Chapter 8

Molecular Genetic Testing for Metabolic Disorders

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Introduction

Inborn errors of metabolism represent a highly diverse group of genetic disorders. Individually the disorders are rare. The most prevalent, phenylketonuria (PKU), affects approximately 1 in 10,000 individuals. However, because numerous metabolic disorders exist, collectively they are estimated to affect as many as 1 in 600 individuals. The clinical consequences of such disorders are broad and can be severe, with progressive neurological impairment, mental retardation (MR), organomegaly, and high morbidity. Their mode of inheritance is usually autosomal recessive but also can be X-linked. Metabolic disorders result from defects in the individual enzymes of pathways that govern many different aspects of metabolism in distinct compartments within the cell.

The onset of disease is most often after birth with the appearance of an apparently normal infant, but in some classes of metabolic disorders multiple congenital anomalies also exist. For most metabolic disorders, disease symptoms present in early infancy or childhood, but in less-severe cases, adolescent or adult onset may occur. Therefore, early recognition with prompt therapeutic intervention when possible is critical for reducing damage due to the metabolic defect. For those diseases that are prevalent and for which early detection and intervention would have a beneficial outcome, neonatal screening is performed in the United States and in several countries around the world. In the United States, each state and the District of Columbia determine the diseases for which newborns are screened and the methods used for screening. With respect to metabolic disorders, all states screen for PKU and congenital hypothyroidism, and all but one screen for galactosemia. A number of states screen for maple syrup urine disease, homocystinuria, biotinidase deficiency, tyrosinemia, and congenital adrenal hyperplasia, or some combination of these. Tandem mass spectrometry has been added to newborn screening programs in many states and can detect more than 20 metabolic disorders, including medium chain acyl CoA dehydrogenase (MCAD) deficiency. DNA testing is currently used as a follow-up to an initial screen for certain disorders, such as MCAD deficiency and PKU.

This chapter discusses the molecular mechanisms of disease and the available genetic testing for selected metabolic disorders. The choice of disorders reflects population prevalence and current availability of molecular testing, as the mutations in many of the metabolic diseases are genetically heterogeneous and diagnoses are still widely dependent on biochemical testing. However, DNA testing is often critical for confirmatory studies, genetic counseling, carrier and prenatal testing, genotype-phenotype correlation, and is widely used for carrier screening for metabolic disorders in certain populations that have a high frequency of specific mutations due to founder effects. As molecular technologies advance, molecular methods will increasingly be used to screen for more metabolic diseases.

AMINO ACIDURIAS (PHENYLKETONURIA)

Molecular Basis of the Disease

PKU is an autosomal recessive disorder caused by the inability of the body to convert phenylalanine to tyrosine. PKU is the most common metabolic disease in caucasians, with an incidence of 1 in 10,000 individuals. About 98% of PKU cases are caused by defects in the phenylalanine hydroxylase (*PAH*) gene. The other 2% are caused by defects in the biosynthesis or regeneration of the cofactor of PAH, 6(R)-L-erythro-tetrahydrobiopterin (BH4). Accumulation of phenylalanine can damage the development of the central nervous system and result in MR. PKU has a spectrum of phenotypes ranging from classic PKU, which is the most severe type with the least tolerance to dietary phenylalanine, to moderate PKU, mild PKU, and mild hyperphenylalaninemia (MHP). Patients with MHP have no clinical symptoms and do not require dietary treatment.

PKU is included in newborn screening programs in all 50 states and is a classic example of a genetic disease that

meets the criteria for newborn screening: relatively high occurrence, availability of fast and economical screening methods, and therapeutic options. With early diagnosis and intervention, including a low-phenylalanine diet, the major disease phenotypes of mental and growth retardation can be prevented.

The *PAH* gene is located on 12q23.2 and spans a genomic region of 90 kilobases (kb). The coding region is about 4 kb and is comprised of 13 exons. More than 400 mutations in *PAH* have been reported to date, most of which are private mutations (http://www.pahdb.mcgill.ca/). The most prevalent European mutations, accounting for approximately two thirds of all mutations, are R408W (31%), IVS12 +1G→A (11%), IVS10–11G→A (6%), I65T (5%), Y414C (5%), R261Q (4%), and F39L (2%).[1]

Clinical Utility of Testing

Molecular diagnosis of PKU serves several purposes, including prognosis, confirmation of clinical and newborn screening results, carrier testing, prenatal diagnosis, and information for genetic counseling. The genotype-phenotype correlations can be used to direct the degree of restriction of phenylalanine in the diet (Table 8-1). Moreover, for patients with mild mutations in the BH4 cofactor-binding region (V190A, R241C, A300S, A313T, E390G, A403V, and P407S), overloading with BH4 can increase PAH activity and may be used as an alternative to dietary restriction.[2] Prenatal diagnosis allows for the termination of an affected fetus or can ensure immediate therapeutic intervention after birth. Proper genetic counseling assists parents in making informed decisions.

