	Cytochrome P450, subfamily IID (debrisoquine, sparteine), polypeptide 6; cytochrome P450, subfamily IID (debrisoquine, sparteine)-like I; debrisoquine 4-hydroxylase; flavoprotein-linked monooxygenase; microsomal monooxygenase; xenobiotic monooxygenase	Amitriptyline, caffeine, cimetidine, citalopram, clomipramine, clozapine, cocaine, codeine, debrisoquine, desipramine, dextromethorphan, diltiazem, flecainide, fluoxetine, fluvoxamine, haloperidol, imipramine, interferon- α , metoprolol, mexiletine, morphine, paroxetine, perhexiline, perphenazine, propafenone, propranolol, ribavirin, risperidone, ritonavir, sparteine, tamoxifen, thioridazine, thiotepa, timolol, tramadol, venlafaxine, xenobiotics, yohimbine, zuclopenthixol	Breast neoplasms, cystic fibrosis, depression, chronic hepatitis C, lung neoplasms, neoplasms, codeine dependence pain, schizophrenia, codeine dependence, psychosis	Susceptibility to parkinsonism, debrisoquine sensitivity	CPD6; <i>CYP2D</i> ; <i>CYP2D6</i> ; <i>CYP2DL1</i> ; P450-DB1; P450C2D
<i>CYP2E1</i>	10q24.3-qter	Cytochrome P450, subfamily IIE (ethanol-inducible)	Cytochrome P450, subfamily IIE (ethanol-inducible); cytochrome P450, subfamily IIE (ethanol-inducible), polypeptide I; flavoprotein-linked monooxygenase; microsomal monooxygenase; xenobiotic monooxygenase	Dexamethasone, ethanol, etoposide, midazolam, nicotine, teniposide, thiotepa, xenobiotics	Alcoholic liver disease, lung neoplasms, nicotine dependency		CPE1; <i>CYP2E</i> ; <i>CYP2E1</i> ; P450-J; P450C2E
<i>CYP3A</i>	7q21.3-q22.1	Cytochrome P450, family 3, subfamily A	Cytochrome P450, subfamily IIIA (nifedipine oxidase)	Dexamethasone, docetaxel, erythromycin, midazolam, rifampin, tamoxifen, thiotepa, xenobiotics	Arrhythmia, lung neoplasms		<i>CYP3</i>

(continued)

Table 10.3 (continued)

Gene	Locus	Name	Alternate names	Related drugs	Related diseases	OMIM phenotype	Alternate symbols
<i>CYP3A4</i>	7q21.1	Cytochrome P450, family 3, subfamily A, polypeptide 4	P450-III, steroid inducible; cytochrome P450, subfamily IIIA (nifedipine oxidase), polypeptide 3; cytochrome P450, subfamily IIIA (nifedipine oxidase), polypeptide 4; glucocorticoid-inducible P450; nifedipine oxidase	Alprazolam, anthracycline, cisapride, citalopram, dexamethasone, docetaxel, epipodophyllotoxin, etoposide, glucocorticoids, interferon- α , irinotecan, losartan, midazolam, nifedipine, omeprazole, ribavirin, rifampin, tamoxifen, teniposide, testosterone, topotecan, vitamin D, xenobiotics	Breast neoplasms, chronic hepatitis C, leukemia, L1 acute lymphocytic leukemia, myeloid leukemia, neoplasms, prostatic neoplasms, <i>Helicobacter pylori</i> gastric ulcers		CP33; CP34; <i>CYP3A</i> ; <i>CYP3A3</i> ; <i>CYP3A4</i> ; HLP; NF-25; P450C3; P450PCN1
<i>CYP3A5</i>	7q21.1	Cytochrome P450, family 3, subfamily A, polypeptide 5	Aryl hydrocarbon hydrolase; cytochrome P450, subfamily IIIA (nifedipine oxidase), polypeptide 5; flavoprotein-linked monooxygenase; microsomal monooxygenase; nifedipine oxidase; xenobiotic monooxygenase	Aflatoxin B1, anthracycline, cisapride, cyclosporine, dexamethasone, etoposide, glucocorticoids, irinotecan, midazolam, simvastatin, tacrolimus, teniposide, vitamin C, warfarin, xenobiotics	Blood coagulation disorders, L1 acute lymphocytic leukemia, myeloid leukemia		<i>CYP35</i> ; <i>CYP3A5</i> ; P450PCN3; PCN3
<i>CYP3A7</i>	7q21-q22.1	Cytochrome P450, family 3, subfamily A, polypeptide 7	Aryl hydrocarbon hydrolase; cytochrome P450, subfamily IIIA, polypeptide 7; flavoprotein-linked monooxygenase; microsomal monooxygenase; xenobiotic monooxygenase	Cisapride, midazolam, vitamin D, xenobiotics			CP37; P450-HFLA

Table 10.3 (continued)

Gene	Locus	Name	Alternate names	Related drugs	Related diseases	OMIM phenotype	Alternate symbols
<i>CYP4B1</i>	1p34-p12	Cytochrome P450, subfamily IVB, polypeptide 1	Cytochrome P450, subfamily IVB, member 1; cytochrome P450, subfamily IVB, polypeptide 1; microsomal monooxygenase	Xenobiotics			P-450HP
<i>CYP11B2</i>	8q21-q22	Cytochrome P450, family 11, subfamily B, polypeptide 2	Steroid 11- β /18-hydroxylase; cytochrome P450, subfamily XIB (steroid 11- β -hydroxylase), polypeptide 2; steroid 11- β -monooxygenase; steroid 11- β /18-hydroxylase	Candesartan		Aldosterone-to-rennin ratio raised, congenital hypoaldosteronism due to CMO I deficit, congenital hypoaldosteronism due to CMO II deficit, low rennin hypertension	ALDOS; CPN2; <i>CYP11B</i> ; <i>CYP11BL</i> ; P-450C-18; P450aldo

Source: Adapted from ref. 20.

Table 10.4 Pharmacological properties of selected acetylcholinesterase (AChE) inhibitors for the treatment of Alzheimer's disease

Properties	Tacrine	Donepezil	Rivastigmine	Galantamine
Class	Aminoacridine	Piperidine	Carbamate	Tertiary alkaloid
AChE inhibition	Reversible Non-competitive	Reversible Non-competitive	Pseudoirreversible Noncompetitive	Reversible Competitive
Dosis (mg/d)	80–160	5–10	6–12	16–24
Duration	Short acting	Short acting	Intermediate acting	Short acting
Brain AChE selectivity IC ₅₀ (nmol/L)	125	33	42,000	3,900
Serum BuChE selectivity IC ₅₀ (nmol/L)	7.2	988	54,000	18,600
BuChE/AChE selectivity	0.06	30	1.3	4.8
C _{max} (µg/L)	5.1 (10 mg) 20.7 (20 mg) 33.9 (30 mg)	7.2 (5 mg) 25.6 (10 mg)	5.07 (2 mg × 2) 14.1 (6 mg × 2)	42 (12 mg × 2) 137 (16 mg × 2)
T _{max} (h)	1–2	3–5	0.5–2	0.9–2
AUC (µg/L/h)	2–4	539	15.4 (3 mg × 2) 55.9 (6 mg × 2)	1.1
T _{1/2} (h)	1.3	50–80	0.6–2	7–8
Bioavailability (%)	17–37	100	35–40	100
Protein binding (%)	55	96	40	18
Clearance (L/h/kg)	2.42	0.13	1.5 (6 mg bid)	0.34
V _d (L/kg)	3.5–7	14	1.8–2.7	2.64
Cytochrome P450 Metabolism	<i>CYP1A2</i> <i>CYP2D6</i>	<i>CYP2D6</i> <i>CYP3A4</i>	Carbomoylation	<i>CYP2D6</i> <i>CYP3A4</i>
Active metabolites	1-Hydroxy-tacrine	6- <i>O</i> -Desmethyl Donepezil	NAP 226-90	Sanguinine
Urine excretion (%)	<3	17	Metabolite	50
Efficacy ADAS-Cog > placebo	4.8	2.9	3.78	2.14
Adverse effects				
Nausea	3+	3+	3+	2+
Vomiting	2+	2+	2+	2+
Diarrhea	2+	2+	2+	1+
Dizziness	2+	1+	2+	1+
Headache	1+	0	1+	0
Abdominal pain	1+	0	1+	0
Anorexia	2+	1+	1+	0
Bradycardia	0	0	0	0
Fatigue	0	1+	1+	1+
Muscle cramps	0	1+	0	0
Agitation	2+	0	0	0
Dyscrasia	0	0	0	0
Liver dysfunction	3+			

AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; *CYP*, cytochrome P450.

Source: (Adapted from refs. ADAS-Cog: Alzheimer's Disease Assessment Scale-Cognition **19** and **20**).

CYP2C9, *CYP2C8*, *CYP2B6*, *CYP2A6*, *CYP1B1*, *CYP1A1/2*, epoxide hydrolase, esterases, NQO1 (NADPH-quinone oxidoreductase), DPD, ADH, and ALDH (aldehyde dehydrogenase). Major enzymes involved in phase II reactions include the following: UGTs (uridine 5'-triphosphate glucuronosyl transferases), *TPMT*, COMT, HMT, STs, GST-A, GST-P, GST-T, GST-M, *NAT2*, *NAT1*, and others (434). Polymorphisms in genes associated with phase II metabolism enzymes, such as *GSTM1*, *GSTT1*, *NAT2*, and *TPMT* are well understood, and information is also emerging on other GST polymorphisms and on polymorphisms in the UDP-GTs and STs.

10.4.2.1 The CYP Gene Family

The typical paradigm for the pharmacogenetics of phase I drug metabolism is represented by the *CYP* enzymes, a superfamily of microsomal drug-metabolizing enzymes. The P450 enzymes comprise a superfamily of heme-thiolate proteins widely distributed in bacteria, fungi, plants, and animals. The P450 enzymes are encoded in genes of the *CYP* superfamily (see Table 10.3) and act as terminal oxidases in multicomponent electron transfer chains that are called P450-containing monooxygenase systems. Some of the enzymatic products of the *CYP* gene superfamily can share substrates, inhibitors, and inducers, whereas others are quite specific for their substrates and interacting drugs (19,20,26–31).

There are more than 200 *P450* genes identified in different species. Saito et al. (435) provided a catalog of 680 variants among eight *CYP450* genes, nine esterase genes, and two other genes in the Japanese population. Mammals have at least 15 P450 families (*CYP1*, 2, 3, 4, 5, 7, 8, 11, 17, 19, 21, 24, 26, 27, 51) and 29 subfamilies. Humans have 36 sequenced *CYP* genes and 10 pseudogenes: *CYP1A1*, *1A2*, *1B1*, *2A6*, *2A7*, *2A7PT* (telomeric), *2A7PC* (centromeric), *2A13*, *2B6*, *2B7P*, *2C8*, *2C9*, *2C18*, *2C19*, *2D6*, *2D7P*, *2D7AP*, *SD8P*, *SD8BP*, *2E1*, *2F1*, *2F1P*, *2J2*, *3A4*, *3A5*, *3A5P*, *3A7*, *4A11*, *4B1*, *4F2*, *4F3*, *5A1*, *7A1*, *8A1*, *11A1*, *11B1*, *11B2*, *17*, *19*, *21A1P*, *21A2*, *24*, *26*, *27A1*, *27B1*, and *51*; some have adopted a new nomenclature (see Table 10.3). Rats have 60 sequenced *CYP* genes and 4 pseudogenes. Mice have 45 sequenced *CYP* genes and 1 pseudogene. Rabbits have 32 *CYP* genes and no pseudogenes. Many other *CYP* genes have been sequenced in other mammalian species, including 15 *CYP* genes in hamsters, 14 in pigs, 11 in guinea pigs, 10 in bovines, 8 in monkeys, 8 in dogs, 6 in sheep, 3 in goats, 2 in horses, and 3 in baboons. Insect sequences show at least 17 *CYP* genes in the mosquito *Anopheles albimanus*, 17 *CYP* genes in *Drosophila melanogaster*, and 16 *CYP* genes in *Musca domestica* (the housefly). In the nematode worm *C. elegans* a gene cluster of 8 *CYP* genes and 1 pseudogene have been identified, with about 80 genes known in *C. elegans*. Yeast has only 3 *CYP* genes (*CYP51*, *CYP56*, *CYP61*); *Candida tropicalis* has 8 *CYP* genes; *Arabidopsis* has at least 51 *CYP* genes; *Zea mays* (maize) has 17 *CYP* genes; eggplant has 8 *CYP* genes in 4 different families; and about 30 families have been designated in plants. Among the

bacterial sequences, *Streptomyces* has 8 *CYP* genes, and *Mycobacterium tuberculosis* has 11 *CYP* genes (436,437).

The microsomal, membrane-associated, P450 isoforms *CYP3A4*, *CYP2D6*, *CYP2C9*, *CYP2C19*, *CYP2E1*, and *CYP1A2* are responsible for the oxidative metabolism of more than 90% of marketed drugs, and *CYP3A4* metabolizes more drug molecules than all other isoforms together. Most of these polymorphisms exhibit geographic and ethnic differences (438–444). These differences influence drug metabolism in different ethnic groups, for whom drug dosage should be adjusted according to their enzymatic capacity, differentiating normal or extensive metabolizers (EMs), poor metabolizers (PMs), and ultra-rapid metabolizers (UMs). Most drugs act as substrates, inhibitors, or inducers of *CYP* enzymes. Enzyme induction enables some xenobiotics to accelerate their own biotransformation (autoinduction) or the biotransformation and elimination of other drugs (19,20). A number of P450 enzymes in human liver are inducible. Induction of the majority of P450 enzymes occurs by an increase in the rate of gene transcription and involves ligand-activated TFs, aryl hydrocarbon receptor, constitutive androstane receptor (CAR), and pregnane X receptor (PXR) (443,445). In general, binding of the appropriate ligand to the receptor initiates the induction process that cascades through a dimerization of the receptors, their translocation to the nucleus, and binding to specific regions in the promoters of *CYPs* (445). *CYPs* are also expressed in the CNS, and a complete characterization of constitutive and induced *CYPs* in the brain is essential for understanding the role of these enzymes in neurobiological functions and in age-related and xenobiotic-induced neurotoxicity (446).

In the PharmGKB database (447), there are 15,195 genes, of which 292 genes contain PharmGKB primary data, 234 genes contain genotype data, and 1619 genes contain literature annotations related to potential pharmacogenetics activities. The number of drugs potentially associated with genes involved in their metabolism account for 4674 chemical names (437 drugs with PharmaGKB primary data and 350 diseases with supporting information). Assuming that the human genome contains about 20,000–30,000 genes, at the present time only 0.31% of commercial drugs have been assigned to corresponding genes with gene products that might be involved in pharmacokinetic and pharmacodynamic activities of a given drug, and only 4% of the human genes have been assigned to a particular drug metabolic pathway. Supposing a theoretical number of 100,000 chemicals in current use worldwide and assuming that practically all human genes can interact with drugs taken by human beings, each gene in the human genome should be involved in the metabolism or biopharmacological effect of 30–40 drugs; however, assuming that most xenobiotic substances in contact with our organism can influence genomic function, it might be possible that, for 1 million xenobiotics in daily contact with humans, an average of 350–500 xenobiotics have to be assigned to each one of the genes potentially involved in drug metabolism or xenobiotic processing. To fulfill this task, a single gene has to possess the capacity of metabolizing many different xenobiotic substances; at the same time, many different genes have to cooperate in orchestrated networks to metabolize a particular drug or xenobiotic under sequential

biotransformation steps (see **Fig. 10.9**). Numerous chemicals increase the metabolic capability of organisms by their ability to activate genes encoding various xenobiotic-metabolizing enzymes, such as *CYPs*, transferases, and transporters.

Many natural and artificial substances induce the hepatic *CYP* subfamilies in humans, and these inductions might lead to clinically important drug–drug interactions. Some of the key cellular receptors that mediate such inductions have been identified, including nuclear receptors such as CAR (NR1I3), the retinoid X receptor (RXR, NR2B1), PXR (NR1I3), and the vitamin D receptor (VDR, NR1I1) and steroid receptors such as the glucocorticoid receptor (GR, NR3C1) (**448**). There is a wide promiscuity of these receptors in the induction of *CYPs* in response to xenobiotics. Indeed, this adaptive system acts as an effective network in which receptors share partners, ligands, DNA response elements, and target genes, influencing their mutual relative expression (**448,449**).

Ethnic Differences

The most important enzymes of the P450 cytochrome family in drug metabolism by decreasing order are *CYP3A4*, *CYP2D6*, *CYP2C9*, *CYP2C19*, and *CYP2A6* (**433–436,444**). The predominant allelic variants in the *CYP2A6* gene are *CYP2A6**2 (Leu160His) and *CYP2A6del*. The *CYP2A6**2 mutation inactivates the enzyme and is present in 1–3% of Caucasians. The *CYP2A6del* mutation results in no enzyme activity and is present in 1% of Caucasians and 15% of Asians (**19,20,434**). The most frequent mutations in the *CYP2C9* gene are *CYP2C9**2 (Arg144Cys), with reduced affinity for P450 in 8–13% of Caucasians, and *CYP2C9**3 (Ile359Leu), with alterations in the specificity for the substrate in 6–9% of Caucasians and 2–3% of Asians (**19,20,434**). The most prevalent polymorphic variants in the *CYP2C19* gene are *CYP2C19**2, with an aberrant splicing site resulting in enzyme inactivation in 13% of Caucasians, 23–32% of Asians, 13% of Africans, and 14–15% of Ethiopians and Saudis, and *CYP2C19**3, a premature stop codon resulting in an inactive enzyme present in 6–10% of Asians and almost absent in Caucasians (**19,20,434,450**). The most important mutations in the *CYP2D6* gene are the following: *CYP2D6**2xN, *CYP2D6**4, *CYP2D6**5, *CYP2D6**10, and *CYP2D6**17 (**18–20,444,451**). The *CYP2D6**2xN mutation gives rise to a gene duplication or multiplication resulting in increased enzyme activity that appears in 1–5% of the Caucasian population, 0–2% of Asians, 2% of Africans, and 10–16% of Ethiopians. The defective splicing caused by the *CYP2D6**4 mutation inactivates the enzyme and is present in 12–21% of Caucasians. The deletion in *CYP2D6**5 abolishes enzyme activity and shows a frequency of 2–7% in Caucasians, 1% in Asians, 2% in Africans, and 1–3% in Ethiopians. The polymorphism *CYP2D6**10 causes Pro34Ser and Ser486Thr mutations with unstable enzyme activity in 1–2% of Caucasians, 6% of Asians, 4% of Africans, and 1–3% of Ethiopians. The *CYP2D6**17 variant causes Thr107Ile and Arg296Cys substitutions that produce a reduced affinity for substrates in 51% of Asians, 6% of Africans, and 3–9% of Ethiopians and is practically absent in Caucasians (**18–20,434,444,451**).

10.4.3 *CYP2D6 Genotypes in Alzheimer's Disease*

The *CYP2D6* enzyme, encoded by a gene that maps on 22q13.1–13.2, catalyses the oxidative metabolism of more than 100 clinically important and commonly prescribed drugs, such as ChEIs (tacrine, donepezil, galantamine); antidepressants; neuroleptics; opioids; some β -blockers; class I antiarrhythmics; analgesics; and many other drug categories, acting as substrates, inhibitors, or inducers with which ChEIs may potentially interact, this leading to the outcome of ADRs (18–20,444,452). The *CYP2D6* locus is highly polymorphic, with more than 100 different *CYP2D6* alleles identified in the general population showing deficient (PMs), normal (EMs) or increased enzymatic activity (UMs) (437,453). Most individuals (>80%) are EMs; however, remarkable interethnic differences exist in the frequency of the PM and UM phenotypes among different societies all over the world (18–20,439,441–444,451). On the average, approx. 6.28% of the world population belongs to the PM category. Europeans (7.86%), Polynesians (7.27%), and Africans (6.73%) exhibit the highest rate of PMs, whereas Orientals (0.94%) show the lowest rate. The frequency of PMs among Middle Eastern populations, Asians, and Americans is in the range of 2–3% (18–20,444). *CYP2D6* gene duplications are relatively infrequent among Northern Europeans, but in East Africa the frequency of alleles with duplication of *CYP2D6* is as high as 29% (29).

The most frequent *CYP2D6* alleles in the European population are the following: *CYP2D6**1 (wild type) (normal), *CYP2D6**2 (2850C>T) (normal), *CYP2D6**3 (2549A>del) (inactive), *CYP2D6**4 (1846G>A) (inactive), *CYP2D6**5 (gene deletion) (inactive), *CYP2D6**6 (1707T>del) (inactive), *CYP2D6**7 (2935A>C) (inactive), *CYP2D6**8 (1758G>T) (inactive), *CYP2D6**9 (2613–2615 delAGA) (partially active), *CYP2D6**10 (100C>T) (partially active), *CYP2D6**11 (883G>C) (inactive), *CYP2D6**12 (124G>A) (inactive), *CYP2D6**17 (1023C>T) (partially active), and *CYP2D6* gene duplications (with increased or decreased enzymatic activity depending on the alleles involved) (18–20,453–455).

In the Spanish population, for whom the mixture of ancestral cultures has occurred for centuries, the distribution of the *CYP2D6* genotypes differentiates four major categories of *CYP2D6*-related metabolizer types: (1) EMs (*1/*1, *1/*10); (2) IMs (*1/*3, *1/*4, *1/*5, *1/*6, *1/*7, *10/*10, *4/*10, *6/*10, *7/*10); (3) PMs (*4/*4, *5/*5); and (4) UMs (*1xN/*1, *1xN/*4, Dupl). In this sample, we have found 51.61% EMs, 32.26% IMs, 9.03% PMs, and 7.10% UMs (42) (see Fig. 10.10). The distribution of all major genotypes is the following: *1/*1, 47.10%; *1/*10, 4.52%; *1/*3, 1.95%; *1/*4, 17.42%; *1/*5, 3.87%; *1/*6, 2.58%; *1/*7, 0.65%; *10/*10, 1.30%; *4/*10, 3.23%; *6/*10, 0.65%; *7/*10, 0.65%; *4/*4, 8.37%; *5/*5, 0.65%; *1xN/*1, 4.52%; *1xN/*4, 1.95%; and Dupl, 0.65% (42) (see Fig. 10.10). These results are similar to others previously reported by Sachse et al. (451), Bernal et al. (455), Cacabelos (18–20), Bernard et al. (456), and others in the Caucasian population (440–444,457–459).

When comparing AD cases with controls, we observed that EMs are more prevalent in AD (*1/*1, 49.42%; *1/*10, 8.04%) (total AD-EMs 57.47%) than in controls (C) (*1/*1, 44.12%; *1/*10, 0%) (total C-EMs 44.12%). In contrast, IMs are more

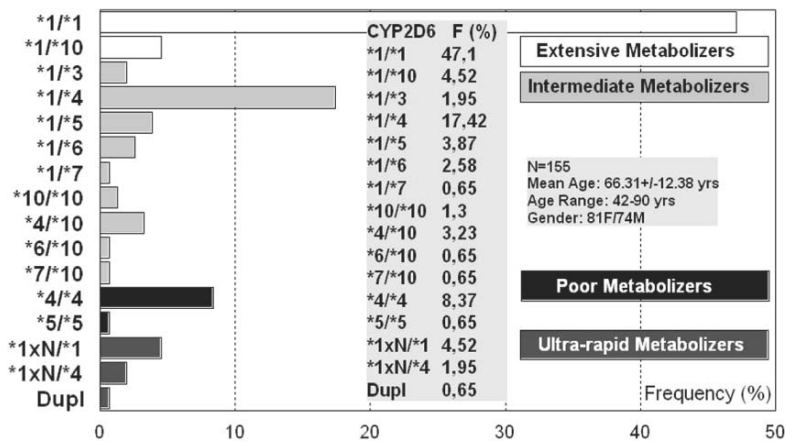


Fig. 10.10 Frequencies of major cytochrome P450 (CYP) 2D5 genotypes in the Spanish population. (Adapted from ref. 42.)

frequent in controls (41.18%) than in AD (25.29%), especially the **1/*4* (C 23.53%; AD 12.64%) and **4/*10* genotypes (C 5.88%; AD 1.15%). The frequency of PMs was similar in AD (9.20%) and controls (8.82%), and UMs were more frequent among AD cases (8.04%) than in controls (5.88%) (42).

Although initial studies postulated the involvement of the *CYP2D6B* mutant allele in Lewy body formation in both Parkinson’s disease and the Lewy body variant of AD, as well as in the synaptic pathology of pure AD without Lewy bodies (460), subsequent studies in different ethnic groups did not find association between AD and *CYP2D6* variants (459,461–466). Notwithstanding, the genetic variation between AD and controls associated with *CYP2D6* genotypes is 13.35% in EMs, 15.89% in IMs, 0.38% in PMs, and 2.16% in UMs, with an AGV of 31.78% between both groups, suggesting that this genetic difference might influence AD pathogenesis and therapeutics (42).

10.4.3.1 Association of *CYP2D6* Variants with Alzheimer’s Disease-Related Genes

We have also investigated the association of *CYP2D6* genotypes with AD-related genes, such as *APP*, *MAPT*, *APOE*, *PS1*, *PS2*, *A2M*, *ACE*, *AGT*, *FOS*, and *PRNP* variants (42) (see Table 10.5). No *APP* or *MAPT* mutations have been found in AD cases. Homozygous *APOE*-2/2 (12.56%) and *APOE*-4/4 (12.50%) accumulate in UMs, and *APOE*-4/4 cases were also more frequent in PMs (6.66%) than in EMs (3.95%) or IMs (0%). *PS1*-1/1 genotypes were more frequent in EMs (45%),

Table 10.5 Distribution of Alzheimer's disease (AD)-related genotypes associated with different cytochrome P450 2D6 (*CYP2D6*) metabolizer types in Alzheimer's disease

Gene	Polymorphic variant	Extensive metabolizers (%)	Intermediate metabolizers (%)	Poor metabolizers (%)	Ultrarapid metabolizers (%)
<i>APOE</i>	2/2	0.00	0.00	0.00	12.56
	2/3	5.26	8.51	20.00	0.00
	2/4	0.00	0.00	0.00	0.00
	3/3	61.84	63.83	46.67	50.00
	3/4	28.95	27.66	26.67	25.00
	4/4	3.95	0.00	6.66	12.50
<i>PS1</i>	1/1	45	23.68	15.39	20.00
	1/2	46.67	63.16	46.15	60.00
	2/2	8.33	13.16	38.46	20.00
PS2	E5(-)	66.67	79.49	66.67	33.33
	E5(+)	33.33	20.51	33.33	66.67
A2Mins/del	II	65.72	70.00	87.50	0.00
	ID	34.28	23.33	12.50	100.00
	DD	0.00	6.67	0.00	0.00
A2Mpol	AA	44.45	32.26	37.50	100.00
A2M-V100I	AG	50.00	51.62	62.50	0.00
	GG	5.55	16.12	0.00	0.00
<i>ACE</i>	II	23.53	3.57	16.67	0.00
	ID	29.41	50.00	50.00	0.00
	DD	47.06	46.43	33.33	100.0
AGT-M235T	MM	0.00	12.50	20.00	16.67
	MT	84.21	41.67	80.00	50.00
	TT	15.79	45.83	0.00	33.33
AGT-T174M	MM	0.00	0.00	0.00	25.00
	TM	15.79	20.00	0.00	25.00
	TT	84.21	80.00	100.00	50.00
cFOS	B/B	2.18	0.00	0.00	0.00
	A/B	23.91	33.33	28.57	25.00
	A/A	73.91	66.67	71.43	75.00
PRNP-M129V	MM	52.94	30.00	100.00	100.00
	MV	41.18	60.00	0.00	0.00
	VV	5.88	10.00	0.00	0.00

APOE, apolipoprotein E; PS, presenilin A2M, α -2-macroglobuline, ACE, Angiotensin converting enzyme; AGT, Angiotensinogen; cFOS; FBJ, murine osteosarcoma viral (v-fos) oncogene homolog; PRNP, Prior Protein gene

Source: Adapted from ref. 42.

whereas *PS-1/2* genotypes were overrepresented in IMs (63.16%) and UMs (60%). The presence of the *PS1-2/2* genotype was especially high in PMs (38.46%) and UMs (20%). A mutation in the *PS2* gene exon 5 (*PS2E5+*) was markedly present in UMs (66.67%). About 100% of UMs were *A2M-V100I-A/A*, and the *A2M-V100I-G/G* genotype was absent in PMs and UMs. The *A2M-I/I* genotype was absent in UMs, and 100% of UMs were *A2M-I/D* and *ACE-D/D*. Homozygous mutations in the *FOS* gene (B/B) were only present in UMs as well. *AGT-T235T* cases were absent in PMs, and the *AGT-M174M* genotype appeared in 100% of

PMs. Likewise, the *PRNP-M129M* variant was present in 100% of PMs and UMs (42) (see Table 10.5). These association studies clearly show that in PMs and UMs there is an accumulation of AD-related polymorphic variants of risk that might be responsible for the defective therapeutic responses currently seen in these AD clusters (42).

10.4.3.2 CYP2D6-Related Biochemical and Hemodynamic Phenotypes in Alzheimer’s Disease

It appears that different *CYP2D6* variants, expressing EMs, IMs, PMs, and UMs, influence to some extent several biochemical parameters, liver function, and vascular hemodynamic parameters that might affect drug efficacy and safety. Blood glucose levels are elevated in EMs (*1/*1 vs *4/*10, $p < 0.05$) and in some IMs (*4/*10 vs 1xN/*4, $p < 0.05$), whereas other IMs (*1/*5 vs *4/*4, $p < 0.05$) tend to show lower levels of glucose compared with PMs (*4/*4) or UMs (1xN/*4) (see Table 10.6). The highest levels of total cholesterol are detected in the EMs with the *CYP2D6**1/*10 genotype (vs *1/*1, *1/*4 and 1xN/*1, $p < 0.05$). The same pattern has been observed with regard to LDL cholesterol levels, which are significantly higher in the EM-*1/*10. In general, both total cholesterol levels and LDL cholesterol levels are higher in EMs (with a significant difference between *1/*1 and *1/*10), intermediate levels are seen in IMs, and much lower levels in PMs and UMs; the opposite occurs with HDL cholesterol levels, which on average appear much lower in EMs than in IMs, PMs, and UMs, with the highest levels detected in *1/*3 and 1xN/*4 (see Table 10.7). The levels of TGs are very variable among different *CYP2D6* polymorphisms, with the highest levels present in IMs (*4/*10 vs *4/*5 and 1xN/*1, $p < 0.02$). These data clearly indicate that lipid

Table 10.6 Cytochrome P450 2D6 (*CYP2D6*)-related blood glucose levels in Alzheimer’s disease

Phenotype	<i>CYP2D6</i>	Glucose (mg/dL)
Extensive metabolizers	*1/*1	101.01 ± 30.90 ^a
	*1/*10	104.85 ± 26.35
Intermediate metabolizers	*1/*3	94.66 ± 13.31
	*1/*4	101.56 ± 36.12
	*1/*5	91.83 ± 5.84 ^b
	*1/*6	99.66 ± 15.27
	*10/*10	99.33 ± 18.14
	*4/*10	127.80 ± 63.38 ^c
Poor metabolizers	*4/*4	96.76 ± 13.37
Ultrarapid metabolizers	*1xN/*1	105.57 ± 23.77
	*1xN/*4	82.61 ± 6.65

Values: mean ± SD.
^a $p < 0.05$ vs *4/*10; ^b $p < 0.05$ vs *1xN/*4; ^c $p < 0.05$ vs *4/*4.

Table 10.7 Cytochrome P450 2D6 (*CYP2D6*)-related blood lipid levels in Alzheimer's disease

Phenotype	<i>CYP2D6</i>	Cholesterol (mg/dL)	LDL cholesterol (mg/dL)	HDL cholesterol (mg/dL)	Triglycerides (mg/dL)
Extensive	*1/*1	223.15 ± 41.58 ^a	147.20 ± 35.00 ^d	52.30 ± 9.98 ⁱ	128.24 ± 76.61 ^k
metabolizers	*1/*10	275.57 ± 77.00 ^{b,c}	196.40 ± 62.70 ^{e-h}	53.28 ± 12.67	129.85 ± 71.58
Intermediate	*1/*3	235.33 ± 47.07	134.86 ± 21.06	64.66 ± 22.12	179.00 ± 149.22
metabolizers	*1/*4	235.39 ± 49.64	158.44 ± 36.33 ^{i,j}	54.37 ± 11.64	121.76 ± 93.76
	*1/*5	222.00 ± 41.45	148.08 ± 35.72	50.40 ± 8.96	154.00 ± 59.33 ^{l,m}
	*1/*6	234.61 ± 32.53	162.75 ± 31.43	57.75 ± 13.81	106.5 ± 47.59
	*10/*10	239.00 ± 22.62	152.30 ± 27.01	52.50 ± 3.50	171.00 ± 40.24
	*4/*10	255.20 ± 52.71	170.15 ± 59.87	45.25 ± 5.43	226.75 ± 124.84 ^{n,o}
Poor	*4/*4	233.85 ± 62.50	148.72 ± 46.51	57.92 ± 17.76	144.76 ± 21.24
metabolizers	*1xN/*1	202.14 ± 52.23	129.71 ± 46.23	53.28 ± 10.25	150.16 ± 33.74
Ultrarapid	*1xN/*4	203.66 ± 19.50	113.21 ± 28.30	63.01 ± 9.20	145.66 ± 31.65
metabolizers					

Values: mean ± SD.

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

^a*p* < 0.004 vs *1/*10; ^b*p* < 0.05 vs *1/*4; ^c*p* < 0.05 vs *1xN/*1; ^d*p* < 0.001 vs *1/*10; ^e*p* < 0.05 vs *1/*4; ^f*p* < 0.05 vs *4/*4; ^g*p* < 0.04 vs *1xN/*1; ^h*p* < 0.05 vs *1xN/*4; ⁱ*p* < 0.05 vs *1xN/*1; ^j*p* < 0.05 vs *1xN/*4; ^k*p* < 0.01 vs *4/*10; ^l*p* < 0.05 vs *4/*4; ^m*p* < 0.04 vs *1xN/*1; ⁿ*p* < 0.008 vs *4/*4; ^o*p* < 0.02 vs *1xN/*1.

metabolism can be influenced by *CYP2D6* variants, or that specific phenotypes determined by multiple lipid-related genomic clusters are necessary to confer the character of EMs and IMs. Another possibility might be that some lipid metabolism genotypes interact with *CYP2D6*-related enzyme products leading to define the phenotype-genotype of PMs and UMs.

No significant changes in blood pressure values have been found among *CYP2D6* genotypes; however, important differences became apparent in brain cerebrovascular hemodynamics (see **Table 10.8**). In general terms, the best cerebrovascular hemodynamic pattern is observed in EMs and PMs, with higher brain blood flow velocities and lower resistance and pulsatility indices, but differential phenotypic profiles are detectable among *CYP2D6* genotypes (see **Table 10.8**). For instance, systolic blood flow velocities (Sv) in the left middle cerebral arteries (LMCAs) of AD patients are significantly lower in *1/*10 EMs, with high total cholesterol and LDL cholesterol levels, than in IMs (*4/*10, *p* < 0.05); diastolic velocities (Dv) also tend to be much lower in *1/*10 and especially in PMs (*4/*4) and UMs (*1xN/*4), whereas the best Dv is measured in *1/*5 IMs. More striking are the results of both the pulsatility index [PI = (Sv-Dv)/Mv] and resistance index [RI = (Sv-Dv)/Sv], which are worse in IMs and PMs than in EMs and UMs (see **Table 10.8**). These data taken together seem to indicate that *CYP2D6*-related AD

Table 10.8 Cytochrome P450 2D6 (CYP2D6)-related brain hemodynamics in Alzheimer's disease

Phenotype	CYP2D6	LMCA- Mv (IU/L)	LMCA- Sv (IU/L)	LMCA- Dv (IU/L)	LMCA- PI (units)	LMCA- RI (units)
Extensive meta bolizers	^a 1/1	44.97 ± 13.62	71.27 ± 20.40	28.29 ± 10.06	0.98 ± 0.22 ^d	0.60 ± 0.07 ^{j,k}
	^a 1/10	38.22 ± 8.85	61.42 ± 12.07 ^a	24.32 ± 7.39	0.99 ± 0.37	0.59 ± 0.11
	^a 1/3	62.30 ± 15.23	87.20 ± 20.12	30.21 ± 10.80	0.67 ± 0.46	0.47 ± 0.12
	^a 1/4	47.73 ± 15.56	76.62 ± 22.91	29.74 ± 11.29	1.03 ± 0.24 ^e	0.61 ± 0.05 ^{l,m}
	^a 1/5	52.16 ± 13.76	81.16 ± 19.30	35.46 ± 10.45 ^b	0.88 ± 0.07 ^f	0.56 ± 0.02 ⁿ
Poor metabolizers	^a 1/6	42.00 ± 15.24	67.00 ± 20.30	24.80 ± 5.30	0.98 ± 0.20	0.61 ± 0.17
	^a 10/10	42.75 ± 6.57	78.85 ± 7.70	22.60 ± 3.67	1.47 ± 0.32 ^{g-i}	0.75 ± 0.07 ^{o-q}
	^a 4/10	47.50 ± 10.84	76.84 ± 11.90	28.00 ± 9.47	1.07 ± 0.31	0.62 ± 0.08
Ultrarapid metabolizers	^a 4/4	42.04 ± 12.24	68.85 ± 18.90	23.68 ± 7.42 ^c	1.06 ± 0.14	0.64 ± 0.07 ^r
	^a 1×N/1	46.32 ± 11.31	71.42 ± 15.41	31.87 ± 9.24	0.86 ± 0.21	0.55 ± 0.10
	^a 1×N/4	39.00 ± 11.26	60.66 ± 16.19	24.00 ± 7.03	0.95 ± 0.07	0.60 ± 0.02

Values: mean ± SD.

Dv, diastolic velocity; LMCA, left middle cerebral artery; Mv, mean velocity; PI, pulsatility index; RI, resistance index; Sv, systolic velocity.

^a*p* < 0.05 vs ^a4/10; ^b*p* < 0.03 vs ^a4/4; ^c*p* < 0.05 vs ^a1×N/1; ^d*p* < 0.003 vs ^a10/10; ^e*p* < 0.02 vs ^a10/10; ^f*p* < 0.04 vs ^a10/10; ^g*p* < 0.006 vs ^a4/4; ^h*p* < 0.05 vs ^a1×N/1; ⁱ*p* < 0.05 vs ^a1×N/4; ^j*p* < 0.05 vs ^a4/4; ^k*p* < 0.01 vs ^a10/10; ^l*p* < 0.003 vs ^a10/10; ^m*p* < 0.05 vs ^a1×N/1; ⁿ*p* < 0.02 vs ^a10/10; ^o*p* < 0.05 vs ^a4/4; ^p*p* < 0.05 vs ^a1×N/1; ^q*p* < 0.03 vs ^a1×N/4; ^r*p* < 0.05 vs ^a1×N/1.

PMs exhibit a poorer cerebrovascular function, which might affect drug penetration in the brain, with the consequent therapeutic implications.

10.4.3.3 Influence of CYP2D6 Genotypes on Liver Transaminase Activity

Some conventional antidementia drugs (tacrine, donepezil, galantamine) are metabolized via *CYP*-related enzymes, especially *CYP2D6*, *CYP3A4*, and *CYP1A2* (467,468), and polymorphic variants of the *CYP2D6* gene can affect the liver metabolism, safety, and efficacy of some ChEIs (18–20,42,469,470). To elucidate whether *CYP2D6*-related variants may influence transaminase activity, we studied the association of glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and γ -glutamyl transpeptidase (GGT) activity with the most prevalent *CYP2D6* genotypes in AD (see Table 10.9). Globally, UMs and PMs tend to show the highest GOT activity and IMs the lowest. Significant differences appear among different IM-related genotypes. The **10/*10* genotype exhibited the lowest GOT activity, with marked differences compared to UMs ($p < 0.05$ vs **1xN/*1*; $p < 0.05$ vs **1xN/*4*) (42). GPT activity was significantly higher in PMs (**4/*4*) than in EMs (**1/*10*, $p < 0.05$) or IMs (**1/*4*, **1/*5*, $p < 0.05$). The lowest GPT activity was found in EMs and IMs (42). Striking differences have been found in GGT activity between PMs (**4/*4*), which showed the highest levels, and EMs (**1/*1*, $p < 0.05$; **1/*10*, $p < 0.05$), IMs (**1/*5*, $p < 0.05$), or UMs (**1xN/*1*, $p < 0.01$) (see Table 10.9). Interesting enough, the **10/*10* genotype, with the lowest values of GOT and GPT, exhibited the second highest levels of GGT after **4/*4*, probably indicating that *CYP2D6*-related enzymes differentially regulate drug metabolism and transaminase

Table 10.9 Cytochrome P450 2D6 (*CYP2D6*)-related liver transaminase activity in Alzheimer’s disease

Phenotype	<i>CYP2D6</i>	GOT (IU/L)	GPT (IU/L)	GGT (IU/L)
Extensive metabolizers	<i>*1/*1</i>	23.49 ± 8.70 ^a	23.77 ± 16.04	31.16 ± 31.26 ^{a,p}
	<i>*1/*10</i>	17.57 ± 6.29 ^b	16.28 ± 7.40 ^k	18.14 ± 6.79 ^q
Intermediate metabolizers	<i>*1/*3</i>	22.33 ± 1.52 ^{c,d}	24.66 ± 10.59	22.00 ± 8.71
	<i>*1/*4</i>	21.76 ± 3.57 ^{e,f}	21.88 ± 8.40	32.23 ± 25.53
	<i>*1/*5</i>	18.33 ± 2.33 ^{g,h}	16.16 ± 5.60 ^{l,m}	18.50 ± 6.47 ^{r,s}
	<i>*1/*6</i>	23.00 ± 4.83	23.25 ± 5.31	33.50 ± 26.41
	<i>*10/*10</i>	16.00 ± 1.41 ^{i,j}	16.50 ± 3.53	39.00 ± 11.31 ^t
	<i>*4/*10</i>	20.00 ± 3.87	20.60 ± 4.03	34.20 ± 16.20
Poor metabolizers	<i>*4/*4</i>	21.78 ± 6.48	17.64 ± 15.05	59.71 ± 113.58 ^u
Ultrarapid metabolizers	<i>*1xN/*1</i>	20.50 ± 3.01	18.00 ± 5.32	21.50 ± 9.22
	<i>*1xN/*4</i>	23.33 ± 4.04	23.00 ± 5.01	25.66 ± 6.02

Values: mean ± SD.
GGT, γ -glutamyl transpeptidase; GOT, glutamic-oxalacetic transaminase; GPT, glutamic-pyruvic transaminase.
^a $p < 0.05$ vs **1/*10*; ^b $p < 0.05$ vs **1/*4*; ^c $p < 0.03$ vs **1/*5*; ^d $p < 0.001$ vs **1/*10*; ^e $p < 0.03$ vs **1/*5*; ^f $p < 0.03$ vs **10/*10*; ^g $p < 0.05$ vs **1/*6*; ^h $p < 0.04$ vs **1xN/*4*; ⁱ $p < 0.05$ vs **1xN/*1*; ^j $p < 0.05$ vs **1xN/*4*; ^k $p < 0.05$ vs **4/*4*; ^l $p < 0.05$ vs **1/*6*; ^m $p < 0.05$ vs **4/*4*; ⁿ $p < 0.05$ vs **4/*4*; ^o $p < 0.01$ vs **10/*10*; ^p $p < 0.01$ vs **4/*10*; ^q $p < 0.05$ vs **4/*4*; ^r $p < 0.01$ vs **10/*10*; ^s $p < 0.05$ vs **4/*10*; ^t $p < 0.05$ vs **1xN/*1*; ^u $p < 0.05$ vs **1xN/*1*.

activity in the liver. These results are also clear in demonstrating the direct effect of *CYP2D6* variants on transaminase activity (42) (see Table 10.9).

10.4.3.4 *CYP2D6*-Related Therapeutic Response to a Multifactorial Treatment

No clinical trials have been performed to date to elucidate the influence of *CYP2D6* variants on the therapeutic outcome in AD in response to ChEIs or other anti-dementia drugs. To overcome this lack of pharmacogenetic information, we performed the first prospective study in AD patients who received a combination therapy (CPND protocol) with (1) an endogenous nucleotide and choline donor, CDP-choline (500mg/d); (2) a nootropic substance, piracetam (1600mg/d); (3) a vasoactive compound, 1,6 dimethyl 8 β -(5-bromonicotinoyl-oxyethyl)-10 α -methoxyergoline (nicergoline) (5 mg/d); and (4) a ChEI, donepezil (5 mg/d), for 1 yr.

With this multifactorial therapeutic intervention, EMs improved their cognitive function (MMSE score) from 21.58 ± 9.02 at baseline to 23.78 ± 5.81 after 1 yr of treatment ($r = +0.82$; a coefficient [coef.] = +20.68; b coef. +0.4). IMs also improved from 21.40 ± 6.28 to 22.50 ± 5.07 ($r = +0.96$; a coef. = +21.2; b coef. = +0.25), whereas PMs and UMs deteriorate from 20.74 ± 6.72 to 18.07 ± 5.52 ($r = -0.97$; a coef. = +21.63; b coef. = -0.59) and from 22.65 ± 6.76 to 21.28 ± 7.75 ($r = -0.92$; a coef. = +23.35; b coef. = -0.36), respectively. According to these results, PMs and UMs were the worst responders, showing a progressive cognitive decline with no therapeutic effect, and EMs and IMs were the best responders, with a clear improvement in cognition after 1 yr of treatment (see Fig. 10.11).

Among EMs, AD patients harboring the $*1/*10$ genotype ($r = +0.97$; a coef. = +19.27; b coef. = +0.55) responded better than patients with the $*1/*1$ genotype ($r = +0.44$; a coef. = +22.10; b coef. = +0.25). The best responders among IMs were the $*1/*3$ ($r = +0.98$; a coef. = +20.65; b coef. = 1.18), $*1/*6$ ($r = 0.93$; a coef. = +22.17; b coef. = +0.44), and $*1/*5$ genotypes ($r = +0.70$; a coef. = +19.96; b coef. = +0.25), whereas the $*1/*4$, $*10/*10$, and $*4/*10$ genotypes were poor responders (see Fig. 10.12). Among PMs and UMs, the poorest responders were carriers of the $*4/*4$ ($r = -0.98$; a coef. = +19.72; b coef. = -0.91) and $*1xN/*1$ genotypes ($r = -0.97$; a coef. = +24.55; b coef. = -0.98), respectively (42) (Fig. 10.12).

From all these data, we can conclude the following: First, the most frequent *CYP2D6* variants in the Spanish population are the $*1/*1$ (47.10%), $*1/*4$ (17.42%), $*4/*4$ (8.37%), $*1/*10$ (4.52%), and $*1xN/*1$ (4.52%), accounting for more than 80% of the population. Second, the frequency of EMs, IMs, PMs, and UMs is about 51.61%, 32.26%, 9.03%, and 7.10%, respectively. Third, EMs are more prevalent in AD (57.47%) than in controls (44.12%); IMs are more frequent in controls (41.18%) than in AD (25.29%), especially the $*1/*4$ (C: 23.53%; AD: 12.64%) and $*4/*10$ genotypes (C 5.88%; AD 1.15%); the frequency of PMs is similar in AD (9.20%) and controls (8.82%); and UMs are more frequent among AD cases (8.04%) than in controls (5.88%). Fourth, there is an accumulation of AD-related genes of risk in PMs and UMs. Fifth, PMs and UMs tend to show higher transaminase

CYP2D6-Related Therapeutic Response in Alzheimer's Disease

Cognitive Performance in Extensive (EM), Intermediate (IM), Poor (PM) and Ultra-rapid Metabolizers (UM)

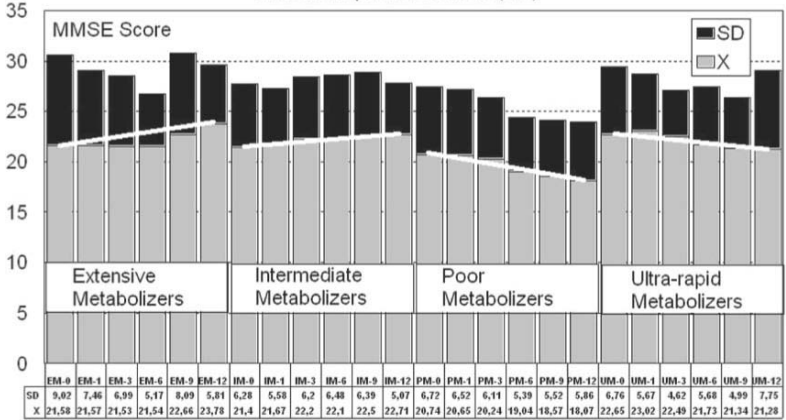


Fig. 10.11 Cytochrome *P450* (*CYP*) 2D6-related therapeutic response to a multifactorial treatment in Alzheimer's disease. (Adapted from ref. 42.)

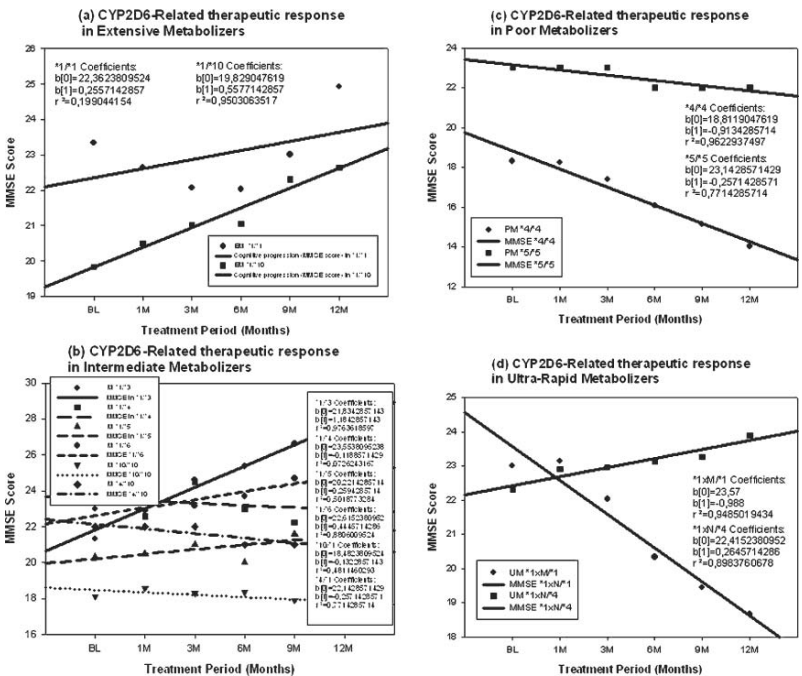


Fig. 10.12 Cytochrome *P450* (*CYP*) 2D6-related therapeutic response to a multifactorial treatment in Alzheimer's disease. (Adapted from ref. 42.)

activities than EMs and IMs. Sixth, EMs and IMs are the best responders, and PMs and UMs are the worst responders to a combination therapy with ChEIs, neuroprotectants, and vasoactive substances. Finally, the pharmacogenetic response in AD appears depend on the networking activity of genes involved in drug metabolism and genes involved in AD pathogenesis (18–20,42).

Taking into consideration the available data, it might be inferred that at least 15% of the AD population may exhibit an abnormal metabolism of ChEIs or other drugs that undergo oxidation via *CYP2D6*-related enzymes. Approximately 50% of this population cluster would show an ultrarapid metabolism, requiring higher doses of ChEIs to reach a therapeutic threshold, whereas the other 50% of the cluster would exhibit a poor metabolism, displaying potential adverse events at low doses. If we take into account that approx. 60–70% of therapeutic outcomes depend on pharmacogenomic criteria (e.g., pathogenic mechanisms associated with AD-related genes), it can be postulated that pharmacogenetic and pharmacogenomic factors are responsible for 75–85% of the therapeutic response (efficacy) in AD patients treated with conventional drugs (12–16,18–20,291).

Of particular interest are the potential interactions of ChEIs with other drugs of current use in patients with AD, such as antidepressants, neuroleptics, antiarrhythmics, analgesics, and antiemetics, that are metabolized by the cytochrome P450 *CYP2D6* enzyme (456). Although most studies predict the safety of donepezil (471) and galantamine (467,468,472,481), as the two principal ChEIs metabolized by *CYP2D6*-related enzymes (468,473), no pharmacogenetic studies have been performed so far on an individual basis to personalize the treatment, and most studies reporting safety issues are the result of pooling together pharmacological and clinical information obtained with routine procedures (452,474–476). In certain cases, genetic polymorphism in the expression of *CYP2D6* is not expected to affect the pharmacodynamics of some ChEIs because major metabolic pathways are glucuronidation, O-demethylation, N-demethylation, N-oxidation, and epimerization. However, excretion rates are substantially different in EMs and PMs. For instance, in EMs, urinary metabolites resulting from O-demethylation of galantamine represent 33.2% of the dose compared with 5.2% in PMs, which show correspondingly higher urinary excretion of unchanged galantamine and its N-oxide (477).

Therefore, there are many unanswered questions regarding the metabolism of ChEIs and their interaction with other drugs (potentially leading to ADRs) that require pharmacogenetic elucidation. It is also worth mentioning that dose titration (a common practice in AD patients treated with ChEIs, e.g., tacrine, donepezil) is an unwise strategy because approx. 30–60% of drug failure or lack of therapeutic efficacy (or ADR manifestation) is not a matter of drug dosage but a problem of poor metabolizing capacity in PMs. In addition, inappropriate drug use is one of the risk factors for ADRs in the elderly. The prevalence of use of potentially inappropriate medications in patients older than 65 years of age admitted to a general medical or geriatric ward ranges from 16% to 20% (478), and these numbers may double in ambulatory patients. Overall, the most prevalent inappropriate drugs currently prescribed to the elderly are amiodarone, long-acting benzodiazepines, and anticholinergic antispasmodics; however, the list of drugs with potential risk also include

antidepressant, antihistaminics, NSAIDs, amphetamines, laxatives, clonidine, indomethacin, and several neuroleptics (478), most of which are processed via *CYP2D6* and *CYP3A5* enzymes (479). Therefore, pretreatment *CYP* screening might be of great help for rationalizing and optimizing therapeutics in the elderly by avoiding medications of risk in PMs and UMs.

10.4.4 *APOE in Alzheimer's Disease Therapeutics*

10.4.4.1 Pathogenic Role of APOE in Alzheimer's Disease

Polymorphic variants in the *APOE* gene (19q13.2) are associated with risk (*APOE*-4 allele) or protection (*APOE*-2 allele) for AD (12,18–20,47,480–482). The three major isoforms of human *APOE* (*APOE*-2, *APOE*-3, *APOE*-4) are coded by the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles. Differences in the amino acid sequence at sites A (residue 112) and B (residue 158) of the *APOE* molecule distinguish the *APOE*-2 (Cys/Cys), *APOE*-3 (Cys/Arg), and *APOE*-4 (Arg/Arg) isoforms. *APOE*-3 is the most frequent isoform (wild type), and *APOE*-4 differs from *APOE*-3 in a Cys-to-Arg change at position 112 (*APOE*-4/Cys112Arg). *APOE*-2 (Arg158Cys) is the most common isoform of the four different mutations at the E2 position with isoelectric focusing. The other three *APOE*-2 isoforms are E2(Lys146Gln), E2(Arg145Cys), and E2(Arg136Ser). The *APOE* gene encodes a 299-amino acid polypeptide (Mr 34,200). This gene is in close proximity with the *APOC1*, *APOC2*, and *GPI* genes in the same region of 19q, suggesting that these genes arose from a common ancestor by gene duplication (12).

Sequence haplotype variation in 5.5 kb of genomic DNA encompassing the whole *APOE* locus and adjoining flanking regions revealed the existence of 22 diallelic sites defining 31 distinct haplotypes. Sequence analysis suggested that haplotypes defining the *APOE*-3 and *APOE*-2 alleles were derived from the ancestral *APOE*-4, and that the *APOE*-3 group of haplotypes had increased in frequency, relative to *APOE*-4, in the past 200,000 years. Substantial heterogeneity is present in the three classes of sequence haplotypes, with interpopulation differences in the sequence variation underlying the protein isoforms, probably explaining conflicting results when interpreting phenotypic associations with variation in the common protein isoforms (483).

ZIC proteins stimulate potent transcriptional activation of *APOE* through binding sites in the –136 to 125, 65–54, and –185 to 174 regions of the *APOE* promoter (484). The ZIC (3q24) is a zinc finger protein that displays a highly restricted expression pattern in the adult and developing mouse cerebellum and is highly homologous to the *Drosophila* pair-rule gene Opa (485).

In addition to the known transcript (*APOE S1*) that translates into *APOE*, there are three additional transcripts in mice. Two of these transcripts, *APOE S2* and *APOE S3*, which are predicted to be TM proteins, are transcribed from the sense strand. *APOE S1* is transcribed from the antisense strand and is complementary to exon 4 of *APOE*

SI. The antisense transcript falls within the region of the human *APOE-4* allele. It has been proposed that these alternate transcripts might be involved in the molecular pathogenesis of CNS disorders and *APOE* expression (486).

The *APOE* alleles show a peculiar distribution in the world (12,47,487). The *APOE-3* allele is the most frequent in all human societies, especially in populations with a long-established agricultural economy, such as those of the Mediterranean basin, where the allele frequency is 0.849–0.898. *APOE-4* is the ancestral allele, with a frequency that still remains higher in Pygmies (0.407) and Khoi San (0.370), aborigines of Malaysia (0.240) and Australia (0.260), Papuans (0.368), some Native Americans (0.280), and Lapps (0.310), for whom an economy of foraging still exists or food supply is scarce or sporadically available. The frequency of the *APOE-2* allele fluctuates with no apparent trend (0.145–0.02), is absent in Native Americans, and is very low (<1%) in southern Europeans (12,20,47,487–489).

The best-known effect of *APOE* is the regulation of lipid metabolism (see Fig. 10.13). *APOE* is a constituent of TG-rich chylomicrons, VLDL particles and their remnants, and a subclass of HDL. In addition to its role in the transport of cholesterol and the metabolism of lipoprotein particles, *APOE* can be involved in many other physiological and pathological processes, including immunoregulation, nerve regeneration, activation of lipolytic enzymes (hepatic lipase, lipoprotein lipase, lecithin:cholesterol acyltransferase), ligand for several cell receptors, neuronal homeostasis, and tissue repair (488,490). *APOE* is essential

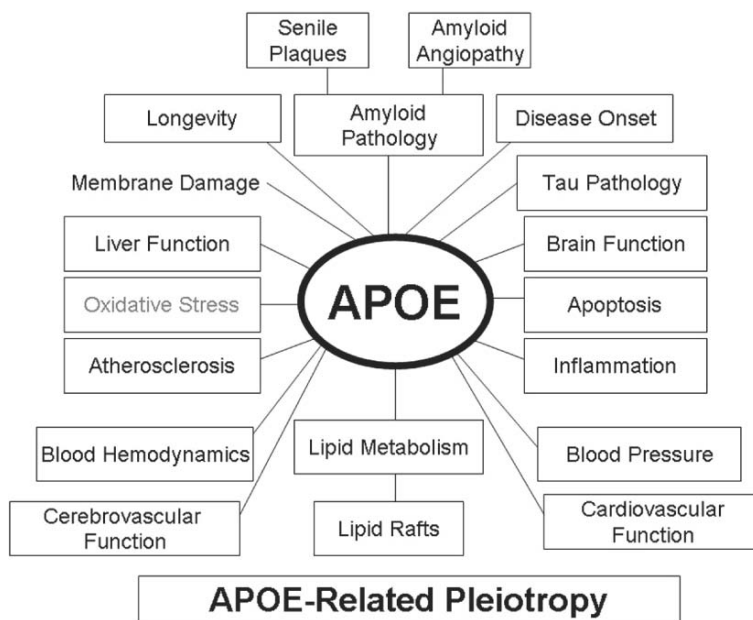


Fig. 10.13 Potential pleiotropic effects of the apolipoprotein E (*APOE*) gene and its products

for the normal catabolism of TG-rich lipoprotein constituents. The interaction of *APOE* and the LDL receptor controls the removal of *APOE*-rich lipoproteins (VLDL, chylomicron remnants, intermediate-density proteins) and determines the homeostasis of cholesterol and TGs. Some studies indicated that *APOE* polymorphism variation may explain 14–17% of the genetic variability of plasma cholesterol concentrations (488). The three *APOE* isoforms have different affinities for the LDL receptor, *APOE*-3 and *APOE*-4 showing similar affinities and *APOE*-2 exhibiting a defective binding activity. *APOE* plays a critical role in lipoprotein metabolism and plasma lipid homeostasis through its high-affinity binding to the LDL receptor family.

In solution, *APOE* is an oligomeric protein and the C-terminal domain causes *APOE*'s aggregation. The aggregation property presents a major difficulty for structural determination of this protein. Using protein engineering techniques, Fan et al. (491) identified a monomeric, biologically active *APOE* C-terminal domain mutant. This mutant replaces five bulky hydrophobic residues in the region of residues 253–289 with either smaller hydrophobic or polar/charged residues (F257A, W264R, V269A, L279Q, V287E). These residues are critical for aggregation but may not be important for maintaining the structure, stability, and lipid-binding activity of this *APOE* domain, suggesting that *APOE* may use different epitopes for its aggregation property, helical structure/stability, and lipid-binding activity (491).

APOE-2-containing remnants and VLDL particles are slowly removed from the plasma and induce an upregulation of the liver LDL receptor and subsequent low concentration of plasma cholesterol. VLDL-*APOE*-4 particles are removed from plasma faster than VLDL-*APOE*-3 particles, inducing a downregulation of the LDL receptor, and thus the VLDL-*APOE*-4 phenotype is associated with a higher concentration of circulating cholesterol (488).

The *APOE* genotype is an important determinant of plasma and CSF *APOE* and lipid levels. The *APOE*-2 allele is associated with high concentrations of *APOE* and the *APOE*-4 allele with lower *APOE* levels (12,20,488). Some authors have found association between *APOE*-2 and *APOE*-4 and high levels of plasma TGs as well as an association of *APOE*-3 and low levels of TGs in the general population (488). It has also been suggested that individuals carrying the *APOE*-4 genotype had significantly greater increase in TGs accompanied by an increase in body weight, suggesting that obese individuals with an *APOE*-4 allele might be at an increased risk for developing hypertriglyceridemia and atherosclerosis (488,492,493); however, recent studies in the AD population clearly indicated that (1) cholesterol levels are markedly increased in *APOE*-4/4 carriers; (2) TG levels are the lowest in *APOE*-4/4; and (3) *APOE*-4/4 carriers show high atherogenic activity (3,12,13,15,16,18–20,25,59,289,290). These differences probably reflect the influence of endogenous factors interacting with *APOE* to induce an alteration in lipid metabolism in patients with AD. In familial *APOE* deficiency, homozygotes have only trace amounts of plasma *APOE*, and accumulation of APOB-4 and APOA-4 in VLDL, IDL, and LDLs. Obligate heterozygotes generally have normal plasma lipids and mean plasma *APOE* concentrations (494).

Atypical dysbetalipoproteinemia, associated with the *APOE*-3/3 phenotype rather than the classic *APOE*-2/2 phenotype, is characterized by severe hypercholesterolemia and hypertriglyceridemia, xanthomatosis, premature vascular disease, and a preponderance of β -VLDL. Subjects with atypical dysbetalipoproteinemia are homozygous for an amino acid substitution in *APOE* at residue 158 (495). In AD, *APOE*-4 carriers show lower levels of *APOE* in the CSF compared to controls (496).

Sullivan et al. (497) studied the pattern of *APOE* expression in the CNS. Immunocytochemistry on brain sections from three human *APOE*-targeted-replacement mouse lines, wild-type mice, African green monkeys, and humans showed a predominantly glial pattern of *APOE* expression. The levels of human *APOE* protein in hippocampus and frontal cortex were similar between targeted replacement mice and nondemented human tissue. Within a given brain region, the levels of *APOE* were very similar among all three isoforms, which contrasts sharply with plasma, where *APOE*-2 levels were 16-fold higher than *APOE*-3 and *APOE*-4 levels. Across brain regions, cerebellar *APOE* levels were significantly higher than cerebral *APOE* levels (497). In human brain, *APOE*-4 dose correlated inversely with dendritic spine density in the hippocampus (498).

APOE is expressed at high levels in hepatocytes, macrophages, fibroblasts and astrocytes. Neurons also express *APOE* at lower levels than astrocytes in response to various physiological and pathological conditions, including excitotoxic stress. Neuronal expression of *APOE* is regulated by a diffusible factor or factors released from astrocytes, and this regulation depends on the activity of the Erk kinase pathways in neurons (499).

Fibroblast *APOE* mRNA and protein levels are upregulated during staurosporine-induced apoptosis, and this correlates with increased caspase-3 activity and apoptotic morphological alterations. The transcription of *APOE* and specific proapoptotic genes is regulated by the nuclear receptor LXR- α . LXR- α increases before *APOE* mRNA induction. The expression of *ABCA1* (ATP-binding cassette transporter A1) mRNA is regulated by LXR- α and increases in parallel with *APOE* mRNA, indicating that LXR- α probably promotes *APOE* and *ABCA1* transcription during apoptosis.

Fibroblast *APOE* levels increase under conditions of serum-starvation-induced growth arrest and hypoxia-induced senescence. In both cases, increased nuclear *APOE* levels are observed, particularly in cells that accumulate lipofuscin. Nuclear *APOE* is translocated to the cytosol when mitotic nuclear disassembly occurs, and this is associated with an increase in total cellular *APOE* levels (500).

For many years, alterations in *APOE* and defects in the *APOE* gene have been associated with dysfunctions in lipid metabolism, cardiovascular disease, and atherosclerosis. During the past 25 years, an enormous amount of studies clearly documented the role of *APOE*-4 as a risk factor for AD, and the accumulation of the *APOE*-4 allele has been reported as a risk factor for other forms of dementia and CNS disorders (1,12,18–20,47,488).

In 1993, Allen Roses and coworkers found a clear association between *APOE* genotypes and AD, demonstrating that the frequency of the *APOE*-4 allele was

significantly higher in LOAD (501–503). Since then, many other studies have confirmed the early findings of Saunders et al. (502,503) and Corder et al. (504) reporting an increased frequency of the *APOE-4* allele in AD and the association of the *APOE-4* allele with LOAD and sporadic forms of AD (1,12,47,480–482,505–509). A protective effect of *APOE-2* for LOAD has also been proposed (509) and confirmed (12,20,47,507). There is also a significant lowering of age of onset for those affected with *APOE-4/4* as compared to one *APOE-2* allele or all others (508,509). *APOE-4* promotes atherosclerosis and is less frequent in centenarians than in controls, and *APOE-2*, which was associated with type III and type IV hyperlipidemia, is more frequent in people with higher longevity rates (510). The risk for AD increases from 20% to 90% and mean age at onset decreases from 84 to 68 yr with increasing number of *APOE-4* alleles (504), confirming the dosage effect of the *APOE-4* allele, which in *APOE-4/4* homozygotes anticipates the age at onset in their 60s (1,12,20,47,507,508,511,512).

In addition to the *APOE-4* haplotype, at least two other polymorphic variants in the promoter of the *APOE* gene (–219G>T and –491A>T) might also contribute to disease susceptibility, and modulate the impact of structural changes in the *APOE* lipoprotein, by altering its expression. Recent studies demonstrated that in the human brain most of the *cis*-acting variance in *APOE* expression is accounted for by the *APOE-4* haplotype, but there are additional small *cis*-acting influences associated with promoter genotype (513).

APOE is found in amyloid plaques and NFTs in AD brains. The accumulation of potentially pathogenic C-terminally truncated fragments of *APOE* depends on both the isoform and the cellular source of *APOE*. Neuron-specific proteolytic cleavage of *APOE-4* is associated with increased phosphorylation of tau and may play a key role in the development of AD-related neuronal deficits (514). Hippocampal *APOE* levels correlate with NFT formation (Braak stages), especially in *APOE-3/3* autopsy samples, but not in *APOE-4* carriers (515).

APOE may affect NFT and ABP deposition in AD (516). *APOE-4*-related proteins may interfere with binding of tau to microtubules, altering tau glycation and phosphorylation (517). The presence of *APOE-4* increases the odds ratio for CAA, and *APOE-4* is strongly associated with increased neuritic plaques and ABP deposition in AD (518–520). The oxidized form of purified *APOE-4* shows a higher affinity binding to synthetic ABP and MAP2 than the *APOE-3* isoform, and probably *APOE* may affect microtubule function and ABP accumulation in AD (501,516). Carriers of *APOE-2* and *APOE-4* alleles are also more prone to recurrent CAA than *APOE-3/3* carriers (521). The frequency of *APOE-4* was also increased in patients with ABP deposition following head injury (522), and the neurologic recovery after brain trauma is poorer in *APOE-4* carriers than in subjects without that allele. AD *APOE-4* carriers showed reduced glucose metabolism in selected brain regions (523). There is also an *APOE*-related cognitive decline in AD patients that is more accelerated in subjects with the *APOE-4/4* genotypes (18–20). *APOE*-related differences in serum *APOE* levels, blood pressure values, and lymphocyte apoptosis have been demonstrated in AD (12,18–20,47). *APOE-4/4* patients are also the worst responders to different

treatments (6,12–16,18–20,47,290,291). *APOE-4* carriers also show poorer brain metabolism (18–20,25,523–525).

The *APOE-4* genotype is accompanied by lower metabolic activity in nucleus basalis of Meynert neurons in AD and controls (526). Dubelaar et al. (526) used the size of the Golgi apparatus as an indicator of metabolic activity. Control subjects harboring the *APOE-4* allele had reduced neuronal metabolism and showed more neurons with smaller Golgi apparatus size compared with *APOE-4* noncarriers. As the disease progresses into later stages of AD (Braak V–VI), neuronal metabolism strongly diminishes, resulting in neurons with extremely small Golgi apparatus size, irrespective of *APOE* genotype (526).

APOE-4 may influence AD pathology, interacting with *APP* metabolism and ABP accumulation; enhancing hyperphosphorylation of tau protein and NFT formation; reducing choline acetyltransferase activity; increasing oxidative processes; modifying inflammation-related neuroimmunotrophic activity and glial activation; altering lipid metabolism, lipid transport, and membrane biosynthesis in sprouting and synaptic remodeling; and inducing neuronal apoptosis (1,6,12,18–20). ABP deposition enhanced by *APOE-4* precedes NFT formation in the frontal cortex (527). The *APOE* protein reduces ABP1–40 levels by 60–80% and ABP1–42 levels by 20–30% in conditioned media from cells in culture, possibly through ABP clearance mechanisms. All three isoforms of *APOE* (ϵ 2, ϵ 3, ϵ 4) have similar effects on ABP and *APP-C* terminal fragments, and the effects are independent of the LDL receptor family (528). However, hyperexpression of human *APOE-4* in astroglia and neurons does not lead to proportional changes in the age of appearance, relative burden, character, or distribution of amyloid deposition in *APP* or PS transgenic mice (529).

Despite abundant information associating *APOE-4* with AD (12), some studies concluded that the *APOE* locus is neither necessary nor sufficient to cause AD (530). In contrast, other studies concluded that homozygosity for *APOE-4* is virtually sufficient to cause AD by age 80 (502–504). A critical review of the international literature provided convincing support to the hypothesis of *APOE* as a major player in AD pathogenesis and risk of dementia (12). Taking into account the information available at present, the major facts demonstrating that *APOE* is associated with AD can be summarized as follows: (1) increased frequency of the *APOE-4* allele in AD and protective effect of *APOE-2*; (2) association of *APOE-4* with an anticipation of the age at onset; (3) negative influence of *APOE-4* on cognitive performance; (4) deleterious associations of *APOE-4* with other genes as potential risk factors for AD; (5) Relationship between *APOE* and sex differences in AD; (6) association of *APOE* with ABP and tau in AD pathology; (7) *APOE* and alterations in lipid metabolism; (h) *APOE* and neuroendocrine function in AD; (8) *APOE* and behavior; (9) *APOE* and brain atrophy; (10) *APOE* and survival; (11) increased frequency of *APOE-4* in other CNS disorders, especially vascular and mixed dementia, psychosis, depression, and anxiety; (12) *APOE* transgenic models; and (13) the impact of *APOE* on AD functional genomics and pharmacogenomics (1,3,6,12–16,18–20,25,42,59,289,290,496).

10.4.4.2 *APOE*-Related Phenotypic Profiles in Alzheimer's Disease

Different *APOE* genotypes confer specific phenotypic profiles to AD patients. Some of these profiles may add risk or benefit when the patients are treated with conventional drugs, and in many instances the clinical phenotype demands the administration of additional drugs that increase the complexity of therapeutic protocols. From studies designed to define *APOE*-related AD phenotypes (12–16,18–20,289,290), several confirmed conclusions can be drawn: (1) The age at onset is 5–10 yr earlier in approx. 80% of AD cases harboring the *APOE*-4/4 genotype. (2) The serum levels of *APOE* are the lowest in *APOE*-4/4, intermediate in *APOE*-3/3 and *APOE*-3/4, and highest in *APOE*-2/3 and *APOE*-2/4. (3) Serum cholesterol levels are higher in *APOE*-4/4 than in the other genotypes. (4) HDL cholesterol levels tend to be lower in *APOE*-3 homozygotes than in *APOE*-4 allele carriers. (5) LDL cholesterol levels are systematically higher in *APOE*-4/4 than in any other genotype. (6) TG levels are significantly lower in *APOE*-4/4. (7) NO levels are slightly lower in *APOE*-4/4. (8) Serum ABP levels do not differ between *APOE*-4/4 and the other most frequent genotypes (*APOE*-3/3, *APOE*-3/4). (9) Blood histamine levels are dramatically reduced in *APOE*-4/4 compared with the other genotypes. (10) Brain atrophy is markedly increased in *APOE*-4/4>*APOE*-3/4>*APOE*-3/3. (11) Brain mapping activity shows a significant increase in slow-wave activity in *APOE*-4/4 from early stages of the disease. (12) Brain hemodynamics, as reflected by reduced brain blood flow velocity and increase pulsatility and resistance indices, is significantly worse in *APOE*-4/4 (and in *APOE*-4 carriers, in general, as compared with *APOE*-3 carriers). (13) Lymphocyte apoptosis is markedly enhanced in *APOE*-4 carriers. (14) Cognitive deterioration is faster in *APOE*-4/4 patients than in carriers of any other *APOE* genotype. (15) Occasionally, in approx. 3–8% of the AD cases, the presence of some dementia-related metabolic dysfunctions (e.g., iron, folic acid, vitamin B₁₂ deficiencies) accumulate in *APOE*-4 carriers more than in *APOE*-3 carriers. (16) Some behavioral disturbances (bizarre behaviors, psychotic symptoms), alterations in circadian rhythm patterns (e.g., sleep disorders), and mood disorders (anxiety, depression) are slightly more frequent in *APOE*-4 carriers. (17) Aortic and systemic atherosclerosis are also more frequent in *APOE*-4 carriers. (18) Liver metabolism and transaminase activity also differ in *APOE*-4/4 with respect to other genotypes. (19) Blood pressure (hypertension) and other cardiovascular risk factors also accumulate in *APOE*-4. (20) *APOE*-4/4 individuals are the poorest responders to conventional drugs. These 20 major phenotypic features clearly illustrate the biological disadvantage of *APOE*-4 homozygotes and the potential consequences that these patients may experience when they receive pharmacological treatment (1,3,6,12–16,18–20,25,42,59,60, 289–291,469).

10.4.4.3 *APOE*-Related Therapeutic Response to Cholinesterase Inhibitors and Multifactorial Treatments

Several studies indicated that the presence of the *APOE*-4 allele differentially affects the quality and size of drug responsiveness in AD patients treated with

cholinergic enhancers (tacrine, donepezil, rivastigmine) (531–533). For example, *APOE-4* carriers show a less-significant therapeutic response to tacrine (60%) than patients with no *APOE-4* (531). In another study, the frequency of *APOE-4* alleles was higher in responders to a single oral dose of tacrine (533). It has been demonstrated that more than 80% of *APOE-4*(–) AD patients showed marked improvement after 30 wk of treatment with tacrine, whereas 60% of *APOE-4*(+) carriers had a poor response (531). Others found no differences after 6 mo of treatment with tacrine among *APOE* genotypes, but after 12 mo the CIBIC scores revealed that *APOE-4* carriers had declined more than the *APOE-2* and *APOE-3* patients, suggesting that a faster rate of decline was evident in the *APOE-4* patients, probably reflecting that *APOE-4* inheritance is a negative predictor of treatment of tacrine in AD (534).

It has also been shown that the *APOE* genotype may influence the biological effect of donepezil on *APP* metabolism in AD (535). Prospective studies with galantamine in large samples of patients in Europe (536) and in the United States (301) showed no effect of *APOE* genotypes on drug efficacy, but a retrospective study with a small number of AD cases in Croatia showed the intriguing result of 71% responders to galantamine treatment among *APOE-4* homozygotes (537).

MacGowan et al. (538) reported that gender is likely to be a more powerful determinant of outcome of anticholinesterase treatment than *APOE* status in the short term. In contrast, other studies do not support the hypothesis that *APOE* and gender are predictors of the therapeutic response of AD patients to tacrine or donepezil (539,540). Petersen et al. (541) showed that *APOE-4* carriers exhibited a better response to donepezil. Similar results have been found by Bizzarro et al. (542); however, Rigaud et al. (540) did not find any significant difference between *APOE-4*-related responders and nonresponders to donepezil. An *APOE*-related differential response has also been observed in patients treated with other compounds devoid of acetylcholinesterase (AChE)-inhibiting activity (CDP-choline, anapsos) (543,544), suggesting that *APOE*-associated factors may influence drug activity in the brain, either directly acting on neural mechanisms or indirectly influencing diverse metabolic pathways (545).

To date, few studies have addressed in a prospective manner the impact of pharmacogenetic and pharmacogenomic factors on AD therapeutics (see Table 10.10). Because *APOE*, *PS1*, and *PS2* genes participate in AD pathogenesis regulating neuronal function and brain amyloidogenesis, in an attempt to envision the potential influence of major AD-associated genes on the therapeutic response in AD patients, we have performed the first pharmacogenomic study in AD using a genetic matrix model (trigenic haplotype-like model) to identify the response of a multifactorial therapy in different AD genotypes combining allelic associations of *APOE* plus *PS1* plus *PS2* genes (14). With this strategy, we have demonstrated that the therapeutic response in AD is genotype specific, with *APOE-4/4* carriers as the worst responders, and that some polymorphic variants exert a dominant effect on treatment outcomes (6,13–16,18–20,29) (see Figs. 10.14 and 10.15). From these studies, we can conclude the following: First, multifactorial treatments combining neuroprotectants, endogenous nucleotides, nootropic agents, vasoactive substances, ChEIs, and NMDA antagonists associated with metabolic supplementation on an individual basis adapted to the phenotype of the patient may be useful to improve

Table 10.10 First generation of pharmacogenomic studies in Alzheimer’s disease with cholinesterase inhibitors, noncholinergic drugs, and multifactorial therapy

Drug	Type	Genotype	Study type	Result	Outcome measure	Comment
Tacrine	Cholinesterase inhibitor	<i>APOE</i>	Retrospective	Genotype dependent	Cognition	Controversial results depending on authors and study model
Donepezil	Cholinesterase inhibitor	<i>APOE</i>	Retrospective	Genotype dependent	Cognition	Controversial results depending on authors and study model
Rivastigmine	Cholinesterase inhibitor	<i>APOE</i>	Retrospective	Unclear	Cognition	Unconclusive data
Galantamine	Cholinesterase inhibitor	<i>APOE</i>	Prospective	Unrelated	Cognition	Controversial results depending on authors and study model
CDP-choline	Choline donor Endogenous nucleotide	<i>APOE</i>	Retrospective Prospective	Genotype dependent Genotype dependent	Cognition Brain blood flow	Replicated in two studies
Anapso	Neuro-immunotrophic agent	<i>APOE</i>	Prospective	Genotype dependent	Brain mapping Bio-chemistry Cognition	Replicated in two studies
Memantine	Natural product NMDA antagonist	<i>APOE</i>	Prospective	Apoptosis related	Immune function Cognition	One study
Cerebrolysin	Neurotrophic factor	<i>PS1</i> <i>PS2</i> <i>APOE</i>	Prospective	Unrelated	Cognition	Gene-to-gene analysis One study Monogenic

CDP-choline	Combination therapy	<i>APOE</i>	Retrospective	Genotype dependent	Cognition	Genomic analysis integrating <i>APOE</i> + <i>PS1</i> + <i>PS2</i> in a trigenic matrix model
Piracetam	Endogenous nucleotide + nootropic agent + neuro-immunotrophin	<i>PS1</i>			Brain blood flow	
Anapsos		<i>PS2</i>		Cluster dependent	Brain mapping Immune markers	
CDP-choline	Combination therapy	<i>APOE</i>	Prospective	Genotype dependent	Cognition	Replicated in two studies One study Monogenic + trigenic cluster analysis
Piracetam	Endogenous nucleotide + nootropic agent	<i>PS1</i> <i>PS2</i>		Cluster dependent	Lipid metabolism	
CDP-choline	Combination therapy	PRNP-M129V	Retrospective	Unrelated	Brain blood flow Brain mapping Immune markers Cognition	One study Monogenic analysis
Piracetam						
CDP-choline	Combination therapy	<i>APOE</i>	Retrospective	Cluster-dependent	Cognition	One study Tetragenic cluster analysis
Piracetam		<i>PS1</i> <i>PS2</i> PRNP			Biochemical markers Hematological markers	

(continued)

Table 10.10 (continued)

Drug	Type	Genotype	Study type	Result	Outcome measure	Comment
CDP-choline	Combination therapy	<i>CYP2D6</i>	Prospective	<i>CYP2D6</i> dependent	Cognition	One study
Nicergoline	Endogenous				Extensive metabolizers	
Donepezil	nucleotide + vasoactive agent + cholinesterase inhibitor				Intermediate metabo- lizers	
					Poor metabolizers	
					Ultrarapid metabolizers	
LipoEsar	Monotherapy	<i>APOE</i>	Prospective	<i>APOE</i> dependent	Lipid metabolism	One study
	Marine lipoprotein					
CDP-choline	Combination therapy	<i>APOE</i>	Prospective	<i>APOE</i> , <i>ACE</i> dependent	Atheroma plaque size Cognition	One study Monogenic + bigenic cluster analysis
Nicergoline	Endogenous nucleotide + vasoactive agent + marine lipoprotein + nutraceutical product	<i>ACE</i>			Anxiety	
LipoEsar					Depression	
Animon					Lipid metabolism	
					Brain hemodynamics	

ACE, angiotensin-converting enzyme; *APOE*, apolipoprotein E; *CYP*, cytochrome P450; *NMDA*, *N*-methyl-D-aspartate; *PS*, presenilin, *CDP*, choline, Citidine-diplospho-choline, *PRNP*, Prior Protein gene.
Source: Adapted from refs. 19 and 20.

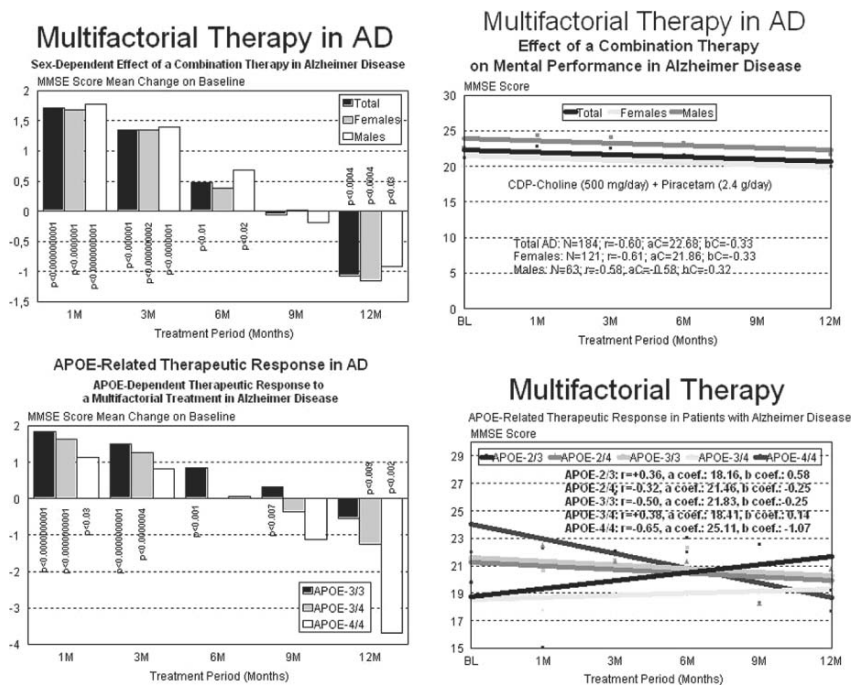


Fig. 10.14 Multifactorial therapy in Alzheimer's disease (AD). Apolipoprotein E (APOE)-related therapeutic response. (Adapted from refs. 13–16 and 18–20.)

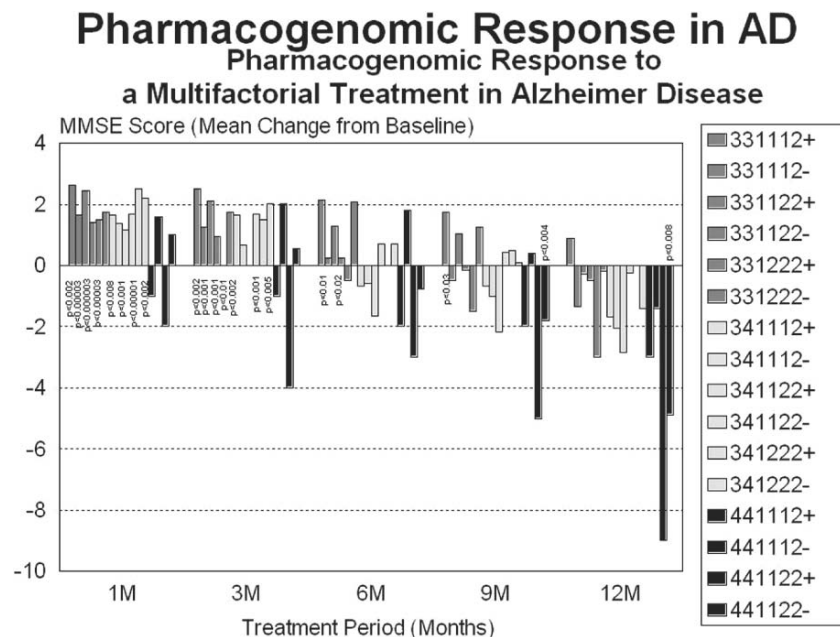


Fig. 10.15 Pharmacogenomic response to a multifactorial therapy in Alzheimer's disease (AD) according to a trigenic cluster integrated by the apolipoprotein E (APOE), presenilin 1 (PS1), and presenilin 2 (PS2) genes. (Adapted from refs. 13, 20, and 291.)

cognition and slow disease progression in AD. Second, in our personal experience, the best results have been obtained combining (1) CDP-choline with piracetam and metabolic supplementation; (2) CDP-choline with piracetam and anapsos; (3) CDP-choline with piracetam and ChEIs (donepezil, rivastigmine); (4) CDP-choline with memantine; and (5) CDP-choline, piracetam, and nicergoline. Third, some of these combination therapies have proven to be effective, improving cognition during the first 9 mo of treatment, and not showing apparent side effects. Fourth, the therapeutic response in AD seems to be genotype-specific under different pharmacogenomic conditions. Fifth, in monogenic-related studies, patients with the *APOE*-2/3 and *APOE*-3/4 genotypes are the best responders, and *APOE*-4/4 carriers are the worst responders (see Fig. 10.14). Sixth, *PS1*- and *PS2*-related genotypes do not appear to influence the therapeutic response in AD as independent genomic entities; however, *APP*, *PS1*, and *PS2* mutations may drastically modify the therapeutic response to conventional drugs. Seventh, in trigenic-related studies, the best responders are those patients carrying the 331222-, 341122-, 341222-, and 441112-genomic clusters (see Fig. 10.15). Eighth, a genetic defect in exon 5 of the *PS2* gene seems to exert a negative effect on cognition, conferring *PS2*+ carriers in trigenic clusters the condition of poor responders to combination therapy. Ninth, the worst responders in all genomic clusters are patients with the 441122+ genotype (see Fig. 10.15). Tenth, the *APOE*-4/4 genotype seems to accelerate neurodegeneration, anticipating the onset of the disease by 5–10 years, and in general, *APOE*-4/4 carriers show faster disease progression and poorer therapeutic response to all available treatments than any other polymorphic variant. Finally, pharmacogenomic studies using trigenic, tetragenic, or polygenic clusters as a harmonization procedure to reduce genomic heterogeneity are very useful to widen the therapeutic scope of limited pharmacological resources (6,13–16,18–20,29).

10.4.4.4 Influence of *APOE*-*CYP2D6* Interactions on Alzheimer's Disease Therapeutics

APOE influences liver function and *CYP2D6*-related enzymes, probably via regulation of hepatic lipid metabolism (42,469). It has been observed that *APOE* may influence liver function and drug metabolism by modifying hepatic steatosis and transaminase activity. There is a clear correlation between *APOE*-related TG levels and GOT, GPT, and GGT activities in AD (469). Both plasma TG levels and transaminase activity are significantly lower in AD patients harboring the *APOE*-4/4 genotype, probably indicating (1) that low TG levels protect against liver steatosis, and (2) that the presence of the *APOE*-4 allele influences TG levels, liver steatosis, and transaminase activity. Consequently, it is very likely that *APOE* influences drug metabolism in the liver through different mechanisms, including interactions with enzymes such as transaminases or *CYP*-related enzymes encoded in genes of the *CYP* superfamily (469).

When *APOE* and *CYP2D6* genotypes are integrated in bigenic clusters and the *APOE* plus *CYP2D6*-related therapeutic response to a combination therapy is ana-

lyzed in AD patients after 1 yr of treatment as in Fig. 10.11, it becomes clear that the presence of the *APOE*-4/4 genotype is able to convert pure *CYP2D6**1/1 EMs into full PMs (see Fig. 10.16), indicating the existence of a powerful influence of the *APOE*-4 homozygous genotype on the drug-metabolizing capacity of pure *CYP2D6* EMs.

10.4.4.5 New Insights into *APOE*-Related Pathogenesis and Therapeutics in Alzheimer’s Disease

APOE is a pleiotropic gene with many polymorphic activities, most of them influencing AD pathogenesis (see Fig. 10.13). In this regard, the influence of *APOE* variants on AD therapeutics cannot be neglected, especially taking into account that (1) *APOE* polymorphic variants by themselves are enough to modify the therapeutic response to conventional antedementia drugs; (2) *APOE* interacts with many receptors and participates in a large number of metabolic cascades and signaling pathways; and (3) the presence of the *APOE*-4 allele can alter the phenotypic profile of *CYP2D6* genotype-related drug metabolizers and probably of other *CYP* enzymes, such as those encoded by the *CYP3A5* gene, which affect more than 50% of the drugs currently prescribed in the clinical setting. Moreover, many metabolic pathways in which *APOE* participates (e.g., lipid metabolism, *APP*/*ABP* processing, cardiovascular and cerebrovascular function, etc.) are involved in pathogenic processes that represent major risk factors of dementia (e.g., atherosclerosis, hypercholesterolemia, hypertension, brain hypoperfusion), which can be potentially predictable and preventable with therapeutic intervention.

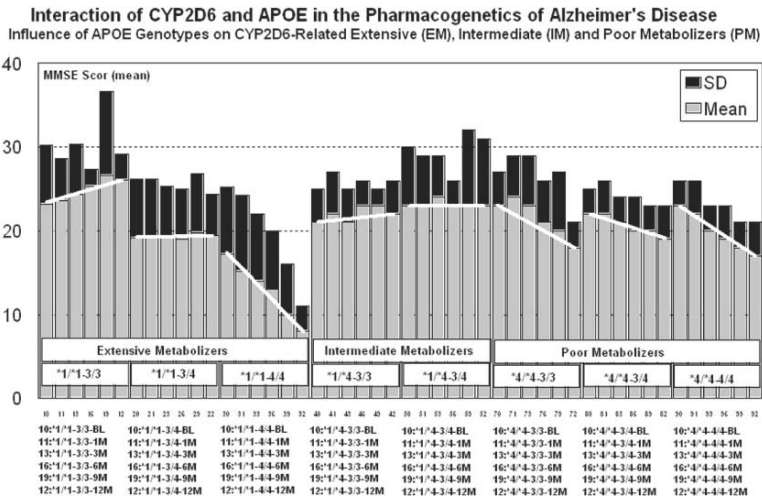


Fig. 10.16 Interaction of cytochrome *P450* (*CYP*) *2D6* and apolipoprotein E (*APOE*) in the pharmacogenetics of Alzheimer’s disease

APOE is a ligand for the seven identified mammalian members of the evolutionarily conserved LDL receptor (LDLR) family: *APOE* receptor 2 (*APOER2*), the VLDL receptor (VLDLR), multiple epidermal growth factor (EGF) repeat-containing protein (MEGF7), megalin, LDL-related protein 1 (LRP1), and LDL-related protein 1b (LRP1b) (546). The LDLR family consists of over ten receptors that function in receptor-mediated endocytosis and cellular signaling. Together with LDLR itself, the family includes LRP/LRP1, megalin/LRP2, VLDLR, *APOER2*/LRP8, SORLA-1/LR11, LRP4, LRP5, LRP6, and LRP1B. Most of the *APOE* receptors have been found in the CNS, where they participate in endocytosis, intracellular signaling, synaptic plasticity, and ABP metabolism (546). *APOE* receptors have been suggested to act as clearance mechanisms for ABP and have also been implicated in the production of ABP. LRP interacts with *APP* through the intracellular adaptor protein FE65 or via direct binding to the KPI domain, and its endocytosis facilitates *APP* endocytic trafficking and ABP production (547–549). SORLA/LR11 alters *APP* trafficking and *APP* processing by β - and γ -secretases (550–552). It has also been suggested that soluble *APOE* receptors could play roles as dominant negative regulators of *APOE*, and thus understanding their generation and actions might be important for understanding normal and pathological functions of *APOE* in the CNS and in AD (546).

It might be possible that normalization of biological parameters associated with *APOE*-related pathogenic pathways contributing to brain dysfunction and neurodegeneration could be beneficial in terms of prevention or slowing the clinical course of dementia. In this strategic category, we can include the following: (1) lipid metabolism dyshomeostasis, (2) *APOE*-related *APP*/ABP processing, (3) blood pressure control, (4) atherogenesis, (5) cerebrovascular hemodynamics, and (6) neuroprotection. Some examples can illustrate the technical feasibility of these interventions and the differential effect of *APOE* allele dosage on efficacy issues as well.

The *APOE*–Cholesterol Binomial Conundrum

Cardiovascular and cerebrovascular disorders associated with lipid metabolism disturbance and atherosclerosis represent major risk factors for dementia (3,25,59). Atherosclerosis is the primary cause of heart disease and stroke in which genetic and environmental factors converge (553). More than 90% of patients older than 70–80 yr with dementia show signs of atherosclerosis in their arteries and a clear cerebrovascular component in their dementia process. It is very likely that pure AD is practically absent in octogenarians, in whom the prevalent diagnosis is vascular or mixed dementia (3,25,59), in which the *APOE*-4 allele also accumulates (18–20,554).

APOE genotypes directly influence lipid metabolism and atherosclerosis (see Fig. 10.17). The presence of the *APOE*-4 allele contributes to the phenotypic manifestation of atherosclerosis, brain amyloid angiopathy, and cerebral white

Effect of LipoEsar (E-SAR-94010) on Atheroma Plaques

APOE-related anti-atherogenic effect of LipoEsar in patients with chronic hyperlipidemia

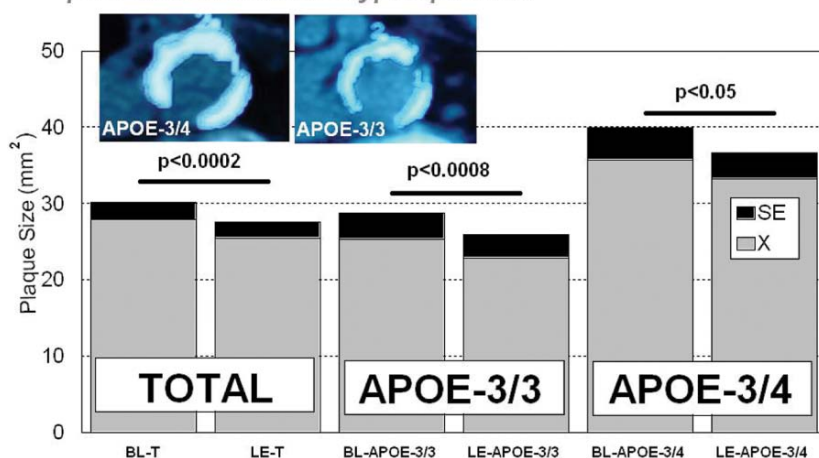


Fig. 10.17 Apolipoprotein E (*APOE*)-related therapeutic efficacy of E-SAR-94010 on atheroma plaques in the abdominal aorta of patients with chronic hyperlipidemia. (Adapted from refs. 19 and 20.)

matter damage (555). The size of atheroma plaques in the abdominal and thoracic aortas of patients with dementia or dyslipidemia is significantly larger in *APOE-4* carriers than in *APOE-3* carriers (18–20) (see Fig. 10.17). In addition, the effect of lipid-lowering agents on atheroma plaques is *APOE* related, with a more effective response in *APOE-3* carriers (18–20) (see Fig. 10.17).

Evidence from epidemiological, in vitro, and in vivo studies suggests that brain cholesterol may play a role in AD. The exact nature and magnitude of this role is unknown, but a number of possibilities have emerged, including modulation of *APP* cleavage pathways and ABP production and clearance, *APOE*-mediated cholesterol transport, and cholesterol efflux from the brain (556–558).

Cholesterol is implicated in the production of ABP, the primary constituent of senile plaques in the AD brain (559–563). In *APP* transgenic mice, hypercholesterolemia correlates with increased ABP levels and more severe amyloid plaque load (564,565). Some retrospective epidemiological studies indicated that statin therapy might decrease AD risk (566), but statins do not alter serum ABP levels (567,568) and in some cases may worsen cognitive function, increase brain ABP load, or activate inflammatory responses involving microglia (3,25,569). Some studies have reported an association between high cholesterol levels and AD risk (570) and increased brain ABP1-42 levels (571). Defective binding of *APOE* to HSPGs is associated with increased risk of atherosclerosis and AD, probably because of the inefficient clearance of lipoprotein remnants from the liver with negative consequences for neuronal repair (572). *CYP46**C (cholesterol 24-hydroxylase) along

with *APOE-4* were associated with higher cognitive decline in AD, and both variants synergistically increase the risk of AD (573–575) as well as brain and CSF ABP load (576). Deficiency of the cholesterol transporter ABCA1 produced by glial cells impairs *APOE* metabolism in the CNS (577).

Some studies also indicated that genetic variants of *ABCA1* modify AD risk and tau- and ABP-related pathogenesis (578); however, other studies have demonstrated that several SNPs in the multidrug resistance (*ABCB1*) gene (*MRD1*) (*C1236T* in exon 12, *G2677T/A* in exon 21, and *C3435T* in exon 26) do not show association with AD (579); in contrast, *ABCA2* has been reported to be a strong genetic risk factor for EOAD (580,581). The ABC superfamily consists of membrane proteins that transport a wide variety of substrates across membranes (582). *ABCA1* and *ABCG1* play a pivotal role in the regulation of neuronal cholesterol to *APOE* disks and in suppression of *APP* processing to generate ABP. *ABCA1* is required for normal brain *APOE* levels and for lipidation of astrocyte-secreted *APOE* (583), and the absence of *ABCA1* decreases soluble *APOE* levels but does not diminish ABP deposition in AD murine models (584). Others have reported increased ABP deposition in *APP23* and *PDAPP* mice in the absence of *ABCA1*, suggesting that despite substantially lower *APOE* levels, poorly lipidated *APOE* produced in the absence of *ABCA1* is strongly amyloidogenic (585,586).

ABCA1 protein expression is induced by ligands of the nuclear hormone receptors of the retinoid X receptor and LXR family. Treatment of neuroblastoma cells with retinoic acid and 22(R)-hydroxycholesterol causes significant increases in secreted ABP40 and ABP42, and treatment with a nonsteroidal LXR ligand, TO-901317, similarly increases ABP40 and ABP42 levels, which can be reduced by RNAi blocking of *ABCA1* expression (587).