Available Assays

Several methods are currently used for the molecular detection of mutations in *PAH* associated with PKU. These methods include:

- Testing for a panel of common mutations with a detection rate of approximately 50%, depending on the number of mutations included.
- Mutation scanning of all 13 exons and the intron-exon junction regions. DNA sequencing detects approximately 94% of mutations; however, this method can be expensive.[3] A recently developed system for mutation scanning, denaturing high-performance liquid chromatography (DHPLC), which also has a high detection rate (~96%) and is more cost-effective than DNA sequencing,[4] may be the method of choice for PKU molecular testing.
- Finally, when molecular analysis fails to detect one or both mutant alleles, linkage studies can be performed and are highly accurate if polymorphic markers within or very closely linked to the *PAH* gene are used.

Table 8-1. Genotype-Phenotype Correlations for the Most Common *PAH* Mutations

Mutation	Prevalence	PAH Activity in COS Cells	Phenotype
R408W	31%	<1%	Classic PKU
IVS12+nt1G→A	11%	<1%	Classic PKU
IVS10–11G→A	6%	Not available	Classic PKU
I65T	5%	26%	Classic PKU Variant PKU Non-PKU PAH
Y414C	5%	50%	Variant PKU Non-PKU PAH
R261Q	4%	<30%	Classic PKU Variant PKU

Source: "PAH Activity in COS Cells" and "Phenotype" from the *PAH*db Phenylalanine Hydroxylase Locus Knowledgebase [database online]. Available at: http://www.pahdb.mcgill.ca/.

Interpretation of Test Results

The heterogeneity of the clinical phenotypes results mainly from the great variety of mutations in the *PAH* gene. Null alleles eliminate almost all the enzyme's activity and cause classic PKU, while mutations with residual PAH activity result in milder forms. Like many single-gene disorders, genotype-phenotype correlations exist in most but not all cases. Environmental factors and/or modifier genes can also play a role in the clinical manifestations of the disease. The correlations of the most common mutations and their biochemical and clinical phenotypes are summarized in Table 8-1.

UREA CYCLE DISORDERS (ORNITHINE TRANSCARBAMYLASE DEFICIENCY)

Molecular Basis of the Disease

Defects in the urea cycle constitute a rare group of disorders resulting in the accumulation of urea precursors, mainly ammonium and glutamine. Ornithine transcarbamylase (OTC) deficiency, the most common inborn error of ureagenesis, is an X-linked disorder. Affected hemizygous males typically present in the neonatal period or later in childhood, with symptoms that include vomiting, lethargy, hypothermia, apnea due to hyperammonemia, and leading to coma or death. Recurrent episodes of metabolic crisis can result in MR. The only available treatment after an acute metabolic episode is liver transplantation, which should be performed as early as possible to prevent brain damage. In 15% to 20% of carrier females, symptoms of disease are evident. Symptomatic carrier females typically have later onset but disease also may be fatal, presumably due to an unfavorable pattern of X-inactivation in the liver.[5]

OTC is a homotrimeric mitochondrial matrix enzyme that catalyzes the synthesis of citrulline from ornithine and carbamyl phosphate, and is found almost exclusively in the liver and intestinal mucosa. Loss of OTC activity results in high plasma glutamine and ammonium, low plasma citrulline and an excess of orotic acid in the urine, a combined metabolic profile that is diagnostic for OTC deficiency. However, a direct assay of OTC activity performed on tissue isolated from a liver biopsy specimen is necessary to obtain unequivocal biochemical results.