Maintenance of an adequate supply of cholesterol is important for neuronal function, whereas excess cholesterol promotes *APP* cleavage and generation of toxic ABP isoforms (588). Impaired recycling of *APOE-4* is associated with intracellular cholesterol accumulation (589). Cholesterol- and sphingolipid-rich membrane microdomains are involved in regulating trafficking and processing of *APP*. In this metabolic pathway, the amyloidogenic processing of *APP* depends on lipid rafts because access of α - and β -secretase to *APP* may be determined by dynamic interactions of *APP* with membrane lipid microdomains (349). γ -Secretase is also located in lipid raft microdomains of post-Golgi and endosomes that are implicated in *APP* processing (590). Methyl- β -cyclodextrin and leptin reduce β -secretase activity in neuronal cells possibly by altering the lipid composition of membrane lipid rafts. This phenotype contrasts treatments with cholesterol and etomoxir, an inhibitor of carnitine-palmitoyl transferase 1. Conversely, inhibitors of acetyl CoA carboxylase and fatty acid synthase mimic leptin's action. Leptin is also able to increase *APOE*-dependent ABP uptake in vitro; thus, leptin can modulate bidirectional ABP kinesis, reducing its levels extracellularly. The chronic administration of leptin to AD-transgenic animals notably reduces the brain ABP load (591).

APOE-4 may affect AD risk by conferring high cholesterol levels and thereby increasing ABP production (592). *APOE-4* carriers with AD have increased levels of brain and CSF ABP and have more extensive plaque pathology (593,594);

however, with a genomic-based approach, by using *APOE* knockin mice, which express each human allele under the endogenous regulatory elements, on a defined C57BL6/J background, Mann et al. (592) demonstrated that the presence of *APOE* significantly increases brain ABP levels, irrespective of genotype, indicating an independent role for *APOE* in cholesterol metabolism in the periphery relative to the CNS.

In humans, probably thousands of genes may regulate lipid metabolism. Some relevant genes, such as *APOE* (50%), *CETP* (28%), *LIPC* (9%), *APOB* (8%), and *LDLR* (5%), may influence variation in LDL; *LIPC* (53%), *CETP* (25%), *ABCA1* (10%), *LPL* (6%), and *LDLR* (6%) may influence HDL variance (595). The *APOE*-2 allele is associated with the lower and the *APOE*-4 allele with the higher total plasma cholesterol and LDL cholesterol levels compared with the *APOE*-3 allele (596). Individuals with *APOE*-2 and *APOE*-3 reduce plasma cholesterol and LDL cholesterol levels more than *APOE*-4 individuals treated with HMG-CoA reductase inhibitors (statins), gemfibrozil and cholestyramine. In contrast, *APOE*-4 carriers might respond better than carriers of other genotypes to probucol. Perimenopausal women with *APOE*-2 or *APOE*-3 genotypes appear to improve plasma lipoprotein-lipid profiles more than *APOE*-4 women under protocols with hormone replacement therapy. Likewise, *APOE*-2 and *APOE*-3 individuals tend to improve plasma lipid profiles with exercise training more than *APOE*-4 individuals (597).

In an attempt to reverse the *APOE* deficit in AD, Poirier (598) reported the identification and characterization of several *APOE* inducer agents using a low-throughput screening assay. The old cholesterol-lowering drug probucol led to significant increases in CSF *APOE* levels and a decrease of CSF ABP1-42, with an effect on CSF tau or lipid peroxides levels (598). In a prospective, dose-finding, 36-wk treatment trial with statins (simvastatin or atorvastatin) conducted in 39 patients with hypercholesterolemia, ABP levels remained unchanged (599). The Heart Protection Study Collaborative Group (600) and the Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) (601) have both reported that neither simvastatin nor pravastatin appeared to slow cognitive decline in the elderly during 5 yr of treatment in the Heart Protection Study and 3.2 yr in the PROSPER.

Since *APOE* can protect against cardiovascular disease (e.g., coronary artery disease) via hepatic removal of atherogenic remnant proteins, sequestration of cholesterol from vessel walls and local antioxidant, antiplatelet, and anti-inflammatory actions, it has been postulated that *APOE* gene transfer might ameliorate a hyperlipidemic profile and exert a beneficial effect at lesion sites to prevent or regress atherosclerosis (602). Using plasmid vectors expressing allelic human *APOE*-2 or *APOE*-3 isoforms, Athanasopoulos et al. (602) demonstrated that skeletal muscle was an effective secretory platform for *APOE* gene augmentation, and that muscle-based expression of *APOE*-2 after intramuscular plasmid injection in *APOE*-/- mice was able to reduce atherosclerotic lesions in proximal aorta by 20–30%, with total abolishment of gross dorsal xanthoma (>5-mm diameter) up to 9 mo following a single *APOE*-2 plasmid administration. The same group of Dickson (603), 2 yr later, with an improved technology, observed an acute regression

of advanced and retardation of early aortic atheroma in immunocompetent *APOE*-deficient mice by administration of a second-generation (E1-, E3-, polymerase-) adenovirus vector expressing human *APOE*. Intramuscular injections resulted in low expression of *APOE* and afforded no sustainable protection against atherogenesis; in contrast, intravenous (liver-directed) injections into *APOE*^{-/-} mice resulted in increased plasma *APOE* levels accompanied by reductions in plasma cholesterol and normalization of lipoprotein profiles. Liver-directed *APOE* gene transfer to these mice retarded progression of atherosclerosis by 38% during the 70-d study period, with a progressive decline in *APOE* levels and no evoked humoral immune response (603).

10.4.5 Angiotensin-Converting Enzyme in Alzheimer's Disease

Angiotensin I-converting enzyme (EC 3.4.15.1) [kininase II; dipeptidyl carboxypeptidase 1, carboxycathepsin, dipeptide hydrolase, peptidase P, peptidyl dipeptidase-4(A), peptidyl-dipeptide hydrolase] is a zinc metallopeptidase with dipeptidyl carboxypeptidase activity that regulates blood pressure, the renin-angiotensin system, the kinin-kallikrein cascade, and electrolytic balance by hydrolyzing angiotensin I into angiotensinogen. *ACE* is the target of the *ACE* inhibitor family of drugs (captopril, enalapril, fosinopril, imidapril, lisinopril) currently used as antihypertensive agents.

The *ACE* gene maps on 17q23 and encodes a 732-residue preprotein with a 31-residue signal peptide and a mature molecular weight of 80,073. *ACE* contains two large homologous active domains, the N- and C-terminal domains. The properties of these two domains differ in different ways: (1) The N-domain is less activated by chloride ions than the C-domain; (2) inhibition kinetics of the two active centers are not identical; (3) both domains cleave bradykinin, angiotensin I, and substance P, but the rates of hydrolysis of these substrates are dissimilar; and (4) the C-domain catalyzes angiotensin I about 3 times faster than the N-domain (604), whereas the N-domain cleaves LHRH 12 times faster than the C-domain (605).

The *ACE* gene encodes two isozymes (somatic *ACE* isozyme and germinal *ACE* isozyme). *ACE* is a membrane-bound enzyme on the surface of vascular endothelial cells that also circulates in plasma and shows great individual variability determined by an I/D polymorphism in intron 16 of the *ACE* gene (*ACE*-I/D polymorphism). More than 160 *ACE* polymorphisms have been reported, 34 of which are located in coding regions, and 18 are missense mutations (606). *ACE*-related polymorphic variants have been associated with hypertension, atherosclerosis, stroke, left ventricular hypertrophy, chronic renal failure in IgA nephropathy, Henoch-Schonlein purpura nephritis, mechanical efficiency of skeletal muscle, intracranial aneurysms, susceptibility to myocardial infarction, diabetic nephropathy, AD, and longevity (12,606,607).

The metalloprotease *ACE*-secretase cleaves *ACE* between Arg1203 and Ser1204 on the extracellular side of the TM domain to generate a C-terminal

truncated soluble or plasma form of the enzyme (608). *ACE* acts on multiple substrates (608). *ACE* isoforms hydrolyze a spectrum of circulating peptides and catalyze the hydrolysis of substance P, angiotensin 1–9, *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), cholecystokinin, hemopressin, the vasodilator peptides bradykinin and kallidin, as well as ABP (608).

Many studies showed association between *ACE-I/D* indel variants and AD (12,609–620). The polymorphism at intron 16 of the *ACE* gene, consisting of a 287-bp *I/D* is associated with *ACE* concentrations, and the *ACE-D/D* genotype is associated with cardiovascular disorders and arterial occlusive disease. The frequencies of the *ACE-D* and *ACE-I* alleles are higher in AD (55.7%, 51.7%) than in controls (44.2%, 48.2%) (613), with an overrepresentation of the *ACE-D/D* genotype in AD (12). In general, *ACE-D* is more frequent in those with dementia than in healthy subjects; however, a significantly higher frequency of the *ACE-D* allele is seen in vascular dementia (0.66, $p < 0.008$) and mixed dementia (0.65, $p < 0.01$) than in controls (0.50) (12,614,615). In contrast, the *ACE-I* allele tends to be more frequent in LOAD (0.59) than in controls, probably indicating that the potential pathogenic role of *ACE* in dementia is more relevant in those cases with a vascular component (615). In fact, enhanced brain renin–angiotensin system immunoreactivity has been detected in cortical vessels with microvascular pathology (621), and *ACE* density was increased in the temporal cortex of AD patients (622).

The distribution of *ACE* genotypes in the Spanish population was as follows: *ACE-I/I* 14.8% in controls and 20.5% in AD; *ACE-I/D* 45.6% in controls and 46.0% in AD; and *ACE-D/D* 39.6% in controls and 33.5% in AD (623). In this study, a small increase in the frequency of *ACE-I* allele was observed in EOAD (623). Nevertheless, as with many other polymorphic genes, some authors did not find association between the *ACE-I/D* polymorphism and AD in different populations (80,617,624–631). However, a meta-analysis of 12 case–control series published up to the year 2000 suggested that *ACE* genotypes are associated with AD (611), and that the *ACE-D* allele is more frequent in Spanish, American, and Russian AD patients than in controls (614,615,617) but not in the Italian and Jewish populations (627,632).

In the Japanese population, the frequency of the *ACE-I/I* genotype is 1.4 times higher in AD than in controls, whereas the *ACE-D/D* genotype is only 0.4 times higher (633). In this population, in whom *ACE-I/I* associates with AD, it has been demonstrated that *ACE* degrades ABP; retards ABP aggregation, deposition, and fibril formation; and inhibits cytotoxicity in a dose-dependent manner, suggesting that *ACE* may affect susceptibility to AD by degrading ABP and preventing the accumulation of ABP in plaques and vessels (634).

In the Chinese population, *ACE* genotypes associated with AD, but the *ACE-I/I* genotype was identified as a risk factor for AD (635), and in other Chinese studies the *ACE-I/I* genotype as a risk factor was restricted only in subjects with hypertension (636). In a study in the United Kingdom, the frequency of the *ACE-I* allele was not increased in AD, and in autopsy-confirmed AD cases, possession of the *ACE-I* allele had no impact on the pathology of AD, at least in terms of the amount of BAP

or tau deposited in the brain (637). In confirmed necropsy LOAD cases, homozygotes for both the *I* and *D* alleles were associated with a higher risk compared to *I/D* heterozygotes (611).

In a large meta-analysis with 39 samples, comprising 6037 cases of AD and 12,099 controls from three ethnic groups (North Europeans, South Caucasians, and East Asians), *D* homozygotes were at reduced risk of AD, *ACE-I* homozygotes showed no association with AD, while heterozygotes were at increased risk (619). These results confirmed the association of *ACE-I/D* with AD across diverse populations, although this might probably be caused by linkage disequilibrium with the true risk factor (619).

White matter hyperintensities in magnetic resonance imaging (MRI) scans reflecting cerebrovascular damage and brain hypoperfusion are more severe in *ACE-D/D* subjects (638). Association of *ACE-D/D* with vascular dementia has also been reported (615,639), although in some studies no association of *ACE-I/D* indel variant with vascular dementia was found (640).

It was demonstrated that the N-terminal active center of human *ACE* degrades ABP1-40 in vitro (641). *ACE*-His at concentrations of 100 and 1000 nM reduced ABP aggregation to 57% and 47% of control values, respectively; the same concentrations of N-His reduced ABP aggregation to 33% and 35%, respectively, and C-His at the same concentrations did not modify ABP aggregation (641). Wild-type *ACE* from human seminal plasma catalyzes degradation of ABP1-40 at the site Asp7-Ser8 (634), and ABP does not affect *ACE* C-domain inhibition (641).

It has been postulated that *ACE* prevents ABP accumulation in the brain (634), and that treatment with *ACE* N-terminal domain-related peptides might be a potential therapeutic strategy in AD (641). Eckman et al. (642) analyzed ABP accumulation in brains from *ACE*-deficient mice and in mice treated with *ACE* inhibitors and found that *ACE* deficiency did not alter steady-state ABP concentration; in contrast, ABP levels were significantly elevated in *ACE* and *NEP* knock-out mice, and inhibitors of these enzymes cause a rapid increase in ABP concentration in the brain (642). In contrast, Hemming and Selkoe (643) reported that ABP is degraded by *ACE* and elevated by *ACE* inhibitors, such as captopril, raising the question of whether currently prescribed *ACE* inhibitors could elevate brain ABP levels in humans.

10.4.5.1 *ACE*-Related Phenotypic Profiles in Alzheimer's Disease

The *ACE*-related biochemical and hemodynamic phenotypes have been studied in patients with AD (12,20). *ACE-I/I* patients tend to be younger than *ACE-I/D* or *ACE-D/D* patients at the time of diagnosis and also show more severe cognitive deterioration. Serum *APOE*, total cholesterol, LDL cholesterol, HDL cholesterol, NO, histamine, and *ACE* levels are higher in *ACE-I/I* carriers than in patients with the other genotypes; in contrast, serum TG and VLDL levels are notably lower in *ACE-I/I* patients compared to patients harboring the *ACE-I/D* or

ACE-D/D genotypes, whereas ABP levels do not show any clear difference among *ACE*-related genotypes. Cerebrovascular function tends to be worse in *ACE-D/D*, with lower brain blood flow velocities and higher pulsatility and resistance indices, than in *ACE-I/D* (intermediate cerebrovascular hemodynamics) or *ACE-I/I* (almost normal cerebrovascular function) (12,20). The correlation between lipid levels and brain hemodynamics is very similar in this study to data observed in *CYP2D6*-related metabolizer profiles in which EM patients with moderate cholesterol and lipoprotein levels (as well as relatively high NO, histamine, *ACE*, and *APOE* levels) tended to show a better cerebrovascular hemodynamic profile than AD patients with lower cholesterol and lipoprotein levels (42). This apparently paradoxical correlation appears to indicate that major players in cerebrovascular homeostasis and hemodynamic brain blood flow are cholesterol, lipoproteins, NO, *ACE*, and histamine, among many other factors, in AD, and that peripheral levels of ABP are indifferent in this concern. On the other hand, it seems likely that low TG levels may facilitate cerebrovascular function. It is also worth mentioning that *ACE-I/I* patients with the highest cholesterol levels are the worst in mental performance. Another interpretation of these data might suggest an association between poor cerebrovascular function with *ACE-D/D* and *ACE-I/D* and an association between alterations in lipid metabolism in *ACE-I/I* (12,20).

10.4.5.2 *ACE*-Related Therapeutic Response to a Multifactorial Treatment in Alzheimer's Disease

No studies have been reported concerning the role of *ACE* in the therapeutic response to specific treatments in AD, with the exception of *ACE* inhibitors in hypertension and cardiovascular disorders. The positive effects of *ACE* inhibitors were thought to be the consequence of reducing angiotensin II levels and the degradation of bradykinin; however, some of the beneficial effects of *ACE* inhibitors can be attributed to novel mechanisms, including the accumulation of the *ACE* substrate *N*-acetyl-seryl-aspartyl-lysyl-proline, which blocks collagen deposition in the injured tissues, as well as the activation of an *ACE* signaling cascade that involves the activation of the kinase CK2 and JNK in endothelial cells and leads to changes in gene expression (608). Because hypertension, cardiovascular disorders, and alterations in cerebrovascular hemodynamics clearly affect brain perfusion, contributing to accelerate neuronal death in susceptible patients, it would be worthwhile to evaluate the effect of different *ACE* variants on cognition in AD patients treated with conventional antidementia drugs. It has also been observed that hypertensive and hypotensive patients are at risk of developing AD, and that the *APOE-4* allele accumulates in hypertensive subjects (1,12–16,18–20). In addition, patients treated with *ACE* inhibitors may show an increased rate of mood disorders, such as depression- and anxiety-like symptoms. Taking into consideration all these observations, the effects of *ACE* polymorphic variants, either alone or in conjunction with *APOE*-related genotypes, on cognitive performance and mood

disorders have been studied, under pharmacogenomic protocols, in AD patients treated with multifactorial therapy.

ACE-Related Cognitive Performance in Response to a Multifactorial Therapy in Alzheimer's Disease

For 1 yr, EOAD and LOAD patients ($N = 463$; 257 females and 206 males; age 63.51 ± 13.12 yr; range 40–98 yr) received a multifactorial therapy (CNLA protocol) integrated by CDP-choline (500 mg/d po), nicergoline (5 mg/d po), E-SAR-94010 (LipoEsar®) (250 mg tid), and Animon Complex® (2 capsules/d). E-SAR-94010 is a marine lipoprotein derivative extracted from *Sardina pilchardus* and with powerful antiatherosclerotic and plasma lipid-lowering activities; its therapeutic properties exhibit an *APOE*-related profile (18–20) (see Fig. 10.17). Animon Complex is a nutraceutical compound integrated by a purified extract of *Chenopodium quinoa* (250 mg), ferrous sulfate (38.1 mg equivalent to 14 mg of iron), folic acid (200 µg), and vitamin B₁₂ (1 µg) per capsule (RGS 26.06671/C). Patients with chronic deficiency of iron (<35 µg/mL), folic acid (<2.5 ng/mL) or vitamin B₁₂ (<150 pg/mL) received an additional supplementation of iron (80 mg/d), folic acid (5 mg/d), and B complex vitamins (15 mg/d B₁; 15 mg/d B₂; 10 mg/d B₆; 10 µg/d B₁₂; 50 mg/d nicotinamide) to maintain stable levels of serum iron (50–150 µg/mL), folic acid (5–20 ng/mL), and vitamin B₁₂ (500–1000 pg/mL) to avoid the negative influence of all these metabolic factors on cognition (12,12–16,18–20). All patients underwent the EuroEspes protocol for dementia, consisting of the following: (1) medical examination (general, psychiatric, and neurological evaluation); (2) psychometric assessment (cognition: MMSE, ADAS-Cog, FAST, GDS; behavior: HRS-A/D; BPRS; Behave-AD; functioning: ADL); (3) blood and urine analyses; (4) chest and neck X-ray; (5) brain computed tomographic (CT) scan; (6) brain mapping (qEEG); (7) electrocardiographic (ECG) and blood pressure monitoring; (8) cerebrovascular assessment (transcranial Doppler ultrasonography); and (9) genetic testing (*APP*, *PS1*, *PS2*, *APOE*, *MAPT*, *FOS*, *A2M*, *ACE*, *NOS3*, *MTHFR*, *CETP*). Psychometric assessment was carried out at baseline and at 1, 3, 6, 9, and 12 mo during the treatment period. Major psychotropic drugs (antidepressants, benzodiazepines, neuroleptics) were avoided during the study in more than 90% of the patients.

The distribution of *ACE* indel variants was as follows: (1) *ACE-D/D* (35.64%) ($N = 165$; 97 females, age 64.86 ± 13.08 yr, range 41–91 yr; 68 males, age 63.49 ± 12.45 yr, range 44–96 yr); (2) *ACE-I/D* (49.46%) ($N = 229$; 122 females, age 63.29 ± 13.77 yr, range 42–91 yr; 107 males, age 63.35 ± 13.10 yr, range 40–98 yr); and (3) *ACE-I/I* (14.90%) ($N = 69$; 38 females, age 64.76 ± 12.57 yr, range 45–90; 31 males, age 59.22 ± 13.06 yr, range 40–84 yr). All *ACE-I/D* variants showed no differences in weight, height, or heart rate. *ACE-I/D* carriers had the highest blood pressure levels (139.06 ± 22.91 mmHg, $p < 0.05$ vs *ACE-D/D*), and *ACE-I/I* males represented the youngest population.

The therapeutic response of AD patients with variable degree of cognitive deterioration (baseline MMSE score = 23.35 ± 7.51 ; range 0–24) to the CNLA protocol showed a clear tendency to the stabilization of mental decline after 1 yr of treatment (MMSE score = 22.32 ± 8.60 ; $r = -0.09$, a coef. 22.90, a coef. -0.02). Among *ACE-I/D* variants, *ACE-D/D* individuals were the worst responders ($r = -0.58$, a coef. 23.03, b coef. -0.17), and *ACE-I/I* individuals were the best responders ($r = +0.26$, a coef. 22.7, b coef. $+0.12$), with those with *ACE-I/I* showing an intermediate positive response ($r = +0.01$, a coef. 23.11, b coef. $+0.007$) (see Fig. 10.18).

Effect of *ACE*–*APOE* Interactions on the Therapeutic Response in Alzheimer’s Disease

Since synergistic effects of *APOE* with many other genes, including *ACE*, *AGT*, *NOS3*, *FOS*, *APP*, *PS1*, *PS2*, *MAPT*, *GTS*, and others, have been documented in the international literature (12), we have characterized bigenic clusters integrating *APOE* and *ACE* genotypes in AD patients to evaluate the impact of different *APOE* genotypes on the *ACE*-related therapeutic response to a multifactorial therapy in AD. In classical studies, the association of *ACE-D* and *APOE-4* alleles was more frequent in AD than in controls (613), and the association of *ACE-D/D* genotypes and the *APOE-4* allele may confer higher risk for cerebrovascular damage (644).

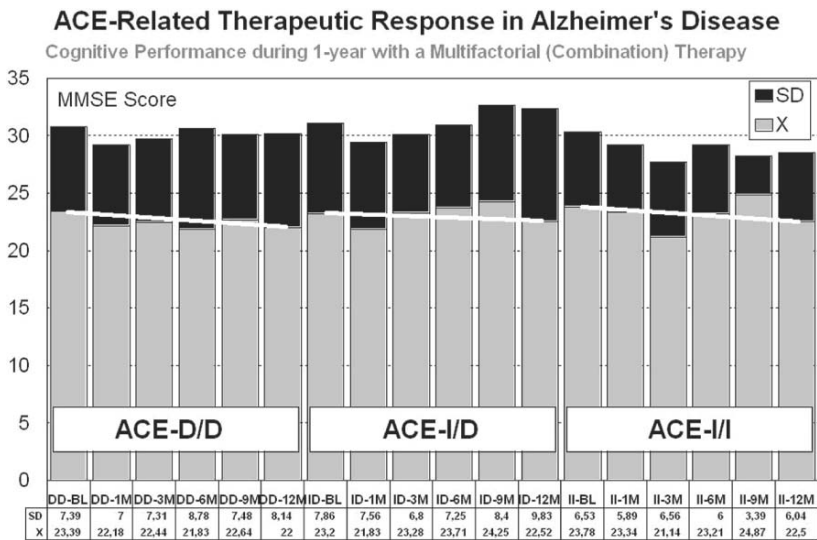


Fig. 10.18 Angiotensin-converting enzyme (*ACE*)-related therapeutic response to a multifactorial treatment in Alzheimer’s disease

As in previous studies (6,12–16,18–20,291), *APOE*-2/4 ($r = +0.79$, a coef. 14.05, b coef. +1.19) > *APOE*-3/4 ($r = +0.64$, a coef. 21.12, b coef. +0.70) > *APOE*-2/3 ($r = +0.01$, a coef. 23.06, b coef. +0.02) were the best responders, whereas *APOE*-4/4 patients were the worst responders ($r = -0.98$, a coef. 25.15, b coef. -1.30) (see Fig. 10.19). The integration of *ACE* and *APOE* genotypes in bigenic clusters yielded 18 different genotypes. The most frequent bigenic genotypes were 33ID (33.74%), 33DD (22.68%), and 33II (9.29%). The frequencies of bigenic clusters integrated by *APOE*-4/4 and *ACE* variants were 0.86% 44DD, 1.51% 44ID, and 0% 44II. Among *ACE*-*APOE* bigenic genotypes, the best responders were ID34 ($r = +0.74$, a coef. 23.95, b coef. +0.49) > ID23 ($r = +0.50$, a coef. 23.85, b coef. +0.45) > II33 ($r = +0.49$, a coef. 24.06, b coef. +0.24) > DD34 ($r = +0.35$, a coef. 19.96, b coef. +0.23), and the worst responders were DD44 ($r = -0.99$, a coef. 21.75, b coef. -1.11) > DD33 ($r = -0.77$, a coef. 24.54, b coef. -0.32) > II23 ($r = -0.71$, a coef. 25.21, b coef. -0.58) > ID33 ($r = -0.46$, a coef. 23.37, b coef. -0.29) > II34 ($r = -0.32$, a coef. 23.45, b coef. -0.58) > DD23 ($r = -0.35$, a coef. 23.92, b coef. -0.62) (see Fig. 10.20). These results clearly show that (1) the worst responders were those AD patients harboring the DD44 bigenic genotype, (2) the presence of the *ACE*-D/D variant transformed potentially good (*APOE*-2/3) or moderately good (*APOE*-3/3) responders into poor responders, and (3) the presence of the *APOE*-4/4 genotype determined a poor therapeutic response when combined with any *ACE* variant (see Fig. 10.20).

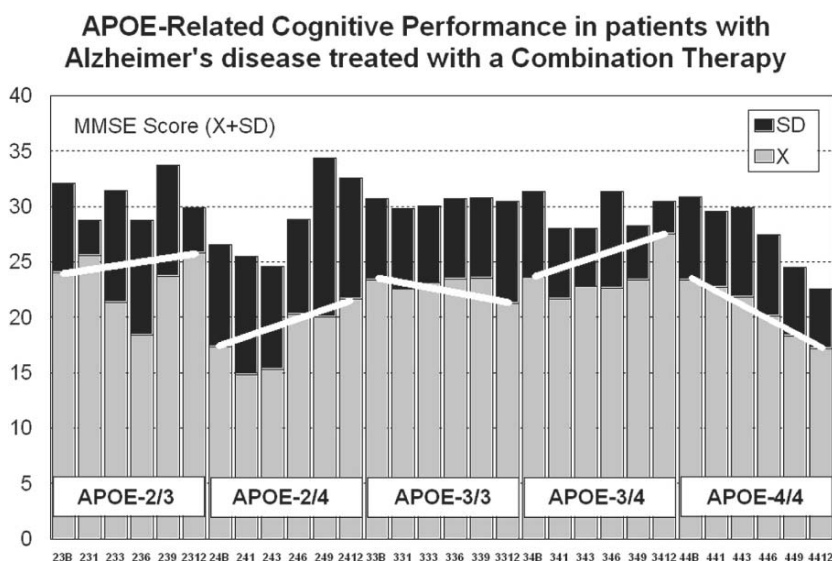


Fig. 10.19 Apolipoprotein E (*APOE*)-related cognitive performance in patients with Alzheimer's disease treated with a combination therapy for 1 yr

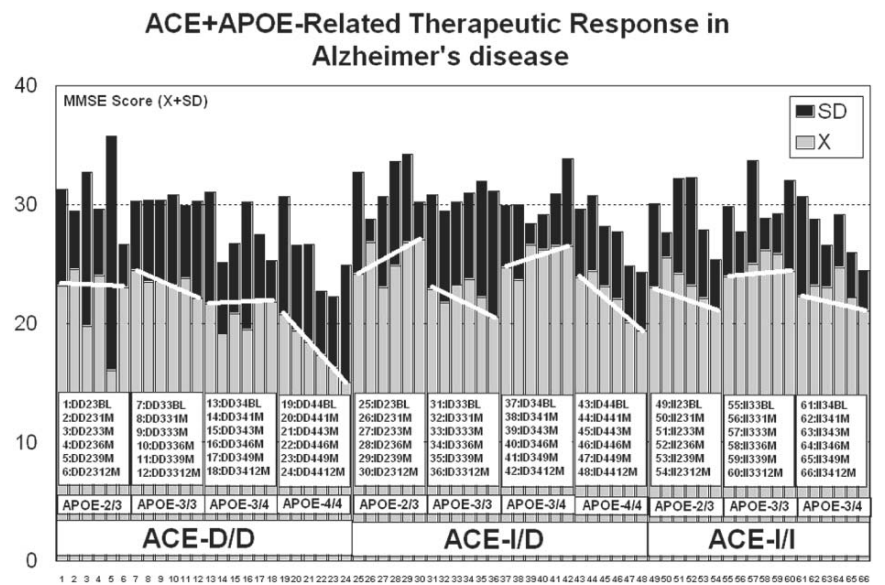


Fig. 10.20 Angiotensin-converting enzyme plus apolipoprotein E (*ACE + APOE*)-related therapeutic response to a multifactorial treatment in Alzheimer's disease

APOE- and *ACE*-Related Anxiety Rate in Alzheimer's Disease

Behavioral disturbances and mood disorders are intrinsic components of dementia associated with memory disorders (37,47,645–647). The appearance of anxiety, depression, psychotic symptoms, verbal and physical aggressiveness, agitation, wandering, and sleep disorders complicates the clinical picture of dementia and adds important problems to the therapeutics of AD and the daily management of patients as well. Under these conditions, psychotropic drugs (antidepressants, anxiolytics, hypnotics, and neuroleptics) are required, and most of these substances contribute to deteriorate cognition and psychomotor functions. Both *APOE*- and *ACE*-related polymorphic variants have been associated with mood disorders (648,649) and panic disorder (650). Differences in anxiety-related behavior have been detected between *APOE*-deficient *C57BL/6* and wild-type *C57BL/6* mice, suggesting that *APOE* variants may affect emotional state (651). *APOE*-4 carriers with deep white matter hyperintensities in MRI show association with depressive symptoms and vascular depression (652). Reduced caudate nucleus volumes and genetic determinants of homocysteine metabolism accumulate in patients with psychomotor slowing and cognitive deficits (653), and older depressed subjects have persisting cognitive impairments associated with hippocampal volume reduction (654,655). Depressive symptoms are also associated with stroke and atherogenic lipid profile (656).

Since 1990s, antipsychotic, antianxiety, and cognitive-enhancing effects have been attributed to *ACE* inhibitors (657,658). It has been reported that some *ACE* inhibitors (captopril, SQ29,852) display similar effects to benzodiazepines in dealing with anxiety-related behaviors in animals (659), and another *ACE* inhibitor (cero-napril) might share with neuroleptic drugs an ability to enhance latent inhibition in learning tasks (657). One SNP (rs4291) located in the promoter region of the *ACE* gene has been associated with unipolar major depression (648).

To understand whether cognitive function and mood disorders are cooperatively influenced by genetic factors in AD and to know the potential impact that conventional neuroprotection can exert on mood disorders, we studied the effect of the therapeutic CNLA protocol on anxiety in AD and the differential *APOE*- and *ACE*-related responses distinguishing the influence of monogenic and bigenic variants on emotional conditions.

Surprisingly, the CNLA protocol was extremely effective in reducing anxiety progressively from the first month to the twelfth month of treatment (see Fig. 10.21). The anxiety rate declined from a baseline HRS-A score of 10.90 ± 5.69 to 9.07 ± 4.03 ($p < 0.0000000001$) at 1 mo, 9.01 ± 4.38 ($p < 0.000006$) at 3 mo, 8.90 ± 4.47 ($p < 0.005$) at 6 mo, 7.98 ± 3.72 ($p < 0.00002$) at 9 mo, and 8.56 ± 4.72 ($p < 0.01$) at 12 mo of treatment ($r = -0.82$, a coef. 10.57, b coef. -0.43) (see Fig. 10.21). From a global perspective, these data might suggest that improvement in mood conditions can contribute to stabilize cognitive function or that neuroprotection (with the consequent stabilization or improvement in mental performance) can enhance emotional equilibrium.

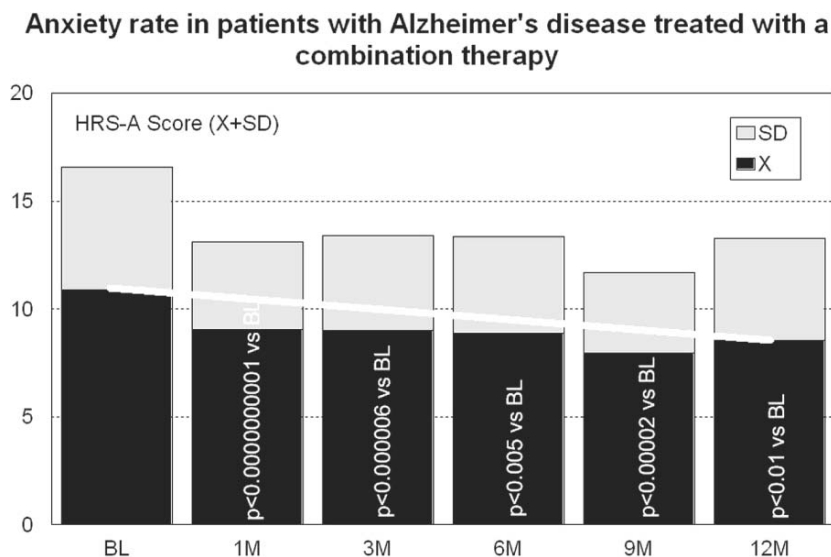


Fig. 10.21 Anxiety rate in patients with Alzheimer's disease treated with a combination therapy

APOE-Related Anxiety Rate

At baseline, all *APOE* variants showed a similar anxiety rate except the *APOE*-4/4 carriers, who differed from the rest in a significantly lower anxiety rate ($p < 0.05$). Remarkable changes in anxiety were found among different *APOE* genotypes (see Fig. 10.22). Practically, all *APOE* variants responded with significant diminution of anxiogenic symptoms except patients with the *APOE*-4/4 genotype, who only showed slight improvement. The best responders were those with *APOE*-2/4 ($r = -0.87$, a coef. 14.80, b coef. -1.03) > *APOE*-2/3 ($r = -0.77$, a coef. 11.04, b coef. -0.45) > *APOE*-3/3 ($r = -0.69$, a coef. 10.8, b coef. -0.39) > *APOE*-3/4 carriers ($r = -0.45$, a coef. 10.93, b coef. -0.30) (see Fig. 10.22). The modest anxiolytic effect observed in *APOE*-4/4 patients ($r = -0.25$, a coef. 7.53, b coef. -0.23) might be caused by the very low anxiety rate observed at baseline. In any case, *APOE*-4/4 carriers are the worst responders, with results similar to those obtained in cognitive performance; however, the potential influence of *APOE* variants on anxiety and cognition in AD does not show a clear parallelism, suggesting that other more complex mechanisms are involved in the onset of anxiety in dementia.

ACE-Related Anxiety Rate

Patients with each of the three *ACE*-I/D indel variants were equally anxiogenic at baseline, and all of them favorably responded to the CNLA protocol by gradually reducing anxiety symptoms during the 12-mo treatment period (see Fig. 10.23).

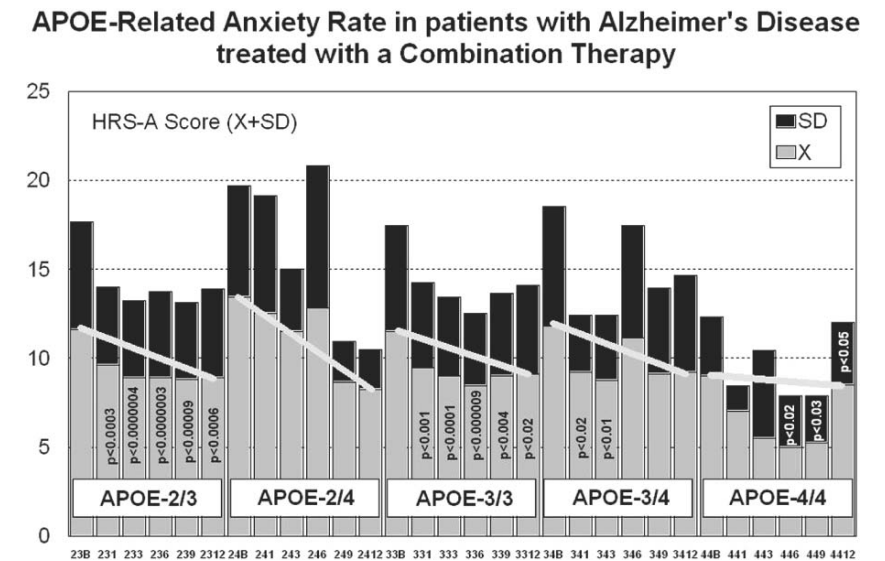


Fig. 10.22 Apolipoprotein E (*APOE*)-related anxiety rate in patients with Alzheimer's disease treated with a combination therapy

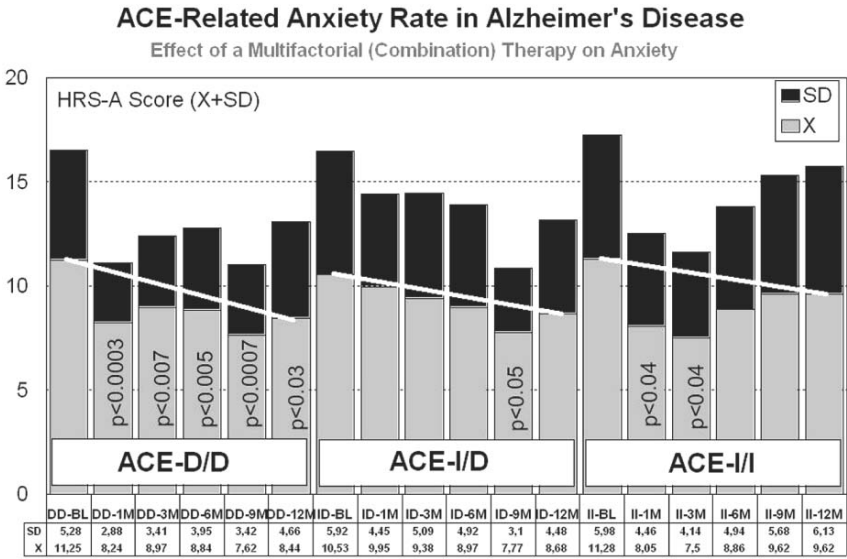


Fig. 10.23 Angiotensin-converting enzyme (ACE)-related anxiety rate in patients with Alzheimer's disease treated with a combination therapy

The best responders were *ACE-I/D* ($r = -0.89$, a coef. 10.83, b coef. -0.46), followed by *ACE-D/D* ($r = -0.68$, a coef. 10.49, b coef. -0.45) and *ACE-I/I* ($r = -0.08$, a coef. 10.57, b coef. -0.06), the AGS-1/1 carriers exhibiting the less-significant change in anxiogenic parameters (see Fig. 10.23). In *ACE-D/D* carriers, the anxiolytic response was faster and more sustainable during the treatment period (1 mo, $p < 0.0003$ vs baseline; 3 mo, $p < 0.007$; 6 mo, $p < 0.005$; 9 mo, $p < 0.0007$; 12 mo, $p < 0.03$) than in the other genotypes, whereas in *ACE-I/D* the response was gradual, reaching significant values after 9 mo of treatment ($p < 0.05$). In contrast, *ACE-I/I* patients showed a very positive response during the first trimester of treatment (1 mo, $p < 0.04$ vs baseline; 3 mo, $p < 0.04$), with an apparent relapse of anxiogenic symptomatology thereafter (see Fig. 10.23). This differential ACE-related anxiety pattern might suggest some influence of ACE-I/D variants on mood disorders in AD.

Effect of APOE-ACE Interactions on Anxiety

The combination of *APOE* and *ACE* polymorphic variants in bigenic clusters yields a quite different anxiety pattern (see Figs. 10.24 and 10.25). The most anxiogenic patients at baseline were those with the *DD23*, *ID44*, and *I134* genotypes, and the less-anxiogenic patients were those harboring the *I123*, *DD44*, and *ID23* genotypes (see Fig. 10.24). All bigenic clusters showed a positive anxiolytic response to the CNLA protocol, except *DD44*, which by a large margin exhibited the worst response ($r = +0.38$, a coef. 8.16, b coef. $+0.19$). The sequence of good responders from better

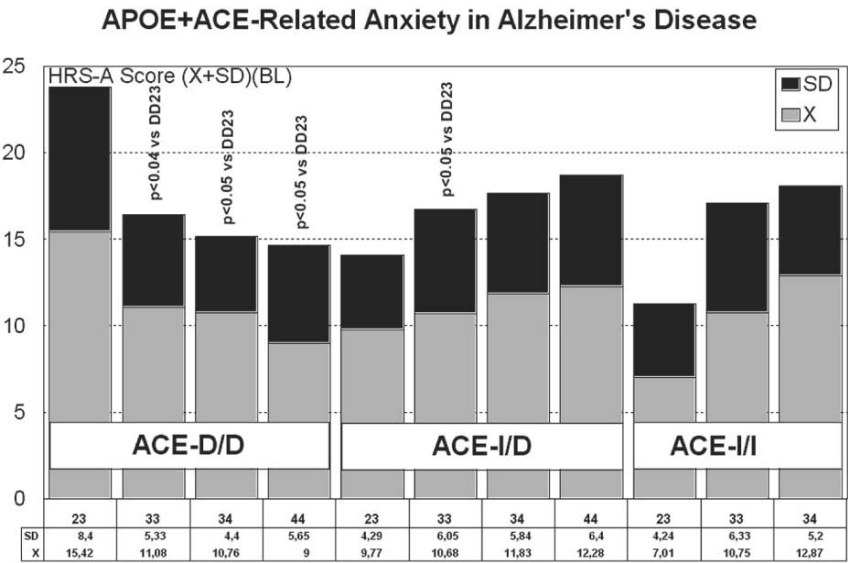


Fig. 10.24 Apolipoprotein E plus angiotensin-converting enzyme (*APOE + ACE*)-related anxiety rate in patients with Alzheimer's disease

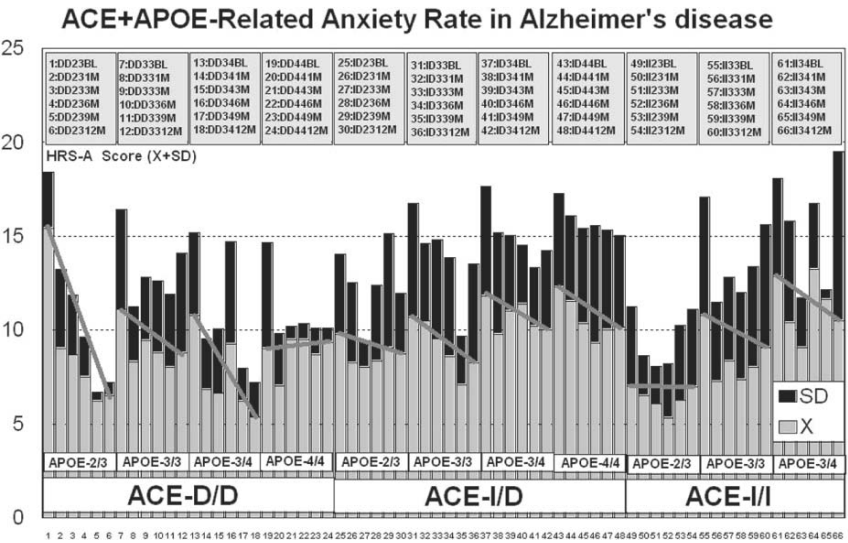


Fig. 10.25 Apolipoprotein E plus angiotensin-converting enzyme (*ACE + APOE*)-related antianxiety effect of a multifactorial treatment in patients with Alzheimer's disease

to worse was as follows: *ID33* ($r = -0.89$) > *DD23* ($r = -0.85$) > *ID44* ($r = -0.79$) > *DD34* ($r = -0.69$) > *DD33* ($r = -0.63$) > *ID34* ($r = -0.47$) > *II33* ($r = -0.29$) > *ID23* ($r = -0.19$) > *II23* ($r = -0.13$) = *II34* ($r = -0.13$) (see **Fig. 10.25**). Again, as in the case of cognition, *DD44* patients represented the poorest responders, clearly indicating that the association of the *APOE-4/4* and *ACE-D/D* genotypes plays a severe deleterious role on mental performance, at least in cognition and anxiety. Another interesting conclusion from these results comes from the fact that the association of *ACE-I/D* with *APOE-4/4* is beneficial in terms of mood improvement, neutralizing the negative influence of *APOE-4/4*.

10.5 Conclusions

The optimization of AD therapeutics requires the establishment of new postulates regarding (1) the costs of medicines, (2) the assessment of protocols for global treatment in dementia, (3) the implementation of novel therapeutics addressing causative factors, and (4) setting up pharmacogenetic/pharmacogenomic strategies for drug development (*12–16,18–20,42*).

The cost of medicines is an important issue in many countries because (1) of the growth of the aging population (>5% disability), (2) AD patients (5–15% > 65 yr) belong to an unproductive sector of the population with low income, and (3) of the high cost of health care systems in developed countries. Despite the effort of the pharmaceutical industry to demonstrate the benefits and cost-effectiveness of available drugs, the general impression in the medical community and in some governments is that the antidementia drugs present in the market are not cost-effective (*9,18–20*). Conventional drugs for AD are relatively simple (and some of them are also very old) compounds with unreasonable prices. There is an urgent need to assess the costs of new trials with pharmacogenetics and pharmacogenomics strategies and to implement pharmacogenetic procedures to predict drug-related adverse events (*17–21*).

Cost-effectiveness analysis has been the most commonly applied framework for evaluating pharmacogenetics. Pharmacogenetic testing is potentially relevant to large populations that incur in high costs. For instance, the most common drugs metabolized by *CYP2D6* account for 189 million prescriptions and U.S.\$12.8 billion annual U.S. expenditures, which represent 5–10% of total utilization and expenditures for outpatient prescription drugs (*660*). Pharmacogenomics offer great potential to improve patients' health in a cost-effective manner; however, pharmacogenetics/pharmacogenomics will not be applied to all drugs available in the market, and careful evaluations should be done on a case-by-case basis prior to investing resources in research and development of pharmacogenomic-based therapeutics and making reimbursement decisions (*661*).

In performing pharmacogenomic studies in AD, it is necessary to rethink the therapeutic expectations of novel drugs, redesign the protocols for drug clinical trials, and incorporate biological markers as assessable parameters of efficacy and

prevention (6,12–16,18–20,289–291,662). In addition to the characterization of genomic profiles, phenotypic profiling of responders and nonresponders to conventional drugs is important (and currently neglected). Brain imaging techniques, computerized electrophysiology, and optical topography in combination with genotyping of polygenic clusters can help in the differentiation of responders and nonresponders. For instance, brain mapping shows a good imaging correlation with *APOE*-related genotypes in AD patients (18–20), and responders and nonresponders to donepezil have different electroencephalographic (EEG) cortical rhythms (663). Age and *AChE*- and *BuChE*-related genotypes can also influence the therapeutic response to donepezil and rivastigmine (664). The early identification of predictive risks requires genomic screening and molecular diagnosis, and individualized preventive programs will only be achieved when pharmacogenomic/pharmacogenetic protocols are incorporated in the clinical armamentarium with powerful bioinformatics support (18–20).

Another important issue in AD therapeutics is that antidementia drugs should be effective in covering the clinical spectrum of dementia symptoms represented by memory deficits, behavioral changes, and functional decline (6,20,432). It is difficult (or impossible) that a single drug will be able to fulfil this criteria. A potential solution to this problem is the implementation of cost-effective, multifactorial (combination) treatments integrating several drugs, taking into consideration that traditional neuroleptics and novel antipsychotics (and many other psychotropics) deteriorate both cognitive and psychomotor functions in the elderly and may increase the risk of stroke (432). Few studies with combination treatments have been reported, and most of them were poorly designed. We also have to realize that the vast majority of dementia cases in people older than 75–80 yr are of a mixed type, in which the cerebrovascular component associated with neurodegeneration cannot be therapeutically neglected (2,3,6,25). In most cases of dementia, multifactorial (combination) therapy appears to be the most effective strategy (12–16,18–20,291). The combination of several drugs (neuroprotectants, vasoactive substances, AChEIs, metabolic supplementation) increases the direct costs (e.g., medication) by 5–10%, but in turn annual global costs are reduced by approx. 18–20%, and the average survival rate increases about 30% (from 8 to 12 yr postdiagnosis).

There are major concerns regarding the validity of clinical trials in patients with severe AD. Despite the questionable experience with memantine (305), similar strategies have been used to demonstrate the utility of donepezil in severe AD (665). This kind of study bears some important pitfalls, including (1) short duration (<1 yr), (2) institutionalized patients, (3) patients receiving many different types of drugs, (4) nonevaluated drug–drug interactions, (5) side effects (e.g., hallucinations, gastrointestinal disorders) that may require the administration of additional medication, (6) lack of biological parameters demonstrating actual benefits, and (7) no cost-effectiveness assessment, among many other possibilities of technical criticism (18–20,666). Some of these methodological (and costly) problems might be overcome with the introduction of pharmacogenetic/pharmacogenomic strategies to identify good responders who might obtain some benefit by taking expensive medications.

Major impact factors associated with drug efficacy and safety include the following: (1) the mechanisms of action of drugs, (2) drug-specific adverse reactions, (3) drug–drug interactions, (4) nutritional factors, (5) vascular factors, (6) social factors, and (7) genomic factors (nutrigenetics, nutrigenomics, pharmacogenetics, pharmacogenomics). Among genomic factors, nutrigenetics/nutrigenomics and pharmacogenetics/pharmacogenomics account for more than 80% of efficacy–safety outcomes in current therapeutics (18–20,667).

Some authors considered that priority areas for pharmacogenetic research are to predict serious ADRs and to establish variation in efficacy (668). Both requirements are necessary in AD to cope with efficacy and safety issues associated with either current AD-related drugs, new drugs, and psychotropic drugs of current use in dementia (669). Because drug response is a complex trait, genomewide approaches (oligonucleotide microarrays, proteomic profiling) may provide new insights into drug metabolism and drug response. Genomewide family-based association studies, using single SNPs or haplotypes, can identify associations with genomewide significance (670).

To achieve a mature discipline of pharmacogenetics and pharmacogenomics in CNS disorders and dementia, it would be convenient to accelerate the following processes: (1) education of physicians and the public on the use of genetic/genomic screening in the daily clinical practice; (2) standardization of genetic testing for major categories of drugs; (3) validation of pharmacogenetic and pharmacogenomic procedures according to drug category and pathology; (4) regulation of ethical, social, and economic issues; and (5) incorporation of pharmacogenetic and pharmacogenomic procedures to both drugs in development and drugs in the market to optimize therapeutics (18–20,671,672).