The *OTC* gene is located on Xp21 and spans a region of 73 kb that contains ten exons and encodes a protein of 354 amino acids. The overall prevalence of the disease is estimated at 1 in 50,000 in the United States, with similar statistics reported in Japan. Mutations have been identified in all ten exons; however, disease-causing mutations are less frequent in exons 1 and 7, the least conserved exons, most likely reflecting their lesser relevance to the function of the enzyme.[6]

Clinical Utility of Testing

Diagnosis of OTC deficiency by molecular testing is preferable to the more invasive liver biopsy that is necessary for the enzymatic test. Molecular screening for *OTC* mutations identifies approximately 80% of mutations, while the remaining undetected mutations are expected to affect promoter function or splicing.[7] The *OTC* gene has an approximately 50:1 male-to-female mutation ratio, and 80% of male probands inherit the mutation from their mothers, while only 23% of manifesting females inherit the mutation. Therefore, any woman who has a son with OTC deficiency has a 20% a priori risk of having another affected son in her next pregnancy. Due to the inheritability and the severity of the disease with the limited treatment available, molecular screening of at-risk couples for the purpose of prenatal testing may be beneficial. Additionally, mutation identification may be of prognostic value in OTC deficiency (see "Interpretation of Test Results," below).

Available Assays

Only a few laboratories in the United States offer clinical molecular genetic testing for OTC deficiency. One method for mutation screening of the *OTC* gene is single-strand conformation polymorphism (SSCP or DHPLC) analysis of polymerase chain reaction (PCR)-amplified exons with direct sequencing of any SSCP-positive exons.[8] Approximately 230 mutations have been reported (http://www.cnmcresearch.org/OTC/). Most mutations (86%) in the *OTC* gene are point mutations, with G→A transitions accounting for 34% and C→T transitions accounting for 21% of the total. Approximately one third of all point mutations are at CpG dinucleotides, and 15% are at splice junctions. Although the CpG sites are recurrent mutation sites, none accounts for more than 4% of the total single-base substitutions.

Interpretation of Test Results

In general, the genotypic spectrum correlates with the severity of the phenotype, and mutations that result in complete loss of function or amino acid changes near the active site of the protein result in neonatal onset of disease. In contrast, amino acid changes that are not close to the active site and result in protein with residual enzymatic activity are associated with later onset and a milder disease course.[8]

LYSOSOMAL STORAGE DISORDERS

Lysosomal storage disorders are a group of diverse inherited metabolic diseases that result from the disruption of the lysosomal system and catabolism of macromolecules (for review, see Reference 9). Mutations in genes encoding hydrolyzing enzymes, activator proteins, lysosomal membrane proteins, or proteins involved in the posttranslational modification or transport of lysosomal proteins can cause such storage disorders. More than 40 lysosomal storage disorders are known, and they have a collective incidence of approximately 1 in 5,000 to 8,000 live births in the United States. Most of the genes responsible for lysosomal storage disorders have been cloned, permitting gene mutation testing once a diagnosis is established by biochemical analyses. This information is valuable for genotype-phenotype correlation, selection of therapy, and genetic counseling. In this section, two lysosomal storage disorders are discussed: Tay-Sachs disease, which serves as a model for population screening, and Gaucher disease, for which much effort has been concentrated on genotype-phenotype correlations.

TAY-SACHS DISEASE

Molecular Basis of the Disease

Tay-Sachs disease (TSD) is a neurodegenerative disorder resulting from deficiency of the lysosomal enzyme hexosaminidase A (HEX A), resulting in accumulation of the cell membrane glycolipid G_{M2} ganglioside within lysosomes (for review, see References 10 and 11). The clinical course of TSD is characterized by normal development for the first few months of life followed by progressive loss of motor skills, macrocephaly, seizures, blindness, and death usually before 4 years of age. Infantile TSD is always fatal, and there is no effective treatment. There are also later-onset forms with slower disease progression. TSD is an autosomal recessive disease and has a carrier frequency of

approximately 1 in 30 Ashkenazi Jewish individuals and 1 in 250 to 300 in most other populations. Genetic isolates such as the French Canadians of Quebec, Cajuns from Louisiana, and the Amish in Pennsylvania also have carrier frequencies similar to that seen in Ashkenazi Jews. The first carrier screening programs began in 1970 and used the measurement of HEX A activity in serum, leukocytes, or tears. When the *HEXA* gene encoding HEX A was cloned in 1987, disease-associated mutations were identified. Current testing for TSD utilizes both biochemical and molecular testing by various methods.

Clinical Utility of Testing

Carrier detection for TSD, which began in the 1970s and was later endorsed by the American College of Obstetricians and Gynecologists (ACOG) and the American College of Medical Genetics (ACMG), has been a paradigm for population screening. As a result, there has been a 90% reduction in the incidence of TSD in the North American Ashkenazi Jewish population, such that the incidence of TSD is now 3- to 4-fold higher in non-Jews by comparison.

Available Assays

Clinical laboratories use several strategies to incorporate mutation studies into their screening programs. Some laboratories initially screen by enzyme analysis and follow with mutation studies for individuals with a result in the carrier or inconclusive ranges, while other laboratories use DNA studies alone for selected populations. DNA studies are performed used a variety of methods, including PCR amplification followed by allele-specific oligonucleotide (ASO) hybridization or restriction enzyme digestion, allele-specific amplification, TaqMan probe technologies, or ligation chain reaction amplification.

In Ashkenazi Jewish individuals, there are two common mutations in *HEXA* associated with infantile TSD and one associated with an adult-onset form of the disease. A 4 base pair insertion (TATC) in exon 11 accounts for approximately 80% of mutant alleles in this population, and a splice defect in intron 12 (IVS12+1G→C) accounts for another 15%. A missense mutation, G269S, leads to an adult-onset form of TSD and accounts for approximately 2% of carriers.

Interpretation of Test Results

A pseudodeficiency allele, R247W, is present in approximately 2% of Ashkenazi Jewish individuals who are carriers by the enzymatic assay. The R247W variant decreases the activity of HEX A for the artificial substrate used in the laboratory but does not cause TSD since it does not affect HEX A activity for its natural substrate, G_{M2} ganglioside. About 36% of non-Jewish individuals who are carriers by enzyme analysis have a pseudodeficiency allele (32% R247W and 4% R249W). In addition, screening for the three common Ashkenazi Jewish mutations and an additional mutation (IVS9+1G→ A) will identify approximately 95% of Ashkenazi Jewish carriers, but only 40% to 50% of disease-causing alleles in non-Jews. Other populations who are at high risk, such as the French Canadians, Cajuns, and Pennsylvania Dutch, have their own common alleles. Therefore, the mutations included for population screening must target the specific mutations of the ethnic background of the individual being tested.

GAUCHER DISEASE

Molecular Basis of the Disease

Gaucher disease (GD) is another prevalent autosomal recessive lysosomal storage disorder that is found with higher incidence in the Ashkenazi Jewish population. The carrier frequency is 1 in 18 in this population and 1 in 100 in other populations[12] (for comprehensive review on GD, see Reference 13). A defect in the enzyme glucocerebrosidase leads to the accumulation of glucocerebrosides in lysosomal compartments in macrophage/monocyte-derived cells, particularly in the liver, bone marrow, spleen, and lung. Several forms of GD exist. Type 1 GD has a wide range of clinical presentations, with some patients being asymptomatic, but can include bone disease, hepatosplenomegaly, anemia, and thrombocytopenia, but without primary central nervous system involvement. Types 2 and 3 have primary central nervous system involvement that varies by age of onset and rate of disease progression. Type 2 GD patients usually have an earlier age of onset than type 3 patients, with acute disease progression and death by approximately 2 years of age. Type 3 patients have onset in early childhood to adolescence and survive into their first to fourth decade of life. A perinatal-lethal form of GD also can occur, as well as a cardiovascular form characterized by aortic and mitral valve calcification, ophthalmologic abnormalities, and hydrocephalus.

The glucocerebrosidase gene (*GBA*) and its transcribed pseudogene (*ΨGBA*) are located on chromosome 1q21. Almost 200 mutations causing GD have been identified. Many of the mutations are most likely due to gene conversion events with the pseudogene.

Clinical Utility of Testing

The demonstration of deficient glucocerebrosidase activity in leukocytes establishes a diagnosis of GD but is unreliable for carrier detection. Therefore, molecular genetic testing is useful for carrier identification, prenatal testing, and genetic counseling.