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Chapter 11

Pharmacogenetics of Asthma

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11.1	Introduction.....	359
11.2	Approaches and Methods for Performing Pharmacogenetic Studies	361
11.3	Asthma Pharmacogenetics: Candidate Gene Analyses.....	362
11.4	Asthma Pharmacogenetics: Pathway Studies	365
11.5	Conclusion	369
	References.....	370

Summary Asthma is a common disease characterized by airway inflammation and bronchorestriction. There are several common categories of medications for treating asthma; however, not all asthmatics have the same response to these medications, some of which are adverse responses that are potentially life threatening. Because interindividual responses to asthma medications can vary considerably, the potential for genetic contributions to variable drug responses is significant. This chapter reviews the most common biological pathways targeted by asthma therapy and briefly discusses the genetic contribution to varied responses to asthma therapy for four common types of asthma medications: β -agonists, anticholinergics, leukotriene modifiers, and corticosteroids.

Keywords Anticholinergics; asthma; β -agonist; corticosteroids; drug response; genetic association; leukotriene; polymorphisms.

11.1 Introduction

Asthma is a phenotypically heterogeneous disease caused by interactions among demographic, social, environmental, and genetic factors (1–4). It is one of the most common diseases, affecting nearly 10% of all North Americans (5) and in excess of 300 million people worldwide (6). Every year, approx. 5000 deaths occur in the United States because of complications of asthma (7,8); worldwide the annual death rate is approaching 200,000 (6). In Westernized, developed countries, the estimated cost to diagnose and treat one asthma patient ranges from \$300 to \$1300

per year, most of which is spent on medications for controlling the disease. Therefore, diagnosing and treating asthma is a worldwide concern and in turn a major financial burden on the health care system.

Because we do not always know what triggers an asthmatic episode, diagnosing and treating asthma can be challenging. Asthma medications affect different response pathways (*see Table 11.1*); therefore, asthma treatments must specifically address the levels of airway inflammation and airway obstruction for each asthma patient. Adding an extra layer of complexity is evidence that nearly 70–80% of asthmatics may have variable responses to the most common asthma medications (*9–16*). For example, a study by Malmstrom et al. found that asthmatics treated with inhaled corticosteroids or leukotriene modifiers demonstrated a broad spectrum of asthma drug responses (*17*). For some of these patients, these differences in drug responses can be adverse and potentially life threatening (*9,12,18–27*). Poor patient adherence, ineffective methods of drug delivery, or incorrect drug selection can all cause poor or adverse treatment responses. However, in most cases the causes of interindividual differences in drug responses cannot be explained, suggesting that a genetic component, unique to specific groups of patients, could play an important part in controlling the interindividual drug response to asthma medications.

Pharmacogenetics is the term used to describe how genetic variations affect interpersonal drug response. The term *drug response* is a generalized term used to describe the cumulative affects of more complicated biological pathways affected by genetic variations, including biological pathways that control the site of action, toxic side effects, rate of drug metabolism, drug transport, and drug excretion and clearance. Thus, an altered drug response may be caused by variations in genes that independently or cooperatively affect one or more biological pathways.

Table 11.1 Examples of asthma drug response pathways and pharmacogenetic target genes

Drug category	Drug names	Drug target	Pharmacogenetic target genes
Corticosteroids	Beclomethasone	Glucocorticoid receptor	<i>NR3C1</i>
	Fluticasone		
β-Agonists	Albuterol	β ₂ -adrenergic receptor	<i>ADRβ2</i>
	Salmeterol		
	Formoterol		
Leukotriene modifiers	Zileuton	5-Lipoxygenase	<i>ALOX5</i>
	Zafirlukast	Cystein leukotriene 1 (CysLT ₁)	<i>CYSLT1</i>
Anticholinergics	Montelukast		
	Tiotropium	Muscarinic receptor 3	<i>CHRM3</i>
	Ipratropium		

11.2 Approaches and Methods for Performing Pharmacogenetic Studies

Pharmacogenetic studies are performed using several basic strategies. The two most common strategies are candidate gene analysis and biological pathway analysis. Both strategies can utilize various population study designs, including case and control populations, family-based populations, and cohorts (28). Candidate gene studies rely on measuring the association of drug responses with genetic variation within a single gene based on that gene's function in the drug response pathway. Biological pathway analysis, in contrast, requires interrogation of multiple genes encoding several components of a drug response pathway. Candidate gene analysis is simpler to perform, primarily because genetic polymorphisms need only be selected from one gene, thus reducing the time and costs of genotyping and data analysis. In addition, a candidate gene analysis reduces the problems associated with multiple testing statistical issues (29) by limiting the number of genetic markers and genetic loci tested for association with the drug response phenotype. The pathway approach, however, can be more comprehensive in identifying whether more than one gene is associated with drug responsiveness and allows gene-by-gene interactions to be evaluated.

A biological pathway study generally involves analysis of a subset of genes that are important for controlling iterative steps in drug response. Biological pathway studies can become quite large because multiple genetic targets involved in the drug response are interrogated simultaneously. The advantage of yielding information on several genetic targets can be counterbalanced by difficulty in interpretation of a single gene's polymorphism's contribution to drug response because multiple genetic loci are tested simultaneously. In addition, because a pathway approach interrogates different steps in the response pathway, multiple phenotypes may have to be considered.

Whether a candidate gene approach or pathway approach is selected, the overall strategy for selecting genetic polymorphisms (single-nucleotide polymorphisms, SNPs) for each study is nearly identical. SNP selection can be facilitated by several genomic Internet sites, including the University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu/>), dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), and HapMap (<http://www.hapmap.org/>). The Santa Cruz Genome Browser is a comprehensive Internet site that links most of the most important genomic Internet sites. This Internet site provides excellent visual and graphic displays of genomic information that can be viewed from the nucleotide to the chromosomal levels.

One of the key genomic databases linked from the Santa Cruz Genome Browser is the database dbSNP. The database dbSNP contains detailed information on most SNPs within a gene, including the validation status of each polymorphism, the location of each SNP, the type of SNP (coding or noncoding), and most important, the minor allele frequency (MAF) (race and ethnic group specific). For most pharmacogenetic studies, SNPs with MAFs above 0.10 are usually selected to gain the most power for detecting genetic associations. SNPs with MAFs less than 0.10 can also be tested, but if a study population is small, the chance that SNPs with low

MAFs will yield informative genetic association data can be quite low. Circumstances for which polymorphisms with low MAFs should be considered in a genetic association study occur when the polymorphism causes functional effects, such as an alteration in gene expression, or when the polymorphism alters the gene coding region and has potential structural or functional effects on the protein. Although these low-frequency functional polymorphisms may not be informative for the whole study population, they can provide circumstantial evidence for a pharmacogenetic effect in subjects with unique drug response characteristics.

The database HapMap has become a powerful new tool in designing pharmacogenetic assays (30–35). HapMap is a compilation of genetic studies designed to define haplotype structure across the human genome in four populations: Centre de'Etude du Polymorphisme Humain (CEPH) (U.S. Caucasian of Western and Northern European ancestry), Chinese, Japanese, and Yoruban (Nigeria). Using these data and the accompanying downloadable software HaploView (36), a linkage disequilibrium structure can be viewed and an optimal set of tagging SNPs can be selected that, when assayed, will capture a majority of the haplotype structure across a gene or chromosomal region. Not all polymorphisms in dbSNP or from published sources have been genotyped and placed in the HapMap database. Therefore, caution should be used if a specific polymorphism of interest is not represented in the HapMap database, especially if the polymorphism has potential effects on gene expression or peptide function. Many investigators feel it is prudent to sequence a gene of interest in a representative number of the target population to ensure both the complete discovery of all important genetic variations in the gene and to determine if the published allele frequencies pertain to the population under study.

In addition to candidate gene and pathway pharmacogenetic studies, new technological SNP genotyping technologies are now available for simultaneously genotyping large numbers of SNPs (50,000 to 1 million) (37–44) using DNA chips from Illumina Inc. (<http://www.illumina.com>) and Affymetrix Inc. (<http://www.affymetrix.com>). Although in its infancy, these large genomewide screening approaches to pharmacogenetic analysis, more appropriately termed *pharmacogenomics*, promise to be powerful tools for identifying unknown genes and biological pathways associated with drug responses. There are, however, several drawbacks to performing these large studies, including the difficulty in interpreting and analyzing large data sets (43,45,46) and the high costs of performing such studies.

11.3 Asthma Pharmacogenetics: Candidate Gene Analyses

11.3.1 β_2 -Adrenergic Receptor

The classic example of an asthma pharmacogenetic candidate gene is *ADR β 2* (β_2 -adrenergic receptor). The β_2 -adrenergic receptor agonists, such as the short-acting

β -agonist (SABA) albuterol and the long-acting β -agonist (LABA) salmeterol, are the most commonly prescribed asthma medications for the treatment of airway obstruction associated with asthma. *ADRB2* is a small gene located on chromosome 5q31. The gene consists of a single exon that encodes a 413-amino acid, G protein-coupled receptor. Several mutations have been identified in *ADRB2* (47,48), including two common nonsynonymous coding changes, Gly¹⁶Arg and Gln²⁷Glu. Both Gly¹⁶Arg and Gln²⁷Glu have been shown to have regulatory effects on receptor function (49), with receptors containing the amino acid combinations Gly¹⁶/Gln²⁷ and Gly¹⁶/Glu²⁷ downregulated by 41% and 39%, respectively, when exposed in vitro to isoproterenol. Receptors containing the amino acid combination Arg¹⁶/Gln²⁷ have also been shown to be downregulated, but to a lesser degree, 26%; receptors containing the amino acid combination Arg¹⁶/Glu²⁷ have not been shown to exhibit downregulation. Because these two mutations are common in all populations studied, they have been the intense focus of numerous asthma and pharmacogenetic studies.

In one of the first β -agonist response studies, asthmatic children homozygous for both Arg¹⁶ and Gln²⁷ were found to be 5.3 times more likely to have positive responses to albuterol based on the change in percent predicted FEV₁ (forced expiratory volume in 1 s) when compared to children heterozygous for both Arg¹⁶ and Gly¹⁶ (all homozygous for Gln²⁷) and 2.3 times more likely to have a positive response than children homozygous for Gly¹⁶ (50). This same study also concluded that the Gln²⁷Glu mutation had no significant effect on a subject's response to albuterol therapy. Follow-up studies have also correlated a positive β -agonist response with the Arg¹⁶ genotype (51–54).

Several conflicting studies, however, have found adverse effects of β -agonist therapy in individuals with the Arg¹⁶ genotype (55–58). Two of these studies pointed out potential problems with the use of β -agonists in individuals homozygous for Arg¹⁶. The first study, performed as part of the Asthma Clinical Research Network (ACRN), found that asthma patients homozygous for Arg¹⁶ and treated with regular β -agonist therapy had a significant decline in their morning peak flow expiratory rates (PFEs) in comparison to asthma patients homozygous for Gly¹⁶ and receiving similar regular β -agonist therapy (58). In contrast, asthma patients homozygous for Arg¹⁶ or Gly¹⁶ and treated with β -agonists only as needed did not experience decreases in morning PFEs. In a more recent study called BARGE (Beta-Agonist Response by Genotype), asthma patients homozygous for Arg¹⁶ or Gly¹⁶, identified prospectively, were given intermittent or regular β -agonist therapy or placebo and then crossed over (56). This study found that patients homozygous for Arg¹⁶ improved when treated with regularly administered placebo compared with regular albuterol. In contrast, asthma patients homozygous for Gly¹⁶ improved when treated with regular albuterol when compared with placebo treatment. When comparing Arg¹⁶ and Gly¹⁶ homozygotes, genotype-specific effects were observed for morning PEF, FEV₁, asthma symptoms, and rescue inhaler use.

As mentioned, ethnic and racial differences can have a significant effect on the frequency and type of polymorphisms within a gene and could therefore affect the results for genetic association studies. A primary example of this effect occurs when considering racial or ethnic groups with broad genetic backgrounds. For

instance, both Mexicans and Puerto Ricans are generally characterized as Latino populations. However, the prevalence of asthma is higher in Puerto Ricans than Mexicans, and as a consequence, Puerto Ricans have a higher morbidity rate associated with asthma (59,60). Further complicating the comparison of these two Latino populations are conflicting reports on β -agonist response. In one study, Burchard et al. (59) reported that Puerto Ricans exhibited a lower response to β -agonist than Mexicans. However, when Choudhry et al. (60) studied similar sets of subjects by comparing differences in β -agonist response between these two Latino populations based on the Gly¹⁶/Arg¹⁶ genotype, Puerto Ricans with the Arg¹⁶ had a greater response to albuterol. Overall, the Gly¹⁶/Arg¹⁶ genotype could predict β -agonist response in Puerto Ricans; however, there was no significant association of Gly¹⁶/Arg¹⁶ genotypes with β -agonist response in Mexicans.

Altered β_2 -adrenergic receptor response has also been associated with genetic variations outside the coding region of *ADRB2* (61–67). A short open reading frame (ORF) in the promoter region of *ADRB2* encodes a 19-amino acid peptide called the β_2 -adrenergic receptor upstream peptide (BUP). This region, also labeled the leader cistron (LC) region, contains an important polymorphism that converts the 19th amino acid of the BUP from Cys to Arg. This mutation in BUP has been shown to regulate the translation rate of *ADRB2* messenger RNA (mRNA), with the Cys form of the BUP increasing mRNA translation (63). In combination with mutations in the coding region of *ADRB2*, the regulatory effect of the BUP mutation indicates that *ADRB2* regulation and expression is complex and should be evaluated using multiple polymorphisms across the gene.

Drysdale et al. (61) were the first to comprehensively evaluate *ADRB2* by genotyping 13 *ADRB2* polymorphisms in four ethnic groups and defining the haplotype structure across the gene. This study identified 12 common haplotypes, five of which represent more than 95% of the haplotypic variation in the four ethnic groups studied. Significant differences in haplotype frequencies were found between U.S. Caucasians and African Americans, especially in haplotypes containing Arg¹⁶. In addition, this study also investigated the potential effects of haplotype pairs on β -agonist response in 121 Caucasians. The results indicated that a subset of individuals with haplotype pairs containing Arg¹⁶/Arg¹⁶ and Gln²⁷/Gln²⁷ (haplotypes pair 4/4) had a lower response to albuterol compared to other haplotype pairs. Individuals with haplotype pairs containing Gly¹⁶/Gly¹⁶ and Glu²⁷/Glu²⁷ (haplotype pair 2/2) had significantly higher responses to albuterol. Individuals with a combination of these two haplotypes (haplotype pair 2/4) had intermediate β -agonist responses. A second haplotype combination, also containing Arg¹⁶/Gly¹⁶ and Gln²⁷/Gln²⁷ (haplotype pair 4/6), however, had the highest response to albuterol, indicating that these haplotype pairs may not have clear predictive value in assessing β -agonist response. These data also suggest that other polymorphisms in the gene that are not represented in these haplotypes may play an important role in regulating β -agonist response. More recently, a study by Taylor et al. (68) also explored the relationship between haplotype pairs and responses to albuterol in 176 asthmatics. They concluded that there was no value in using haplotype pairs to predict β -agonist response.

A more recent *ADR 2* haplotype study may explain some of the discordance between these two haplotype pair studies. Hawkins et al. (47) performed highly detailed DNA sequencing of an approx. 5.3-kb region of *ADRβ2* in 429 Caucasians and 240 African Americans. Their results indicated that the haplotype structure of *ADRβ2* is more complex than previously described (61), and that variation in a polycytosine repeat in the 3 β untranslated region of *ADRβ2* not only increases the number of *ADRβ2* haplotypes, but also may play an important role in gene function, possibly affecting lung function (47).

Other coding mutations have been identified in *ADRβ2* (47,48). The mutation Val³⁴Met is very rare and appears to have little effect on β_2 -adrenergic receptor function. The mutation Thr¹⁶⁴Ile is also a rare coding change, but functional studies indicated that this mutation can have significant effects on receptor function by decreasing the binding affinity of β -agonist by two- to threefold (69). The coding change Ser²²⁰Cys has been identified (47) in African Americans, but there is no functional information on this mutation.

11.3.2 Muscarinic Receptors and Anticholinergics

Muscarinic receptors (M2 and M3) also participate in regulating airway tone and caliber (70,71). Anticholinergics, such as ipratropium and tiotropium, block the activity of muscarinic receptors, and are commonly used for treating patients with chronic obstructive pulmonary disease or, in combination with β -agonists, as a rescue medication for asthmatics. Very little is known about the pharmacogenetic responses of muscarinic receptors to anticholinergics, although several potentially functional polymorphisms have been identified in the genes for M2 and M3 (*CHRM2* and *CHRM3*, respectively) (71–75). *CHRM2* and *CHRM3* are very large genes (~148 and 280kb, respectively) and thus are daunting targets for genetic analysis, especially considering the potential haplotype structure across such large genes. Functional studies of muscarinic receptors indicated that molecular synergy may exist between the anticholinergic and β_2 -agonist pathways (76). These results indicated that some asthmatics who have a low β -agonist response may benefit from treatment with anticholinergic agents, especially when the β -agonist has been shown to provide a less-than-optimal clinical response.

11.4 Asthma Pharmacogenetics: Pathway Studies

11.4.1 Leukotriene Modifiers

A classic example of a biological pathway with implications for asthma pharmacogenetics is the leukotriene synthesis pathway. Cysteinyl leukotrienes

(CysLTs) are proinflammatory molecules synthesized primarily by basophils, neutrophils, and mast cells and are potent mediators of airway inflammation and bronchoconstriction (77,78). There are two classifications of drugs in this category that can regulate the effects of CysLTs: inhibitors of the 5-lipoxygenase enzyme, such as zileuton, and CysLT receptor antagonists, such as montelukast and zafirlukast.

Leukotrienes (LTA_4 , LTB_4 , LTC_4 , LTD_4 , and LTE_4) are synthesized from arachidonic acid by a cascade of enzymes that include 5-lipoxygenase (5-LOX), 5-lipoxygenase-activating protein (FLAP), and leukotriene C₄ synthase (LTC_4 synthase) (79,80). The leukotriene LTA_4 is synthesized by 5-LOX in the first step and is an unstable precursor that is then enzymatically converted to LTB_4 or LTC_4 (80,81), which can subsequently be metabolized to LTD_4 and LTE_4 . LTC_4 , LTD_4 , and LTE_4 are the components of the slow-reacting substance of anaphylaxis. These moieties, particularly LTC_4 and LTD_4 , are active forms of CysLTs that interact with the G protein-coupled cysteinyl leukotriene receptors (CysLtr1 and CysLtr2) (70,81,82). Once engaged, the activated CysLtr receptors stimulate the secretion of mucus and induce edema and bronchoconstriction (81).

CysLT₁ and CysLT₂ (83,84) are encoded by the genes *CYSLT1* and *CYSLT2*. Genetic studies of *CYSLT1* and *CYSLT2* are limited and have generally been confined to studies involving associations with atopy, asthma severity, and aspirin-intolerant asthma (AIA) (85–91). There are, however, two studies that have investigated the potential pharmacogenetic effects of these receptors. The first study examined the function of wild-type *CYSLT2* and a mutated form of *CYSLT2* containing Val instead of Met at amino acid position 201 (90). This study concluded that *CYSLT2* receptors containing Val were fivefold less sensitive to the CysLTD₄, indicating that asthma patients with this mutation could have altered responses to leukotriene modifiers. A more recent study measured the association of four *CYSLT1* polymorphisms with improvement in FEV₁ in asthma patients treated with montelukast for a period of 6 months (92). No significant association between these polymorphisms and improvement in FEV₁ were measured.

The CysLT synthesis pathway occurs in a series of steps, beginning with the activation of 5-LOX by FLAP, allowing 5-LOX to catalyze the first enzymatic step that converts arachidonic acid to hydroperoxyeicosatetraenoic acid (5-HPETE) and LTA_4 (80,93,94). FLAP is encoded by the gene *ALOX5AP*. Although there have been several genetic studies of *ALOX5AP*, these studies have concentrated more on how the gene confers risk for myocardial infarction, asthma, and obesity (95–101) and not on pharmacogenetics. There are no coding mutations in *ALOX5AP*; therefore, there are no functional variants of this enzyme to investigate.

The gene for 5-LOX, *ALOX5*, has been evaluated as a risk factor for several diseases (93,97,102–109) in addition to its potential pharmacogenetic effects on leukotriene modifier treatment (13,92). The promoter region of *ALOX5* contains a variable DNA sequence consisting of a variable number of insertions or deletions of an Sp1/Egr1 transcription factor-binding sequence GGGCGGG (13,95,110–114). The number of tandem Sp1/Egr1 repeats ranges from three to seven, with five the most common number of repeats. In vitro analyses of this region indicated that

varying the number of Sp1/Egr1 repeats alters the levels of *ALOX5* transcription (95,113).

Two pharmacogenetic studies investigated the effects of variable Sp1/Egr1 repeats. The first compared treatment with the zileuton derivative ABT-761 or placebo with percent improvement in FEV₁ compared to baseline FEV₁, found that patients with the most common number of Sp1/Egr1 repeats (five repeats) had an approx. 15% improvement in FEV₁ compared to patients with longer or shorter repeats (13). A study by Lima et al. (92), however, did not observe the same effect for the *ALOX5* repeat. In this study, subjects were treated for 6 mo with montelukast, resulting in changes in FEV₁ vs baseline measurement. This study indicated that subjects homozygous for Sp1/Egr1 five repeats (the most common allele) had an increased risk of asthma exacerbations while treated with montelukast. The study was limited, however, because although 252 subjects (69% white and 26% African American) participated in the clinical trial, only 61 whites were genotyped to obtain the final association results. Based on these conflicting observations, it appears that additional genetic variants, as well as haplotypic variation, will have to be considered to determine the pharmacogenetic contribution of *ALOX5*.

LTC₄ synthase catalyzes the conversion of LTA₄ to LTC₄ (80). Because LTA₄ is unstable, this is a critical step in the production of LTC₄, LTD₄, and LTE₄. There are two polymorphisms in the LTC₄ synthase gene (*LTC4S*) that have been repeatedly scrutinized for pharmacogenetic effects and effects on asthma risk (15,92,99,115–121). One of these polymorphism, -444 (A-444C), is located in the promoter region of *LTC4S* within a putative activator protein 2 (AP2)-binding site and has been associated with changes in CysLT production (118). A more recent study found that individuals with A-444C heterozygosity had a 73% reduced risk of having an asthma exacerbation compared to A-444C AA homozygotes when treated with montelukast over a 6-mo period (92).

One key phenotype that may be associated with the leukotriene synthesis pathway is AIA. AIA patients have been found to produce increased quantities of CysLTs, which have been quantitated in both their urine and lung secretions (122). However, several association studies comparing the A-444C polymorphism and the risk of AIA have not been conclusive (117,121).

11.4.2 Corticosteroid Pathway

Because of their potent effects on airway inflammation, corticosteroids are one of the most prescribed medication types for controlling asthma, either alone or in combination with a bronchodilator such as a β_2 -agonist. Although the effectiveness of corticosteroids in treating airway inflammation is well documented, the mechanism of corticosteroid action is quite complex and not completely understood (123). The primary target of corticosteroids is the cytoplasmic glucocorticoid receptor. However, the glucocorticoid receptor does not act independently but is part of a large multiprotein glucocorticoid receptor complex (124–126). In addition

to the glucocorticoid receptor, a minimal glucocorticoid receptor complex is composed of heat shock protein (hsp) 90, hsp70, hsp40, hsp organizing protein (hop; hsp70/hsp90 organizing protein), and p23 (*126–135*). Correct assembly of the glucocorticoid receptor is essential for proper receptor responsiveness, and it occurs in a series of highly regulated steps. Once activated, the glucocorticoid receptor complex is transported through the nuclear membrane with the aid of an additional set of proteins called *immunophilins* are peptidylprolyl isomerases that are targets of immunosuppressive drugs such as FK-506 (FKBP51 and FKBP52) (*136*). Once within the nucleus, activated glucocorticoid receptor dimers bind to glucocorticoid receptor response elements (GREs) that lie in regulatory regions of genes, causing increases or decreases in gene expression (*137–141*).

Although each component of the glucocorticoid receptor complex is required for proper steroid response, very little information is available concerning the relationship between genetic variants in genes encoding the glucocorticoid receptor complex and corticosteroid response or sensitivity, except for the gene encoding the glucocorticoid receptor (*NR3C1*). *NR3C1* is a large, very complex gene consisting of three forms of exon 1 and two forms of exon 9 dispersed over approx. 160kb of genomic DNA (*142–146*). Three isoforms of the glucocorticoid receptor, GR α , GR β , and GR γ , are produced by *NR3C1* (*143,147,148*). GR α and GR β are produced by differential splicing of exons 9 α and 9 β , respectively; GR γ contains an additional amino acid inserted by variable splicing between exons 3 and 4 that adds one additional codon. In the glucocorticoid complex, the primary active form of the glucocorticoid receptor exists as GR α dimers. GR β is not functional; however, it has been proposed that GR β acts as a dominant negative inhibitor of GR α transactivation (*149*). Thus, when expression of GR β is increased, cells can become insensitive to corticosteroids, an effect that has been confirmed in several asthma studies (*150–156*). Little is known about the activity of GR γ , but one study suggested that GR γ does not have a significant effect on steroid sensitivity (*157*).

Several mutations have been found in *NR3C1* that affect the receptor's ability to effectively bind steroids (*158–166*). However, these gene mutations are associated with corticosteroid resistance and not attenuated corticosteroid responsiveness. There are two *NR3C1* genetic variations that may have potential effects on corticosteroid response. The first genetic variation is a relatively rare coding mutation, Asn³⁶³Ser (rare variant Ser), which has been identified in several populations (*167–171*). Lymphocytes from individuals with receptors with the ³⁶³Ser mutation were shown to have a greater sensitivity to dexamethasone compared to lymphocytes from noncarriers (*172*). In addition, the Asn³⁶³Ser carriers tended to have higher body mass and lower bone density, indicating a possible metabolic affect of the mutation.

One additional *NR3C1* genetic variation, located +1308 bp in the 3' untranslated region (UTR) after the stop codon of exon 9 β , has potential pharmacogenetic implications. This polymorphism lies within a mRNA stability motif (AUUUA) and converts the motif to the sequence GUUUA. Gene expression studies have shown that the presence of the G allele in this mRNA stability motif increases the stability of GR β mRNA, thus allowing increased rates of GR β translation (*173*). Asthmatics possessing this G allele could therefore produce more GR β , thus competing with

functional GR α in the steroid dimer complex. These individuals could also have an attenuated response to exogenous and endogenous corticosteroids.

The complexity of the corticosteroid pathway is one reason that investigations to date have yielded little pharmacogenetic information with practical clinical utility. Therefore, broad approaches to finding genes associated with corticosteroid response may be necessary. A key requirement for identifying genes with pharmacogenetic potential is the replication of positive findings. Tantisira et al. (174), for example, identified one gene, *CRHR1*, that was associated with improved lung function in patients taking corticosteroids; the identification was in three different populations from three different clinical trials. In their strategy, polymorphisms from 14 genes were genotyped in a primary population. Polymorphisms that were significant in the primary population were subsequently analyzed in primary and secondary replicate response populations. In the case of *CRHR1*, haplotypic analysis, as well as analyses of individual polymorphisms, identified a common *CRHR1* haplotype that was associated with corticosteroid response. This study further speculated that patients with specific *CRHR1* genetic variations may be limited in making endogenous corticosteroids, thus increasing the chance that a more favorable response to exogenously administered corticosteroids would be observed.

Several factors made this study particularly robust. First, all three clinical trials were performed separately, thus ensuring that observations from each study were independent from other clinical trials. Second, different corticosteroids were utilized in each clinical trial, indicating that the associations observed were independent of the corticosteroid used in each trial. Finally, although two of the clinical trials used adult patients, one study used children (average age 9 years), indicating that the associations observed may have utility in predicting corticosteroid responses in both children and adults.

11.5 Conclusion

The birth of pharmacogenetics has promised a new way to approach the treatment of groups of patients who share important genetic elements. The field is moving rapidly away from analysis of a single or a few polymorphisms in a single gene to more complete haplotypic analyses of multiple genes that comprise complete pathways important for specific drug responses. Optimum approaches to pharmacogenetics will begin with complete SNP discovery followed by haplotype definition, which will then permit the creation of the most efficient genotyping strategy. Robust populations of different ethnicities must be available for both initial association studies and for replication of positive findings. Finally, positive findings should also be confirmed in prospective studies.

Although genetic variation in many genes may be associated with differential drug responses, it is also apparent that other factors, such as epigenetics (DNA methylation, interference RNA, RNA editing, histone acetylation/deacetylation,

etc.), will also play an important role in determining individual and group responses to drugs. We are rapidly moving from an era of evidence-based medicine (the best medicine for the population as a whole) to personalized medicine (medicine for an individual or group of individuals who share key genetic and other traits in common).

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Chapter 12

From SNPs to Functional Studies in Cardiovascular Pharmacogenomics

Sharon Cresci

12.1	Introduction.....	379
12.2	Choosing Candidate Genes.....	381
12.3	Choosing Candidate SNPs.....	383
12.4	Functional Studies to Suggest Potential Mechanism.....	385
12.5	Notes.....	390
	References.....	391

Summary Functional studies can be utilized to give importance/relevance to clinical associations. Once a clinical genetic or pharmacogenetic association is found, molecular studies can be utilized to explore the mechanism for the association. By employing cells in culture or transgenic mice modified with specific variant genes or sequence polymorphisms of interest, pathophysiological processes and response to pharmacological agents may be tested under conditions that are not approachable in human patients. These mechanistic studies may be particularly important when it comes to pharmacogenetic associations by providing significant, clinically relevant insights into the variable responses patients show to drug therapy.

Keywords Candidate gene; cardiovascular; functional genomics; pharmacogenomics; single-nucleotide polymorphism (SNP).

12.1 Introduction

Cardiovascular medicine has advanced rapidly in the last 25 years, I would prefer not to say “since xxxx” as this is a general statement and to place a start time on these advances would be erroneous. providing major advances in pharmacotherapeutics to reduce morbidity and mortality from cardiovascular diseases. These advances have been driven by the results of clinical trials, which test the average rates of efficacy and safety of medical therapies. Such clinical trial evidence has provided strong indications for the widespread and routine application of many agents for a variety of cardiovascular diagnoses, such as β -adrenergic blockers for

heart failure or acute coronary syndromes. Although many patients likely derive substantial benefit from these agents, others may not benefit, and some may even be harmed. The reasons for this variability in response are poorly understood, but emerging evidence suggests that genetic factors may play an important role in whether a drug treatment is effective and safe in a particular individual (1,2). Furthermore, knowledge of relevant genetic variation that affects drug responsiveness may allow a clinician to select or withhold certain drugs for the treatment of a particular condition and thereby personalize an optimal therapeutic regimen while avoiding or minimizing hazard. The charge of cardiovascular genomics and pharmacogenomics is to design ways of investigating relevant genetic influences on pathophysiology and responses to drug treatments to refine our approach to treating the disease and to advance the goal of personalized therapeutics.

A fundamental challenge for the geneticist and pharmacogeneticist is how to determine the specific genetic variation of interest for study in relation to a particular phenotypic marker or treatment outcome. This problem is solved by one of two general approaches: (1) an unbiased, genomewide association approach using chromosomal markers at relatively evenly interspaced positions throughout the genome and analyzing the association of the severity of a phenotype (linkage) with these chromosomal markers; and (2) a candidate gene approach by which candidate genes are selected based on the evidence driven from molecular biological studies implicating a specific molecule in the pathogenesis of the trait. Even if a genomewide association approach is utilized, once linkage with a particular chromosomal region is identified, the investigator often needs to proceed to candidate gene selection to focus on the causative gene.

An elegant example of beginning with a genomewide association approach and proceeding to candidate gene selection was published by Topol and colleagues (3). These investigators studied a large single family, of whom 13 members had coronary artery disease (CAD) diagnosed at age 35–65, and 9 members had sustained an acute myocardial infarction. Although multiple known risk factors, including dyslipidemia, hypertension, and cigarette smoking, were present in some family members, the presence of CAD in this family showed an autosomal dominant pattern of inheritance (3). The investigators used DNA from members of the family to perform a genomewide linkage scan for presence of CAD. Using 382 markers spanning chromosomes 1–22 with an average interval of 10 cM, they demonstrated positive linkage with marker D15S120, located on chromosome 15q26 (LOD or logarithm of the odds ratio for linkage score of 4.19). Haplotype analysis with nearby markers confirmed the linkage and the locus was designated adCAD1 (autosomal dominant CAD and MI locus 1) (3). The region surrounding the locus (adCAD1 region) contained approx. 93 genes (43 known and 50 hypothetical). The nuclear transcription factor MADS (MCM1-agamous-ARG80-deficiens-SRF) box transcription enhancer factor 2 α (also known as myocyte enhancer factor 2 α or MEF2 α) was one of the known genes and was felt to be a strong candidate gene for the observed phenotype because, based on animal studies, MEF2 α had been previously shown to play an important role in vascular morphogenesis (4,5).

With this knowledge of its potential functional relevance from previous studies, MEF2 α was then selected by Wang and colleagues for more detailed analysis,

essentially transitioning this study to a candidate gene approach. Within the identified family with premature CAD, systematic mutational screening of their candidate gene (the MEF2 α gene) in the affected family members demonstrated a 21-bp deletion in exon 11 of the MEF2 α gene in all ten living affected members, resulting in a protein that was missing seven amino acids (3). Functional studies demonstrated that the variant MEF2 α protein lacking the seven amino acids (Δ 7aa MEF2 α protein) had decreased transcriptional activity in cotransfection studies, the Δ 7aa MEF2 α protein had blocked entry into the nucleus compared to wild type, and the Δ 7aa MEF2 α protein localized to the endothelium of the coronary arteries (3). The authors proposed that this resulted in a defective coronary endothelium that in turn may have promoted atherosclerotic CAD. Their elegant analysis exemplifies how functional studies can powerfully support a candidate gene approach to understanding the influence of gene variation on phenotype.

With that example as a starting point, this chapter provides an overview of the candidate gene approach, including (1) the selection of candidate genes and relevant clinical populations in which to assess genomic and pharmacogenomic associations using information, both from bioinformatics analysis and from animal models, that suggest that a particular gene is important in a particular disease state; (2) the selection of candidate single-nucleotide polymorphisms (SNPs) to analyze in functional studies; and (3) a discussion of how functional studies can be utilized to provide possible mechanisms for (or to give clinical relevance to) the clinical associations found from genomic and pharmacogenomic studies. I predominantly use cardiovascular examples to illustrate each of these topics. This chapter is not meant to provide an exhaustive review of the available literature and bioinformatics databases or to suggest that there is only one scientific approach to testing a pharmacogenetic hypothesis. Instead, it is offered to suggest one potential approach among others for the design and interpretation of genomic and pharmacogenomic association studies.

12.2 Choosing Candidate Genes

Candidate genes for genomic and pharmacogenomic studies are often selected based on the evidence driven from molecular biological studies implicating (1) a specific molecule in the pathogenesis of a trait; (2) the molecular pathway for the biological mechanism of action of a drug; or (3) the pathways involved in the activation, deactivation, or elimination of the drug. Observations in animal models can be utilized to support the selection of candidate genes and relevant clinical populations in which to assess genomic and pharmacogenomic associations. Transgenic mouse models in which a gene is overexpressed or “knocked out” (null) or “knocked down” can provide important information about the physiological relevance of specific genes. This information may be readily apparent on inspection of the animal or may be apparent only on exquisitely detailed phenotyping. For example, in the ob/ob mouse, in which a mutation leads to absence of the 16-kDa protein leptin,

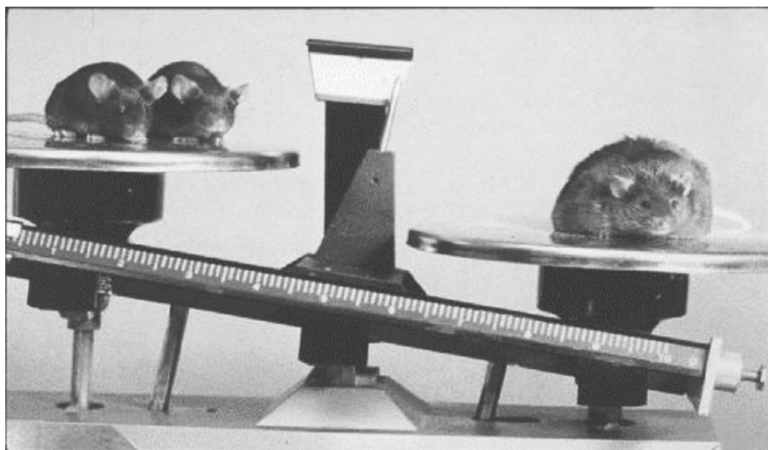


Fig. 12.1 Leptin-deficient (*ob/ob*) mouse. (From <http://www.obesity-news.com/abs01-00.htm>.)

transgene littermates weigh more and have much more body fat compared to nontransgene littermates (see **Fig. 12.1**) (6). Investigators therefore speculated that the gene encoding leptin may be involved in weight gain, feeding/satiety, and the regulation of body weight and body fat (7,8). This hypothesis was confirmed when recombinant leptin protein administered to *ob/ob* mice led to restoration of weight and feeding patterns similar to that of nontransgene littermates (9–12), and a human form of congenital leptin deficiency was subsequently found (13).

Similarly, transgenic mice with selective skeletal muscle overexpression of the transcription factor peroxisome proliferator-activated receptor delta ($\text{PPAR}\delta$) (14) demonstrated obvious differences in skeletal muscle fibers. The investigators observed that the transgenic mice had redder muscles with increased numbers of type I muscle fibers and therefore hypothesized that $\text{PPAR}\delta$ was involved in fiber-type transformation as is seen in endurance exercise (14). The investigators confirmed this hypothesis by demonstrating that these mice were able to run continuously for up to twice the distance of nontransgene littermates. In a related example, a cardiomyocyte-restricted $\text{PPAR}\delta$ deletion mouse (15) also showed obvious cardiac abnormalities, including myocardial hypertrophy and lipid accumulation that led to cardiac dysfunction and congestive heart failure with reduced survival (15), suggesting to those investigators that $\text{PPAR}\delta$ has a critical role in maintaining normal myocardial fatty acid oxidation and normal cardiac function.

In contrast to the obvious abnormalities seen in the aforementioned cases, mice with cardiac-specific overexpression of the fatty acid transport protein 1 (FATP-1) (16) appear grossly normal compared to their nontransgene littermates. However, sophisticated cardiac phenotyping methods, such as micro positron emission tomography (microPET), echocardiography, and ambulatory electrocardiographic (ECG) monitoring demonstrated subtle abnormalities of the FATP-1 transgenic mouse, such as increased cardiac fatty acid uptake and utilization, diastolic dysfunction,

and prolonged QT interval (**16**). These observations led the investigators to conclude that FATP-1 is involved in free fatty acid import into the heart and is implicated in the metabolic pathway that contributes to early myocardial diastolic dysfunction similar to that seen in diabetic cardiomyopathy (**16**).

With a wealth of gene–disease phenotype associations already catalogued, there are innovative Web sites that have compiled all available information that relates a gene’s relevance to a given disease state. A particularly user-friendly site that allows one to insert a candidate gene and provides a compilation of every related disease state or allows one to insert a disease or symptom and provides a compilation of every related gene is SNPs3D (<http://www.snps3d.org>). This site also provides invaluable information about gene pathways and allows one to develop gene pathway approaches to genomic and pharmacogenomic association studies.

12.3 Choosing Candidate SNPs

When utilizing a candidate gene approach to genetic association studies, once a candidate gene is selected, coverage of the candidate gene using a “tagSNP” approach is traditionally utilized for the selection of SNPs to genotype. This approach takes advantage of the fact that one does not need to genotype all existing common polymorphisms because genotypes at many polymorphic sites are strongly correlated and form linkage disequilibrium (LD) blocks. Genotyping one SNP that “tags” or tracks with many others is advantageous because it provides the maximum coverage for the lowest cost per SNP. (Various algorithms have been developed to select tagSNPs, and many are freely available; **17,18**.) A tagSNP approach will establish whether there is an association with the gene of interest in a particular study population but will not, necessarily, identify the “causative” SNP. There are several tools, both bioinformatics and molecular, that can be employed to assess the functional relevance of a candidate SNP.

12.3.1 Bioinformatics Information

There are many computer-based approaches that provide evidence of possible functional relevance.

12.3.1.1 Homology

In contrast to the random drift that accounts for genetic diversity among species and individuals within a species over the course of evolution, there is felt to be selective pressure to conserve sequences that have important biological function. (In fact, “ultraconserved” regions of 200 or more nucleotides that are identical in human,

rat, and mouse genomes and that have analogs in the dog, chicken, and fish genomes have been described; *19,20*.) Therefore, the degree of homology between the SNP and the sequence surrounding the SNP is often informative about how functionally relevant a SNP is.

The $\Delta 7\text{aa}$ MEF2 α protein found by Wang and colleagues (*3*) and discussed in the introduction may help illustrate this point. The seven amino acids deleted in the dysfunctional protein are located in a region that is highly conserved between MEF2A and a MEF2A homolog (MEF2C) and are conserved among MEF2 α proteins in the human, the mouse, the pig, and the Chamek spider monkey (*3*). Several open-access databases and Web-based bioinformatics programs allow assessing if a candidate SNP is located within a conserved sequence (see [Table 12.1](#)).

12.3.1.2 Functional Role

The location of the SNP often determines the functional role that it is likely to have. SNPs located in the coding region may change the structure or function of the resulting protein; SNPs in the 5' and 3' untranslated and intronic regions may interfere with regulatory protein (e.g., transcription factor/enhancer/repressor protein) binding or nucleic acid (e.g., microRNA) binding; SNPs located in intron–exon junctions may interfere with a splice site.

Many bioinformatics programs exist that provide information to allow determining if the candidate SNP may interfere with a potential functional site (see [Table 12.1](#)). Polymorphism Mining and Annotation Programs (PolyMAPr) (*21*) provides this information in a particularly efficient way once a file containing the annotated gene sequence has been entered. PolyMAPr will then map all SNP sequences taken

Table 12.1 User-Friendly Web Sites that May Help Prioritize SNP

	Bioinformatic SNP Web Sites	Reference
Homology/conserved sequence	http://genome.ucsc.edu/	<i>35,36</i>
	http://mordor.cgb.ki.se/cgi-bin/jaspar2005/jaspar_db.pl	<i>37,38</i>
	http://www.phylofoot.org/	<i>39,40</i>
	http://polly.wustl.edu/regulign/default.html	<i>41</i>
	http://evolution.genetics.washington.edu/phylip/software.html	
Functional	PolyMAPr (CREATE site: http://create.unc.edu/)	
	http://www.gene-regulation.com	<i>42–44</i>
	http://ural.wustl.edu/software.html	
	(RNA regulatory) Links to several sites can be found at http://www.rnaiweb.com/RNAi/MicroRNA/MicroRNA_Tools__Software/MicroRNA_Target_Scan/index.html	<i>45–47</i>

from public databases (dbSNP, CGAP, and JSNP), or from the investigators' own SNP discovery efforts, to the annotated gene sequences and name them according to suggested nomenclature guidelines. The functional effects of nonsynonymous coding-region SNPs, putative transcription factor-binding site SNPs, and SNPs that might alter exon splicing enhancer (ESE) sites or intron–exon splice sites are also predicted. Databases also exist to determine if the intronic SNP interferes with an RNA regulatory element (*see* [Table 12.1](#)).

12.4 Functional Studies to Suggest Potential Mechanism

Functional studies may help clarify a mechanism of clinical association and are often chosen based on the location of the SNP. The technique that is chosen obviously depends on the mechanism that is being assessed. This section provides examples of how molecular techniques have been used to provide functional information about a SNP and how that information suggested a possible mechanism for the reported clinical association. In case of unfamiliarity with the technique used in the examples, brief descriptions of these techniques are provided at the end of this section. These descriptions are not intended to provide a comprehensive explanation of the technique. Detailed description of how to perform these techniques, as well as a summary of the technical issues relevant to them, can be found in texts such as *Current Protocols in Molecular Biology* (22).

12.4.1 Example 1: PPAR δ Gene (PPARD) Promoter SNP

As discussed, transgenic mouse models with tissue-specific overexpression or deletion of PPAR δ implicated PPAR δ in fiber-type transformation, as seen in endurance exercise and in the maintenance of normal myocardial fatty acid oxidation and cardiac function (14). In addition, PPAR δ knockout mice demonstrated decreased metabolic rates and were found to be glucose intolerant (15). Data such as these led Vanttinen and colleagues to ask whether candidate SNPs in the PPAR δ gene (PPARD) would be associated with whole-body, skeletal muscle, and subcutaneous adipose tissue glucose uptake in healthy individuals (23). The investigators found that three such SNPs, and their haplotypes, were associated with whole-body and skeletal muscle glucose uptake (23). One of the three SNPs was located in the PPARD promoter (named +294T/C by these authors as it is located 294 nucleotides downstream of the transcription start site but is 73 nucleotides upstream of the translation start site) and was found to be associated with differences in cholesterol metabolism in two independent cohorts (24).

Skogsberg and colleagues analyzed the sequence surrounding this SNP and found that the rare (C) variant formed a consensus sequence for binding to the transcription factor steroidogenic factor-1 (Sp1) (24). To explore this, they performed

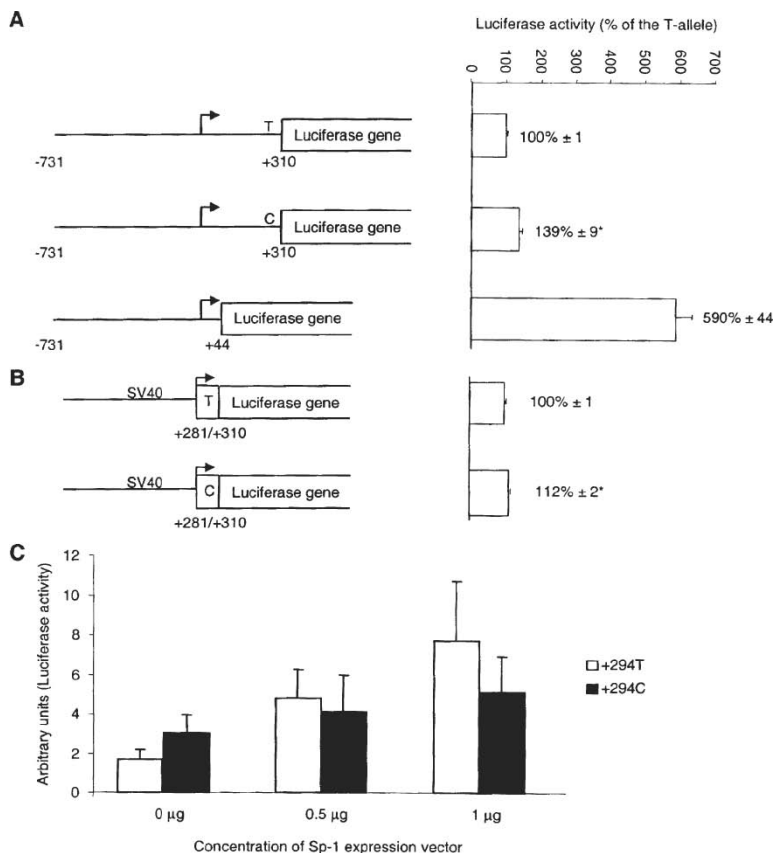


Fig. 12.2 Differences in transcriptional activity between the T and C alleles of the +294 polymorphism (24). **A** DNA constructs covering the region -731 to +310 of either T or C genotype or the proximal promoter, from position -731 to +44, were transfected into human monocytic U937 cells. The activity of the T allele is in every experiment set to 100%, and the activities of the C allele and the proximal promoter are always compared with the activity of the T allele construct ($n = 6$). **B** DNA constructs covering the region +281 to +310 of either T or C genotype placed downstream of the SV40 promoter were transfected into U937 cells ($n = 3$). **C** DNA constructs covering the region -731 to +310 of either T or C allele were transfected into SL2 cells ($n = 3$). All values are given as mean ± SE. * $p < 0.05$

transient transfection studies and demonstrated that the rare variant had higher transcriptional activity compared to the common variant (see Fig. 12.2 and Note 1) (24). Electromobility shift assays (EMSAs) further demonstrated that the binding of Sp1 to a probe containing the C variant sequence was increased when compared to binding to a probe containing the T variant sequence (see Fig. 12.3 and Note 2) (24). Thus, innovative functional studies can point to the biologic mechanism by which a variant gene influences a clinically relevant phenotype.