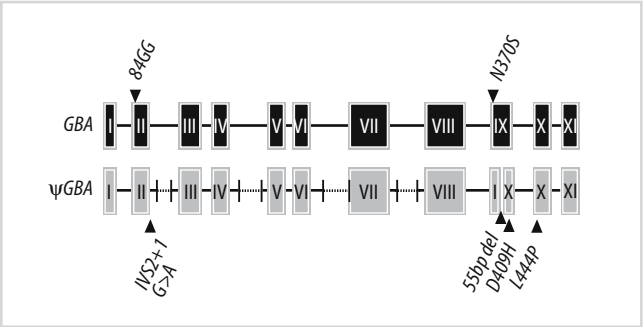

Figure 8-1. Exon/intron structure of the *GBA* gene and the pseudogene copy *ΨGBA*. Exons are indicated by boxes with Roman numeral designations, and introns are indicated by lines. Dotted lines are deletions within the pseudogene relative to the gene. Positions of mutations are indicated by arrow heads. Neither 84GG nor N370S is present in the pseudogene, whereas IVS2+1G→A, 55 bp del, D409H, and L444P are pseudogene specific.

Available Assays

Four mutations (N370S, IVS2+1 G→A, 84GG, L444P) are responsible for approximately 95% of disease-causing alleles in Ashkenazi Jewish individuals and 50% of disease-causing alleles in non-Jewish individuals (Figure 8-1).[12] Most laboratories performing GD mutation studies test for at least these four mutations using standard laboratory techniques. There are several factors to be aware of when designing molecular testing for GD. Primers must be selected that avoid amplification of the pseudogene located 16 kb downstream and approximately 96% identical to the functional gene (Figure 8-1). Recombinant alleles, which are thought to have resulted from unequal crossovers between exons 9 and 10 of the functional gene and pseudogene, contain two or more point mutations, including L444P. If L444P alone is tested, misdesignation of the genotype may occur. This may be important, as the recombinant allele is typically associated with a more severe genotype. Mistyping is also possible when a 55 base pair (bp) deletion in exon 9 is present in combination with the common N370S allele. Homozygosity of N370S would be observed even though the true genotype is N370S/55 bp deletion. Therefore, the 55 bp deletion should be analyzed in patients who are found to be homozygous for N370S.

Interpretation of Test Results

Genotype-phenotype correlations have been widely investigated in GD.[14] While overlaps occur, some generalizations can be made. The presence of an N370S allele is predictive of type 1 disease. Individuals with L444P in the presence of a null allele will usually have type 2 GD, while homozygosity for L444P typically results in type 3 GD. Homozygosity for the D409H allele has been associated with the rarer cardiovascular form of GD. The phenotypic variation observed in GD is due to factors other than genotype alone, presumably involving the interplay of other genes, including modifier genes and environmental factors.

DISORDERS OF CARBOHYDRATE METABOLISM

Galactosemia and the glycogen storage diseases are discussed in this section, which addresses disorders of carbohydrate metabolism.

GALACTOSEMIA

Molecular Basis of the Disease

Galactosemia is an autosomal recessive disorder caused by deficient or absent activity of one of three enzymes involved in the metabolic pathway to convert galactose to glucose: galactokinase (GALK), galactose-1-phosphate uridyl transferase (GALT), and UDP-galactose 4′-epimerase (GALE). The predominant form is classic galactosemia, which is due to a severe reduction or absence of the GALT enzyme, and has an incidence of 1 in 40,000 to 60,000 in European newborns (for review, see Reference 15).

The symptoms of classic galactosemia in neonates include poor feeding, vomiting, failure to thrive, lethargy, jaundice, occasionally diarrhea, and *E. coli* sepsis. The symptoms in an affected newborn can be obviated if a lactose-free diet is initiated within the first two weeks of life. Newborn screening for galactosemia is included in most states in the United States. Newborns with a positive screen are followed up with immediate dietary treatment and confirmatory biochemical analysis.

The *GALT* gene is located at 9q13, is about 4 kb in length, and consists of 11 exons. More than 150 mutations in the *GALT* gene have been reported, most of which are private mutations.[16] Q188R is the most frequent mutation associated with classic galactosemia in many populations, and accounts for 64% of disease alleles in Europeans, 60% to 70% in Americans, and 50% to 58% in Mexican Hispanics.[16,17] Ethnic-specific mutations include K285N, S135L, IVS2–2A→G, and a 5 kb deletion in Caucasian, African American, Hispanic, and Jewish patients, respectively. Due to differing ethnic backgrounds in different regions of the United States, disease allele prevalence may vary in different regions of the United States. For example, Q188R, S135L, K285N, L198P, Y209C, and F171S were reported to be the most prevalent mutations in a study based on individuals from the state of Georgia, while Q188R, K285N, IVS2–2A→G, S135L, and T138M are the most common mutant alleles observed in Texas newborns.[18,19]

Clinical Utility of Testing

Molecular testing is used for confirmation of diagnosis, carrier detection, prenatal diagnosis, prognosis, and genetic counseling. Because the detection rate of molecular testing is less than 100% and biochemical testing is highly accurate, mutation detection is carried out in parallel with biochemical analysis.

Prenatal diagnosis for galactosemia can provide the opportunity for immediate dietary restriction of the newborn. Although galactosemia is considered "treatable," symptoms such as mental and growth delays, speech dyspraxia, abnormal motor function, and premature ovarian failure in women may still occur even with early intervention and lifetime dietary restrictions. These long-term phenotypes are often associated with specific mutations; for example, Q188R can cause premature ovarian failure and speech dyspraxia. Genetic counseling is very important for parents of affected fetuses before a choice regarding pregnancy outcome is made.

Available Assays

Initial molecular testing for the diagnosis of galactosemia focuses on the most prevalent mutations or the N314D mutation associated with Duarte galactosemia (Duarte-2) by quick and cost-effective methods, such as multiplex PCR followed by restriction enzyme digestion.[18] If only one mutation or no mutations are found, screening of all 11 exons and exon-intron boundaries by PCR amplification plus SSCP analysis with confirmation of positives by DNA sequencing or direct DNA sequencing alone is performed.[18] A detection rate of 96% can be achieved by a combination of testing for prevalent mutations and direct DNA sequencing.[19] The remaining 4% of undetected mutations may be due to unknown sequence changes within intronic regions that affect splicing or in the 5′ or 3′ untranslated regions of the *GALT* gene that may affect transcriptional or translational efficiency or both.

Interpretation of Test Results

Genotype-phenotype associations have been established for some mutations.[16] For example, Q188R, K285N, and L196P alleles have undetectable GALT activity and are associated with severe phenotypes, whereas S135L and T138M are less severe and are usually associated with a good prognosis. In addition to the classic form of galactosemia, which has less than 5% of the normal GALT activity, the Duarte-2 variant associated with the N314D variant has 50% of the normal GALT activity. The N314D allele is in linkage disequilibrium with four polymorphisms, IVS4–27G→C, IVS5–24G→A, IVS5+62G→A, and c.-119_-116delGTCA. The symptoms of Duarte-2 patients are mild compared to the classic type, but Duarte-2 still results in long-term phenotypes such as mental and growth delays, speech dyspraxia, abnormal motor function, and premature ovarian failure in women.

GLYCOGEN STORAGE DISEASES

Molecular Basis of the Disease

Glycogen storage diseases (GSD) are a group of heterogeneous genetic disorders characterized by the accumulation of glycogen in tissues. Eight types of GSD that vary significantly in clinical phenotypes, age of onset, and affected organs have been identified, with an overall incidence of 1 in 20,000 to 25,000 live births.[20] They are caused by defects in one of eight genes in glycogen metabolism. Glycogen storage diseases type I to type VII are inherited in an autosomal recessive pattern, and GSD IX is X-linked recessive. A summary of the eight GSD types is presented in Table 8-2. GSD I, II, III, and IV, which are the most common and severe types, are discussed.

GSD I (von Gierke disease) is characterized by hepatomegaly, kidney enlargement, growth retardation, hypoglycemia, hyperuricemia, and hyperlipemia. GSD I has two major subgroups, GSD1a and GSD1b. The subgroup GSD1a is caused by deficiency of glucose-6-phosphatase

Table 8-2. Glycogen Storage Diseases

Disorder(s)	Defective Enzymes	Gene Location	Inheritance Pattern
GSD I (von Gierke disease)	GSD1a: Glucose-6-phosphatase	17q21	AR
	GSD1b: Glucose-6-phosphate translocase	17q23	AR
GSD II (Pompe disease)	Lysosomal acid α-1,4-glucosidase	17q25	AR
GSD III (Cori disease)	Amylo-1-6-glucosidase	1p21	AR
GSD IV (Andersen disease)	Branching enzyme (α-1,4 to α-1,6)	3p14	AR
GSD V (McArdle disease)	Phosphorylase (muscle)	11q13	AR
GSD VI (Hers disease)	Phosphorylase (liver)	14q21–q22	AR
GSD VII	Phosphofructokinase	12q13	AR
GSDIX (GSDVIII)	Phosphorylase kinase	Xp22	XR