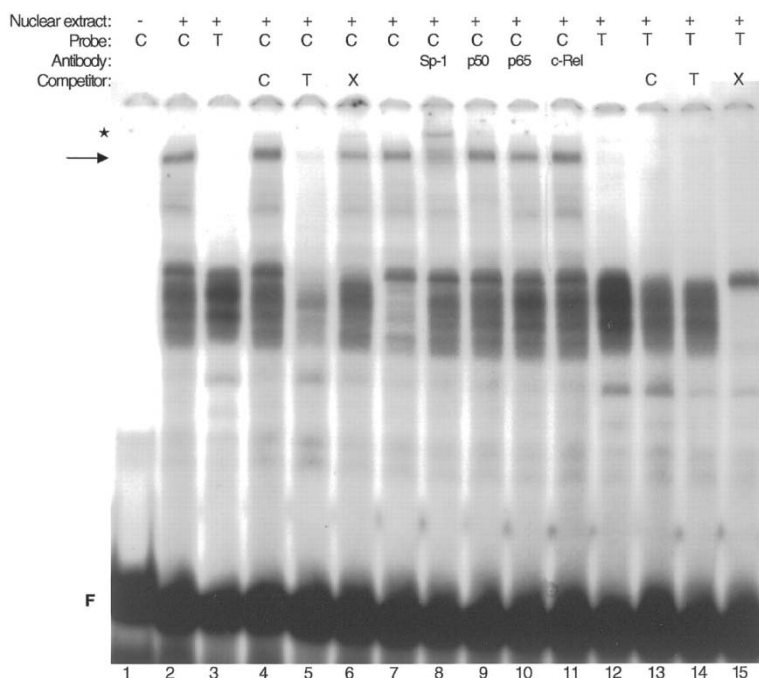


Fig. 12.3 The +294T/C polymorphic site in *PPARD* influences the binding of transcription factor Sp1 (24). Electromobility shift assays (EMSAs) of nuclear extract derived from human monocytic U937 cells using double-stranded 25-mer oligonucleotides corresponding to the sequence from position +281 to +305 of the common T allele and the rare C allele. Arrow refers to the +294C allele-predominant factor. F denotes free DNA. Lane 1, without extract; lanes 2 to 4, 12, with 10 µg nuclear extract in the absence of competitor; lanes 5 to 7, 13 to 15, with 10 µg nuclear extract in the presence of 150-fold excess of unlabeled DNA as competitor. Competitors used were +294C probe (lanes 5, 13; indicated with C); +294T probe (lanes 6, 14; indicated with T); and a nonspecific probe (lanes 7, 15; indicated with X). *Sp1 complex. Lanes 8 to 11, with 10 µg nuclear extract in the presence of antibodies directed against Sp1, p50, p65, and c-Rel, respectively

12.4.2 Example 2: β_1 -Adrenergic Receptor Gene (*ADRB1*) Nonsynonymous Coding SNP

An elegant example of an experimental program designed to test the functional relevance of a nonsynonymous coding SNP (Arg389Gly) in the gene encoding the β_1 -adrenergic receptor (*ADRB1*) can be found in the investigations of Liggett, Dorn, and colleagues (25–28). Based on previous data, these investigators proposed that *ADRB1* was an important candidate gene for pharmacogenomic associations with variable responses to β -adrenergic stimulation and variable clinical outcomes in response to β -adrenergic blocking drugs among treated patients. To assess the functional importance of the nonsynonymous Arg389Gly coding SNP, these

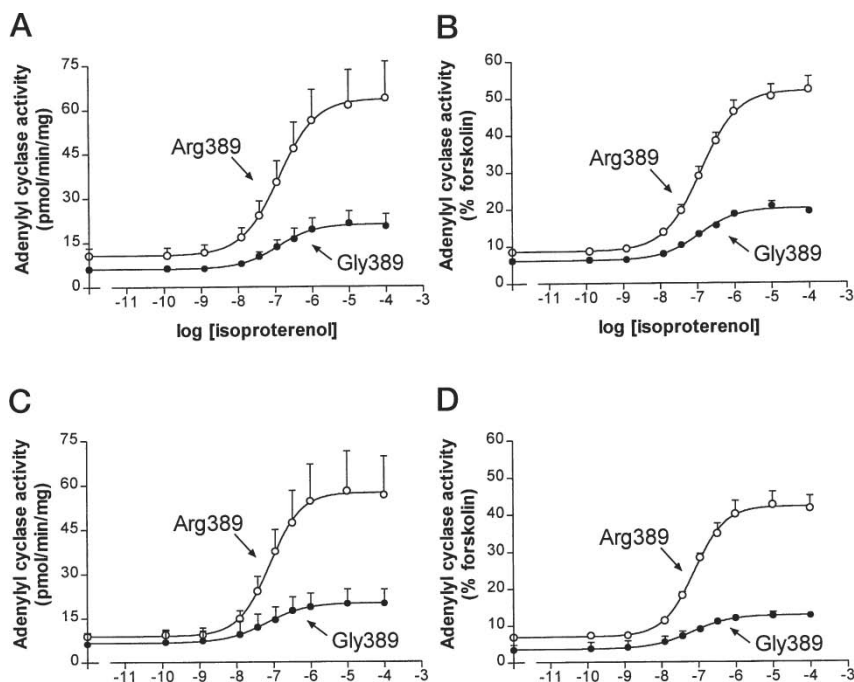


Fig. 12.4 Functional coupling of the Gly389 and Arg389 receptors to adenylyl cyclase. (Reproduced from ref. 26 with permission of the American Society for Biochemistry and Molecular Biology.) Shown are the results from studies with clonal lines expressing each receptor at matched levels and the data presented as absolute activities (A) and normalized to the stimulation by forskolin (B). The results of similar studies with two other clonal lines are shown in C and D. The Arg389 demonstrated small increases in basal activities and marked increases in agonist-stimulated activities compared with the Gly389 receptor. Shown are the mean results from four independent experiments carried out with each line. Absent error bars denote that standard errors were smaller than the plotting symbol

investigators performed stable transfections in CHW fibroblasts with a mammalian expression vector expressing either the Arg389 or the Gly389 β_1 -adrenergic receptor and found that, in cell lines expressing each receptor at matched levels, the Arg389 receptor had increased adenylyl cyclase activity in response to the β -adrenergic agonist isoproterenol compared to the Gly389 receptor (see Fig. 12.4) (26). They also performed transient transfection assays, using the same mammalian expression vector constructs, in COS-7 cells and found that, in the presence of the β -adrenergic agonist isoproterenol, GTP γ S binding was greater to the Arg389 receptor compared to the Gly389 receptor (see Fig. 12.5) (26). Cardiac-targeted transgenic mice expressing human Arg389 or Gly389 in their ventricles were generated to further test the functional relevance of the Arg389Gly SNP, and the response to β -adrenergic blockade was tested in these mice and compared to patients with heart failure (27). Transgenic mice expressing the Arg389 receptor

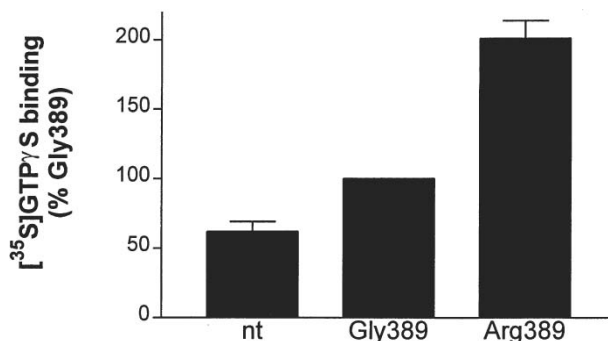


Fig. 12.5 [³⁵S]GTPγS binding to the two polymorphic β₁-adrenergic receptors. (Reproduced from ref. 26 with permission of the American Society for Biochemistry and Molecular Biology.) Binding in the presence of 10 μM isoproterenol was greater ($p < 0.05$) with the Arg389 than with the Gly389 receptor. Because of day-to-day variation in the absolute levels of binding, data are presented as a percentage of binding to the wild-type (Gly389) receptor (mean absolute values were $7.7 \pm 1.4 \times 10^5$ dpm/mg for Gly389). Basal levels of binding were not different between the two receptors. nt, nontransfected cells

were more sensitive, as determined by (–) chronotropic and (–) contractile response, to β-adrenergic blockade. Notably, patients with congestive heart failure homozygous for the Arg389 genotype had significant improvement in left ventricular ejection fraction (LVEF) in response to the β-adrenergic blocker carvedilol (27). These studies clearly showed how, by a carefully designed candidate gene approach combined with functional studies, one can gain important insights into not only the functional relevance but also into the possible molecular mechanism of pharmacogenomic associations.

12.4.3 Example 3: Antiviral Enzyme 2'5"-Oligoadenylate Synthetase Gene (*OAS1*) Splice Site SNP

Given that predisposing viral illnesses have been implicated in the development of type 1 diabetes and that animal studies have shown that the murine equivalent of *OAS1* is important in resistance to viral infections (29–31), Bonnevie-Nielsen and colleagues hypothesized that the gene encoding the antiviral enzyme 2'5"-oligoadenylate synthetase, a critical component of human resistance to viral infections, was an excellent candidate gene for investigating associations with type 1 diabetes (32,33). To investigate the functional relevance of an A/G SNP located at the *OAS1* exon 7 splice-acceptor site, they performed enzymatic activity assays in individuals and found that enzyme activity varied in a dose-dependent manner (individuals homozygous for the GG allele demonstrated the highest enzymatic activity) (33). They then demonstrated that individuals with type 1 diabetes had higher frequencies of *OAS1* GG and GA genotypes compared to their healthy siblings, and that the risk

of developing diabetes for siblings with the GG or GA genotypes was increased compared to those with the AA genotype (32).

These brief examples may serve to illustrate how studies exploring the functional consequences of a variant gene can provide information that may help clarify the biologic mechanism of the association observed between an SNP and a clinically important disease phenotype or response to drug therapy (see [Note 3](#)).

12.5 Notes

1. Transient transfections are useful for assessing transcriptional activity from SNPs located in the gene promoter region or coding region. Plasmid constructs comprised of “full-length” gene promoter or coding sequences upstream of a reporter (e.g., luciferase reporter) are transfected into a cell line chosen to yield the desired information (e.g., if interested in a promoter element thought to be important in the heart, rat cardiomyocytes may be chosen as the cell type). Transcriptional activity is determined by reporter activity of the cotransfected plasmid containing the promoter (or coding) sequence compared to that of a cotransfected plasmid containing the plasmid backbone alone. If interaction between promoter elements and different transcription factors and coactivators is desired, cells “null” for the transcription factors of interest are often used (e.g., CV1 cells for cardiac-relevant transcription factors). In this case, plasmids overexpressing or constitutively expressing the protein (e.g., transcription factor) of interest are cotransfected into the cell alone and in combination, and the reporter activities are compared.
2. An EMSAs is a simple assay to determine if a DNA sequence is able to bind a specific protein. Purified, cellular, or nuclear protein extract is incubated with labeled double-stranded oligonucleotide DNA probes, usually 20–25 nucleotides long. Each probe contains the SNP nucleotide (one per probe) and the identical sequence corresponding to the sequence around the SNP. The mixture is then run on a nondenaturing gel. If the DNA sequence binds the protein, there is a shift in the mobility of the complex (e.g., lane 2, [Fig. 12.3](#)). Specific and nonspecific competitors are often added to demonstrate specificity of the shift (lanes 5–7, [Fig. 12.3](#)). If an antibody

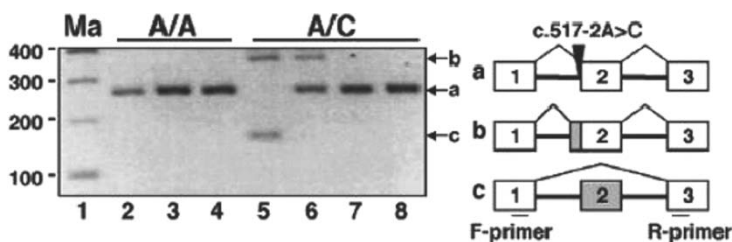


Fig. 12.6 Detection of the messenger RNA (mRNA) transcripts of the *ORCTL4* gene by reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. (Reproduced from ref. 34 with permission of the Indian Academy of Sciences, publisher and copyright holder of *Journal of Genetics*, and by the courtesy of Hidetaka Yamada and colleagues.) The left shows the results of the RT-PCR analysis. Genotypes are indicated above the panels. The diagrams on the right show the genomic structures of the normal (diagram a) and abnormal (diagrams b and c) splicing forms. Inserted and deleted sequences are indicated by closed squares. The location of splice-site polymorphism is represented by the inverted arrowhead. The locations of RT-PCR primers are represented by bars

to the protein exists, additional lanes with antibody (demonstrating a “supershifted” complex) and unrelated antibody are included on the gel (lanes 8–11, [Fig. 12.3](#)).

3. Regarding reverse transcriptase polymerase chain reaction (RT-PCR) analysis to assess a splice site variant, as seen in [Fig. 12.6](#), if one designs forward (F-) and reverse (R-) RT-PCR primers to span the SNP (which in turn creates or abolishes a splice site), one will have different size PCR products (labeled a, b, and c in the figure) that can easily be resolved on a gel.

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Chapter 13

Pharmacogenomics in Gastrointestinal Disorders

Michael Camilleri and Yuri A. Saito

13.1	Introduction: Pharmacogenetics and Pharmacogenomics.....	395
13.2	Acid-Related Gastrointestinal Disease.....	396
13.3	Azathioprine and 6-Mercaptopurine Use in Inflammatory Bowel Disease	398
13.4	Irritable Bowel Syndrome	401
13.5	Liver Transplantation	402
13.6	Use of Chemotherapy in Colorectal Cancer.....	404
13.7	Conclusion.....	405
	References.....	408

Summary It is anticipated that unraveling the human genome will have a direct impact on the management of specific diseases. Variations or mutations in genes involved in drug metabolism or disease pathophysiology in gastroenterology and hepatology are expected to have effect on response to therapy. The spectrum of diseases is vast. Thus, we focus this review on clinical pharmacogenetics of inflammatory bowel disease, *Helicobacter pylori* infections, gastroesophageal reflux disease, irritable bowel syndrome, liver transplantation, and colon cancer. Although only a few genotyping tests are used regularly in clinical practice, we anticipate that in the future there will be more routine use of many of the tests described in this review.

Keywords Colon cancer; functional dyspepsia; functional gastrointestinal disorders; gastroesophageal reflux disease; *Helicobacter pylori* infection; inflammatory bowel disease; irritable bowel syndrome; liver transplantation; pharmacogenetics.

13.1 Introduction: Pharmacogenetics and Pharmacogenomics

Pharmacogenetics and pharmacogenomics are related fields of study of how genes influence drug metabolism and response in diseases. *Pharmacogenetics* is the field that evaluates the genetic contribution of one or a few genes on drug response.

The term *pharmacogenomics* usually refers to the broader study of multiple genes or the entire genome in characterizing drug response. Thus, pharmacogenomics examines many genomic loci, including large biological pathways and the whole genome to identify variants that together influence response to drug therapy (1). Future progress in whole genome scanning will likely take advantage of *haplotypes*, blocks of polymorphisms that are in linkage disequilibrium with one another so that only one polymorphism may need to be genotyped to know the genotype of the other polymorphisms.

Genes can affect response to a drug if they encode proteins that are responsible for drug processing or metabolism. These include genes that encode drug transporters, receptors, or enzymes in the metabolic pathway of a drug. Variability in drug response results from genetic variations, either insertions or deletions of nucleotides in the DNA or, more commonly, substitution of a single or multiple nucleotides. If these variants are infrequent (<1% of the general population), they are termed *mutations*. In pharmacogenetics, the variants of interest are usually present in more than 1% of the population and are referred to as *polymorphisms*. Many of these polymorphisms may be *nonfunctional*, not affecting protein function or structure. However, if the polymorphism is “functional,” resulting in altered protein quantity or function, the genetic variants may result in therapeutic failure or unwanted drug side effects.

Routine clinical testing of genetic variants to predict therapeutic dosing or response to therapy has been applied to certain areas of gastroenterology and hepatology. This review assesses the gastroenterological diseases in which genes have an impact on drug response. Our focus is on patient genetics rather than tissue (e.g., somatic mutations in colon cancer) or infectious pathogen genetics (e.g., hepatitis C viral genotypes). Ultimately, the goal is that such studies lead to “personalized” care to maximize therapeutic responses while minimizing adverse side effects.

13.2 Acid-Related Gastrointestinal Disease

13.2.1 Proton Pump Inhibitor Use in Gastrointestinal Disease

Proton pump inhibitors (PPIs), such as omeprazole, esomeprazole, lansoprazole, pantoprazole, and rabeprazole, are commonly prescribed to treat symptoms of heartburn, acid reflux, chest pain, dyspepsia, and chronic cough. PPIs inhibit the transfer of protons into the stomach lumen. Pharmacological acid suppression is thus used to treat gastroesophageal reflux disease (GERD) and esophagitis, peptic ulcers, and *Helicobacter pylori* infection as well as to prevent ulcer development with concurrent nonsteroidal anti-inflammatory drug use.

GERD symptoms are caused by the reflux of stomach acid into the esophagus, but the acidic material may also ascend higher into the throat, airways, or mouth. GERD is a common disorder: 20% of the population experiences symptoms weekly, and 44% of the population experiences symptoms at least monthly (2).

Ulcers of the gastrointestinal tract typically affect the stomach or the duodenum, and risk increases with advancing age to the higher prevalence of *Helicobacter pylori* infections as well as higher usage of nonsteroidal anti-inflammatory drugs (3). *Helicobacter pylori* is a spiral-shaped bacterium that resides in the stomach mucosa. Infected individuals have a six- to tenfold increased risk of developing a gastric ulcer compared to noninfected individuals. This organism is the most common cause of ulcer disease in the world. In the United States, it is estimated that 10% of people aged 18–30 years are infected, but the prevalence increases to 50% for individuals over age 60 (4). Failure to adequately eradicate the bacterium results in recurrent ulceration (5,6).

Reflux is generally treated with lifestyle modifications followed by acid suppression in the form of histamine-2 receptor antagonists (e.g., ranitidine, famotidine) or PPIs, which are more efficacious (7–10).

Treatment of *H. pylori* typically consists of two antibiotics, a PPI, and/or bismuth.

13.2.2 CYP2C19 Polymorphisms and Proton Pump Inhibitor Metabolism

Cytochrome P450 (CYP) 2C19 is the main liver enzyme responsible for PPI elimination (11). Rabeprazole is unique in that it is also metabolized by CYP3A4 and degraded by “nonenzymatic” pathways (12,13). It is conceivable that genotype may not influence rabeprazole efficacy. A formal efficacy–genotype study has shown that, when compared to other PPIs, rabeprazole had the greatest efficacy on the time when pH is 4.0 higher in CYP 2C19 extensive metabolizers (14). The *CYP2C19* gene contains several polymorphisms that result in poor metabolism of drugs, including PPIs. The molecular defects range from a G-to-A conversion on nucleotide 681 on exon 5 (allele *2; 15), a G-to-A conversion at nucleotide 656 on exon 4 (allele *3; 16), an A-to-G conversion in the initiation codon (allele *4; 17), and a C-to-T conversion at nucleotide 1297 on exon 9 (allele *5; 18).

The molecule alteration results in decreased microsomal S-mephenytoin 4'-hydroxylase activity in the liver and further results in reduced metabolism and higher levels of drug. Poor metabolism occurs in 13–23% of Oriental populations (typically *2 and *3 alleles; 19) and 2–5% of Caucasian populations (typically *4 allele; 20). Poor metabolizers (homozygous for variant alleles) demonstrate area-under-the-curve (AUC) values that are 3 to 13 times higher than extensive metabolizers, consistent with a gene–dose effect (11). Those who are heterozygous for the variant alleles demonstrate AUC values that are two to four times higher than extensive metabolizers. These enhanced pharmacokinetics translate into better pharmacodynamics, with median 24-h intragastric pH values in poor metabolizers higher than extensive metabolizers, with heterozygotes demonstrating a median pH between the two groups (11).

Endoscopic cure rates for esophagitis differ for poor metabolizers, heterozygotes, and extensive metabolizers. In one study, cure rates at 8 weeks were 85%, 68%, and 46%, respectively (21), and in another study were 100%, 95%, and 77%, respectively (22). In patients infected with *H. pylori*, the wild-type genotype for *CYP2C19* is associated with a 20% lower cure rate compared to PPI plus two antibiotics in two meta-analyses of 12 and 8 studies (23,24).

In summary, genotyping of *CYP2C19* (25) may only be relevant in people of Asian extraction, of whom up to 20% may be slow metabolizers. In practice, one could also avoid the need to genotype by using a PPI with lower metabolism by *CYP2C19* (e.g., rabeprazole).

13.3 Azathioprine and 6-Mercaptopurine Use in Inflammatory Bowel Disease

The prevalence of ulcerative colitis and Crohn's disease is estimated at 37 to 246 cases per 100,000 persons and 26 to 199 cases per 100,000, respectively (26). Both forms of inflammatory bowel disease (IBD) aggregate in families; the risk to siblings is 13–36 times for ulcerative colitis and 7–17 times for Crohn's disease relative to the general population (27). Specific genes may increase susceptibility to Crohn's disease. For example, linkage studies have consistently identified the caspase-activating recruitment domain-15 (CARD15; also known as NOD₂) gene (28).

In patients with relapsing or refractory IBD, steroid-sparing agents such as azathioprine (AZA) or 6-mercaptopurine (6-MP) may be used as they decrease steroid requirements in 70% of patients who are steroid dependent (29). AZA is converted into 6-MP by sulfhydryl-containing substances such as glutathione found in red blood cells (RBCs) and other tissues. 6-MP is metabolized in the liver and gut by one of three enzymes: thiopurine-S-methyltransferase (TPMT), xanthine oxidase, and hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) (see Fig. 13.1). 6-MP and the 6-thioguanine nucleotides are produced by HGPRT metabolism of 6-MP and are responsible for cytotoxic effects that lead to decreased production of T lymphocytes and plasma cells. These effects may limit drug dosing. In the short or longer term, 3–10% of patients can develop hepatotoxicity, and 5–16% develop leukopenia and thrombocytopenia, which are dose dependent (29,30). Because of these side effects, these agents are started at low dose initially and the dose is gradually increased over months, if well tolerated, while monitoring blood counts and liver tests.

Factors that affect drug metabolism will influence the development of serious adverse effects. TPMT, one of the three enzymes that metabolize 6-MP, is encoded by a gene on chromosome 6 that contains functional polymorphisms. Multiple variants have been described (*2, *3A, *3B, *3C, *3D, *4, *5, *6, *7, *10; 31,32) that result in lower TPMT activity, leading to preferential metabolism of 6-MP by the HGPRT enzyme. This results in an increase in the amount of cytotoxic thioguanine nucleotides and greater myelotoxicity. There appears to be an allele dose-dependent

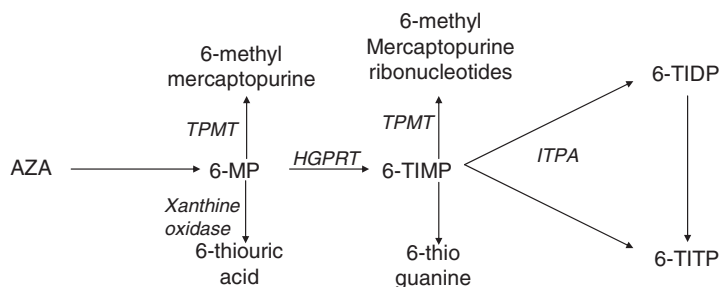


Fig. 13.1 Pathways of thiopurine metabolism. The positions of two polymorphically expressed enzymes, TPMT (thiopurine methyl transferase) and ITPA (inosine triphosphate pyrophosphatase), are shown. HGPRT, hypoxanthine guanine phosphoribosyl transferase; 6-TIDP, 6-thioinosine diphosphate; 6-TIMP, 6-thioinosine monophosphate; 6-TITP, 6-thio inosine triphosphate

effect: Homozygous wild-type patients metabolize AZA and 6-MP normally, heterozygotes are intermediate metabolizers, and homozygotes for the variant alleles have negligible TPMT activity. The estimated genotype frequencies in the general population are 89%, 11%, and 0.3%, respectively (33).

Because there is good phenotype (TPMT activity on assay)–genotype correlation, there is no clinical need for genotyping assays or direct sequencing of the *TPMT* gene. Rather, TPMT activity is directly measured, and the phenotype serves as a surrogate for genotype. However, there is no consensus yet to the utility of determining on a routine basis the TPMT status in patients with IBD. One decision analysis has suggested that pretreatment testing for TPMT activity is cost-effective (34). On the other hand, in another study, the majority (73%) of individuals who developed bone marrow suppression were not carriers for the variant allele (35), suggesting that neither phenotype assay nor genotype assay can replace simple monitoring of white blood counts (see Fig. 13.2). A study of 72 pediatric IBD patients concluded that pharmacogenetic assessment before starting thiopurine therapy was not warranted (36).

Although the discussion on TPMT pharmacogenomics often focuses on toxicity, it is also relevant to note that some studies suggested that *TPMT* genotype correlates with 6-thioguanine nucleotide (6-TGN) concentrations (37), and that a 6-methylmercaptopurine ribonucleotide (6-MMPR)/6-TGN ratio below 11 is associated with therapeutic efficacy (37), confirming an earlier report that therapeutic 6-TGN levels are necessary to bring resistant Crohn's disease into remission (38). Ansari et al. (39) showed that very high TPMT activity (>14 U/mL RBCs) was associated with failure to respond to AZA (see Fig. 13.3).

Other genotypes may be relevant to pharmacotherapeutics in IBD. These are summarized here.

1. Inosine triphosphate pyrophosphatase (ITPA) deficiency may lead to accumulation of 6-thioinosine triphosphate (ITP) in those treated with AZA or 6-MP. The gene encoding ITPA is located on chromosome 20, and two of five single-nucleotide

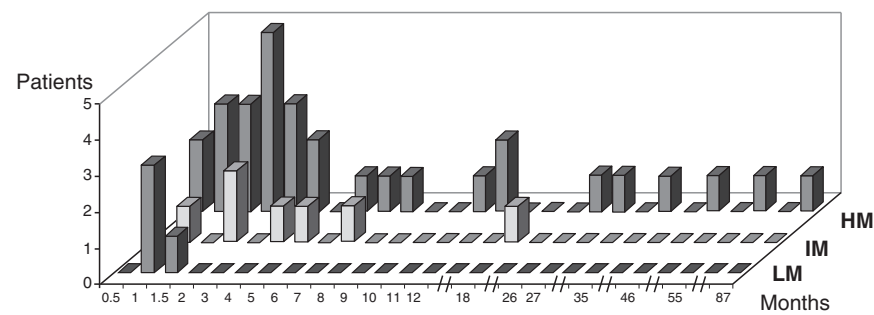


Fig. 13.2 Thiopurine methyl transferase (TPMT) methylator genotypes in Crohn’s disease during azathioprine/6-mercaptopurine (AZA/6-MP) treatment influences the time in months to development of severe myelosuppression. However, only 27% of patients with Crohn’s disease and myelosuppression during AZA therapy had mutant alleles of the TPMT gene associated with enzyme deficiency. Myelosuppression is more often caused by other factors. Continued monitoring of blood cell counts remains mandatory in patients treated with AZA. (Reproduced from ref. 35.)

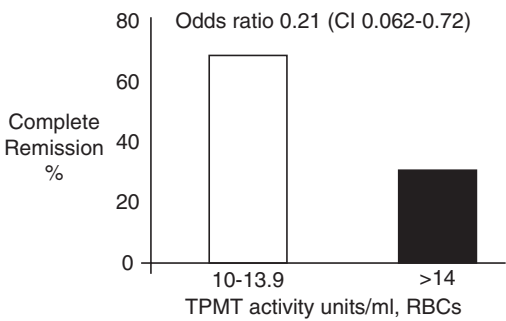


Fig. 13.3 Clinical response, adverse effects, and hematological parameters were determined and correlated with thiopurine methyl transferase (TPMT) enzyme activity and genotype in 106 patients with inflammatory bowel disease. The odds of achieving complete remission (CR) to azathioprine is approx. five times lower if TPMT is greater than 14 units/mL red blood cells (RBCs). (Reproduced from ref. 39.)

polymorphisms (SNPs) identified are associated with reduced ITPA function (40). In one series, 94C>A SNP was associated with flulike symptoms, rash, and pancreatitis (41) but not with leukopenia (41); other studies have not linked this polymorphism with adverse effects from AZA (42–44). A report (45) showed *ITPA* 94C>A predicted leukopenia with an odds ratio of 3 to 504 (CI 1.119–10.971, $p = 0.046$).

2. Although the multidrug resistance (*MDR-1*) gene and the glucocorticoid receptor (*GCR*) gene have been evaluated, to date no clear link exists between genetic variations on these genes and IBD response, or toxicity to corticosteroids has not been documented.

3. Similarly, polymorphisms on the *N*-acetyltransferase 1 and 2 genes (*NAT1*, *NAT2*) that encode the enzymes responsible for inactivating 5-aminosalicylate and sulfapyridine did not influence response to therapy or toxicity (46).
4. Other genetic studies have evaluated the role of variation on the tumor necrosis factor (*TNF*) gene (47,48) and the *TNF* receptor system (49) on predicting response to infliximab, but no association has been discovered to date. The Leuven group showed association between the Fas ligand-843TT genotype and nonresponsiveness to infliximab. However, the unfavorable effect of that genotype was overcome by concomitant treatment with 6-MP/AZA (50). A multicenter study found no association between the *TNF* gene and response to infliximab; however, one haplotype in a distintegrin and metalloproteinase domain 17 (*ADAM17*), which plays a role in *TNF*- α shedding region, was significantly associated with response to infliximab in Crohn's disease (51).
5. Two studies evaluating three SNPs on the *CARD15* gene found no significant prediction of response to infliximab (52,53).

13.4 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) affects 10–22% of the general population (54) and results in high health care utilization (55). The pathophysiology is not clearly defined; diet, psychological distress, infection, altered mucosal immunity, visceral hypersensitivity, intestinal dysmotility, and abnormal brain–gut interactions are potential mechanisms for this disorder (56). Treatment options vary and are generally selected based on the patient's primary symptom.

Over 95% of the body's serotonin (5-HT) is found in the gastrointestinal tract in enterochromaffin cells and neurons. There are 18 known serotonin receptor subtypes, of which 5-HT₁, 5-HT₂, and 5-HT₄ are located in the gut and modulate gut secretion, motility, and sensation (57). The 5-HT in the synaptic spaces stimulates these receptors until it is actively cleared by a 5-HT transporter protein located on the presynaptic neuronal endings.

Alosetron, a 5-HT₃ receptor antagonist, retards colonic transit (58) and is used to treat diarrhea-predominant IBS. Although genetic variants in the 5-HT receptors have been discovered, their physiological relevance has yet to be determined.

Alteration of 5-HT transporter function may affect gut function. Mice lacking the 5-HT transporter initially exhibited accelerated colonic motility (59). The gene for the 5-HT transporter is called *SLC6A4*, for solute carrier family 6, member 4. It is also referred to as SERT or 5-HTT (T for transporter). The *SLC6A4* gene spans 31 kb and consists of 14 exons (60). Several allelic variants have been discovered on this gene, but a 44-bp insertion/deletion in the promoter region has functional consequences. The short allele results in lower transcriptional efficiency of the 5-HT transporter, decreased transporter expression, and therefore decreased uptake of 5-HT in a lymphoblast cell line (61). The homozygous short genotype may be associated with diarrhea-predominant IBS (62). The SS genotype should result

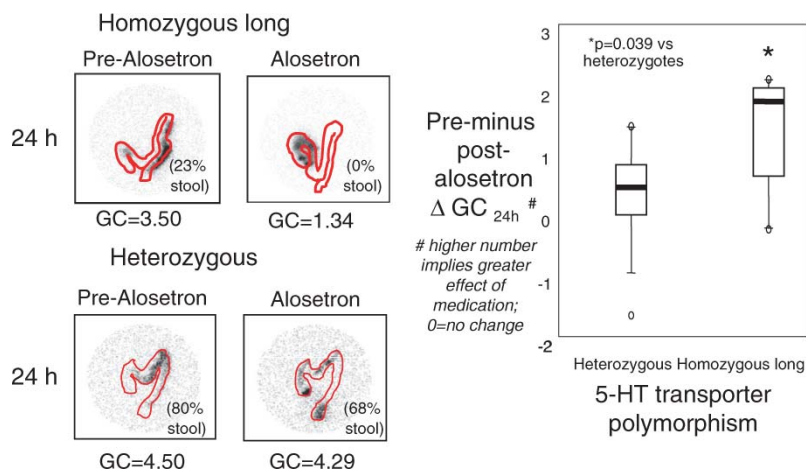


Fig. 13.4 Response of colonic transit to treatment with alosetron is greater in homozygous long vs heterozygous SERT-P genotype. 5-HT, serotonin. (Reproduced from ref. 63.)

in increased 5-HT in the synapse and may contribute to diarrhea (e.g., by stimulating 5-HT receptors on excitatory myenteric or submucosal neurons).

We performed a small pharmacogenomics study showing that the L allele of the SLC6A4 promoter was associated with increased response to treatment with alosetron (63). The hypothesis is that, consistent with in vitro studies by Lesch et al. (60), the L allele results in efficient transporter production, reducing 5-HT in the synapse and allowing greater 5-HT₃ receptor antagonism with alosetron. However, IBS patients homozygous for the S alleles did not exhibit the worst response to alosetron, arguing against a gene-dose effect. Conversely, in a study of 41 patients with constipation-predominant IBS, the efficacy of tegaserod treatment with the L/L genotype was lower (36.4%) than with S/L (70%) and S/S (85%) genotypes (64) (see Fig. 13.4). The results from these studies are limited by the small sample size, the inadequate number of patients with homozygous S alleles in the U.S. study on alosetron, and the small number who were homozygous L in the tegaserod study from China. Future studies are needed to define whether genotyping has a clinical role in the management of these patients.

13.5 Liver Transplantation

Decompensated liver disease is complicated by jaundice, refractory ascites, bacterial peritonitis, coagulopathy, and variceal bleeding and may require liver transplantation. The number of liver transplants for decompensated cirrhosis doubled from 1990 to 2004, when 5845 cadaveric (orthotopic) liver transplants were performed (65).

The main indications for liver transplantation include chronic hepatitis C, alcoholic liver disease, nonalcoholic fatty liver disease, and cryptogenic cirrhosis.

After transplantation, immunosuppression must be used to prevent host rejection of the graft liver, usually with prednisone and tacrolimus or cyclosporine. Tacrolimus and cyclosporine are calcineurin inhibitors and require drug level monitoring because of a narrow therapeutic range and significant toxicity, including renal failure and neurotoxicity.

13.5.1 CYP3A4 Polymorphisms and Cyclosporine and Tacrolimus Dosing

Cytochrome P450 3A4, subfamily IIIa, polypeptide 4, is the main P450 enzyme expressed in the liver, but it is also expressed in intestinal epithelial cells (enterocytes) (66). An estimated 60% of drugs are cleared by this P450 enzyme. The gene is called *CYP3A4* and has been localized to chromosome 7. Two genetic variants are commonly studied: *CYP3A4-V* is located in the promoter region of the nifedipine-specific response element of the gene (67), and *CYP3A4*1B* results in increased gene transcription (68), which results in lower drug levels. Thus, *CYP3A4*1B* carriers need higher doses of tacrolimus compared to noncarriers (69), but the same has not been shown for cyclosporine (70).

Several other genes are involved in metabolizing cyclosporine and tacrolimus in transplant patients. *CYP3A5* is expressed in 33% of Caucasians and 60% of African Americans (71). *CYP3A5* represents up to 50% of total *CYP3A* activity and may therefore be responsible for interindividual variability in *CYP3A* drug-metabolizing activity. Different alleles, including *1, *3 (intron 3 6986A>G), and *6 (exon 7 G>A), have been reported. The *1 allele is the wild type that results in expression of large amounts of *CYP3A5*, whereas alleles *3 and *6 result in absence of *CYP3A5* expression in the liver. When cyclosporine and tacrolimus trough levels were checked in 167 kidney transplant patients and compared to genotype, tacrolimus (but not cyclosporine) levels were higher, and dose (in mg/kg) was lower in those homozygous for the *3 allele compared with those carrying at least one *1 allele (72).

In liver transplant practice, genotyping of the donor (not the recipient) may be necessary to assess *CYP3A* expression in the liver. The *CYP3A5* genotype of the recipient may be relevant because the intestinal expression of this CYP enzyme will also have an impact on the circulating levels of cyclosporine or tacrolimus. Donor and recipient genotyping may be necessary to optimally manage dosage of these drugs.

The gene encoding the membrane-bound adenosine triphosphate (ATP)-binding cassette, subfamily B, member 1 transporter protein (P-glycoprotein) is formally named the *ABCB1* gene but is more commonly known as the multidrug resistance 1 (*MDR1*) gene. P-glycoprotein is expressed in the epithelial cells in the gut, liver, and kidney and contributes to drug excretion (73). A functional SNP on exon 26 (3435C>T) is associated with decreased duodenal expression in homozygous TT (74), which would result in lower drug excretion. One study in 44 liver transplant recipients

showed that the TT genotype was associated with higher drug levels, and TT genotype patients required 50% lower weight-adjusted doses than wild-type patients (75). In another study of 50 liver transplant patients, those with the CC genotype required higher doses of tacrolimus to achieve target blood concentrations (76). Thus, there may be a role for genotyping transplant recipients to tailor immunosuppressant regimens. However, this remains an area of controversy, with contradictory data on the effect of MDR1 3435C>T polymorphism in most disease areas; hence, information from this test should be applied with great caution in clinical practice.

13.6 Use of Chemotherapy in Colorectal Cancer

Colorectal cancer is the third most common cancer and the second leading cause of cancer-related deaths in the United States. (77). The incidence is approx. 40 per 100,000 in men and 25–30 per 100,000 in women (78). For those with stage III disease with presumed micrometastatic disease, adjuvant chemotherapy is used, typically 5-fluorouracil (5-FU) and leucovorin for 6–8 mo, with a 30% reduction in disease recurrence and 22–32% reduction in mortality (79,80).

Two genes are known to influence 5-FU drug metabolism or drug efficacy. The first, *DPYD*, encodes the enzyme dihydropyrimidine dehydrogenase (DPD), which converts 5-FU into inactive dihydro-5-FU in the liver. Functional genetic variations of *DPYD* affect tumor response and predict severe toxicity. Low enzyme activity predicts response to 5-FU (81); extremely low DPD activity levels result in severe toxicity, including mucositis, granulocytopenia, and neuropathy (82,83). There are over 20 known functional polymorphisms or mutations of this gene, and approx. 3–5% of the general population are heterozygous for DPD functional variants. Another 0.1% are homozygous carriers (84). A well-described functional variant is *DPYD**2A, and diagnostic kits are available for clinical use.

A second relevant enzyme to 5-FU metabolism is thymidylate synthetase (TYMS). If this enzyme is complexed with 5-FU metabolites along with 5,10-methylene-tetrahydrofolate, it cannot maintain a thymidine-5'-monophosphate pool required for DNA replication and repair. A tandem repeat polymorphism in the 5'-promoter region of the *TYMS* gene can increase enzyme expression (85,86). Tumors carrying the repeats have higher enzyme expression, resulting in lower response to chemotherapy compared to wild type (87).

For irinotecan, there are severe dose-limiting side effects, such as myelosuppression and diarrhea from its active metabolite SN-38, which is normally metabolized by the hepatic uridine diphosphate (UDP)-glucuronyl-transferase. The gene for this enzyme, *UGT1A1*, is located on chromosome 2. Over 30 allelic variants have been discovered. The *UGT1A1**28 allelic variant results in a longer TA repeat in the TATA sequence of the gene promoter, leading to lower hepatic expression and thus lower glucuronidation of SN-38, which is associated with diarrhea and bone marrow toxicity in the setting of treatment with irinotecan (88,89). Other variants, *UGT1A1**6/*6 and *UGT1A9*–118(dT)9/9 also showed a trend for high incidence of

severe diarrhea, but not tumor response. These findings suggest that UGT1A1*6 and UGT1A9*22 genotypes may be important for SN-38 glucuronidation and associated with irinotecan-related severe toxicity (**90,91**). A UGT1A1 molecular assay has been approved (**92**).

Tumor response to platinum agents such as cisplatin, carboplatin, and oxaliplatin appears to be affected by polymorphisms on three genes that affect DNA repair as well as a gene involved in drug metabolism. The X-ray cross-complementing group 1 protein (XRCC1), the xeroderma pigmentosum group D protein (ERCC2, formerly XPD), and excision repair cross-complementing enzyme (ERCC1) are important for the repair of DNA and influence drug efficacy. The glutathione S-transferase 1 (GSTP1) protein inactivates platinum drugs, and an SNP, Ile105Val, on codon 105 demonstrates a dose–response relationship with survival that was related to decreased enzyme activity (**93**).

To date, these genes have not been evaluated as part of routine clinical practice in treating patients with colorectal neoplasia. However, drug selection, dosing, and prognosis are influenced by these genetic variants. When accurate tests are available, they will likely allow for individualized cancer regimens based on genetic background.

13.7 Conclusion

Extensive study has been performed evaluating the role of genetic variation and its impact on the clinical response to drugs in gastrointestinal disease. In this review, we demonstrated that pharmacogenetics has the potential to impact a wide variety of disease, ranging from gastroesophageal disease, IBD, and functional gastrointestinal disorders, to liver transplantation and colon cancer. A summary of the discussed conditions, drugs, genes, polymorphisms, and their clinical effects is provided in **Table 13.1**. Routine use of TPMT enzyme measurement as a proxy for *TPMT* genotype represents a good example of how pharmacogenetics can have a direct impact on clinical management of patients.

Clinical utilization of pharmacogenetics is currently limited in gastrointestinal disease. Of the conditions, drugs, and genes discussed in this review, only TPMT activity is measured on a routine basis to aid in AZA and 6-MP dosing to minimize serious potential side effects. Nonetheless, with expanding understanding that the role of genetic variation plays in disease progression and response to therapy, the role of pharmacogenetics in this field will likely grow. In the near future, emphasis of study should be placed on evaluating toxic medications with serious adverse events, such as tacrolimus use in liver transplantation or chemotherapy use in colon cancer treatment.

On the other hand, the usefulness of pharmacogenetics in dosing safe and effective medications, such as pump inhibitors in patients with GERD, is less certain. Unless genotyping can be performed very quickly and inexpensively, most clinicians will likely dose based on clinical response rather than on a genotype.

Table 13.1 Gastrointestinal conditions, drugs, genes, and polymorphisms with clinical effects

Condition	Drug	Protein (gene)	Alleles or polymorphisms	Clinical effects
Inflammatory bowel disease (IBD)	Azathioprine, 6-MP	Thiopurine-S-methyltransferase (TPMT)	*2, *3A, *3B, etc.	High, intermediate, low metabolizers predict drug dosing needs
	Methotrexate	Methylenetetrahydrofolate reductase (MTHFR)	1298A>C	CC patients experience more side effects
Gastroesophageal reflux disease (GERD), <i>Helicobacter pylori</i> infection	Proton pump inhibitors (PPIs)	Cytochrome P450 2C19 (CYP2C19)	*2, *3, *4, *5 alleles result in decreased metabolism of drug	Wild-type predicts slower healing of esophagitis, lower cure rates of <i>H. pylori</i> infections
Irritable bowel syndrome (IBS)	Alosetron	Serotonin transporter (SLC6A4)	5-HTT LPR	Patients with diarrhea and LL homozygotes may predict better response and slowing of colonic transit
Functional dyspepsia (FD)	Multiple drugs (PPIs, prokinetics, spasmolytics, tricyclic antidepressants)	G-protein β_3 -subunit (GN β 3)	825C>T	CC genotype predicts response to therapy
Liver transplantation	Cyclosporine, tacrolimus	Cytochrome P450 3A4 (CYP3A4)	*1B	*1B carriers require higher tacrolimus doses
		Cytochrome P450 3A5 (CYP3A5)	*3, *6	Alleles predict higher tacrolimus doses and lower dosage
Colorectal cancer	5-fluorouracil (5-FU), capecitabine	Dihydropyrimidine dehydrogenase (DPYD)	*2A	Low DPD activity predicts decreased response and increased toxicity

Irinotecan	Thymidylate synthase (TYMS)	TSER3 repeats	Carriers have lower response to therapy
	Uridine diphosphate-glucuronyl transferase (UGT1A1)	*28	Carriers of one or two copies associated with dose-limiting diarrhea, myelosuppression
	X-ray cross-complementing group 1 protein (XRCC1)	399Arg>Gln	Carriers have lower response to 5-FU/platinum regimens
Cisplatin			
Carboplatin Oxaliplatin	Xeroderma pigmentosum group D protein (ERCC2)	751Lys>Gln	Carriers have poorer response to chemotherapy and survival
	Excision repair cross-complementing enzyme (ERCC1)	N118N	T carriers have lower protein expression, poorer survival
	Glutathione S-transferase (GST)	105Ile>Val	Dose response: wild type showed survival

DPD, dihydropyrimidine dehydrogenase; 6-MP, 6-mercaptopurine.
5HTTLPR = 5-hydroxytryptamine transporter long promoter region.

Although genotyping *CYP2C19* for GERD therapy guidance is unlikely to be helpful in general clinical use in the near future and given that the genotype is immutable, it is conceivable that a one-time CYP genotyping that is available in the medical record could enhance the choices of medications and doses for drug classes with limited efficacy or toxicity in standard doses (e.g., prokinetics, antikinetics, antispasmodics, and antidepressants in functional gastrointestinal diseases).

A study showed the potential value of genotyping 14 polymorphisms of eight genes encoding drug-metabolizing enzymes and transporters using a customized oligonucleotide array (TPMT, NQ01, MTHFR, GSTP1, CYP1A1, CYP2D6, ABCB1 [also called MDR1], and SCL19A1), which were screened in 371 cord blood samples from healthy newborns (94).

Ultimately, such assays may lead to studies to address the cost-utility and cost-efficacy of genotyping as a means to individualize guidelines for management of gastrointestinal and liver disease.

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Chapter 14

Pharmacogenomics in Rheumatoid Arthritis

Prabha Ranganathan

14.1	Introduction	413
14.2	Pharmacogenetics of Disease-Modifying Antirheumatic Drugs.....	414
14.3	Conclusions and Future Directions	430
	References.....	431

Summary Rheumatoid arthritis (RA) is a systemic inflammatory arthritis that leads to severe joint damage and is associated with high morbidity and mortality. Disease-modifying antirheumatic drugs (DMARDs) are the mainstay of treatment in RA. DMARDs not only relieve the clinical signs and symptoms of RA but also inhibit the radiographic progression of disease. Recently, a new class of disease-modifying medications, the biologic agents, has been added to the existing spectrum of DMARDs in RA. However, patients' response to these agents is not uniform, with considerable variability in both efficacy and toxicity. There are no reliable means of predicting an individual patient's response to a given DMARD prior to initiation of therapy. In this chapter, the current published literature on the pharmacogenomics of traditional DMARDs and the newer biologic DMARDs in RA is highlighted. Pharmacogenomics may help individualize drug therapy in patients with RA in the near future.

Keywords Azathioprine; methotrexate; pharmacogenetics; polymorphisms; rheumatoid arthritis; sulfasalazine; tumor necrosis factor antagonists.

14.1 Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory arthritis that occurs in about 1% of the population worldwide. Untreated, RA is associated with joint destruction, disability, and increased mortality (**I**). Early detection and therapy with disease-modifying antirheumatic drugs (DMARDs) is critical in preventing these sequelae of RA. With the recent advent of biologic DMARDs, which are effective but expensive therapies for RA, there has been a focus on developing methods that

include those based on pharmacogenomics to predict a priori an individual patient's response to a given DMARD.

This chapter highlights some of the recent major publications in the field of pharmacogenomics in RA and describes the implications of this field for future research and clinical care. The pharmacogenetics of three major DMARDs (methotrexate [MTX], azathioprine [AZA], and sulfasalazine [SSZ]) and one class of biologic DMARDs, the tumor necrosis factor (TNF) antagonists, in RA, are reviewed.

14.2 Pharmacogenetics of Disease-Modifying Antirheumatic Drugs

14.2.1 Pharmacogenetics of Methotrexate

For over the past two decades, MTX has been the first-line DMARD in RA because of its well-established efficacy and safety (2–4). However, the response among patients to MTX can be quite variable, ranging from 46% to 65% (5,6). The exact mechanism of action of the drug in RA remains unclear; however, it is believed that MTX's effects in RA occur because of its effects on the intracellular folate and adenosine pathways.

MTX is actively transported into the cell by solute carrier 19A1 (SLC 19A1), also called reduced folate carrier 1 (RFC1) (see Fig. 14.1). MTX is pumped out of the cell by members of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters, also known as multidrug resistant (MDR) transporters, and multidrug resistance-associated proteins (MRPs) (7). Intracellular MTX is polyglutamated by the enzyme folylpolyglutamyl synthase (FPGS). This process can be reversed by gamma glutamyl hydrolase (GGH). Polyglutamation of MTX (MTXPGs) helps retain MTX within the cell, preventing drug efflux by the ABC transporters. MTXPGs inhibit dihydrofolate reductase (DHFR), which reduces dihydrofolate to tetrahydrofolate (THF) (8). THF is converted to 5,10-methylene tetrahydrofolate (5,10-CH₂-THF) and subsequently to 5-methyl THF (5-CH₃-THF) by methylenetetrahydrofolate reductase (MTHFR). 5-methyl THF is a biologically active folate cofactor that functions as a one-carbon donor for many important cellular reactions, including the conversion of homocysteine to methionine (9). MTXPGs also inhibit thymidylate synthase (TYMS), which converts deoxyuridylate to deoxythymidylate in the de novo pyrimidine synthetic pathway (10).

MTX also has several effects on the purine synthetic pathway. MTXPGs inhibit the enzyme aminoimidazole carboxamide ribonucleotide (AICAR) transformylase, which in turn causes intracellular accumulation of AICAR. AICAR and its metabolites can then inhibit two enzymes in the adenosine pathway: adenosine deaminase and adenosine monophosphate (AMP) deaminase, which leads to intracellular accumulation of adenosine and adenine nucleotides. Subsequent dephosphorylation of these nucleotides results in increased extracellular concentrations of adenosine, which is a powerful anti-inflammatory agent (11).

In a study by Dervieux et al., the effect of the G80A single-nucleotide polymorphism (SNP) on response to MTX in 105 RA patients was examined. Patients within the top 25th percentile of MTX responders were identified using a visual analog scale (VAS) measuring the patients' and physicians' assessment of response to MTX. Patients homozygous for the *RFC* SNP 80A/A had a greater response to MTX compared to patients carrying the wild-type 80G/G SNP. Patients homozygous for the A allele were three times more likely to be within the top 25th percentile of MTX responders (OR=3.0, CI 1.3–8.4; $p < 0.01$) compared to the patients with the wild-type G allele. Thus, the *RFC* 80AA SNP may be a marker of increased response to MTX in RA (15).

14.2.1.2 ABCB1

The *ABCB1* gene is a 209-kb gene located on chromosome 7 (7q21.1). P-glycoprotein (P-gp), the product of the *ABCB1* gene, is a membrane transporter important in the transport of several drugs. SNPs in the *ABCB1* gene have been identified and their effects on P-gp expression studied (16). The C3435T SNP is a synonymous SNP in exon 26 of the *ABCB1* gene. It is often linked to a G2677T SNP in exon 21 that results in substitution of alanine in position 893 by serine or threonine (17,18). It is unclear whether variations in *ABCB1* or P-gp expression have an impact on MTX efflux from the cell. Although there is lack of published data to support that *ABCB1* SNPs influence MTX cellular transport directly, some studies suggested that higher P-gp expression may mediate MTX resistance (19); other studies did not support this (20,21). Considering the linkage of the two SNPs, haplotype analyses may be more helpful in examining the role of these genetic variants in influencing MTX's effects.

In one study, 92 RA patients and 97 healthy controls were genotyped for the C3435T polymorphism. Patients who had active RA ($n = 62$) after 6 months of treatment with MTX (7.5–15 mg/wk) and prednisone (5–10 mg daily) and those who responded after 6 months of the same treatment ($n = 30$) were classified as two groups and studied. The *ABCB1* genotypes were distributed similarly among the RA patients and controls. Patients with the 3435CC and 3435CT genotypes were more likely to have active RA compared to patients with the 3435TT genotype (OR 2.89, CI 0.87–9.7; $p < 0.05$). Thus, the presence of the 3435T allele seemed to be protective in that patients homozygous for this allele had less severe RA that was more responsive to MTX and prednisone (22).