(G6Pase), which converts glucose-6-phosphate to glucose and phosphate, the last step in glycogenolysis. The *G6PC* gene encoding G6Pase is located on 17q21. The subgroup GSD1b results from deficiency in glucose-6-phosphate translocase, encoded by *G6PTL* gene located on 11q23. Common mutations vary in different ethnic groups.[20] The prevalent mutations for GSD1a in different ethnic groups are: R83C and Q347X in caucasians; R83C in Ashkenazi Jews; 459insTA and R83C in Hispanics; V166G in Muslim Arabs; R83H and G727T in Chinese; and G727T in Japanese. For GSD1b, two common mutations, G339C and 1211delCT, are present in whites, while W118R is prevalent in Japanese.

GSD II, also known as Pompe disease, is a lysosomal storage disease caused by the inability to degrade glycogen due to defects in acid α-1,4-glucosidase. The phenotypes range from the most severe infantile disorder to juvenile- and late-onset adult myopathy. Patients with the infantile form usually die from cardiomyopathy before they reach 2 years of age. Acid α-1,4-glucosidase is encoded by a gene (*GAA*) located at 17q25, and different forms of the protein are obtained by different proteolytic processing. Common mutations have been identified in different ethnic groups.

Patients affected with GSD III, also known as Cori disease, have symptoms similar to but milder than those associated with GSD I. The gene encoding amylo-1-6-glucosidase has 35 exons with 4596 bp of coding region and a long 3′ UTR of 2371 bp. Molecular testing of GSD III is difficult and impractical due to the large size of the gene and the lack of predominant mutations.

GSD IV, also known as Andersen disease, is caused by glycogen branching enzyme deficiency that results in glycogen that is abnormal and insoluble. Intracellular accumulations occur in the liver, brain, heart, skeletal muscles, and skin fibroblasts. Neonates with GSD IV appear normal at birth but develop hepatomegaly and failure to thrive in the first year of life. Patients develop progressive cirrhosis and usually die of liver failure by 2 to 5 years of age. Mutations in the branching enzyme (α-1,4 to α-1,6) have been identified in a limited number of patients.

Clinical Utility of Testing

Due to the complex nature of molecular testing for GSD (large genes with numerous mutations), enzyme assays are usually used for the diagnosis of GSD. However, for prenatal diagnosis of GSD I, gene-based mutation testing or linkage analysis is the preferred method, since the enzymes are not present in amniocytes or chorionic villi, requiring a liver biopsy to obtain tissue containing the relevant enzyme. For prenatal diagnosis of GSD II, III and IV, DNA testing can be used to complement and confirm biochemical results. Therapy for GSD I, II, III, and IV includes dietary management, and for GSD II, enzyme replacement is available (for complete review on treatment for GSD, see References 20 and 21).

Available Assays

Molecular diagnosis for GSD I by direct DNA testing and GSD II by linkage studies is available clinically.

FATTY ACID OXIDATION DISORDERS (MCAD DEFICIENCY)

During periods of fasting or prolonged aerobic exercise when glycogen stores are depleted, fatty acids become a main energy source by β-oxidation in the liver, and skeletal and cardiac muscles. The pathway for fatty acid oxidation occurs in the mitochondria and is complex, involving as many as 20 steps. A number of disorders involving different enzymes in the pathway have been identified. Although the symptoms of the disorders have phenotypic overlap, there are several biochemical measurements that can aid in the diagnosis of these disorders, including plasma carnitine levels which are usually low, plasma acylcarnitines, and urine acylglycines (for review, see Reference 22). The most common of these disorders by far is medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, which is discussed in detail below.

Molecular Basis of the Disease

MCAD is an intramitochondrial enzyme that is encoded by a nuclear gene. The normal function of MCAD is the initial dehydrogenation of acyl-CoAs with chain lengths of 4 to 12 carbons. Defective function leads to the accumulation of metabolites of the medium-chain fatty acids, mainly the dicarboxylic acids, acylglycine in urine, and acylcarnitine in plasma. At present, many states within the United States employ tandem mass spectrometry in their newborn screening program, which allows detection of the abnormal plasma acylcarnitine profile characteristic of MCAD deficiency. These metabolites are at their highest concentration in the blood in the first few days of life, making the newborn period the ideal time for detection. Accordingly, the specificity of this testing is 100%, as no false negatives have been reported. MCAD enzymatic activity also can be assayed in several different tissue types.