14.2.1.3 MTHFR/TYMS/DHFR

The *MTHFR* gene is a 19-kb gene located on chromosome 1 (1p36.3). Of the several *MTHFR* polymorphisms that have been identified (23), two polymorphisms, the C677T and A1298C polymorphisms, have been well studied for their influence on MTX's clinical effects. The C677T polymorphism leads to an alanine-to-valine

substitution in the codon at nucleotide 677 of the *MTHFR* gene (24). This change leads to a thermolabile variant of MTHFR to be encoded, with resultant decreased enzyme activity. The A1298C polymorphism causes a glutamine-to-alanine substitution in the codon at nucleotide 1298 and also leads to reduced MTHFR enzyme activity (25). As MTHFR is important in the generation of 5-methyl THF (see Fig. 14.1), which acts as the carbon donor for the remethylation of homocysteine to methionine, these two SNPs by reducing MTHFR activity can increase plasma homocysteine levels (26).

Elevated plasma homocysteine levels mediated by these two SNPs may exacerbate MTX's toxic effects. Several studies have evaluated the effects of these SNPs on MTX response. One study examined 105 patients with RA, 35 of whom were treated with MTX (7.5–15 mg/wk), 34 with SSZ (2–3 g/d), and 36 with MTX and SSZ. All patients were genotyped for the C677T SNP and their plasma homocysteine levels measured. Patients on MTX had higher plasma homocysteine levels than those on SSZ alone, but those on both MTX and SSZ had the highest levels. Patients heterozygous for the C677T SNP had higher plasma homocysteine levels after 1 year than patients without the SNP. Patients homozygous for the SNP had a higher plasma homocysteine level at baseline that did not change significantly. Elevated plasma homocysteine levels (17%, $p < 0.05$) were found in patients with gastrointestinal (GI) side effects from MTX, such as nausea, abdominal pain, and discomfort, compared to patients without side effects. Patients on MTX and SSZ had the highest homocysteine levels and the highest incidence of GI side effects. However, the presence of the C677T SNP was not directly associated with the occurrence of GI events. This study suggested that plasma homocysteine levels (exacerbated by the presence of the *MTHFR* C677T SNP) may influence the GI toxicity of MTX (27).

In another study, 236 patients with RA on MTX were genotyped for the C677T SNP. MTX was initiated at 7.5 mg/wk and titrated to a maximum dose of 25 mg/wk. Patients were assessed for MTX toxicity and disease activity periodically. Of the 236 patients, 122 (52%) did not have the SNP; 19 patients (8%) were homozygous and 95 patients (40%) were heterozygous for the polymorphism. Patients who were homozygous and heterozygous for the C677T SNP had an increased risk of discontinuing MTX because of adverse events (RR 2.01; CI 1.09–3.70), particularly hepatotoxicity (RR 2.38; CI 1.06–5.34). This effect of the genotype on MTX toxicity was also evident in patients on folate supplementation in this study. However, there was no effect of the C677T genotype on MTX efficacy (28).

In a cross-sectional study, 93 RA patients treated with MTX (average dose 11.9 mg/wk) and 377 healthy controls were genotyped for the C677T and A1298C polymorphisms and assessed for RA disease activity and MTX toxicity. Serum folate and plasma homocysteine levels were measured. More RA patients carried the 1298CC genotype (24.7%) than the controls (12.8%), and this was statistically significant ($p < 0.001$). There were interesting effects of the 1298CC genotype on MTX toxicity but not efficacy. Homozygotes for the 1298C SNP appeared to be protected from MTX toxicity; 33% did not experience toxicity, and only 9.1% had adverse reactions ($p = 0.035$). In contrast, patients with the AA genotype were five

times more likely to develop toxicities than those with the CC genotype (OR 5.24; CI 1.38–20). Also, patients carrying the CC genotype had higher plasma homocysteine levels than patients with the AA or AC genotype, and this was not influenced by serum folate levels. The C677T polymorphism had no effects on MTX toxicity or efficacy in this study. This study suggested that homozygosity for the C allele increases susceptibility to RA but also protects from MTX toxicity, presumably via a homocysteine-dependent mechanism (29).

Retrospectively, 106 RA patients who had been treated with MTX were assessed for MTX efficacy and toxicity. The MTX dose ranged from 2.5 to 12.5 mg/wk. All patients were genotyped for the C677T and A1298C SNPs. As this was a retrospective study, no direct assessment of MTX efficacy (as measured by disease activity scores) was possible. However, patients carrying the A1298C polymorphism (homozygous or heterozygous) were more likely to be on lower doses of MTX compared to those without the polymorphism (RR 2.18, CI 1.17–4.06; $p < 0.05$). Patients carrying this polymorphism also showed improvement in their inflammatory markers, such as ESR (erythrocyte sedimentation rate) and CRP (C reactive protein), suggesting clinical efficacy of MTX ($p < 0.05$). Such changes were not seen with the presence of the C677T polymorphism. The presence of the C677T polymorphism was associated with an increased likelihood of side effects from MTX (RR 1.25, CI 1.05–1.49; $p < 0.05$) but not with indicators of MTX efficacy, such as lower doses of the drug or improvement in inflammatory markers. Thus, the C677T polymorphism appeared to be a marker for MTX toxicity and the A1298C polymorphism for MTX efficacy (30).

TYMS is an important enzyme in the de novo synthesis of pyrimidines. It converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) and is a direct target of polyglutamated MTX. The *TYMS* gene is a 15-kb gene located on chromosome 18 (18p11.32). A polymorphic tandem 28-bp repeat sequence has been described in the 5' untranslated region (TSER) of the *TYMS* gene with a variable number of repeat elements (31). This repeat element may function as an enhancer as in vitro studies have shown that TYMS messenger RNA (mRNA) expression and enzyme activity are increased with an increasing number of these repeat sequences (31–33). Patients homozygous for the triple-repeat allele (*TSER**3/3) have higher TYMS mRNA expression compared to patients homozygous for the double-repeat allele (*TSER**2/2) (33,34). Deletion of a 6-bp sequence at nucleotide 1494 in the 3' UTR of *TYMS* has also been described and may be associated with decreased TYMS mRNA stability and expression (35,36).

In another study, 167 patients with RA, of whom 115 were treated with MTX, were genotyped for the following polymorphisms: *TYMS* 5' UTR enhancer repeat (*TSER*), 3' UTR deletion, *MTHFR* C677T, and A1298C. The mean weekly MTX dose in this study was 5.7 ± 2.3 mg. Information on MTX toxicity data was collected retrospectively. Both MTX-treated and untreated groups displayed similar frequencies of these SNPs. The *TYMS* and *MTHFR* polymorphisms were not associated with toxicity, although a significant percentage (45%) of patients on MTX experienced adverse effects. Weekly MTX dose (rather than standardized disease activity measures) was used as a marker of efficacy in this study. A dose greater than 6 mg/wk was considered indicative of less efficacy, and that below 6 mg/wk

was considered indicative of greater efficacy. Homozygotes for the *TSER**2 allele (*TSER**2/*2) required lower doses of MTX (had greater efficacy) than homozygotes for the *TSER**3 allele ($p = 0.033$). The *TYMS* 3' UTR deletion and *MTHFR* polymorphisms had no effect on MTX efficacy. The authors speculated that the repeat enhancer *TSER**3 polymorphism, by increasing *TYMS* mRNA expression, may lead to decreased MTX efficacy. Based on the findings of this study, it also appeared that *MTHFR* polymorphisms did not influence MTX toxicity or efficacy (37).

DHFR reduces dihydrofolate to THF in the intracellular folate pathway. It is directly inhibited by polyglutamated MTX and encoded by the *DHFR* gene, which is a 28-kb gene located on chromosome 5 (5q11.2–q13.2). *DHFR* gene polymorphisms have been studied in 205 MTX-treated RA patients. MTX was started at 7.5 mg/wk and increased to 15 mg/wk (with folic acid supplementation) after 4 weeks based on response to the drug. MTX efficacy and toxicity (GI side effects, elevated liver enzyme levels, skin and mucosal disorders, pneumonitis, and overall adverse drug events) were assessed periodically. Genotyping for the *MTHFR* 677C>T, *MTHFR* 1298A>C, *DHFR* –473G>A, *DHFR* 35289G>A, and *RFC* 80 G>A SNPs was performed. At 6 months, patients carrying the *MTHFR* 1298AA and *MTHFR* 677CC (wild-type) genotypes showed a greater response to MTX compared to patients carrying the heterozygous or homogeneous genotype (OR 2.3, CI 1.18–4.41 and OR 2.73, CI 1.03–7.26, respectively). Haplotype analysis for the *MTHFR* 1298A and 677C SNPs revealed that patients with two copies of the haplotype had greater improvement than those with one or no copies of the haplotype (OR 3.0, CI 1.4–6.4). Patients homozygous and heterozygous for the *MTHFR* 1298 SNP (*MTHFR* 1298AC+CC) had an increased number of overall adverse drug events at 3 and 6 months (OR 2.55, CI 1.20–5.41 and OR 2.5, CI 1.32–4.72, respectively) compared to those with other genotypes. The *RFC* and *DHFR* SNPs were not associated with MTX toxicity or efficacy. Thus, patients with the wild-type *MTHFR* alleles (*MTHFR* 1298AA and 677CC) responded better to MTX; those with the 1298C allele had an increased risk for MTX toxicity (38).

Based on the literature cited (see Table 14.1), the C677T SNP in *MTHFR* appears to have effects on MTX toxicity, presumably through its effects on homocysteine metabolism (27,28,30), and on MTX efficacy (38). The effects of the A1298C polymorphism on MTX are less clear, with data suggesting that it may increase (30) or decrease (38) patients' response to MTX and possibly protect them from MTX toxicity (29). The seemingly inconsistent results of these studies may stem from the fact that these SNPs may have effects other than those on homocysteine metabolism that may influence response to MTX. Some of these studies were retrospective, which may also have led to inaccuracies in the assessment of MTX effects, particularly adverse effects. Although one of the studies (37) concluded that *MTHFR* SNPs did not affect MTX efficacy or toxicity, it is worth pointing out that the doses of MTX used in this study were small (6 mg/wk), which may have masked the differences in MTX response between patient groups. Also, MTX efficacy was not assessed using standardized measures of disease activity in this study; rather, MTX dose was used as a surrogate marker of MTX efficacy. Ethnicity may have been another factor that influenced the results in this Japanese study (37).

Table 14.1 Pharmacogenetics of methotrexate (MTX) in rheumatoid arthritis

Gene	Role in MTX pathway	Polymorphism	Postulated effect of polymorphism	Clinical effects	Reference
RFC1	Active transport of MTX into cell	G80A	Increased transcriptional activity of RFC1 gene; increased MTX entry into cell	Increased response to MTX	15
ABCB1	MTX efflux from the cell	C3435T	Unclear; may increase MTX entry into cell	Increased response to MTX	22
MTHFR	Important in regeneration of reduced folate; indirectly inhibited by MTX	C677T	Thermolabile MTHFR with decreased activity; increased plasma homocysteine	Increased GI side effects Increased hepatic toxicity, GI toxicity, alopecia, stomatitis, and rash No effect on toxicity No effect on efficacy or toxicity	27 28, 30 29 37
MTHFR	As above	A1298C	Decreased MTHFR activity; increased plasma homocysteine	Increased MTX efficacy Increased susceptibility to RA; decreased MTX toxicity No effect on efficacy or toxicity	30 29 37
DHFR	Reduction of dihydrofolate to tetrahydrofolate	G473A G35289A	Decreased DHFR activity	No effect on MTX efficacy or toxicity	38
ATIC	Conversion of AICAR to 10-formyl AICAR; directly inhibited by MTX	C347G	Decreased ATIC activity; AICAR accumulation; increased adenosine	Increased MTX efficacy	39

(continued)

Table 14.1 (continued)

Gene	Role in MTX pathway	Polymorphism	Postulated effect of polymorphism	Clinical effects	Reference
TYMS	Conversion of dUMP to dTMP; directly inhibited by MTX	5' UTR 28-bp repeat (TSER) 3' UTR 6-bp deletion	Increased TYMS enzyme activity Decreased TYMS mRNA stability and expression	Decreased MTX efficacy Increased MTX efficacy	37 37

dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; GI, gastrointestinal; mRNA, messenger RNA; UTR, untranslated region. See text for gene abbreviation definitions.

14.2.1.4 TYMS/AICAR Transformylase/RFC1

Polygenic analyses of the MTX pathway genes have also been performed. This is particularly relevant in the case of MTX as the drug exerts its effects by influencing several different genes in the intracellular pathways. AICAR transformylase (ATIC) converts AICAR to 10-formyl AICAR and is directly inhibited by MTX (see Fig. 14.1). This leads to accumulation of AICAR and adenosine, a purine with anti-inflammatory properties. Adenosine may be an important mediator of the anti-inflammatory effects of MTX (11).

The *ATIC* gene is a 37-kb gene located on chromosome 2 (2q35). A C347G nonsynonymous SNP leading to a threonine-to-serine substitution in codon 2 has been described in this gene. A study examined the combined effects of the C347G SNP in *ATIC*, *TSER**2, and G80A polymorphism in *RFC1* on MTX efficacy; 108 RA patients on MTX at a dose of 15 mg/wk (range 5–25 mg/wk) were examined. Red blood cell (RBC) long-chain MTX polyglutamate (MTXPG) concentrations were measured, and a pharmacogenetic index was calculated from the sum of homozygous variant genotypes (*RFC1* 80AA, *ATIC* 347GG, *TSER**2/*2). Patients were categorized as MTX responders or MTX nonresponders using a VAS. The presence of a homozygous variant genotype or a higher pharmacogenetic index correlated with increased MTXPG levels and increased response to MTX. Patients with one homozygous variant were 3.7 times as likely to respond to MTX than patients without a homozygous variant (OR 3.7, CI 1.7–9.1; *p* = 0.01) (39).

14.2.2 Pharmacogenetics of Azathioprine

Azathioprine is used in the treatment of several rheumatic diseases, including systemic lupus erythematosus (SLE) and RA. About 10–30% of RA patients discontinue AZA because of side effects (40). AZA is a prodrug that after oral intake is

converted into 6-mercaptopurine (6-MP), an active purine antimetabolite that affects the purine de novo synthetic and salvage pathways (*see Fig. 14.2*). 6-MP is converted by hypoxanthine phosphoribosyl transferase (HPRT) to cytotoxic 6-thioguanine nucleotides (6-TGNs) via the intermediary metabolite 6-thiosine monophosphate (6-TIMP). By a parallel pathway, 6-MP can be inactivated by thiopurine methyltransferase (TPMT) to 6-methylmercaptopurine (6-MMP) or by xanthine oxidase (XO) to thiouric acid (TU). Thus, a relative deficiency of TPMT leads to accumulation of cytotoxic TGN and significantly increased AZA toxicity. Common, significant toxicities of AZA are hematologic and gastrointestinal.

The *TPMT* gene is a 26-kb gene located on chromosome 6 (6p22.3). Allelic variants of this gene determine the level of TPMT activity in erythrocytes. TPMT activity in erythrocytes can be classified into high activity, intermediate activity, and low or no activity. Population studies have shown that approximately 90% of the population has high activity, 10% has intermediate activity, and 0.3% has little to no activity (*41*). Standard doses of AZA, when given to patients with low TPMT activity, can lead to severe hematologic toxicity, which may be fatal. Of patients with low TPMT activity, 80–95% usually possess one of the three common allelic variants of the *TPMT* gene: *TPMT**2, *TPMT**3A, or *TPMT**3C (*42–44*). Different frequencies of these allelic variants have been described in different populations worldwide; thus, ethnicity influences the occurrence of these variants (*45,46*). Patients with low TPMT activity require lower AZA doses to avoid toxicities (*47*).

In one study, 68 patients with rheumatic disease on AZA (2 to 3 mg/kg/d) were genotyped for *TPMT**2 and *TPMT**3A alleles. All patients were assessed for side effects from AZA, such as leukopenia, liver function abnormalities, and GI intolerance. Of these patients, 6 (9%) patients were heterozygous for *TPMT**3A, of whom 5 discontinued AZA within 4 weeks of starting the medication because of hematologic toxicity (*48*). In another study, 40 RA patients on AZA (0.7 to 1.4 mg/kg/d) were genotyped for the *TPMT* alleles. Of the 40 patients, 6 discontinued

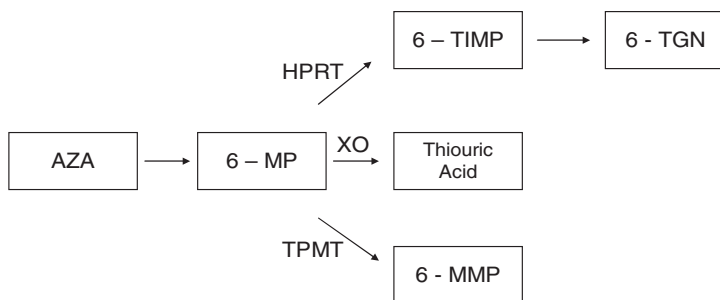


Fig. 14.2 Scheme of thiopurine drug metabolism. HPRT, hypoxanthine phosphoribosyl transferase; 6-MMP, 6-methylmercaptopurine; 6-TGN, 6-thioguanine nucleotides; 6-TIMP, 6-thiosine monophosphate; TPMT, thiopurine methyltransferase; XO, xanthine oxidase

AZA because of toxicity. Of the 6 patients with severe GI toxicity, 3 were heterozygous for the *TPMT**3A allele; the remainder possessed the wild-type *TPMT* allele. The correlation between the *TPMT* allele and AZA toxicity was significant, $p = 0.018$. Based on the results of this study, the positive predictive value for a *TPMT* polymorphism carrier was 60% (49).

Other studies have examined the association between the activity of TPMT and other enzymes in the purine pathway and AZA toxicity. In one study, TPMT, HPRT, 5'-nucleotidase, and purine nucleoside phosphorylase activity in the RBCs of 33 RA patients on AZA (dose of approximately 2 mg/kg/d) and 66 controls was measured. Compared to patients with normal TPMT activity, 14 RA patients with low ($p = 0.004$) and 7 patients with intermediate TPMT activity (RR 3.1) developed AZA toxicity(40). None of the patients were genotyped. Another study measured TPMT activity in 3 RA patients who had experienced AZA-induced hematologic toxicity and 16 RA patients without AZA toxicity. In this study, 2 patients with AZA-induced hematologic toxicity were TPMT deficient, one partial and the other complete (50). Patients were not genotyped in either of these studies.

Thus, both TPMT genotyping and measurement of TPMT activity in RBCs may be useful in predicting and preventing AZA toxicity. Clearly, large, prospective studies are needed to validate the observations from the smaller studies described (see Table 14.2). Of note, TPMT genotyping is available to clinicians currently to

Table 14.2 Pharmacogenetics of azathioprine (AZA) in rheumatoid arthritis

Polymorphism	Amino acid change in enzyme	Population prevalence of polymorphism (%)	Effect of polymorphism on enzyme activity	Biochemical effect of polymorphism	Clinical effects	Reference
TPMT*2 G238C	Alanine to proline	0.2–0.5	Low to intermediate because of enhanced degradation of enzyme	Decreased methylation of AZA to inactive compounds	Hematologic and GI toxicity	
TPMT*3A G460A, A719G	Alanine to threonine and tyrosine to cysteine, respectively	3.2–5.7	As above	As above	Hematologic toxicity	48, 49
TPMT*3C A719G	Tyrosine to cysteine	0.2–0.8	As above	As above	Hematologic toxicity	

GI, gastrointestinal; TPMT, thiopurine methyltransferase.

screen patients prior to the initiation of AZA and is the first commercially available assay for pharmacogenetic testing in rheumatology.

14.2.3 Pharmacogenetics of Sulfasalazine

Sulfasalazine is another DMARD often used in the treatment of RA. It is estimated that 20–30% of RA patients on SSZ report adverse drug reactions. Adverse drug events of SSZ are GI and hematologic.

SSZ is a combination of sulfapyridine and 5-aminosalicylic acid (5-ASA). After ingestion, colonic bacteria split SSZ into these two compounds. 5-ASA remains in the large bowel; most of sulfapyridine is completely absorbed and undergoes acetylation, hydroxylation, and glucoronidation in the liver. Acetylation of sulfapyridine is carried out by the enzyme *N*-acetyltransferase 2 (NAT2), which acetylates sulfapyridine into *N*-acetylsulfapyridine. The NAT2 gene is 9 kb, is located on chromosome 8 (8p22), and can be polymorphic. NAT2 gene polymorphisms may alter the acetylator phenotype of an individual (slow vs fast acetylator) and thus have effects on an individual's susceptibility to SSZ toxicity. Slow acetylators have been shown to be more prone to SSZ toxicity, such as abdominal discomfort, nausea, rash, and headaches, compared to fast acetylators (51,52).

Two studies have evaluated the effects of NAT2 polymorphisms on SSZ toxicity in RA patients. One retrospective study assessed 144 RA patients on SSZ at a dose range of 500 to 1500 mg/d. NAT2 genotyping was carried out in all patients. Slow acetylators lacking the wild-type NAT2*4 allele experienced adverse reactions more frequently (63%) compared to fast acetylators with at least one NAT2*4 allele (8%). This association between the NAT2 genotype and SSZ toxicity was clinically significant (OR 7.73, CI 3.54–16.86; $p < 0.001$). In fact, 25% of the slow acetylators had to be hospitalized for their toxicities (53). In the second study, 114 patients with inflammatory bowel or joint disease treated with SSZ (mean dose of 2 gm per day) were studied. Patients were genotyped for 5 allelic variants, NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6, and NAT2*7 (encoding slow acetylator status) and the wild-type NAT2*4 allele (encoding rapid acetylator status). Of 39 patients, 27 (69%) who developed agranulocytosis within 3 month of starting treatment with SSZ were slow acetylators compared to 34 of 75 patients (45%) who received SSZ without a hematological adverse event (OR 2.7; $p = 0.002$). Patients with SSZ-induced agranulocytosis had higher frequencies of the NAT2*6 alleles among other allelic variants (36%) compared to patients without agranulocytosis (23%) ($p = 0.033$) (54).

Thus, the acetylator status of a patient as determined by the NAT2 genotype appears to be an important determinant of the risk for SSZ toxicity based on the limited data published so far (see Table 14.3). Although more studies and data are clearly needed, this suggests that prospective screening of patients for the NAT2 genotype prior to initiation of SSZ may be a useful tool to prevent SSZ toxicity.

Table 14.3 Pharmacogenetics of sulfasalazine (SSZ) in rheumatoid arthritis

Polymorphism	Effect of polymorphism	Biochemical changes associated with SSZ therapy	Clinical effects	Reference
NAT2*5A	Decreased activity of NAT2 enzyme, leading to slow acetylation (slow acetylator)	Increased concentrations of SSZ intermediates because of slow acetylation	Agranulocytosis Fever, rash	54 53
NAT2*5B	As above	As above	As above	As above
NAT2*5C	As above	As above	As above	As above
NAT2*6	As above	As above	As above	As above
NAT2*7	As above	As above	As above	As above

NAT, *N*-acetyltransferase.

14.2.4 Pharmacogenetics of Tumor Necrosis Factor Antagonists

The tumor necrosis factor- α (TNF α) antagonists, a class of biological DMARDs, have dramatically altered the treatment of RA in recent years. These agents not only ameliorate the signs and symptoms of RA but more importantly are highly effective in slowing the radiographic progression of disease (6,55). In spite of their well-established efficacy, about 20–40% of RA patients do not respond adequately to these agents (56,57).

Three TNF antagonists are currently approved for the treatment of RA: etanercept (ETN), infliximab (INF), and adalimumab. ETN a fusion protein of two identical chains of the recombinant human TNF receptor, p75, fused with the Fc portion of human immunoglobulin (Ig) G1 binds to soluble TNF- α in vivo. INF and adalimumab are both monoclonal antibodies to TNF- α ; INF is chimeric, and adalimumab is fully humanized. Both bind to soluble TNF- α , preventing TNF- α from binding to its receptors on cell surfaces. INF can also bind transmembrane TNF- α , fix complement, and cause cell lysis.

The TNF family, consisting of TNF- α and lymphotoxin A (LTA) and B (LTB) has vital functions in immune regulation. The TNF- α gene is located on chromosome 6 and lies within the human major histocompatibility complex (MHC) III region (see Fig. 14.3). The *TNF* locus is a 7-kb region where the *TNF*, *LTA*, and *LTB*, genes are arranged in tandem and lies in close proximity to the *HLA B* and *MHC III DR* regions.

Polymorphisms in the *TNF* gene, TNF receptor genes, and DNA microsatellites have been studied to help predict response to the TNF antagonists in RA patients. Polymorphisms of the *TNF* gene at positions –308, –238, and +489 have been well studied in this respect. *TNF* –308 and –238 polymorphisms are located in the promoter region of the *TNF* gene, whereas the +489 polymorphism is located in the

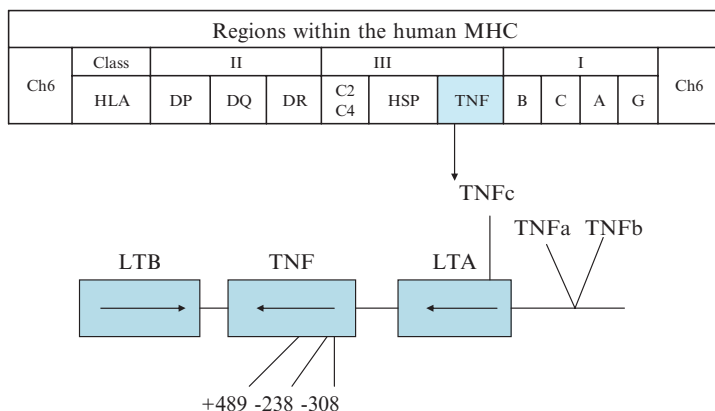


Fig. 14.3 Tumor necrosis factor (TNF) locus with some of the polymorphic sites known within the TNF locus. C2, C4, complement C2, C4; Ch, chromosome; HLA, human leukocyte antigen; HSP, heat shock protein; LTA, lymphotoxin A; LTB, lymphotoxin B; MHC, major histocompatibility complex. (Reproduced from ref. 74 by permission of Future Medicine Ltd.)

intronic portion of the gene. The promoter polymorphisms may increase the transcriptional activity of the *TNF* gene, although this remains controversial (58). The functional significance of the +489 polymorphism is presently unknown.

The *TNF* locus has five DNA microsatellites, *TNFA* through *TNFE*, which are highly polymorphic. The exact functional role of the DNA microsatellites is unclear, although they may be important in DNA folding and conformation. Alternatively, they may have no functional effects but may be markers of genetic variants in close proximity that have real functional effects. Nonetheless, certain *TNF* microsatellites may influence TNF- α levels. *TNFA2* and *TNFD* microsatellite polymorphisms have been associated with high levels of TNF- α and *TNFA6* with low levels of TNF- α in vitro (59).

Polymorphisms in the TNF- α receptors also appear to be important. Soluble TNF- α binds to two transmembrane receptors: p55, also known as CD 120a or TNF receptor type 1 (TNFRSF1A), and p75, also known as CD 120b or TNF receptor type 2 (TNFRSF1B). Local production of soluble TNFRs and their upregulation is important in the modulation of TNF- α activity in RA joints. The *TNFRSF1B* gene is located on chromosome 1 and has ten exons and nine introns. A SNP has been described in exon 6 of the gene, a single-base substitution at codon 196 (T to G, ATG AGG) that leads to a nonconservative amino acid substitution, methionine to arginine (M to R) within the fourth extracellular domain of TNFRSF1B (60). The 196R allele may augment interleukin 6 (IL-6) (a strong proinflammatory cytokine) production compared to the 196M allele (61). The 196R allele may also affect membrane receptor shedding or ligand binding (61).

Several studies have examined the relationship between these polymorphisms and the severity of RA and response to anti-TNF agents. They are discussed next.

14.2.4.1 *TNF* –308

The *TNF* –308 polymorphism may be predictive of an individual patient's response to INF. In one study, 59 RA patients treated with INF after genotyping were classified into homozygotes for the *TNF* –308A allele (A/A; 1.7%), heterozygotes (A/G; 22%), and wild-type (G/G; 76.3%) carriers of the allele. After 22 weeks of treatment with INF, the disease activity score (DAS; a standardized measure of disease activity in RA) was used to assess response to INF. Patients without the A allele (81%) compared to patients with the A allele (42%) had a better response to INF as demonstrated by an improvement in the DAS (p 0.0009). Thus, this study suggested that RA patients who are carriers of the *TNF* –308 G allele may have a more favorable response to INF (62).

Cuchacovich et al. proposed an interesting explanation for the results of the above study based on the results of their own study. In the study by Cuchacovich et al., 132 patients with RA were genotyped for the *TNF* –308 promoter polymorphism. From these 132 patients, 10 patients with the *TNF* –308 G/A and 10 with the *TNF* –308 G/G polymorphism were selected and received INF. The American College of Rheumatology (ACR) 20 and 50 response rates (another standardized measure of disease activity in RA) were then used to assess response. Although both groups showed a similar response and demonstrated an increase in TNF- α levels after INF treatment, the increase in TNF- α levels correlated with the ACR50 response only in patients with the G/A polymorphism ($p < 0.03$) (64). It has been shown previously that circulating TNF- α levels increase after a single dose of an anti-TNF- α monoclonal antibody (63). The authors postulated that the *TNF* –308 polymorphism influences response to INF by its effects on circulating TNF- α levels (64).

14.2.4.2 *TNFRSF1B* 196T/G

The *TNFRSF1B* 196T/G polymorphism (in TNF receptor type 2) was studied in 175 RA patients for its effects on response to TNF antagonist therapy. All patients were genotyped for the polymorphism. Of the 175 patients, 97 had mild-to-moderate disease with partial or complete response to MTX (10–25 mg/wk) after a minimum of 6 months of therapy. There were 78 patients who had severe disease and did not respond to MTX and other DMARDs. Of these 78 patients, 66 were treated with either ETN or INF, and their response to treatment was assessed using the DAS. Of the 66 patients on TNF antagonist therapy, 38 had the TT, 22 had the TG, and 6 had the GG genotypes. Patients with severe RA carried the GG genotype more often (6.4%) than those in the mild-to-moderate group (3.1%). Patients carrying the TT genotype had a better response to therapy over 24 weeks compared to the patients with the TG or GG genotype as measured by the DAS, with the greatest difference seen at 12 weeks (OR 5.1, CI 1.3–19.96; $p = 0.03$). Thus, based on the results of this study, the presence of the *TNFRSF1B* 196G/G genotype appeared to correlate with more severe disease that was less responsive to anti-TNF therapy (65).

14.2.4.3 MHC Gene Polymorphisms, TNF, and MHC Microsatellites

The close proximity of the “*TNF* locus” to the *HLA B* and *HLA DR* genes (*MHC* genes) on chromosome 6 and the fact that there is a strong link between specific *HLA DRB1* alleles (also called the shared epitope alleles) and susceptibility to RA and its severity (66) make it likely that *MHC* gene polymorphisms may influence response to anti-TNF agents. Two studies examined this association. In one study, 78 RA patients treated with INF were genotyped for *HLA DRB1*, *HLA DQA1*, *HLA DQB1*, *MHC* class I chain-related gene A (*MICA*) transmembrane polymorphism alleles, microsatellites *TNFA-e*, *D6S273*, *HLA B*-associated transcript 2 (*BAT2*), and *D6S2223*. Some microsatellite haplotypes have been linked with either susceptibility to RA or *TNF* promoter region SNPs. For example, the *TNFA*2;b3;c1;d1;e3 haplotype is linked to the -308 *TNF* polymorphism (67); the *TNFA*6;b5;c1;d3;e3 haplotype confers an increased risk for RA (68). *BAT2* and *D6S273* are *HLA* class III microsatellites, whereas *D6S2223* is a microsatellite marker located telomeric to the *HLA* class I genes. Also, 342 healthy controls were genotyped to detect linkage disequilibrium between pairs of markers. Response to INF after 3 months was defined based on at least 50% improvement in two or more clearly defined criteria or a 25% improvement in the DAS.

None of the alleles influenced response to INF, including the *TNFA/b* microsatellites (linked to the *TNF* -308 promoter polymorphism), implying that this *TNF* promoter variant may not be important in determining response to INF. However, there were some interesting associations observed between certain microsatellite haplotypes and response. Among the microsatellite haplotypes, the *D6S273_4/BAT2_2* pair was a marker of the INF responder group, both among patients and when compared to controls (46% vs 11% in nonresponders, $p = 0.001$; 46% in responders vs 17% in controls, $p = 0.00002$), indicating that this microsatellite pair may occur on the haplotype that carries the “response gene,” or each microsatellite allele could serve as a marker of a response gene in proximity. The frequency of one of the *TNFA/b* haplotypes was increased in responders compared to nonresponders (41% vs 16% in nonresponders, $p = 0.01$). Thus, some microsatellite haplotypes were associated with response to INF in this study; single alleles did not reveal similar associations (69).

In a second study, patients were genotyped for specific *HLA DRB1* alleles that are the shared epitope (SE) alleles and categorized as having no, one, or two copies of the SE. SNPs at positions -308, -238, and +488 of the *TNF* gene and +249, +365, and +720 of the *LTA* gene were also examined. (These 6 *LTA-TNF* SNPs mark haplotypes spanning the *TNF* locus region). Five *TNF* microsatellites (*TNFA-e*); SNPs in *TNFRSF1A* at positions -609, -580, and -383; and the 196T/G polymorphism in *TNFRSF1B* were also examined. As the Fc receptor (FcR) pathway appears important in the degradation of ETN-TNF complexes, three *FcR* polymorphisms were also examined.

In the study, 457 patients with early active RA (duration of ≤ 3 years) treated with MTX and ETN were genotyped and response to therapy measured by ACR50

response rates after a year of treatment. The number of copies of *HLA DRB1* (SE) alleles, but not SNPs in *TNF*, *LTA*, *TNFRSF1A*, *TNFRSF1B*, and *FcR* genes correlated with response to treatment. Patients with two copies of the SE alleles had a better response to ETN compared to those with no or one copy of the allele (OR 4.3, 95% CI 1.8–10.3). Haplotypes defined by the six *LTA–TNF* SNPs and *DRB1* alleles were deduced for the 16 most common *DRB1* alleles in a subset of 224 Caucasian patients. Among 448 haplotypes thus examined, 2 haplotypes, *HLA DRB1* *0101-GGGAGG and *HLA DRB1* *0404-GGAAGG, strongly correlated with response (76% and 61% ACR50 response at 12 months, respectively). Thus, the number of copies of *HLA DRB1 SE* alleles inherited and specific haplotypes spanning the *HLA DRB1* region and SNPs in the *LTA–TNF* region may be associated with response to ETN, at least in the Caucasian population (70). It is unknown whether these results will be applicable to other ethnic populations.

As is obvious from the studies described, pharmacogenetic studies of biological therapies in RA offer conflicting results (see Table 14.4). Some studies showed that *TNF* promoter polymorphisms such as –308 are markers of response to anti-TNF

Table 14.4 Pharmacogenetics of the tumor necrosis factor (TNF) antagonists in rheumatoid arthritis (RA)

Genes/polymorphisms	Postulated effect of gene/ polymorphism	Clinical effects	Reference
TNF promoter –238 G/G	May increase transcription of TNF-α gene	No effect on response to ETN	70
TNF +489 G/G	Intronic polymorphism; function unknown	No effect on response to ETN	70
TNF promoter –308 G/G	May increase transcription of TNF-α gene	Increased response to INF	62
	May increase circulating TNF-α levels	No effect on response to INF	64
TNFRSF1A –609, –580, –383	May affect ligand binding	No effect on response to ETN	70
TNFRSF1B 196 T/T	May affect receptor shedding and ligand binding; may increase synthesis of IL-6	Increased response to INF, ETN	65
		No effect on response to ETN	70
TNF microsatellites a, b, c, d, and e	May influence production of TNF-α by PBMC; linked to TNF-α –308 SNP; increased susceptibility to RA	Specific TNFa/b haplotype associated with response to INF	69
		No effect on response to ETN	70

(continued)

Table 14.4 (continued)

Genes/polymorphisms	Postulated effect of gene/ polymorphism	Clinical effects	Reference
HLA DR, DQ alleles	May affect response to TNF blockade and increase susceptibility to and severity of RA because of close proximity to TNF locus	No effect on response to INF	69
		Specific individual HLA DRB1 alleles and haplotype markers of increased response to ETN	70
MHC class I chain-related gene A transmembrane polymorphism	As above	No effect on response to INF	69
HLA microsatellites BAT2, D6S273, D6S2223	Haplotype may carry "response gene"	BAT2-D6S273 haplotype associated with increased response to INF	69
FcR polymorphisms	Involved in degradation of ETN–TNF complexes	No effect on response to ETN	70

ETN, etanercept; IL, interleukin; INF, infliximab; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; SNP, single-nucleotide polymorphism.

therapies (**62,64**), but others contradict this finding (**69,70**). Similarly, the role of the *MHC* alleles in determining response to anti-TNF agents is not clear-cut. One study showed a strong association between certain *HLA DRB1* haplotypes and response to ETN (**70**); another did not demonstrate such an association (**69**). *MHC* microsatellite polymorphisms also appear to influence response to these agents (**69**).

14.3 Conclusions and Future Directions

Thus, there is a growing body of literature on the pharmacogenetics of therapies used in RA. Clearly, inherited differences in drug-metabolizing enzymes, drug receptors, and drug targets are important in determining an individual's response to a given drug. Nonetheless, several caveats need to be considered before pharmacogenetics can be brought in to the clinic.

In several of the studies reviewed, the strength of the association between genotype and phenotype can be questioned for several different reasons. Whether many of these studies were adequately powered is questionable; most of the studies

described had small sample sizes, and associations observed in one study were not necessarily reproducible in another. Some of them were retrospective and may have over- or underestimated drug effects, particularly adverse effects. Moreover, ethnicity may have strong influences on pharmacogenetic associations, and the populations examined in most of the above studies were ethnically homogeneous. Our study examining the frequencies of SNPs in the MTX pathway genes in different racial groups demonstrated significant differences in the allele frequencies of several SNPs between Caucasians and African Americans with RA (71). Hence, genotype–phenotype associations may differ significantly in ethnically diverse populations. For example, in the study by Criswell et al., although certain *MHC/TNF* haplotypes were predictive of response to ETN in a Caucasian population, whether these results will apply to other populations remains unknown (70).

As many of the drugs in RA (such as MTX, TNF antagonists) work through several different cellular (and genetic) pathways, examination of SNPs in different metabolic pathways rather than a single pathway may be more predictive of response (39). It is also worth noting that if a variant is only weakly associated with response, this may be because this variant may occur in tandem or in close proximity to the gene that is the actual marker of response. For reasons described, haplotype analyses may be more useful than single-SNP analyses in predicting response (69,70). Finally, the cost-effectiveness of pharmacogenetic testing is an important issue to consider before pharmacogenetics can be incorporated in daily clinical practice (72). Drugs with a narrow therapeutic index, severe side effects, a well-established association between a specific genotype and phenotype (usually toxicity), and for which the frequency of the genetic variant of interest is high are the ideal candidates for pharmacogenetic testing.

Notwithstanding these caveats, as genotyping becomes more readily available and less expensive and major funding agencies display an increasing commitment to pharmacogenetic research (International HapMap Consortium, www.hapmap.org; Pharmacogenetics Research Network, <http://www.nigms.nih.gov/pharmacogenetics/>, by the National Institutes of Health), it is quite likely that genotype-guided therapy of patients with RA will happen in the not too distant future.

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Chapter 15

Cancer Pharmacogenetics

Sharon Marsh

15.1 Introduction..... 437

15.2 Materials 439

15.3 Methods..... 443

15.4 Notes 444

References..... 444

Summary Cancer pharmacogenetics is a burgeoning field. There are now many published associations between genotype and outcome or toxicity from chemotherapy treatment. Performing pharmacogenetics studies in cancer requires careful consideration of the sample type to be used (germline vs tumor); the genotyping platform to be used (medium, low, or high throughput); and the analysis and reporting of associations and observations.

Keywords Cancer; genotyping; germline; mutation; polymorphism; tumor.

15.1 Introduction

Oncology represents a challenge for pharmacogenetics research. With multiple treatments available for many cancer types, there is a need for tools to inform decision making on therapy selection (1). Studies of childhood acute lymphoblastic leukemia (ALL) have highlighted the practical value of improving the use of existing therapy. Overall survival in patients with childhood ALL has improved from less than 10% in the 1960s to over 90% in the present day without the introduction of any new chemotherapy drugs in the past 30 years (2). Studies such as this demonstrate the utility of optimizing existing chemotherapy strategies. Pharmacogenetics offers one possibility for rationalizing therapy/dose selection.

15.1.1 Cancer Pharmacogenetics

There is a growing number of published examples of the utility of screening patients for pharmacogenetic markers prior to chemotherapy selection (*1*). Many published markers of association with chemotherapy outcome or toxicity still require validation in prospective studies. Examples of well-characterized pharmacogenetic markers include thiopurine methyltransferase (TPMT), UGT (uridine diphosphate [UDP]-glucuronosyltransferase) 1A1, and epidermal growth factor receptor (EGFR).

15.1.1.1 Thiopurine Methyltransferase

Thiopurine methyltransferase methylates 6-mercaptopurine, a commonly used treatment for childhood acute lymphocytic leukemia, reducing its conversion to the active form of the drug. Approximately 10% of patients have intermediate enzyme activity, and 0.3% are deficient for TPMT activity. Intermediate activity patients have a greater incidence of thiopurine toxicity, whereas TPMT-deficient patients have severe or fatal hematological toxicity from 6-mercaptopurine therapy. In one study, patients deficient for TPMT tolerated only 7% of a 2.5-yr mercaptopurine treatment regimen. Patients with intermediate TPMT activity tolerated 65% of total weeks of therapy and patients with normal TPMT activity tolerated 84% of total weeks of therapy (*3*).

There are several known polymorphisms in TPMT (*4*). Alleles TPMT*2, TPMT*3A, and TPMT*3C account for up to 95% of reduced TPMT activity. Patients heterozygous for these alleles have intermediate TPMT levels (*5*), and patients homozygous for the variant TPMT alleles are at high risk for severe, sometimes life-threatening, toxicity requiring significant reductions in mercaptopurine doses (*5*).

15.1.1.2 UGT1A1

The active form of irinotecan, a commonly used drug for the treatment of colorectal cancer, can be inactivated through glucuronidation by a member of the UDP-glucuronosyltransferase family, UGT1A1. A polymorphic dinucleotide repeat has been identified in the UGT1A1 promoter TATA element (UGT1A1*28) and consists of five, six, seven, or eight copies of a TA repeat [(TA)_nTAA], with the (TA)₆TAA allele the most common and (TA)₇AA (*28) the most frequently recorded variant allele (*6*). The longer the repeat allele, the lower the corresponding UGT1A1 gene expression. Consequently, patients with the seven and eight alleles have significantly lower UGT1A1 expression and subsequently can experience severe toxicity from irinotecan therapy caused by excessive accumulation of the active metabolite of the drug (*7,8*). The Food and Drug Administration (FDA) has requested the inclusion of UGT1A1 genotype information in the drug package insert (*8*), with

dosing guidelines based on genotype. In addition, the FDA approved a clinical test for the UGT1A1*28 allele, which is performed by Third Wave Technologies (*9,10*), highlighting that the integration of cancer pharmacogenetics into clinical practice is now becoming a reality.

15.1.1.3 Epidermal Growth Factor Receptor

Although TPMT and UGT1A1 represent polymorphisms that can be screened in the patient's germline DNA, some cancer pharmacogenetic markers will require screening of the patient's tumor genome.

EGFR inhibitors gefitinib (Iressa) and erlotinib (Tarceva) are used to treat patients with metastatic non-small-cell lung cancer (NSCLC) who had previously failed standard therapies. Approximately 10% of patients experience a response from these drugs when no other treatment has been effective. Response is more frequently seen in females, nonsmokers, and individuals of Asian descent (*11,12*). Screening the exons of the EGFR gene in tumor DNA from patients who responded favorably to gefitinib or erlotinib identified somatic mutations in the EGFR gene. Conversely, no mutations were identified in patients who did not respond to gefitinib or erlotinib (*13–15*). In addition, mutations were only found in the tumor, not the normal tissue from the same patients (*13,14*). No mutations were found in 108 cancer cell lines from diverse cancer types, suggesting these EGFR mutations are lung cancer specific (*14*). The presence of beneficial somatic mutations in EGFR leads to the possibility of screening patients in advance of EGFR inhibitor therapy to select patients likely to respond. As the response rate is low but important (occurs in patients who have failed all other therapy), screening tumor tissue should enable the identification of patients who are likely to benefit from gefitinib or erlotinib therapy and reduce unnecessary treatment for patients who would only experience adverse reactions or no response.

Studies such as these highlight the usefulness of pharmacogenetics to prescreen patients for therapy/dose selection. There are many methodologies available for pharmacogenetics studies. Some of the criteria specific to cancer pharmacogenetics are highlighted in this chapter.

15.2 Materials

15.2.1 Sample Source

DNA from any source can be utilized for pharmacogenetics research, including germline and tumor sources. Immortalized cell lines can provide an almost infinite DNA resource, and genotyping of Epstein–Barr virus (EBV)-transformed immortalized lymphoblastoid cells has demonstrated similar allele frequencies to equivalent

populations (*16*). However, EBV transformation can lead to problems, such as the induction of EBV lytic replication by some chemotherapy drugs, which can interfere with phenotype measurements (*17*). Cancer cell lines do not share the problems caused by EBV transformation, but many cancer cell lines have chromosome instability (CIN), and multiple passages of CIN cell lines lead to different amounts of chromosome losses and gains (*18*), which can affect subsequent genotype or phenotype assessment.

15.2.1.1 Germline DNA

Germline DNA represents by far the most easily accessible source material (*19–21*). In addition, good-quality, high molecular weight DNA can be obtained from the majority of germline sources, allowing a greater range of assays to be performed. The question remains whether germline DNA is an accurate enough representation of the cancer genome to allow genotype information to be applicable in cancer pharmacogenetics. Germline DNA studies may be more relevant to identifying associations with toxicity. A combination of germline and tumor DNA analysis may be necessary to identify polymorphisms and mutations associated with chemotherapy response (*22*). Sources of germline DNA include the following:

1. Whole blood. DNA from whole blood is one of the most stable and easily obtainable sample sources. The DNA is usually high yield and high molecular weight and suitable for long-term storage. In addition, the DNA is usually suitable for whole-genome amplification.
2. Saliva. The extraction of DNA from mouthwash samples provides high-yield, good-quality DNA that is also suitable for whole-genome amplification (*23*). The advantage of this sample source over blood is that the procedure for collecting samples is noninvasive, and the mouthwash samples are stable at room temperature for extended periods of time.
3. Tissue. DNA can be extracted from normal (nontumor) tissue cells. Frozen tissue provides good-quality DNA. Paraffin embedded or formalin-fixed tissue provides low-yield DNA that is often highly fragmented. Although it is still a useful resource, care should be taken to limit polymerase chain reaction (PCR) amplicon length to improve the likelihood of success of genotyping assays.

15.2.1.2 Tumor DNA

There are many intraindividual differences between the cancer and germline genomes (*24*). As well as differences at the DNA level, alterations in RNA expression between tumor and normal cells have been documented (*25,26*).

Alterations in the tumor genome could have an effect on the presence of functional alleles (e.g., through gene amplification/loss, microsatellite instability, loss of heterozygosity or allelic imbalance) or their expression (e.g., through epigenetic regulation such as methylation). Consequently, although the germline genome is useful for many pharmacogenetics studies, it may not provide the complete picture for cancer pharmacogenetics. Assessment of tumor DNA may be necessary to build a comprehensive pharmacogenomic profile (24). Sources of tumor DNA include the following:

1. Plasma and serum. Circulating DNA can be extracted from plasma and serum. Circulating DNA is mainly tumor DNA, although some germline DNA will be present. Studies have suggested a strong correlation between epigenetic markers in circulating DNA compared to matched tumor tissue, suggesting this is a useful source of tumor DNA (27,28). The DNA is highly fragmented and care should be taken to limit PCR amplicon size to maximize the likelihood of successful genotyping.
2. Tissue. As with using tissue for germline DNA, frozen tissue provides good-quality DNA. Paraffin-embedded or formalin-fixed tissue provides low-yield DNA that is often highly fragmented. Macro or microdissection needs to be performed to ensure minimal contamination of the sample with normal tissue. Care should be taken to limit PCR amplicon size to maximize the likelihood of successful genotyping.

15.2.2 Marker Selection

The selection of polymorphisms to screen in a cancer pharmacogenetic study is dependent on several factors:

1. The treatment the patients received: Different chemotherapy drugs are likely metabolized and undergo transport by different genes (29). In addition, the drug target and the subsequent pharmacodynamic genes involved will be different for most drugs. A short list of candidate genes should be generated based on the drug of interest prior to marker selection.
2. The type of outcome data that are available from the patient samples will influence the choice of genes to be assessed. If only pharmacokinetic data are available, then there is no need to include pharmacodynamic genes in the study. However, pharmacokinetic genes could have an influence on outcome/toxicity and should be included with pharmacodynamic genes if outcome/toxicity data are available.
3. Haplotype analysis may be necessary if the candidate genes do not have known functional polymorphisms. Prior knowledge of haplotype tag single-nucleotide polymorphisms (SNPs) for each gene is essential to limit the number of assays required to avoid wasting finite patient samples.

15.2.3 Genotyping System

There are many genotyping systems commercially available (30–33). No system is perfect, and selection of the best system is largely dependent on the throughput needed for the individual lab. Examples of genotyping systems include the following:

1. Dye-terminator sequencing. Direct sequencing of PCR amplicons is still considered the “gold standard” for genotype analysis and is often used to verify the presence of polymorphisms or for quality control if other genotyping assays are yielding unpredictable or unclear results. In addition, it is an invaluable resource for identifying novel polymorphisms/mutations.
2. Low-throughput systems. PCR/restriction fragment length polymorphism (RFLP) and allele-specific PCR are low-cost, low-throughput methods ideal for small numbers of samples that require a small number of genotyping assays. These methods are also ideal for a quick quality control test for the accuracy of other methodologies. However, they are inefficient for large sample sizes/large number of assays, and the DNA sequence around the polymorphism is not always amenable to assay development.
3. Medium-to-high-throughput systems. The Taqman, Sequenom, and Pyrosequencing systems require specialized equipment and training. The use of 96-well or 384-well platforms allows a large number of samples to be processed rapidly. The majority of pharmacogenetic gene–outcome association studies are ideally suited to one of these systems. Measurements of allelic imbalance, methylation, and gene copy number are possible for some, but not all, medium-throughput systems.
4. High-throughput systems. In the Affymetrix and Illumina systems, the use of chips/beads for large-scale SNP genotyping is usually more suited for narrowing down genome regions of interest where all of the candidate genes are not already known. These systems are cheap per SNP; however, they are costly in terms of the amount of DNA required for each chip/bead array.

15.2.4 Data Storage System

Data storage and data coordination are important aspects of project design. Depending on the genotyping system used, it is possible to collect thousands of data points per day. Consequently, a consistent and reliable method of data input and storage is essential. The use of spreadsheets rapidly becomes cumbersome and unwieldy with large amounts of data and increases the likelihood of human error during data input. Care should be taken to identify a useful method of data storage from which the data can be readily retrieved and applied to downstream applications (e.g., for haplotype analysis or statistical analysis) with a minimum of human intervention (34).

15.3 Methods

Guidelines for reporting tumor marker studies (Reporting Recommendations for Tumour Marker Prognostic Studies, REMARK) have been developed and published (35) to reduce inconsistency between studies of the same markers. These guidelines are applicable to all forms of tumor marker, including genetic markers in both the tumor and the germline genomes. The following methods suggest a flow for performing pharmacogenetic analysis on DNA from patients treated with chemotherapy drugs, and they closely follow the published REMARK guidelines for reporting tumor marker studies (35).

1. The study objectives, hypothesis/aim of the study, and the genes or polymorphisms to be assessed should be stated in advance of the study (see [Note 1](#)). It should be made clear whether the study is retrospective or prospective. If REMARK (35) guidelines have been followed, this should be stated and referenced.
2. Patient characteristics (e.g., demographics, gender, age, smoking status) should be described. This should also include the criteria for inclusion in the study. Appropriate internal review board approval and informed consent must have been obtained and documented.
3. The treatment regimen the patients received must be explained in detail, including randomization criteria.
4. Sample collection, including source of sample (e.g., blood, mouthwash, tissue), whether the sample represents the germline or tumor genome, sample storage, DNA extraction methodology (see [Note 2](#)), and DNA storage (see [Note 3](#)) need to be specified. The sample size should be calculated based on the number of genotypes to be assessed, the frequency of incidence of the endpoints that will be examined in association with genotype, and the frequency of the variant alleles in the patient population (see [Note 4](#)).
5. All outcome measurements (e.g., response to therapy, disease progression, toxicity) need to be defined and clearly listed. In addition, the length of follow-up period, including the median follow-up time, must be defined.
6. The genotyping system used needs to be specified and referenced appropriately. Any deviations from standard published methodologies should be stated in full. Appropriate positive controls to aid in the genotype assignment for individual samples and negative controls to control for assay contamination or background noise should be included for each polymorphism to be assessed. Appropriate quality control should be performed on all data, including repeating random samples to double-check genotype assignment.
7. Statistical analysis should be appropriate to the types of outcome data collected and the number of genotypes used in the analysis. The handling of missing data should be clearly stated. Corrections for multiple comparisons (e.g., controlling for false discovery rates; 36) should be performed if multiple statistical tests are carried out.

8. All data, including the flow of samples through the steps listed, should be presented clearly, including the number of samples and reasons for missing data involved in each step (see [Note 5](#)).
9. All statistics derived from the data should be presented in the form of tables and diagrams, including confidence intervals.
10. If available, a validation sample set of patients receiving the same treatment regimen should be assessed for any positive associations identified in the initial study to help confirm the validity of the data.
11. The limitations of the study and the future applications of the study should be discussed, along with any positive or negative associations identified in the analysis. The distinction between the tumor genome (and if so, the specific tumor type should be stated) or the germline genome should be clearly stated for any association.

15.4 Notes

1. If a commercial chip/array is used, then this should be referenced, and a Web link or reference to the genes and polymorphisms included on the chip/array should be included if available.
2. Commercial kits are available for DNA extraction from the majority of sources, including blood, mouthwash, plasma, serum, frozen tissue, and formalin-fixed tissue. Most kits work very well if carried out in accordance with the manufacturer's instructions. In addition, they reduce the likelihood of variability in the quality and quantity of DNA between batches of samples. Published protocols for the extraction of DNA from paraffin-embedded tissue are also available ([37](#)).
3. Care should be taken to store samples correctly. Blood, plasma, serum, and tissue should all be stored frozen. To minimize DNA degradation, -80°C is preferable. However, blood can be stored at -20°C if necessary, and the DNA will survive intact for short-term storage at 4°C . Extracted DNA should be stored in Tris/EDTA (TE) buffer and frozen for long-term storage. DNA from degraded/fragmented sources such as plasma, serum, formalin-fixed tissue, and paraffin-embedded tissue should be stored frozen to prevent further degradation. However, repetitive freeze-thawing will cause damage to the DNA; consequently, a working dilution of the DNA stored for the short term at 4°C is recommended for projects in progress.
4. Significant ethnic variability is observed with many pharmacogenetic markers ([38](#)). Consequently, the demographics of the study population need to be taken into account when performing power analyses to determine the appropriate sample size. If the frequency of the polymorphism to be studied is unknown in any of the ethnic groups included in the study, the polymorphism should be screened in relevant population controls to determine their allele frequency. This will help to narrow down the number of polymorphisms to be included and prevent using finite patient DNA to assess markers that may have low frequency or be absent from the study population.
5. This may be best represented in the form of a flow diagram.

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Chapter 16

Pharmacogenomics in the Preclinical Development of Vaccines

Evaluation of Efficacy and Systemic Toxicity in the Mouse Using Array Technology

Karin J. Regnström

16.1. Introduction.....	447
16.2 Materials	448
16.3 Methods.....	451
16.4 Notes	464
References.....	466

Summary The development of vaccines, conventional protein based as well as nucleic acid based vaccines, and their delivery systems has been largely empirical and ineffective. This is partly due to a lack of methodology, since traditionally only a few markers are studied. By introducing gene expression analysis and bioinformatics into the design of vaccines and their delivery systems, vaccine development can be improved and accelerated considerably. Each vaccine antigen and delivery system combination is characterized by a unique genomic profile, a “fingerprint” that will give information of not only immunological and toxicological responses but also other related cellular responses e.g. cell cycle, apoptosis and carcinogenic effects. The resulting unique genomic fingerprint facilitates the establishment of molecular structure – pharmacological activity relationships and therefore leads to optimization of vaccine development.

Keywords Bioinformatics; efficacy; formulation; genomics; microarray; toxicity; vaccine;.

16.1 Introduction

When new vaccines are developed, new pharmaceutical delivery systems and additives with properties suitable for this new class of vaccines must be developed. Promising additives and delivery systems have to be evaluated regarding their efficacy and toxicity. These vaccine formulations do not simply act as carriers but are also used to enhance the immune response against the administered antigen (as an

adjuvant) (1). Thus, apart from evaluation for toxicity, new adjuvant delivery systems also have to be evaluated for immunostimulatory and immunosuppressing properties (2). Traditionally, cytokine protein detection methods based on immunochemical techniques such as enzyme-linked immunosorbent assay (ELISA) are used to assess the immunostimulatory effects of various vaccines. However, these methods are capacity limited and therefore give an incomplete picture of the overall *in vivo* response to a vaccine (3–7).

DNA microarrays offer the advantage of sensitivity, flexibility in the choice of genes for analysis, and power of analysis if combined with appropriate bioinformatics tools. Using DNA microarrays, the expression of thousands of genes can be measured simultaneously and in a high-throughput manner.

Comparison of the complementary DNA (cDNA) array technology with traditional cytokine ELISA assays in samples obtained from clinically used alum-based vaccines showed that the immunological response profiles obtained by both methods agreed with each other in accurately measuring cytokine levels and in differentiating between a Th1 and Th2 immune response (4). The results also suggested potential surrogate markers for the Th1/Th2 cytokine analysis. Furthermore, differential gene expression analysis distinguished vaccine- and adjuvant-mediated gene regulation with respect to both the immune response and other pharmacological responses important to vaccine research, such as inflammation, apoptosis, stress response, and oncogenesis (4).

It has been known for quite some time that administration of aluminum adjuvants or of other adjuvants coated with antigen leads to the formation of inflammatory foci that attract immunocompetent cells to the administration site (4). Furthermore, the secondary lymphoid organs are affected and show inflammatory responses (4). *Apoptosis* is a part of the adaptive immune response in the antigen-induced cell death (AICD), which is involved in memory development and effector expansion of rare antigen-specific T cells (4). Many toxicity, stress, and oncogenesis markers are upregulated in spleen lymphocytes after administration of the adjuvant alone, but only a few of these markers are usually activated in the presence of antigen (4). Because the spleen is located far from the administration site, the potential toxicological implications of such possible upregulation are important. It is therefore important to include the adjuvant without antigen in the study of vaccines as described here.

Finally, it is the choice of bioinformatics software that enables the researcher to quickly assess obtained array data. Clustering using self-organizing maps and literature networks are ideally suited for this task.

16.2 Materials

16.2.1 Animal Studies

1. Female Balb/c mice, 10 wk old (Charles River, Wilmington, MA).
2. Forane® Isoflurane anesthetic gas (Baxter Healthcare Corp., Deerfield, IL).
3. Continuous-flow gas anesthesia system (2Biological Instruments, Besozzo, Italy).

4. Tetanus toxoid on aluminum phosphate vaccine.
5. Aluminum phosphate adjuvant solution.
6. Tetanus toxoid antigen solution.
7. Contract laboratory for the measurements of antibody levels (TGA Sciences Inc., Medford, MA).

16.2.2 Isolation, Cell Culture, and In Vitro Restimulation of Lymphocytes

1. Dulbecco's modified phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride (Pierce Biotechnology, Rockford, IL).
2. Tris buffer Nine parts 8.3 g/L ammonium chloride added to one part 20.5 g/L Trizma base, pH 7.2 (*see* [Note 1](#)) (Sigma-Aldrich, St. Louis, MO).
3. RPMI-1640 growth medium with L-glutamine (Irvine Scientific Co., Irvine, CA) supplemented with 10% heat-inactivated fetal bovine serum, 10 IU/mL penicillin, 100 µg/mL streptomycin (PEST), 50 µM 2-mercaptoethanol, 292 µg/mL L-glutamine, 10 mM HEPES buffer (all from Sigma-Aldrich).

16.2.3 Total RNA Isolation

The following materials are provided with the ToTALLY RNA™ RNA isolation kit (Ambion, Austin, Tx):

1. Phenol/chloroform/isoamyl alcohol.
2. Acid phenol/chloroform.
3. 3.0M sodium acetate solution, pH 4.5.
4. 7.5M lithium chloride, 50 mM ethylenediaminetetraacetic acid (EDTA).
5. 5M potassium acetate.
6. Denaturation solution.
7. Elution solution (0.1M EDTA).

In addition, the following materials are needed:

1. Isopropanol of at least ACS grade (meets purity requirements of American Chemical Society).
2. 70% (v/v) ACS grade ethanol.
3. Liquid nitrogen.
4. Glass, polyallomer, or polypropylene centrifuge tubes (*see* [Note 2](#)).
5. RNaseZAP® (Ambion) (*see* [Note 3](#)).
6. Ribonuclease (RNase)-free pipet tips (Ambion).
7. RNase-free plastic tubes (Ambion).
8. Nuclease-free water (not DEPC (diethylpyrocarbonate) treated) (Ambion).
9. LE (low electroendosmosis) agarose (Ambion).
10. Running buffer: 5X TBE: 45 mM Tris base, 45 mM boric acid; adjust to pH 8.0 with 0.5M EDTA.

11. 10X native agarose loading buffer: 15% Ficoll, 0.25% xylene cyanol, 0.25% bromophenol blue.
12. Ethidium bromide solution: 10 mg/mL (*see* [Note 4](#)).
13. RNA Millenium Markers™ (Ambion).
14. Deoxyribonuclease (DNase) I (Promega, Madison, WI).
15. Polymerase chain reaction (PCR) Master Mix containing Taq DNA polymerase, deoxynucleotide 5'-triphosphate (dNTP), MgCl₂, and reaction buffers (Promega).
16. Mouse genomic DNA (Promega).
17. DNA sample (primer pair) for mouse β -actin (Biosource™) (Invitrogen, Carlsbad, CA).
18. Nuclease-free mineral oil.
19. Quanti™ RiboGreen® RNA assay kit (Molecular Probes, Eugene, OR).
20. TE buffer: 10 mM Tris, 1 mM EDTA.

16.2.4 Array Analysis

For the array analysis, the Atlas Plastic Mouse 5K Microarrays (Clontech, Mountain View, CA) is used. The following materials are provided in the kit:

1. dNTP mix
2. Power Script reaction buffer.
3. 100 mM dithiothreitol (DTT).
4. cDNA synthesis control.
5. 10X random primer.
6. Reverse transcriptase.
7. 10X termination mix.
8. Plastic 5 K mouse arrays.

The following materials are not provided in the kit:

1. Sterile nuclease-free 0.5-mL PCR tubes (Ambion, Austin, TX).
2. ³³P-dATP (2'-deoxyadenosine, 5'-triphosphate) (10 μ Ci/ μ L, > 2500 Ci/mmol) (Amersham Biosciences, Piscataway, NJ) (*see* [Note 5](#)).
3. 20X SSC buffer: 3M sodium chloride (NaCl), 0.3M sodium citrate (Na₃C₆H₅O₇·2H₂O). pH 7.0 (Sigma-Aldrich, St. Louis, MO)
4. High-salt wash solution: 2X SSC, 0.1% sodium dodecyl sulfate (SDS).
5. Low-salt wash solution 1: 0.1X SSC, 0.1% SDS.
6. Low-salt wash solution 2: 0.1X SSC.

16.2.5 Image Analysis of Array Data

1. Typhoon Variable Mode Imager (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).
2. Mounted screen (general-exposure type) and exposure cassette (GE Healthcare Bio-Sciences).

3. AtlasImage program version 2.7 (BD Biosciences: Clontech, Mountain View, CA).
4. Microsoft Excel software (Microsoft Corp., Redmond, WA).

16.2.6 *Clustering of Data*

1. GeneCluster 1.0 software (Broad Institute, Cambridge MA; available at: <http://www.broad.mit.edu/cancer/software/software.html>).
2. Treeview 1.60 software (Department of Molecular and Cell Biology, University of California at Berkeley; available at: <http://rana.lbl.gov/EisenSoftware.htm>).

16.2.7 *Data Mining of a Cluster of Genes*

1. PubGene online data mining tool (available at: www.pubgene.org).
2. GeneCards (available at: www.genecards.org).
3. PubMed (available at: www.ncbi.nlm.nih.gov).

16.3 **Methods**

To analyze newly developed vaccines, it is of interest to study the pharmacological and immunostimulatory effects of the delivery system (adjuvant) and to compare them to the effects of the combination between the adjuvant and the antigen of interest. This is accomplished by comparing three groups of mice ($n =$ at least 6): Mice immunized with the vaccine (antigen formulated with adjuvant; VAC) (to study efficacy and type of immune response); mice administered with the adjuvant only; ADJ) (to elucidate the contribution of the adjuvant on immune response, mechanism of action, and possible side effects); and nonimmunized mice (N).

The experimental approach is outlined in **Fig. 16.1**. The administration of vaccine to the VAC group (*see Fig. 16.1B*) and administration of adjuvant only to the ADJ group (*see Fig. 16.1A*) are performed in parallel according to a standard immunization scheme concerning administration route, dosing, and time interval for booster vaccination (*see Note 6*). Spleen lymphocytes are collected from all three groups and pooled for each group (*see Note 7*). Lymphocytes derived from the VAC group are restimulated with antigen (*see Fig. 16.1B*). This stimulates antigen-specific T cells to proliferate and therefore mirrors the most *in vivo*-like situation of immunized mice after challenge with the antigen. Nonimmunized mice with and without *in vitro* restimulation with the antigen under investigation do not differ significantly in their gene expression except for some minor mitogenic effects (4) (*see Note 8*). This allows for direct comparison between the three groups of mice. In experiment A (*see Fig. 16.1*), the adjuvant samples are

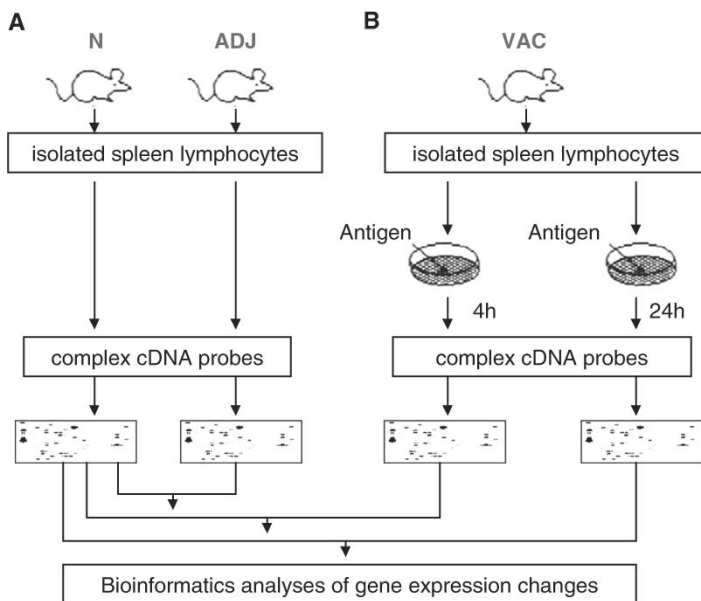


Fig. 16.1 Experimental setup. **A** Control experiments: nontreated mice (N), adjuvant-treated mice (ADJ), and **B** mice vaccinated according to a standard immunization scheme. Spleen lymphocytes are isolated and *in vitro* restimulated for 4 and 24 h with the antigen used in the vaccination. cDNA, complementary DNA

not restimulated *in vitro* with antigen to avoid both potential nonspecific and specific effects of the antigen.

The incubation times for *in vitro* restimulation are performed according to earlier studies (4–8), which established that gene expression for Th1 and Th2 cytokines is highest at 4 and 24 h after *in vitro* restimulation with antigen. The spleen lymphocytes are pooled for each group after the exclusion of animals with confirmed health problems. The data sets obtained by array experiments are very large, and doing replicates from many biological samples (e.g., individual animals) would increase random error (9,10).

Total RNA is isolated from the lymphocytes according to standard procedures and used as a template for radioactive labeled cDNA synthesis. The purified cDNA is used as probe for cDNA expression arrays. The advantages of this method as compared to other array systems are as follows: (1) Radioactive-labeled probes are more sensitive than fluorescent-labeled probes and therefore need less sample RNA. (2) The primers used in the cDNA synthesis match the genes represented on the array. (3) The primer sequences are longer compared to other array systems, which increases the hybridization fidelity of RNA to the matching correct set of genes and therefore reduces mismatch reactions.

The image data of the array experiments are then processed using AtlasImage software. The spots are checked for integrity, and signals lower than the average

background signal are filtered out. The adjusted intensity of each gene is obtained by subtracting the average background signal from the average signal intensity (double spots). Array analyses are performed twice to ensure intrasample reproducibility. To compare two or more arrays, the adjusted signal intensities of all genes on the arrays are normalized using the global mode and the sum method. Briefly, the ratio of the adjusted signal intensity of gene Z on array 1 to the adjusted intensity of the same gene Z on array 2 is calculated for all the genes on the array and averaged. To filter for genes with significant gene expression changes, the following criteria should be used: Only genes with a difference in signal strength of more than 100 and a more than twofold expression difference in at least one sample are included (**11**).

To identify genes with significant gene expression changes and to group them according to similar expression levels across all samples, the data are clustered using the software GeneCluster (**12**). This software uses self-organizing map (SOM) algorithms and is suited to analyze large data sets. By setting the number of nodes, the user can apply partial structuring to the data set. The cluster results can be further visualized using the Treeview software (**13**). Data-mining tools from the World Wide Web such as GeneCards (**14**) and PubGene (**15**) are used to assess biological functions and the importance of the genes for the vaccine's efficacy and safety:

Single-gene expression data can be verified and studied using a large variety of Internet-based search tools, such as PubMed, to find references for single genes and proteins in the literature. Another useful tool is GeneCards. This data-mining tool allows the researcher to find aliases of a gene of interest, search for their function, chromosome location, and a description of encoded proteins, including structure. This tool can also be used to find all transcripts and the single-nucleotide polymorphisms (SNPs) of a given gene and contains links to a database of diseases and display of pathways in which the gene is involved.

Data mining of a larger number of genes (more than ten genes) as found in a single cluster is not feasible by looking up single genes because this is too time consuming. Therefore, an online data-mining tool, PubGene, is used to find and organize relationships between large numbers of genes by extracting gene-to-gene cocitations from over 25 million Medline literature records. Their relationship is presented as a network, and the main association of the network with MeSH terms can be extracted. Furthermore, associations with genes that were not represented on the array but have a relationship to the network in the literature can be queried.

The following instructions assume the use of a tetanus vaccine, here tetanus toxoid formulated with aluminum phosphate. These instructions can easily be adapted to other vaccines by following the standard formulation procedures and immunization protocols for these vaccines in mice.

16.3.1 Animal Studies

1. Animal studies require the approval of a local institutional animal care and use committee and have to be performed in special animal facilities. Often, the

university provides a centralized animal care program with disease surveillance and vendor monitoring. This should be checked with the university administration before proceeding further (see [Note 9](#)).

2. Female Balb/c mice, 10 wk old, are randomly separated into groups of at least six animals.
3. The mice are placed under a heat lamp to avoid heat loss and anesthetized with isoflurane (see [Note 10](#)).
4. A dose of vaccine containing 1 Lf (2–2.5 μ g) tetanus toxoid in aluminum phosphate adjuvant at pH 6.0 (1:1) (TT_{ADJ}) or a dose of adjuvant alone in a dose corresponding to the dose given with the vaccine (ADJ) is administered intramuscularly. The administration site is the left hind leg quadriceps (see [Note 11](#)).
5. The animals are dosed with painkillers as required. The animals are placed in cages to regain consciousness and supplied with water and food. The animals should be monitored daily to ensure health. Animals showing signs of clinical distress, such as lack of grooming, anorexia, weight loss, and diarrhea, are excluded from the study.
6. The same doses of vaccine are administered 3 wk later (booster) by repeating steps 3–5.
7. Two weeks after the booster, the mice are sacrificed by exposing them to CO₂ gas, and a blood sample is taken from all mice by tail artery puncture for measurement of the systemic antibody response.
8. The blood samples are sent to a contract laboratory for measurement of antigen-specific (in this case, tetanus toxoid) antibodies.
9. The spleens are aseptically removed and washed in ice-cold PBS (see [Note 12](#)).

16.3.2 Isolation of Spleen Lymphocytes from Immunized Mice

These instructions are only for the isolation of lymphocytes from mice immunized with the vaccine (VAC) (see [Fig. 16.1B](#)). Spleens of nonimmunized mice and adjuvant-administered mice are processed as described in **Subheading 16.3.4**.

1. Each spleen is treated separately. The spleen is placed in a Petri dish on ice containing ice-cold PBS solution (see [Note 11](#)). Using sterile forceps to hold the spleen on one edge, the outer organ layer is ripped apart with a second pair of forceps. Lymphocytes and erythrocytes are squeezed out into ice-cold PBS solution by gently brushing with the forceps over the spleen. The outer organ layers are removed.
2. The spleen cells of mice with sufficient antibody titers are pooled at this stage if the results are known (see [Note 13](#)).
3. The cell mixture is stored on ice for 10 min.

4. The PBS solution containing the cell mixture is centrifuged at 270g for 10 min at 4°C.
5. The pellet is resuspended in 1 mL Tris and stored on ice until the red color has disappeared. This procedure removes the red-color erythrocytes by lysis.
6. The remaining cells, the lymphocytes, are pelleted by centrifugation at 270g for 10 min at 4°C.
7. The lymphocytes are washed twice with RPMI growth medium by carefully mixing them with RPMI growth medium and centrifuging at 270g for 10 min at 4°C (see [Note 14](#)).

16.3.3 Growth and Restimulation of Lymphocytes

1. The lymphocyte samples of the mice with sufficient antibody response (see [Note 13](#)) are pooled if this was not performed in **Subheading 16.3.2, step 2**.
2. The lymphocytes are resuspended in fresh RPMI growth medium.
3. The number of cells is counted in a counting chamber such as a hemocytometer.
4. The cell suspension is then aliquoted into cell culture flasks in portions of 1.25×10^6 cells/mL. Tetanus toxoid solution is added to each culture flask in quantities of 1 Lf per spleen (see [Note 15](#)). The flasks are incubated at 37°C in 5% CO₂ and 90% humidity. One-half of the suspension in the lymphocyte culture flasks is incubated for 4 h and immediately further processed as described in **step 5**. The other half of the lymphocyte culture is incubated for 24 h.
5. The lymphocytes are centrifuged at 270g for 10 min at 4°C. The supernatant is removed by aspiration and stored at 4°C (see [Note 16](#)). The lymphocytes in the pellet are washed once with ice-cold PBS and recentrifuged at 270g for 10 min at 4°C (see [Note 14](#)). The pellet containing the restimulated lymphocytes is used for total RNA isolation.

16.3.4 Isolation of Total RNA

These instructions assume the use of the ToTALLY RNA Kit (see [Note 17](#)).

1. Whole spleens from the nonimmunized mice and adjuvant-administered mice (see [Fig. 16.1A](#)) are perfused with ice-cold PBS to eliminate erythrocytes. The tissue is then quickly cut into small pieces, frozen in liquid nitrogen, and pulverized on dry ice under 5–10 mL liquid nitrogen using mortar and pestle (see [Note 18](#)).
2. Lymphocytes (see [Fig. 16.1B](#)) are used as a fresh pellet (see **Subheading 16.3.3**) and do not need to be frozen and pulverized. Frozen lymphocyte pellets are thawed on ice.
3. The cells are rapidly mixed with denaturation solution (10 mL/g tissue or 5×10^7 to 10^8 cells) under the hood (see [Note 19](#)) by vortexing. If the mixture is not

homogeneous, an electronic homogenizer may be used. If the mixture is more viscous than 50% glycerol, add 1–2 mL/g tissue or 5×10^7 to 10^8 cells denaturation solution. The volume is measured, a note is made of it, and the mixture is transferred to centrifugation tubes with-standing phenol/chloroform (see [Note 20](#)).

4. An equal volume of phenol/chloroform/isoamyl alcohol is added; the mixture is vortexed for 1 min and stored on ice for 15 min (5 min for the isolation from lymphocytes). The mixture is centrifuged at 12,000g for 15 min (5 min for the isolation from lymphocytes) at 4°C. The uppermost aqueous layer, which contains the sample RNA, is transferred to a new RNase-free tube (see [Note 21](#)). The extraction is performed at least twice with phenol/chloroform/isoamyl alcohol using RNase-free plastic tubes.
5. The volume of the aqueous solution is measured, and one-tenth of the volume sodium acetate solution is added. An equal volume of acid phenol/chloroform is added as described in [step 3](#), and the mixing, incubation on ice, and centrifugation are repeated once (see [Note 22](#)). The upper aqueous RNA-containing phase is transferred to a new RNase-free tube, and the volume is measured.
6. The RNA is now precipitated by adding an equal volume of isopropanol, mixing it well, and storing it at –20°C for at least 1 h or overnight. The precipitate is centrifuged at 12,000g for 20 min (15 min for the RNA isolation from lymphocytes), and the isopropanol-containing supernatant is gently aspirated and removed with a pipet, which should not touch the nucleic acid-containing pellet (see [Note 23](#)). The tube is recentrifuged for 1–2 min and the remaining supernatant removed in an identical manner as in [step 6](#).
7. The pellet is washed by adding 2–3 mL (200 µL for the RNA isolation from lymphocytes) room temperature 70% ethanol to remove residual salts, gently mixing with a slow-speed vortex for up to 3 min. The RNA is pelleted by centrifuging at slow speed (3000g) at room temperature. The ethanol is removed by aspiration with a pipet as described in [step 6](#).
8. The RNA pellet is redissolved in 100 µL to 1 mL nuclease-free water/100 mg spleen or 10^7 lymphocyte cells (see [Note 24](#)). EDTA is added to the RNA solution to a final concentration of 0.1 mM to chelate ions.
9. The quality of the isolated RNA is assessed by loading samples on a 1% native agarose gel (see [Note 25](#)). Heat 0.25 g LE agarose in 25 mL 1X TBE using a microwave oven until dissolved; add and disperse 1.25 µL of ethidium bromide solution (10 mg/mL). The solution is poured into an electrophoresis tray and let stand to polymerize. Mix 1–2.5 µg RNA solution with 2 µL of 10X native agarose loading buffer for RNA to give a final concentration of 1X and applied to the loading slot using a pipet (see [Note 26](#)). As a reference, 5 µL of the RNA marker is mixed with 0.5 µL of loading buffer for RNA and applied to the same gel in the reference slot. As an additional reference, a sample of intact RNA should be run in another slot to allow for direct comparison. The electrophoresis is performed in 1X TBE at 5–6 V/cm until the bromophenol dye band reaches two-thirds the length of the gel. The gel is now visualized on a UV transilluminator, and a photo should be taken as well. An intact RNA sample will show the two ribosomal RNAs (rRNAs) as distinct bands, one at 1500 bp

and one at 900 bp, that correspond to the 28s and 18s rRNAs. A degraded RNA sample will only show a smear at low molecular weight. Genomic DNA contamination can be identified by an additional visible band, which is not seen in the reference RNA sample.

10. Genomic DNA disturbs the accuracy of array experiments. In case DNA contamination is suspected but not clearly visible on the gel, the sample can be tested further by PCR: 2 μ L RNA sample is mixed with 21 μ L distilled H₂O, 25 μ L PCR master mix, and 2 μ L primer pair (50 pmol) in PCR tubes and overlaid with nuclease-free mineral oil. As a positive control, replace the RNA sample with mouse genomic DNA to a final concentration of 10 ng/ μ L. As a negative control, run the PCR reaction without sample by replacing it with distilled water. The samples are incubated for 15 min at 95°C and 35–45 cycles of 45 s at 94°C, 45 s at 63°C, and 2 min at 72°C in a preheated PCR thermocycler. A final incubation for 7 min at 72°C and storage at 4°C are added at the end of the PCR reactions. Run the PCR samples on a 1% agarose gel as described in **step 8**. If no DNA band is visible in the RNA sample PCR reaction, the next step is omitted.
11. Verified DNA contamination in the RNA sample is removed using the RQ1 RNase-free DNase kit according to the manufacturer's instructions: The RNA sample is diluted with RNase-free water to a final volume of 8 μ L, which is mixed with 1 μ L 10X reaction buffer and DNase of 1 U/ μ g RNA. The mixture is incubated for 30 min at 37°C and the reaction stopped by adding 1 μ L stop solution and incubating the mixture for 5 min at 65°C. PCR is used to check for the complete removal of genomic DNA as described in **step 10**. Repeat **steps 10** and **11** until the RNA sample is DNA free.
12. RNA quantities are determined using the RiboGreen assay (*see* **Note 27**): A high-range standard curve as well as a reagent blank are prepared by mixing the standard samples from the kit according to the provided pipeting scheme and measuring fluorescence at 480-nm excitation and 520-nm emission. Subtracting the value of the blank from each standard sample generates the standard curve, which is used to determine the RNA sample's quantity. Add 1.0 mL RiboGreen solution to the RNA sample and mix; fluorescence is measured at 480 and 520 nm in a disposable cuvette. The value of the blank is subtracted, and the RNA concentration is determined by comparing the value with the standard curve. The total RNA samples are adjusted to a final concentration of 1 μ g/ μ L by dilution in TE buffer, divided into portions of 5–10 μ L, and stored at –80°C until further use (*see* **Note 28**).

16.3.5 Array Analysis

These instructions assume the use of Atlas Plastic Mouse 5 K Microarrays containing sequences from approx. 5000 known mouse genes. Array analyses are performed twice for each sample to ensure intrasample reproducibility.

16.3.5.1 ³³P-Labeled cDNA Synthesis from Total RNA

1. 5 μ L 5X Power Script reaction buffer is mixed with 2.5 μ L 10X dNTP mix, 1.5 μ L DDT (100 mM), and 7 μ L ³³PdATP isotope (10 μ Ci/ μ L; > 2500 Ci/mmol) (see [Note 5](#)) in a sterile nuclease-free 0.5-mL PCR tube. This nucleotide mixture is kept at room temperature (see [Note 29](#)).
2. In a second sterile 0.5-ml PCR tube, 1 μ L total RNA (1 μ g/ μ L) is combined with 1 μ L cDNA synthesis control and 1 μ L 10X random primer mix.
3. The primer mix is annealed to the sample RNA at 65°C for 2 min using a PCR heating block. The temperature is lowered to 42°C, and the RNA–primer mixture is incubated for another 2 min.
4. During the incubation, 2 μ L reverse transcriptase are added to the nucleotide mixture from **step 1** and carefully mixed using a pipet.
5. The nucleotide mix is now added to the tube containing the RNA sample with the annealed primers from **step 3** (see [Note 30](#)). The contents are mixed using a pipet.
6. The mixture is incubated at 42°C for 30 min to allow for cDNA synthesis.
7. The reaction is stopped by adding 2 μ L 10X termination mix and carefully mixing it with a pipet (see [Note 31](#)).
8. 180 μ L buffer NT2 are added to dilute the probe synthesis reaction to 200 μ L total volume. Mixture is obtained by pipeting.
9. A spin column from the kit is inserted into a 2-mL collection tube, and the sample is pipeted onto the column. The column is centrifuged at 11,000 *g* for 1 min, and the flowthrough is saved to evaluate the cDNA synthesis efficiency. The collection tube is discarded in an appropriate container for radioactive waste.
10. The spin column is now washed three times by inserting the column into a fresh 2-mL collection tube, adding 400 μ L buffer NT3 to the column, and centrifuging as described in **step 9**. Both the collection tube and the flowthrough are discarded.
11. To elute the cDNA synthesis probe, the spin column is inserted into a clean 1.5-mL microcentrifuge tube; 100 μ L buffer NE are added to the column and soaked into it for 2 min. The column is centrifuged as described in **step 9**, with the flowthrough now containing the labeled cDNA probe.
12. The efficiency of the cDNA probe synthesis is determined by scintillation counting of the flowthroughs from **steps 9** and **11**: 5 μ L of each flowthrough are added to 5 mL scintillation fluid in separate scintillation counter vials. The samples are counted on the ³³P-channel, and the obtained counts are multiplied by a dilution factor of 20. Probes should yield a total of 5–25 $\times 10^6$ cpm (see [Note 32](#)).

16.3.5.2 Hybridization

1. Heat 30 mL hybridization solution to 60°C in a separate container. A hybridization box is filled to approx. 80% full with water and warmed to 55–60°C.
2. The microarray is placed with the printed surface facing up into the hybridization box containing the prewarmed water.

3. The water in the hybridization box is replaced by 15 mL prewarmed hybridization solution and rotated for 10–30 min at 60°C.
4. The radioactive-labeled sample (*see Note 5*) is now denatured by incubating it in a boiling (95–100°C) water bath for 2 min and then transferring it on ice for 2 min. The denatured probe is combined with 15 mL prewarmed hybridization solution in a disposable 50- or 15-mL plastic tube and carefully mixed together.
5. Pour off the hybridization solution from **step 3** and replace it with the mixture from **step 4**. Hybridization is performed overnight at 60°C (*see Note 33*).
6. Heat 300 mL high-salt wash solution and 300 mL low-salt wash solution 1 58–60°C. A 500-mL beaker is filled with room temperature low-salt wash solution 2. The hybridization solution containing the radioactive probe from step 5 is emptied in an appropriate container for radioactive waste and, without letting the array membrane dry, is replaced with 40–50 mL prewarmed high-salt wash solution. The container is rotated for 5 min at 58°C, and the washing procedure is repeated once more. The washing steps are repeated twice with 40–50 mL low-salt solution 1.
7. The temperature of the hybridization oven is reduced to 25–30°C. The wash solution is replaced by low-salt wash solution 2 to approx. 80% capacity of the container and rotated for 5 min.
8. The microarray is removed from the hybridization box using forceps and immediately transferred to the beaker of room temperature low-salt wash solution 2. The microarray is dipped several times into the wash solution.
9. The microarray is slowly removed and allowed to drain completely (*see Note 34*). The microarray is air-dried completely and exposed to a phosphoimaging screen suitable for ³³P-detection.

16.3.6 Image Analysis of Array Data

1. The arrays are exposed to phosphor imager screens for 2 d (*see Note 35*).
2. The screens are scanned in transparent mode at 200-dpi resolution with a Typhoon Variable Mode Imager and stored in TIFF format (*see Notes 36 and 37*).
3. An overall alignment of the Atlas Array phosphor image to the AtlasImage Grid Template is made using the Atlas Image software.
4. The alignment is fine-tuned using Auto-Align and manual adjustment options until the open circles of the AtlasImage Grid Template cover all gene spots (*see Note 38*). All genes that have to be excluded because of background problems should be listed for each array and marked on the exported gene lists.
5. Under most circumstances, the default background calculation can be used. However, the background calculation needs to be adjusted if the background of the array (as measured in white boxes between gene spots) shows a varying pattern (*see Note 39*). Signal thresholds are not changed; that is, the default setting of twice the background value should be used.
6. To assess the intrasample reproducibility, replicate arrays are compared to each other as described in **steps 8 and 9**. “Ratio” and “difference” thresholds are adjusted according to the researchers’ criteria for reproducibility.

7. Replicate arrays that have passed the reproducibility test are averaged using the Average Multiple Arrays mode in the Analysis tool and saved as an aligned composite array.
8. The aligned composite arrays obtained from vaccinated mice (VAC) at 4 or 24 h after *in vitro* restimulation or mice administered with adjuvant (ADJ) are compared to the aligned composite control sample array (N) (as outlined in [Fig. 16.1](#)) with the software's Analysis tool. Both arrays are normalized using the global normalization method in the sum mode; the expression values above background signal of all genes on the arrays are used in this calculation.
9. A customized report containing the gene expression changes between the two samples is generated and saved as a text file.

16.3.7 Analysis of the Data Set Quality

1. To examine the quality of the data set, each data set is plotted against the data set of nonimmunized mice (N) using Excel software.
2. Lines indicating twofold gene expression changes are drawn using the Excel software. The majority of the data points should lie within these lines. For low-quality data, many data points would be spread outside the line for the twofold gene expression changes at lower intensity values ([9,10](#)).

16.3.8 Clustering of Data

1. An Excel file is created containing the data of the whole study by copying the output text files from the AtlasImage analysis. The array coordinates or the gene names are placed into column 1; the GeneBank accession number (last column in AtlasImage) should be placed in column 2 and the expression values in column 3 and upward, starting with the control sample (N) in column 3 (see [Note 40](#)). The number of data rows is typed in cell 1 of row 1, and the number of columns excluding the first two description columns is typed in cell 2 of row 1.
2. A tab-delimited file is created from the Excel file.
3. The program GeneCluster is launched and the file loaded in the File Menu.
4. In the Dataset menu the columns (called panels by the software) are chosen for preprocessing. Because the software has automatically excluded the identifier columns, all columns are chosen, and the icon "Add" is clicked. The default thresholds are accepted by clicking "Add." Under row variation, a maximum/minimum of two and a maximum minus minimum of 100 is chosen. This sets the filter at twofold expression change and 100 above background. The icon "Add" is clicked. The data are normalized to have mean = 0 and variance = 1

- (default values), and the icon “Add” is clicked. The icon “Create and Dismiss” is clicked to start the normalization-and-filtration procedure.
5. In the Algorithms menu, “SOM” is chosen, and the number of clusters for display is selected by changing the numbers in “SOM rows” and “SOM columns” (see [Note 41](#)). The advanced parameters are left at default value. The icon “Run” is clicked, which activates the processing of the data and the display in clusters. Standard deviations are displayed as red lines outside the centroids and displayed as blue lines connecting the dots, which each represent a sample (see [Fig. 16.2A](#)).
 6. If satisfied with the clustering results, the data are saved by clicking on “save current.” This creates two text files, one displaying the standard deviation data for each cluster and the other containing the output data for all genes in each cluster (see [Note 42](#)).
 7. The Treeview program is launched and the output file from GeneCluster loaded in the file menu. This creates a thumbnail image of the cluster results with upregulations in shades of red and downregulations in shades of green (see [Fig. 16.2B](#)), (see [Note 43](#)).

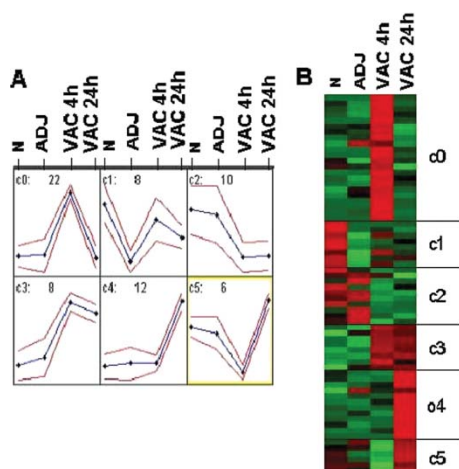


Fig. 16.2 Gene expression differences in mouse spleen lymphocytes between nontreated mice (N), adjuvant-treated mice (ADJ), and diphtheria toxoid vaccinated mice (VAC) 4 and 24 h after *in vitro* restimulation. **A** Self-organizing map clusters (c0–c5). Dark gray lines connecting dots indicate the mean expression profiles; light gray lines indicate the standard deviation. **B** Clustergram of the gene expression profiles from the six clusters (c0–c5) using the Treeview program. The sample is indicated at the top of each column. Shades of dark gray: light gray upregulation, black: unchanged gene expression down regulation.

16.3.9 Verification of Gene Expression Data Using RT-PCR (Optional)

If verification of gene expression data from the array analyses is desired, this can be performed according to a method presented elsewhere (*14*).

16.3.10 Data Mining on the Internet

The following instructions refer to the use of the 2.6 version of the PubGene tool.

1. A list of genes and their expression values as derived from the array experiments and clustering is compiled in Excel, with the GeneBank accession number in column 1 and the expression values in column 2 and upward. In row 1, the titles for each column can be given. This is saved as a text file in the tab-delimited format.
2. The software uses primary gene symbols (the symbols of the Jackson Laboratory for mouse and of the Human Genome Organization (HUGO) for human genes) and can recognize most gene names automatically. If genes are assigned as symbols by the array manufacturer and a GeneBank accession number is not available, it can be a good idea to check the correct primary gene symbol used by PubGene by searching for it in the Names Search tool of PubGene: Use gene names as names source, enter the symbol at the search expression line, and choose all search fields and case insensitive.
3. The text file containing the list of gene symbols is submitted to the Network Browser tool by clicking on the upload file option in the advanced option. As settings, the organism “mus musculus” (“homo sapiens” for vaccine evaluation during clinical studies as described in the next chapter) and no associations in the annotate graph option (“Literature” is the default association) are chosen. The keyword window is left empty to start a broad search. As a query term type, “genes” is chosen. After the tab-delimited file is uploaded in the ID window, the “submit” icon is clicked, which starts the search.
4. The output is comprised of two parts; the upper part contains textual background information of the query genes as a row; the lower part is a graphical description. The textual part contains the symbol of the gene, its full name according to which database, the number of articles with at least one cocitation (*see Note 44*), the number of network neighbors, the different synonyms of the gene, and clickable links to external databases for each gene. The graphical part is displayed as a network, in which lines connect the genes of the network, represented as nodes. The number along the lines displays the number of cocitations between two genes (*see Note 44*). The numbers can be clicked to retrieve the corresponding articles in the literature (*see Note 45*). To the left of the graphics is a list of the literature network neighbors.
5. It is advised to first examine the graphical display of the literature network. All genes contained in the uploaded file are represented as light red nodes. Other

genes, which are cocited with them, are represented in dark red nodes or black nodes. The list of all the literature network neighbors is exported by clicking the “Export list” option on top of the table and saved as a file.

6. The number connecting two genes of interest is clicked to retrieve all articles of cocitation between these two genes.
7. The graphics is saved as a .jpg or .gif postscript file by choosing the correct export option to the right side of the graphics.
8. A subset network is created for genes of interest by checking the boxes in front of the genes of interest on the scrollable list to the right of the graphics. The subsets of interest are explored in the literature or as MeSH associations according to the National Center for Biotechnology Information’s (NCBI’s) nomenclature by choosing the right option on top of the list.
9. The submission is now repeated from **step 2** with a keyword relevant to the study by typing it in the window for keyword in the advanced option menu. An example of the results produced is shown in **Fig. 16.3**.

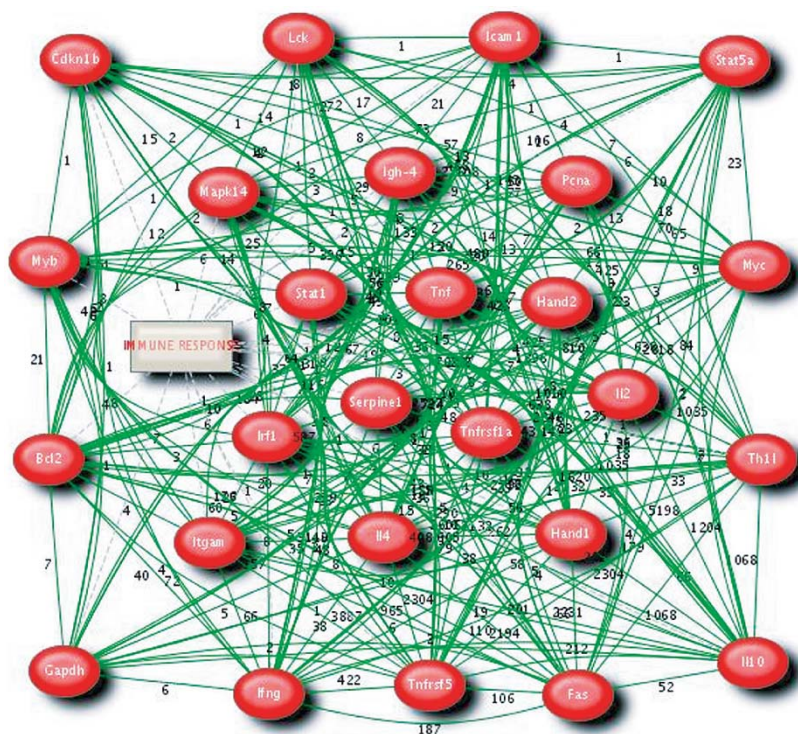


Fig. 16.3 Literature network of 26 genes shared in mice, which were immunized with diphtheria toxoid or tetanus toxoid and different adjuvants (5). The 26 shared genes were submitted to the PubGene software (version 2.6) together with the key word “immune response.” The gene names were translated into primary symbols by the software. These primary symbols formed a literature network with a functional relationship to immune response, confirming their role in immune response after vaccination. The number of cocitations ranged from 1 to 5198

10. The submission is repeated to annotate the results to diseases (MeSH) by choosing this option in the Annotate Graph window. This is repeated with annotations for Function or Process or Component or Chemical Substance as appropriate for the study.

16.4 Notes

1. For solution preparation, only water of 18.2 M Ω -cm and total organic content of less than five parts per billion should be used.
2. Centrifugation tubes of these materials will withstand phenol/chloroform solutions and 12,000 *g* forces.
3. RNaseZap inactivates RNA-degrading RNases, which are found on surfaces in the laboratory.
4. Ethidium bromide is carcinogenic. Wear gloves and glasses for protection.
5. ³³P-dATP isotope emits radioactivity. Shielding, disposal, and protective measures have to be performed according to local government regulations.
6. If a newly developed vaccine or adjuvant is to be tested, it is important to perform array analyses of samples after administration of a dose range, which has biological relevance in relation to antibody titer, T-cell proliferation, and cytokines. This dose range needs to be established before proceeding with the array analysis.
7. Lymphocytes from other sources (e.g., lymph nodes, etc.) are not feasible to use because of their low RNA yield and the large amount of RNA needed for the array experiments.
8. Lymphocytes from nonimmunized mice can also be restimulated and analyzed as a control to eliminate the possibility that the mice had been exposed to the antigen before the onset of this study and to eliminate potential nonspecific *in vitro* effects of the antigen from the gene expression analysis.
9. It is important to use animals in good health and without concurrent infections because this would affect the immune response provoked by the vaccine used and therefore skew the gene expression results.
10. A continuous flow gas anesthesia system is used to deliver isoflurane to the mice. The mice are placed in an induction chamber for initial anesthetization and then supplied the gas through a mouse mask.
11. Aseptic techniques are used to avoid the possibility of infection of the animals or cell cultures. These include the preparation of the vaccines and spleens under aseptic conditions in a class 100 clean room equipped with a laminar airflow hood, sterilization of instruments, and treatment of work surfaces with disinfectant before and after use, washing of the investigator's hands with an antiseptic surgical scrub preparation, and wearing of sterile gloves, face mask, and eyeglasses.
12. The spleens can be frozen in liquid nitrogen and stored at -80°C at this stage.
13. If the antibody response results are not available before growing the lymphocytes, the lymphocytes of each mouse are kept separate, and pooling can be performed after growth and restimulation of the lymphocytes. Antibody type and titers are vaccine specific and should be determined prior to this experiment if not previously known.
14. The lymphocytes can be stored at this point by resuspending the cells in ice-cold PBS and centrifugation at 270 *g* for 10 min at 4°C. Store the pellet at -80°C.
15. Lf is a quantity of toxoid used for vaccines and is assessed by flocculation (limit of flocculation), which may vary among different products.
16. After incubation, the supernatants of the cell cultures can be used for the cytokine assay by capture enzyme-linked immunosorbent assay (ELISA) using specific antibody pairs and recombinant cytokine standards and ELISA plates.

17. The quality of the RNA is the most important factor for the success of array analysis, and great care should be taken to ensure top quality. It is essential to work as quickly as possible during cell disruption to denature RNAses found in tissue cells before they can degrade the sample RNA. RNAses are also found on lab benches and on hands. Therefore, RNase decontamination of the bench area used for isolation of the RNA is recommended. This is done using RNaseZap. Gloves should be changed frequently.
18. Because the denaturation solution inactivates RNAses, mortar and pestle are cleaned with detergent only. Prechill the equipment with liquid nitrogen before usage. Once frozen in liquid nitrogen, the sample can also be stored in an airtight container at -80°C .
19. The lysis solution contains guanidine thiocyanate (GuSCN), which is an irritant to the respiratory tract and skin. Experimental steps involving this solution should therefore be performed under a hood, and gloves should be worn.
20. After homogenization, the lysates can be stored at -80°C for several weeks or months.
21. The interface layer contains contaminants such as proteins, including RNAses, and should not be carried over. To avoid this, the tube is tipped at a 45° angle and the pipet tip carried along the outermost tube wall to be able to pick up the last drop of aqueous solution.
22. If the volume of the aqueous solution recovered from the different extractions is increased compared to the volume before the extractions, still only 1 volume corresponding to the volume before the extractions is added.
23. Depending on the quantity of isolated RNA, the pellet can have either a whitish flocculent or a transparent appearance. The pellet does not adhere well to the walls of the tube. Therefore, the tube should not be inverted to pour off the supernatant.
24. The RNA can be difficult to dissolve at this step. Warming the tube and its content up to 70°C can improve this. It can take up to 30 min to dissolve the pellet.
25. Native agarose gels are sufficient to quickly assess the overall quality of the isolated total RNA by inspecting the 18s and 28s rRNA bands. However, these rRNA bands do not move according to their size in native agarose gels.
26. No preheating is required for RNA samples to run on native agarose gels.
27. RiboGreen is light sensitive and needs to be protected with aluminum foil before use. DNA also binds to RiboGreen and needs to be completely removed from the RNA sample to ensure correct measurement of RNA concentrations.
28. Frequent freezing and thawing can cause strand breaks in nucleic acids (RNAs) and should therefore be avoided by dividing the sample into smaller aliquots.
29. Precautionary measures have to be taken to shield against the radioactivity from the ^{33}P dATP isotope according to each country's own legislation. This applies for the remainder of the experiment until image data have been acquired.
30. This step has to be performed as rapidly as possible to keep the temperature at 42°C . Alternatively, the tube can be left in the heating block. A lower temperature would have a negative impact on the enzymatic activity of the reverse transcriptase.
31. The mixture can be kept on ice for up to 2 h before proceeding. However, it is recommended to continue to ensure the highest quality possible.
32. In case of low enzymatic activity, the majority of the counts would be found in the flow-through of **step 9**.
33. It is critical that the mixture of hybridization solution and radioactive-labeled probe touches the whole surface of the array and that the movement of the container provides for continuous mixture.
34. If droplets still are present on the array, repeat the dipping and removal. Droplets should be removed completely. Dust-free tissue can be used to adsorb them if drainage is not sufficient. Remaining droplets distort the image.
35. It is a good idea to expose arrays over a range of 1 to 5 d depending on the age of the radioactive label and the signal strength/quality of the probe.
36. All arrays are scanned with the exact same scanner settings. Otherwise, it can result in image data errors between samples.
37. The TIFF files should not be compressed.

38. If the same array membrane has been stripped and reprobed, the grid template alignment from the previous experiment can be imported to save time.
39. The software lets you choose other regions for background calculation if required or import external backgrounds.
40. The output of this software contains column 2 as identifiers. The Genebank accession numbers are necessary for data mining using PubGene. If PubGene is not used, gene names in column 2 are also accepted by the software.
41. It is advisable to use the default settings the first time; after studying the results, the settings can be changed to display fewer clusters depending on the number of samples included and the variation of gene expression patterns across samples.
42. The output data can be opened in Excel and sorted according to the cluster number for easier viewing.
43. The size and colors of the image can be changed in the settings menu of the Treeview program.
44. If the software finds only one cocitation between two genes, this cocitation could be caused by location of the two genes adjacent to each other on the chromosome. The number should therefore be clicked to retrieve the reference and check the reason for cocitation.
45. The graphics is interactive and needs a SVG (scalable vector graphic) plug-in, which can be downloaded from the Adobe home page.

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Chapter 17

Pharmacogenomics in the Evaluation of Efficacy and Adverse Events During Clinical Development of Vaccines

Lennart J. Nilsson and Karin J. Regnström

17.1	Introduction.....	469
17.2	Materials	471
17.3	Methods.....	472
17.4	Notes	478
	References.....	478

Summary The understanding of vaccine-induced immune responses in adults and infants is limited. Current vaccination schedules for infants are frequently debated. Especially, the relationship among the timing, the frequency of the dosing, and the generation of an immunological memory are debated. Vaccine antigen-induced cytokine responses to vaccinations given in infancy are of particular interest because little is known about cellular responses in this age, and the information available is based on antibody responses. Pharmacogenomics is ideally suited to study cellular responses related to immune response; in addition, toxicity, inflammation, apoptosis, stress, and oncogenesis can be monitored, since the expression of thousands of genes can be measured in a single experiment.

Keywords Adverse reactions; clinical study; efficacy; microarray; PBMCs; pharmacogenomics; vaccine.

17.1 Introduction

New vaccines have to be evaluated regarding their immunostimulatory and immunosuppressing properties (1). When traditional methodologies are used, this evaluation is demanding and yet incomplete, with only a few biological responses measured. Consequently, the development and introduction of new powerful techniques that allow rapid toxicological and immunological evaluation are essential (2).

Functional genomics has been described as a method that will be critical for the discipline of infectious diseases (3) and will revolutionize vaccine design and formulation (4,5). By measuring the gene expression changes using a pharmacogenomics approach after the administration of vaccines, hundreds to thousands of biological processes, such as immune response, inflammation, toxicity, carcinogenicity, stress, and apoptosis, can be studied in a single experiment (6–9). For each sample, a unique gene expression profile can be obtained.

Furthermore, by comparing the gene expression profiles of healthy clinical subjects with those clinical subjects who experienced adverse reactions after administration of the vaccine, it will be possible to find differences in their immune responses and to identify biomarkers for these adverse reactions. These biomarkers can then be utilized in future vaccine development, diagnosis, and risk calculations.

The evaluation of efficacy and adverse reactions during clinical study phases is an important area for the application of pharmacogenomics (10). Together with supervised bioinformatics approaches, transcriptional profiling can be used to assess efficacy and adverse events (11). This has been recognized by the Food and Drug Administration (FDA), which has advocated incorporation of pharmacogenomics into drug development and regulatory submissions since June 2001 (12). A guidance for the industry about pharmacogenomics data submission was published outlining the type of data needed for submission of marketing applications, the format for submission, and which data will be used for regulatory decisions (13). With the FDA's acknowledgment of the importance of pharmacogenomics, it is anticipated that these guidelines will have a significant impact on the incorporation of this technology into the development of drugs, including vaccines.

Fortunately, several of the newly developed clinically used vaccines in children have fewer side effects today than those used a few years ago (e.g., acellular pertussis vaccines have considerably fewer side effects compared to whole-cell pertussis vaccines) (14,15). Vaccines today contain purer antigens, some components have been excluded (e.g. mercury), and vaccines have fewer local side effects because of administration intramuscularly instead of subcutaneously. As a consequence, the type of questions raised during the development of vaccines is different from earlier years; the discussion today is often focused on the effect of the antigen (often produced as a recombinant protein) or aluminum used as an adjuvant in the vaccines on the induction of type I hypersensitivity and production of immunoglobulin E (IgE) antibodies, which were discussed as involved in the development of allergy (16,17). Therefore, the questions regarding side effects were earlier more about toxic effects of the vaccines but have to some extent changed to questions around immunological diseases.

The basal immunological mechanisms are complex but can now be analyzed with refined and more powerful laboratory methods, such as the microarray technique. With the help of this technique, additional information besides that concerning IgE and cytokine levels about the association between adaptive and innate immune response as well as other cellular responses can be gathered (6–8).

17.2 Materials

The same materials are used as described in Chapter 16. Only additional materials are stated next.

17.2.1 Vaccination

For the combination vaccine use the diphtheria–tetanus–acellular pertussis (DTPa), inactivated polio (IPV) and *Haemophilus influenzae* type b (Hib) (Infanrix-Polio+H_{ib}) vaccine (GlaxoSmithKline, Rixensart, Belgium).

17.2.2 Isolation of Lymphocytes and Cell Culture

1. Heparin-treated tubes (Vacuette, Greiner Labortechnik, Kremsmünster, Austria).
2. Ficoll Paque density gradient (GE Healthcare, Piscataway, NJ).
3. Freezing medium: 50% fetal calf serum, 40% RPMI-1640 growth medium, and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO).
4. Trypan blue solution (Sigma-Aldrich).
5. Serum-free medium: AIM-V medium (Invitrogen, Carlsbad, CA), 20 μ M β -mercaptoethanol (Sigma-Aldrich).
6. Pertussis toxin (PT) (Glaxo Smith Kline Beecham, Rixensart, Belgium).
7. Dulbecco's modified phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride (Pierce Biotechnology, Rockford, IL).
8. Growth medium: RPMI-1640 with L-glutamine (Irvine Scientific Co., Irvine, CA), supplemented with 10% heat-inactivated fetal bovine serum, 10 IU/mL penicillin, 100 μ g/mL streptomycin (PEST), 50 μ M 2-mercaptoethanol, 292 μ g/mL L-glutamine, and 10 mM HEPES buffer (all from Sigma-Aldrich).
9. Wash medium: RPMI-1640, 2% fetal calf serum.
10. Türks solution: 0.04 g gentian violet (Sigma-Aldrich), 50 g 25% HAc (Sigma-Aldrich), water to 150 mL.

17.2.3 Array Analysis

Atlas plastic human 8K microarrays (Clontech, Mountain View CA). For additional materials, refer to the Chapter 16.

17.3 Methods

The pharmacogenomics analysis of samples from a clinical study with an aluminum hydroxide-adsorbed vaccine (diphtheria–tetanus–acellular pertussis/polio/*Haemophilus influenzae*) in infants is described. These instructions can easily be adapted to clinical studies of other vaccines with changes to the type of administered vaccine, administration route, vaccination ages, and so on. This method is therefore also suitable for assessing vaccine responses in adults and to study the cellular reactions in clinical subjects who have experienced adverse reactions.

Venous blood samples are collected from clinical subjects after approval by the regulatory authorities for the evaluation of medicines and the local ethical committees for the sampling and the experiments to be performed. The identities of the clinical subjects are blinded. The collection should contain samples of healthy infants or children of both genders and minorities, with at least five clinical subjects per group. If adverse event reactions are to be studied, the collection should also contain at each time-point of the study one group of clinical subjects who were reported to experience adverse events after vaccination. The clinical subjects are vaccinated intramuscularly with one dose of the vaccine at 3, 5, and 12 mo of age. Samples are collected at 3 mo before the first vaccination and at 6 and 13 mo, with the second samples taken after their second vaccination at 5 mo of age and the third vaccination at 12 mo of age, respectively.

Lymphocytes are isolated from blood samples using a Ficoll Paque density gradient, which is standard methodology. Because of the smaller amount of blood taken from the infants, the quantity of cells is not large enough to allow for sorting. However, array technology is able to differentiate between the different cell types because the expression of cell-type-specific receptors can be assessed (6).

The cells are cultured and *in vitro* restimulated with antigen (here PT) for 12 h to mimic the encounter with the specific antigen in the body. Earlier studies have shown that an *in vitro* restimulation of mouse spleen lymphocytes with antigen for 4 and 24 h, can be used to study immune response as well as other cellular processes, such as apoptosis, inflammation, and so on (see **Chapter 16**). Our experiments, however, showed that immune response markers are expressed at later time-points as compared to mouse, and that 12 h of *in vitro* restimulation is the ideal time-point for the assessment of immune response genes (see **Table 17.1**) (see **Note 1**).

As negative control 1, an aliquot of lymphocytes is cultured for the same time-period without the antigen restimulation. This stimulates the cells to start mitogenesis (6). The genes, which are associated with mitogenesis only, are excluded from further analysis. The *in vitro* restimulation of lymphocytes from 3-mo-old clinical subjects (before vaccination) with antigen is another negative control (control 2) to eliminate the possibility that the clinical subjects were exposed to the antigen before the onset of this study and to eliminate potential nonspecific *in vitro* effects of the antigen from the gene expression analysis (see **Note 2**).

After *in vitro* restimulation with the antigen, the cells are collected by centrifugation, washed in cold PBS, and used for RNA isolation (see **Note 3**). The methods

Table 17.1 Immune response genes expressed at moderate (+) or high (++) levels at 12 h in human peripheral blood mononuclear cells after *in vitro* restimulation with pertussis toxin antigen for 4, 12, 24, and 48 h

Gene name	4 h	12 h	24 h	48 h
CCR1		++		
CCR2	+	+		
CD27 (T-cell antigen)		+		
CD33		+		
CD40L	++	+	+	
CD70		+		
CD72		+		
CD106		+		
ICAM-1 (CD54)		+		
IL-2		++	+	
IL-2R β (CD122)	+	+		
IL-2R γ		++	+	+
IL-3		++		
IL-4R α (CD124)		+		
IL-5 R alpha		+		
IL-7		++	+	
IL-8		++		
IL-10	++	++		
IL-13		++	+	
IL-16		++	+	
INF beta		++		
INF gamma	++	++		
ITGB8	+	+		
MCP4	+	+		
PAX-5		++	+	

for RNA isolation, array analysis, image data analysis, assessment of the data quality, verification of the gene expression data, and data mining are the same as described in **Chapter 16**.

However, the procedure for sample comparison by bioinformatics analysis differs. The main differences are that (1) the samples from each clinical subject are treated separately during the first two phases of analysis, and (2) the method to compare samples is different:

In the first phase, each with antigen *in vitro* restimulated sample (+) is compared to the negative control 1, the nonstimulated but cultured sample (–), at the same age as the clinical subject (3, 6, and 13 mo) (see **Fig. 17.1A**). The aim is to (1) find the antigen-induced gene expression changes after *in vitro* restimulation with antigen at each age of the clinical subject and (2) to exclude gene expression changes that are not immune response related, such as those caused by mitogenesis (6).

In the second phase, the remaining genes from the gene expression changes in **step 1** at 6 and 13 mo of a clinical subject are compared to the corresponding gene expression changes at 3 mo for the same clinical subject, the negative control 2 (see **Fig. 17.1B**). Because the samples at 3 mo were taken before vaccination, the

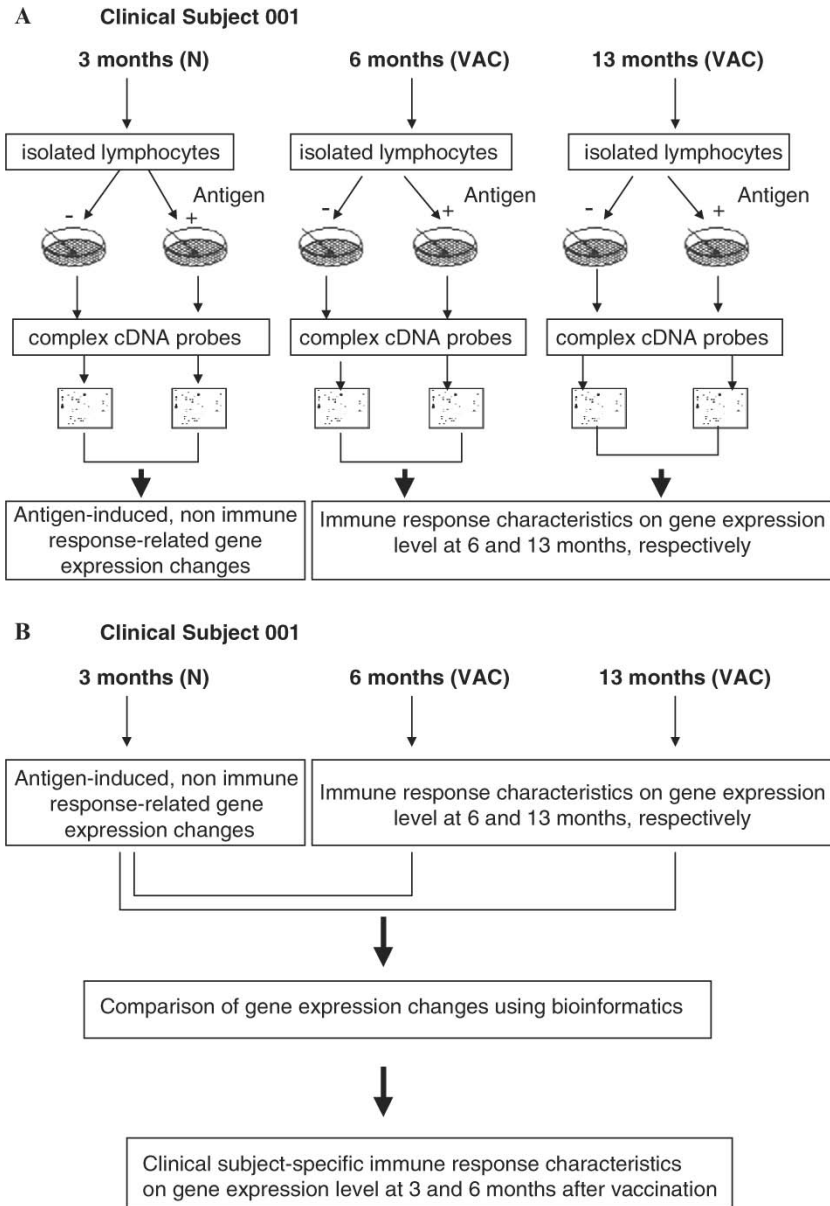


Fig. 17.1 (continued)

differences in gene expression in these comparisons are caused by vaccination at 5 and 12 months, respectively. They therefore reflect the immune response characteristics of the specific vaccine in a specific clinical subject. As observed, each

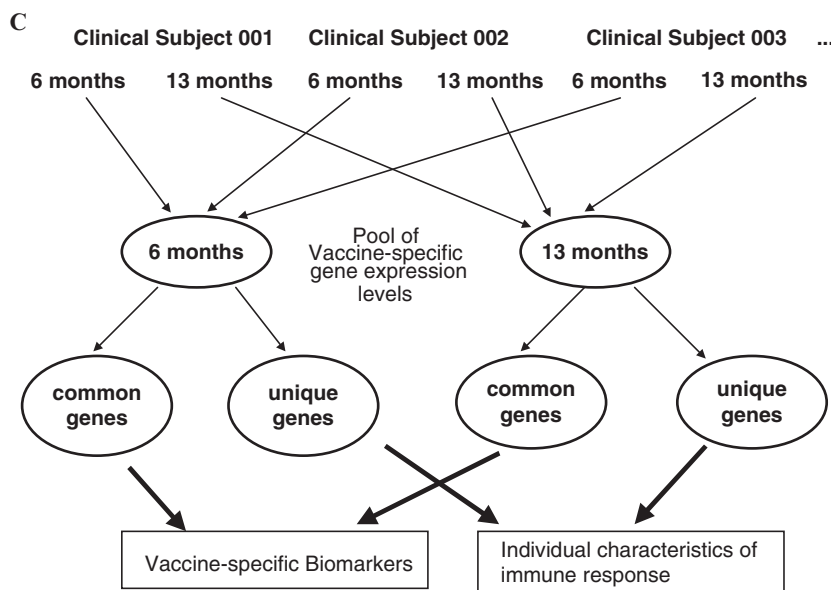


Fig. 17.1 Experimental setup. **A** Lymphocytes of 3-mo-old unvaccinated clinical subjects (N) and of 6- and 13-mo vaccinated clinical subjects (VAC), respectively, were *in vitro* restimulated with antigen (+) for 12h or cultured without antigen (–) for 12h. Total RNA was isolated and analyzed using microarrays, and its gene expression levels were compared between (+) and (–) groups at each age and within each clinical subject using clustering. Only genes with significant expression differences were saved. **B** Comparison of expression levels of saved genes from **A** between (VAC) at each age and (N) within each clinical subject using clustering. Only genes with significant expression differences were saved. **C** Using clustering, the expression levels of saved genes from **B** were compared between all clinical subjects but within each age group. Genes with significant expression differences between individuals were saved as unique genes. The remaining genes from the gene pool form the common genes and are used to assess the overall immune response of the vaccine (efficacy) studied.

antigen–adjuvant combination results in a combination-specific gene expression profile, a fingerprint (7).

Now, in the third phase, a comparison of gene expression changes between the different clinical subjects at each age can be performed (see Fig. 17.1C). Expression changes of genes that are common to all clinical subjects (common genes) are reminiscent of the vaccine-specific immune response. These shared genes are either known immune response markers or can be considered candidates for vaccine-specific biomarkers that need to be validated (10). Genes with significant expression changes specific to only one clinical subject (unique genes) represent individual variations. These unique genes are candidates for adverse reaction markers, if such were observed during the clinical study, or marked for individual immune response reactions.

The following instructions only describe the differences in methodology compared to Chapter 16.

17.3.1 Isolation of Lymphocytes from Blood Samples

1. Venous blood samples are drawn into heparin-treated tubes. The blood is diluted 1:2 with RPMI-1640.
2. The diluted blood is carefully layered onto a Ficoll Paque density gradient and centrifuged at 400 g for 30 min at room temperature without braking to separate the mononuclear cells from erythrocytes and granulocytes. The mononuclear cells at the interface are carefully removed and washed three times in wash medium.
3. The cells are resuspended in 800 μ L wash medium, and 10 μ L of the cell solution are diluted 1:10 in Türks solution and counted in a Bürkner chamber. The cells are then diluted in serum-free medium to a concentration of 1×10^6 cells/mL (see [Note 4](#)).

17.3.2 Growth and Restimulation of Lymphocytes

1. Frozen cells are thawed in a 37°C water bath and resuspended in growth medium. The cells are centrifuged at 400 g for 15 min at room temperature, resuspended, counted, and diluted as described in **Subheading 17.3.1, step 3**.
2. 1 mL lymphocytes is cultivated for 12 h at 37°C in a humidified atmosphere with 5% CO₂, with serum-free medium alone (negative control 1) (N) or with 1 μ g/mL PT, respectively (see [Notes 2](#) and [5](#)).
3. The cells are pelleted by centrifugation at 400 g room temperature, washed in cold PBS, snap frozen in liquid N₂, and stored at -80°C until RNA isolation.

17.3.3 Isolation of Total RNA

Follow the description from **Subheading 16.3.4, step 2**, of Chapter 16.

17.3.4 Array Analysis and Image Data Analysis

The procedure is essentially the same as described in the Chapter 16 (**Subheadings 16.3.5** and **16.3.6**) except that the Atlas human 8 K microarray is used.

17.3.5 Bioinformatics Analysis

17.3.5.1 Comparison of Cultured (-) and *In Vitro* Restimulated (+) Lymphocytes

1. The instructions in the Chapter 16, **Subheading 16.3.8**, are followed from **step 1** through **step 6**, separately for each clinical subject and for each age (3, 6, and 13 mo).

2. The data for genes with different expression as compared to negative control 1 (either up- or downregulated) are saved in an Excel file for each clinical subject (see also [Fig. 17.1A](#)) and sorted alphabetically.
3. The procedure is repeated for each clinical subject.

17.3.5.2 Comparison of Lymphocytes After Vaccination with Lymphocytes Before Vaccination

1. The saved data for gene expression changes of each 6- and 13-mo-old clinical subject (after vaccination) from **Subheading 17.3.5.1** are compared separately with the saved data for gene expression changes of the same clinical subject at 3 mo (before vaccination) (negative control 2) (see [Fig. 17.1B](#)). The template guide in the Excel data menu is used to copy the original image data as well as the array coordinates and the Genebank accession numbers of the genes obtained in **Subheading 17.3.5.1** from its Excel file (as described in Chapter 16, **Subheading 16.3.8, step 1**) into a new Excel file to be used for the subsequent comparison. The array coordinates or the gene names are placed in column 1, the Genebank accession number (last column in Atlas Image) should be placed in column 2, the expression values in column 3 and upward, starting with the control sample (age of 3 mo) in column 3.
2. The instructions in **Subheading 17.3.5.1** are then followed from **step 1** through **step 3**.
3. The process is repeated for each clinical subject.

17.3.5.3 Comparison of Gene Expression Changes Between Clinical Subjects

1. An Excel file is created containing all saved data on genes and their expression changes obtained in the previous step (**Subheading 17.3.5.2**) using the template guide in the Excel data menu to copy the original image data of the genes obtained in **Subheading 17.3.5.1** as well as the array coordinates and the Genebank accession numbers from the Excel file (as described in Chapter 16, **Subheading 16.3.8, step 1**) into a new Excel file used for the subsequent comparison: The array coordinates or the gene names are placed in column 1, the Genebank accession number (last column in Atlas Image) should be placed in column 2, the expression values in column 3 and upward, with one column for each clinical subject and grouping each age group together.
2. The instructions in **Subheading 17.3.5.1** are then followed from **step 1** through **step 2**.
3. The genes and their data as obtained by GeneCluster are saved as described in **Subheading 16.3.8** and used for visualization in Treeview. These genes differ between individual clinical subjects (unique genes) and can be used to study the gene expression differences between clinical subjects.

4. The remaining genes, which have the same gene expression pattern in each age group (common genes), can be used to analyze the overall immune response and possible signs of adverse events.

All subsequent data treatments and further analyses of the results are performed as described in Chapter 16.

17.4 Notes

1. If other vaccines than the one described here are used, the ideal time-points for *in vitro* restimulation with antigen should be assessed before proceeding.
2. Pertussis toxin has a high mitogenic potential. This can be avoided by heat inactivation for 20 min in an 80°C water bath.
3. The supernatant from the centrifugation can be stored for cytokine analysis by enzyme-linked immunosorbent assay (if desired).
4. At this time-point, the cells can be cryopreserved by suspending them in freezing medium to a concentration of 5×10^6 cells/mL, freezing them in isopropanol to -70°C for 24h, and then transferring them to a liquid nitrogen tank (-196°C).
5. If another antigen than PT is used, it can be a good idea to determine the optimal antigen concentration prior to the array experiment.

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Index

A

- 5-fluorouracil (5-FU), 63, 404, 406
- 5-lipoxygenase activating protein (FLAP), 366
- 5-lipoxygenase (5-LOX), 366
- 6-mercaptopurine, 63, 67, 398, 400, 407, 422, 438
- 6-methylmercaptopurine ribonucleotide, 399
- 6-thioguanine, 68, 398–399, 422
- Accessory proteins, 77–78, 81, 84–87, 97–98, 115, 140, 146
- Acute lymphocytic leukemia (ALL), 67, 278, 438
- Adenosine triphosphate (ATP)-binding cassette (ABC) family, 42, 414
 - ABCB1, 24, 41–43, 46–48, 50–56, 69, 310, 403, 408, 415–416, 420
 - ABCC1, 42–44, 46–47, 50, 56, 415
 - ABCC2, 42–43, 45–47, 50, 56, 415
 - ABCG2, 42–44, 46–48, 50–53, 55–56, 70–71
- Adenylyl cyclase (AC), 81, 113, 167
- Admixture, 31–38, 232
- Adrenal cortex, 84
- Adrenergic receptor, 94–95, 124, 127, 140, 149–153, 162, 360, 362, 364–365, 387–389
- Adverse drug reactions (ADRs), 21, 23, 274, 424
- Adverse Event Reporting System (AERS), 14–15
- Agonist, 90, 162, 363
- Agouti (Ag), 121
 - agouti-related proteins (AgRPs), 121
- Albright's hereditary osteodystrophy (AHO), 85, 87
- Albuterol, 153, 162, 360, 363–364
- Algorithm, 18, 32–33, 38
- Alleles, 67, 406, 438
 - Allele Frequencies Database, 10, 12
 - minor allele frequency (MAF), 232, 261
- Alzheimer's disease, 13, 17–18, 83, 87, 148, 164–165, 213–214, 224, 231, 233, 246–247, 257–260, 269, 273, 280, 286–292, 302, 305, 307, 317–323, 326
- American Association of Clinical Chemists (AACC), 23
- AmpliChip, 27
- Amyloid precursor protein (APP), 220, 234
- Amyloid scavengers, 257, 265–266
- Angiotensin, 88, 142–143, 165, 221, 286, 312, 317, 319, 322
 - angiotensin II receptor, 142
 - angiotensin-converting enzyme (ACE), 143, 213, 217, 304, 323
- Antagonist, 125, 128, 143, 153, 156, 227, 260, 302, 401, 427
- Anticholinergics, 360, 365
- Anticonvulsants, 24, 214, 216, 274
- Antidepressants, 24, 147–148, 214, 216, 274, 284, 293, 316, 319, 406, 408
- Antigen, 221, 265, 426, 447–449, 451–452, 464, 470, 472–475, 478
- Antigen-induced cell death (AICD), 448
- Antipsychotics, 24, 144, 146, 148, 325
- Antithrombotics, 24
- Antiviral, 160, 389
- Anxiety, 299–300, 304, 315, 319–324
- Anxiolytics, 24, 214, 216, 274, 319
- Apolipoprotein E (APOE), 217, 221, 257–260, 286, 295, 304–305, 307, 309, 318–319, 321, 323
- Aquaporin 2 gene (AQP2), 126
- Arachidonic acid, 366
- Area under the concentration (AUC), 55–56, 67
- Arginine vasopressin (AVP), 113, 126
 - V2 vasopressin receptor (AVPR2), 113, 126–128

Array, 450, 453, 457, 459, 471, 476
 Assays, 47, 51–52, 57, 241, 362, 386–389,
 399, 408, 440–442, 448
 Association study, 32, 34, 36, 231, 362
 Assortative mating, 34–36
 Asthma, 113, 129, 152, 162, 166, 359–367
 Autogenomics, 27
 Autosomal dominant hypocalcemia (ADH),
 116–117, 119
 Azathioprine, 24, 67–68, 398, 400, 406, 414,
 421, 423

B

Bartter's/Gitelman's syndrome (BS/GS), 89
 Basic Local Alignment Search Tool (BLAST),
 4–6, 8–9, 18
 Benzodiazepines, 24, 265, 293, 316, 320
 Binomial conundrum, 308
 Biochips, 21–22, 25, 29, 273
 BioFilm, 27–28
 Bioinformatics, 1, 2–7, 10, 13–18, 199, 255,
 272, 325, 381, 383–384, 448, 452, 470,
 473–474, 476
 Bipolar disorder (BD), 146, 163,
 187–188, 192
 Bisulfite, 194–195, 198–199, 202–204
 Bleeding disorder, 109, 113, 129, 156
 Blood–brain barrier, 53–54
 Brain-derived neurotrophic factor (BDNF),
 187, 190, 192, 197
 Breast cancer resistance protein (BCRP), 64, 70

C

Caco-2, 50
 Calcium-sensing receptor (CASR), 109, 111,
 113, 116, 118, 139, 141, 167
 Cancer, 9, 17, 70, 160, 228, 404, 437–441
 breast, 64, 70
 colon, 67, 161, 395–396, 405
 colorectal, 404, 406
 Cancer Genome Anatomy Project, 9
 Candidate gene, 17–18, 71, 129, 146, 149,
 361–362, 380–381, 383, 387, 389
 Cardiovascular diseases, 18, 379
 Catechol-O-methyltransferase (COMT),
 187–188, 192, 197, 204
 Cerebrovascular disorders, 263, 308
 Chaperone, 222–223, 233, 240, 249–250, 253,
 269–270
 Chemoattractant receptor, 155
 Chemokines, 157, 160
 receptors, 157, 160

Chemotherapy, 25, 63–64, 67, 70, 404–405,
 407, 437–438, 440–441, 443
 Cholecystokinin (CCK), 160, 313
 Cholinesterase inhibitors, 226, 260, 300, 302
 ClustalW, 4, 6, 8
 Clustering, 18, 33, 37, 90, 448, 451, 460–462,
 475
 Cognition, 214, 255, 280, 291, 302–304, 306,
 315–316, 319, 321, 324
 Cognitive performance, 256, 299, 315–316,
 318, 321
 Combination therapy, 291, 293, 303–304,
 306, 318, 320–322, 325
 Concentrative nucleoside transporters
 (CNTs), 46
 Confounding, 31, 33–38
 Constitutively active mutants (CAMs),
 96, 110
 Coronary artery disease (CAD), 311, 380
 Corticosteroids, 359–360, 367–369, 400
 CpG islands, 193, 199–200
 Cyclic adenosine monophosphate (cAMP), 81,
 88, 93, 110, 113, 156, 190, 192, 219
 cAMP response element binding protein
 (CREB), 190, 219
 Cyclosporine, 43–44, 50, 278, 403, 406
 Cysteinyl leukotriene (CysLT), 79–80, 139,
 145, 147, 150–151, 153–154, 165, 366
 Cytochrome P450 (CYP450), 16, 23, 64, 220,
 275–280, 287–290, 292, 397, 403, 406
 CYP2B6, 23, 276, 281
 CYP2C9, 23–24, 28, 274, 276, 281–283
 CYP2C19, 23, 27–28, 274, 276, 282–283,
 397–398, 406, 408
 CYP2D6, 23–24, 27–28, 64–65, 213, 217,
 223, 274, 277, 280, 282–291, 293–294,
 304, 306–307, 315, 324, 408
 CYP3A5, 23, 65, 274, 278, 294, 307,
 403, 406
 Cytokines, 152, 157, 159, 264, 452
 Cytomegalovirus (CMV), 159

D

Data integration, 4
 Data mining, 18, 223, 451, 453, 462, 466, 473
 Data quality, 460, 473
 Data storage, 442
 Database, 4–10, 12–18, 119, 200, 218, 270,
 282, 361–362, 453, 462
 Database of Interacting Proteins (DIP), 13
 dbGaP, 5
 dbSNP, 4–5, 9–12, 16, 361–362, 385
 Desensitization, 90–91, 163

Dihydropyrimidine dehydrogenase (DPD), 65, 406
Dipeptide transporters (PEPTs), 46
Disease-modifying antirheumatic drugs, 413–414
DNA methylation, 187–191, 193–194, 199–200, 204, 207, 209, 222, 369
Dopamine, 144, 227
 dopamine transporter 1 (DAT1), 187–188, 192
 receptor, 88, 144, 146–147, 163–164, 188, 197
Dopaminergic (DAergic), 143–144, 187–188, 192
Down syndrome, 222, 235, 246, 250, 252
Drug absorption, 16, 53
Drug ADME, 15
Drug efficacy, 23, 57, 63–64, 110, 139, 141, 147, 150, 214, 271, 273, 287, 301, 326, 404–405
Drug excretion, 360, 403
Drug metabolism, 22–23, 55, 213–214, 216, 272, 274, 281–283, 290, 293, 306, 326, 360, 395, 398, 404–405, 422
 drug-metabolizing enzymes, 63–64, 71, 223, 272, 281, 408, 430
Drug monitoring, 23
Drug response, 3, 23–24, 64, 83, 87, 110–111, 140, 144, 146, 148–149, 152–153, 159, 168, 216, 272, 326, 360–362, 395–396

E
Electrocardiographic (ECG), 316, 382
Electrophoresis, 25, 195, 201, 202, 207, 456
Endothelin 1 (ET1), 128, 154
Entrez, 5–7, 17
Environmental Genome Project (EGP), 10, 12
Enzyme Commission (EC), 5
Enzyme-linked immunosorbent assay (ELISA), 448, 478
Epidemiology, 89, 272
Epidermal growth factor receptor (EGFR), 438–439
Epigenetic, 22, 187–189, 191–194, 199, 209, 222, 233–234, 270, 272, 441
 aberrations, 188–189, 192
Epstein Barr virus, 160, 439
Erlotinib, 439
Etanercept (ETN), 425, 430
Ethnic differences, 282–283
European Bioinformatics Institute (EBI), 14
Exon splicing enhancer (ESE), 385
Expression-Based SNP Imagemaps, 9–10

F
Familial hypocalciuric hypercalemia (FHH), 109, 113, 116–117, 119
Familial male precocious puberty, 112, 123
Fasta, 9–10
Fatty acid transport protein 1 (FATP-1), 382
Fingerprinting, 25, 189
Fluorescence resonance energy transfer (FRET), 50
Follicle-stimulating hormone, 109, 121
 follicle-stimulating hormone receptor (FSHR), 112, 121
Food and Drug Administration (FDA), 2, 42, 71, 216, 438, 470
Formulation, 22, 453, 470
Functional dyspepsia, 406
Functional genomics, 2, 213, 216, 222, 250, 255, 259, 271–272, 299, 379, 470

G
G protein-coupled receptor (GPCR), 77–79, 81–82, 88, 110–114, 129, 139, 158, 162, 166
 activator of G protein signaling (AGS), 77, 86, 98
 G protein associated with asthma (GPRA), 109, 111, 113, 129
 G protein-coupled receptor kinases (GRKs), 77, 83, 91, 95, 97–98, 114
 regulator of G protein signaling (RGS), 77, 82, 87
Gain of function, 81, 95–96, 111, 117, 119–120, 140, 162, 165, 233, 239
Gametogenesis, 121–122
Gastroesophageal reflux disease, 276, 395–396, 406
Gastrointestinal disorders, 325, 395, 405
 carcinoma, 160
Gefitinib, 44, 439
GenBank, 5, 9
Gene Expression Omnibus, 14
Gene expression profiling, 22, 26
Gene Ontology (GO), 5
Gene silencing, 270–271
Genetic association, 17–18, 31, 36, 189, 250, 362–363, 383
GeneView, 9, 12
Genomic control (GC), 36
Genotype, 16, 255, 302–304, 363
 genotyping, 26, 46, 383, 419, 442
GENSCAN, 6, 8
Germline, 440
Gilbert's syndrome, 67

Glucocorticoid receptor, 190, 283, 360,
367–368, 400
Glycosylation, 79, 219, 239, 248
Gonadotropin-releasing hormone (GnRHR),
109, 124
Guanosine triphosphatase (GTPase), 82, 84

H

Haplotype, 162, 380, 419, 430, 441
HaploView, 362
HapMap, 9–10, 16, 361–362, 431
Heat shock organizing protein (Hop), 368
Heat shock protein, 248, 368, 426
 heat shock protein 40 (Hsp40), 368
 heat shock protein 70 (Hsp70), 269, 368
 heat shock protein 90 (Hsp90), 269, 368
HEK293 cells, 51, 53, 240
HeLa cells, 52
Helicobacter pylori infection, 396, 406
Herpes simplex virus (HSV), 160
Hirschsprung's disease, 112, 128
Homology, 5–6, 8–10, 84, 95, 116, 383–384
Human Gene Mutation Database (HGMD),
9–10
Human Genome Database (GDB), 18
Human Genome Variation Society (HGVS),
9–10
Human immunodeficiency virus (HIV), 24,
50, 157
Human Protein Reference Database
(HPRD), 13
Hydroperoxyeicosatetraenoic acid, 366
Hypercalcemia, 116
Hypercalciuria, 117, 119, 142
 hypocalcemic, 117
Hypertension, 17–18, 87–88, 97, 142, 162
Hypocalcemia, 116–117
Hypoparathyroidism, 117, 119
Hypophysectomized, 121–122

I

Idiopathic hypogonadotropic hypogonadism
(IHH), 109, 112, 124
Immunity, 157, 401
Immunization, 262, 266–267, 451–453
Immunophilins, 368
Immunoprecipitation, 194, 198, 207
Infectious diseases, 18, 470
Infertility, 122
Inflammation, 155–156, 159, 161, 236,
266, 299, 359–360, 366–367, 448,
469–470, 472

Inflammatory bowel disease, 24, 395, 398,
400, 406
Infliximab (INF), 401, 425, 430
Inosine triphosphate pyrophosphatase, 399
Interaction, 15, 154, 307
 drug–drug interactions (DDIs), 23, 64,
217, 283, 325–326
 protein–protein interaction, 4, 14
Interleukin (IL), 159, 219–220, 426, 430
Internalization, 93–94
Irinotecan, 43–45, 67, 407
Irritable bowel syndrome, 401, 406
Isolated glucocorticoid deficiency (IGD),
112, 125

K

Kallmann syndrome, 124
Kaposi sarcoma (KS), 159
 Kaposi herpes virus, 159
Knockout mice, 53–54, 89, 121, 314, 385
Kyoto Encyclopedia of Genes and Genomes
(KEGG), 4, 12–13

L

Leukotriene, 153, 360, 365
 leukotriene C4 synthase (LTC4 synthase),
366
Linear regression, 37
Linkage disequilibrium (LD), 32, 46, 65, 89,
143, 155, 163, 233, 314, 362, 383,
396, 428
Liquid chromatography/mass spectrometry
(LC-MS), 51
Literature network, 463
Liver, 229, 280, 290, 300, 312
 transplantation, 395, 402–403, 405–406
Loss of function, 81, 85, 110, 112–113, 127,
150, 165, 167, 233, 239
Luteinizing hormone, 109, 111–112, 123
Lymphocyte, 259, 298, 300, 455–456

M

Major histocompatibility complex (MHC),
425–426, 430
MapView, 9, 11
Mass spectrometry, 26, 51
McCune–Albright's syndrome (MAS),
84, 87
MDCK, 50
Melanin-concentrating hormone (MCH), 120
Melanocortin, 112, 121

Metabolizer, 27, 68, 284, 286, 315
 extensive metabolizer (EM), 24
 poor metabolizer (PM), 24, 68, 276
 ultrarapid metabolizers (UMs), 213, 287,
 289–290, 304
Metabolomics, 255, 272
Metabotropic glutamate (mGluR), 80
Methotrexate, 44–45, 406, 414–415
Methylenetetrahydrofolate reductase
 (MTHFR), 23, 25, 219, 406, 414
Methylome, 191, 193, 208
Methylomics, 189
Microarrays, 21, 25–27, 29, 223, 273, 326,
 448, 450, 457, 471, 475
MicroRNAs (miRNAs), 270
Microsatellites, 35, 37, 223, 425–426,
 428–430
Microtubule-associated protein tau (MAPT), 245
Modulators, 228, 263, 269
Monoamine oxidase A (MAOA), 187–188,
 192, 197, 207
Monocarboxylate transporters (MCTs), 46
Monogenic disease, 77, 109, 111, 122,
 130, 140
Motif, 6, 8–9, 91–92, 95, 113, 116, 127, 159,
 166–167, 237, 244, 267, 368
Motif Scan, 6, 8
Multi-drug-resistance (MDR1), 23, 403–404
Muscarinic receptors, 143, 226, 365
Mutations, 81, 110, 115–116, 119–129, 160,
 239–240, 242, 249, 255
Myasthenia gravis (MG), 152, 162

N

N-acetyltransferase 2 (NAT2), 23–24, 424
NanoChip, 28
National Center for Biotechnology
 Information (NCBI), 5–6
National Human Genome Research Institute
 (NHGRI), 14
Nephrogenic diabetes insipidus (NDI), 126
Neurotransmitter, 141, 143–144, 149, 223,
 233, 261
Night blindness, 88, 112, 114, 141

O

Obesity, 120–121, 162–163
Oguchi disease, 88–90, 95–96, 115
Oligoadenylate Synthetase Gene (OAS1), 389
Oncology, 64, 72, 437
Online Mendelian Inheritance in Man
 (OMIM), 4, 17, 218

Open reading frames (ORFs), 8, 222, 252
 Open Reading Frame Finder
 (ORF Finder), 6, 8
Opioids, 24, 284
Organic anion transporting proteins (OATPs),
 41, 46
 OATP1B1, 43, 45–48, 50–53, 56–57
 OATP1B3, 43, 45–50, 53, 56
Organic cation transporters (OCTs), 46
Ovarian dysgenesis, 112, 121–122
Ovarian hyperstimulation syndrome
 (OHSS), 122

P

P-Glycoprotein (P-gp), 24, 42, 64, 69,
 403, 416
Parathyroid hormone (PTH), 85, 87, 109, 113,
 116, 119
 parathyroid hormone receptor 1 (PTHr1),
 85, 119
Pathogenesis, 233, 307
Pathway, 13, 18, 365, 367
Patterns, 2, 4, 8, 22, 85, 90, 140, 187,
 189–191, 232, 255, 259, 300, 382, 466
Peripheral blood mononuclear cells (PBMC),
 87, 89, 152, 473
Peroxisome proliferator-activated receptor
 (PPAR), 382
Personalized medicine, 2, 23, 141–142, 150,
 153, 370
Pfam, 4, 6, 8
Pharmacodynamics, 22, 63, 65, 214, 216–217,
 274, 293, 397
Pharmacogenetics, 22, 41, 139–141, 143, 149,
 153–154, 274, 359–360, 362, 365, 395,
 414, 420–421, 423–425, 429, 431,
 437–438
Pharmacogenomics, 1–3, 5, 12, 21, 23, 63–64,
 77–78, 125, 140, 152, 213, 271–272,
 324, 379, 395, 413, 447, 469
Pharmacokinetics, 15, 22, 42, 46, 51, 53–55,
 57, 63, 65, 69–71, 216, 397
Pharmacotherapy, 69
Phenotypes, 83, 116, 119, 287
 hemodynamic, 287, 314
 phenotypic profiles, 256, 288, 300, 314
Phospholipase C (PLC), 83–84, 156
Phosphorylation, 92, 96–97, 114–115,
 243, 268
Pituitary somatotrophs, 84
Platelet-activating factor (PAF), 159
 platelet-activating factor receptor
 (PAFR), 159

Pleiotropy, 188–189
 Polyacrylamide, 193, 195, 201–203
 Polygenic inheritance, 188–189
 Polymerase chain reaction (PCR), 27, 49–50, 191, 195, 197, 203, 205, 207, 390–391, 440, 450
 Polymorphisms, 10, 51, 53, 63, 71, 89, 97, 142, 146–152, 157–159, 163, 281, 369, 397, 403, 415, 425–426, 428
 PolyPhen, 9–10
 Population structure, 31–38
 PredictProtein, 6, 8
 Presenilins, 237, 239
 Pro-opiomelanocortin (POMC), 121
 Promoter, 112, 164–166, 199, 385
 PROSITE, 4, 6, 8
 Prostaglandin D₂ (PDG2), 155
 prostaglandin D₂ receptor (DP), 155
 Protease-activated receptors (PARs), 161
 Proteasome, 253, 269
 Protein Data Bank (PDB), 4, 6
 Protein kinase A (PKA), 90, 155, 248
 Protein kinase C (PKC), 90
 Proteomics, 2, 255, 272
 Proton, 396–397, 406
 Pseudopseudohypoparathyroidism (PPHP), 85, 87
 PSORT, 13–14
 Psychiatric disorders, 144, 187–189, 191, 193–194, 204, 209
 Psychiatry, 188, 193
 Purinergic (P2RY12), 109, 113, 129

Q

Quantitative Multiplex MSP (QM-MSP), 195, 197, 204–207, 209–210
 Quantitative structure–activity relationship (QSAR), 15–16

R

R (PSMIX), 38
 Reactome, 13
 Reelin (RELN), 191–194, 197, 205, 209
 REMARK guidelines, 443
 Retinitis pigmentosa, 112, 114
 Rheumatoid arthritis, 413, 420, 423, 425, 429
 Rhodopsin, 79, 88, 95–96, 111–112, 114–115, 145, 147, 154
 RNA interference (RNAi), 224, 241, 261, 270, 384
 RxList, 14–15

S

Salmeterol, 360, 363
 Sample source, 439–441
 Scanning, 199
 Schizophrenia (SCZ), 146, 148, 163–165, 188, 191–194
 Secondary structure, 8
 protein, 6, 8
 RNA, 6, 8
 Secretase, 220, 224, 234–235, 238–243, 261–266, 310
 Self-organizing map (SOM), 453, 461
 Sequencing, 25, 48–49, 193, 195–197, 199–200, 202–204, 442
 Serotonin (5-HT), 148, 190, 209, 227, 261, 401–402, 406
 receptor, 147–148
 Signaling, 77–78, 80–91, 110–111, 114–115, 141, 147–151, 154–157, 160–161, 191–192, 237–243, 248, 263–265, 268, 307–308
 signal transduction, 29, 81, 96, 265
 Single nucleotide polymorphism (SNP), 4–5, 8–12, 16, 18, 26, 47, 87–88, 113, 144, 153–155, 167, 231–232, 361–362, 383–385, 387–391, 416–417, 419, 421, 429–430, 442
 SNP-Fasta, 9–10
 Single-base extension (SBE), 26
 Skeletal dysplasias, 119–120
 Software, 5–6, 14–16, 362, 384, 451–453, 459–460, 462–463, 466
 Statistical, 33, 57, 67, 361, 442–443
 adjusted test statistic, 36
 statistics, 9, 444
 Sulfasalazine, 414, 424–425
 SWISS-MODEL, 4, 6
 System for Integrative Genomic Microarray Analysis (SIGMA), 17–18
 Systems biology, 1–3, 5, 7, 18

T

Tacrolimus, 43–44, 403–406
 Tamoxifen, 43, 65, 277–278
 Testotoxicosis, 123
 Therapeutic response, 291–293, 300–301, 306, 315, 317, 319
 Thiopurine methyltransferase (TPMT), 67–69, 214, 274, 422–423
 Three-dimensional (3-D), 6, 223
 Thrombin, 161, 168
 Thromboxane receptors, 155

Thyroid, 84–85, 87, 113, 115, 116
 disease, 113, 115
Thyrotropin (TSH), 85, 111, 115, 123
 TSH receptor (TSHR), 113, 115–116
Toxicity, 53–55, 57, 64, 67–68, 71–72,
 236–237, 404–406, 417–420, 422–424,
 438, 447–448
Transaminase, 290–291, 306
Transcriptome, 22, 29
Transcriptomics, 272
Transmembrane (TM), 78–80, 118, 145, 147,
 154, 234, 415
Transmission disequilibrium test (TDT), 35
Transport, 41–42, 47, 50–57, 238–239, 245,
 415–416
 drug transporters, 57, 63–64, 69,
 71, 272
Treatment, 22–25, 50, 54–57, 65–71, 143,
 260, 273, 280, 291–293, 300–301,
 310–311, 315–317, 319–324, 363,
 365–368, 380, 400–402, 416,
 424–425, 427, 438–439
 multifactorial, 291–292, 315, 317,
 319, 323
Tumor, 439–441, 443–444
Tumor necrosis factor (TNF),
 219–220, 426
 antagonists, 425
Tyrosine hydroxylase (TH), 188,
 191, 196

U

Ubiquitin, 223, 236–237, 240, 249–255,
 269–270
Untranslated region (UTR), 89, 143–144,
 155, 421
Uridine diphosphate glucuronosyltransferase
 (UGT), 63–64, 66
 UGT1A1, 23–24, 66–67, 404, 438–439

V

Vaccination, 261, 267, 472, 477
Variant, 32–33, 53, 55–56, 65, 70, 78, 83–84,
 87–89, 110–114, 116, 122–127,
 141–143, 145, 147–154, 156–159,
 161–168, 249–250, 285–287, 318,
 385–386, 390–391, 397, 399, 404,
 421, 431, 438
Very large scale immobilized polymer
 synthesis (VLSIPSTM), 25
Vitamin K epoxide reductase complex 1
 (VKORC1), 24, 28

W

Warfarin, 24, 28, 276, 278

X

Xenopus laevis, 53