Because fatty acid oxidation fuels hepatic ketogenesis, the symptoms of the disorder appear after periods of prolonged fasting or intercurrent infections and include hypoketotic hypoglycemia, lethargy, seizures, coma, and, without treatment, death. Complications of the disease can include hepatomegaly, acute liver disease, and brain damage. The disease typically presents before 2 years of age but after the newborn period. However, individuals have been described who present with symptoms within the first few days of life as well as those who present as adults.

MCAD deficiency is an autosomal recessive disorder that is prevalent in individuals of northwestern European ancestry, with the highest overall frequency of 1 in 4,900 in

Table 8-3. Genotypes of 57 MCAD-Deficient Newborns Detected Using MS/MS to Screen More Than 1.1 Million Newborns (Neo Gen Screening, Pittsburgh, PA, USA)

Mutation Position and Type	Number of Patients Identified
985 A→G/985 A→G	35
985 A→G/199 T→C (exon 3)	8
985 A→G/deletion 343–348	2
985 A→G/other*	5
985 A→G/unidentified	5
799 G→A/254 G→A	1
Unidentified/unidentified	1

Source: Reprinted with permission from Chace DH, Kalas TA, Naylor EW. The application of tandem mass spectrometry to neonatal screening for inherited disorders of intermediary metabolism. *Annual Review of Genomics and Human Genetics* 3:17–45, © 2002 by Annual Reviews, www.annualreviews.org.
*Other mutations:
244 insertion T (exon 4)
362 C→T (exon 5)
489 T→G (exon 7)
IVS 5+1 G→A
IVS 8+6 G→T

northern Germany. The incidence in the United States is somewhat lower and is estimated to be 1 in 15,700. The MCAD gene, *ACADM*, spans a 44 kb region on chromosome 1p31 and contains 12 exons encoding a protein of 421 amino acids. A single founder mutation in exon 11, 985A→G, which results in the substitution of the acidic amino acid, glutamate, for the basic amino acid, lysine (K304E), represents 90% of all alleles in the northern European population. However, recent studies of the US population, attributable to the expansion of newborn screening for MCAD deficiency, indicate that this mutation accounts for 79% of the total mutant alleles in the US population (Table 8-3).[23] The discrepancy between the two results is presumably due to the greater ethnic diversity of the US population.

Clinical Utility of Testing

Molecular genetic testing for MCAD mutations usually is offered as confirmatory testing after the initial diagnosis by biochemical testing. In addition, carrier testing for MCAD deficiency cannot be performed using biochemical metabolite profiles and must be done by molecular genetic testing or by direct assay of MCAD activity in cultured fibroblasts.

MCAD deficiency is a disease that can be treated if promptly diagnosed in the early postnatal period. Precautions, such as avoidance of fasting and saturated fats and ingestion of carbohydrates prior to bedtime, can eliminate the symptoms and related complications of the disease. Although prenatal diagnosis on chorionic villus sampling or cultured amniocytes using biochemical or molecular genetic testing, or both, is possible, with the inherent risks of the procedures, it may offer no advantage to postnatal testing of acylcarnitines and other metabolites characteristic of the disease. Therefore, genetic counseling and discussion of the issues related to this disease are warranted when prenatal testing is being considered.

Available Assays

Because of its high prevalence among individuals with MCAD deficiency, molecular testing for the K304E allele is performed initially by PCR amplification followed by restriction enzyme digestion or other methods that can discriminate between single nucleotide changes, such as ASO hybridization or ligation chain reaction amplification. Clinical testing for this mutation is widely available. When an affected individual is found to be heterozygous for the K304E mutation or in the rare instance when an affected individual is negative for the mutation, gene sequencing is performed on all 12 exons of the *ACADM* gene; however, relatively few laboratories offer screening of the entire *ACADM* gene. Additional mutations have been identified throughout the gene with no obvious mutation hotspot.

Interpretation of Test Results

The majority of mutations identified in *ACADM* are missense mutations located away from the active center of the enzyme, and are thought to affect the overall stability of the protein by affecting proper protein folding (Figure 8-2).[24] Most patients exhibit the classic MCAD phenotype; however, a small subset of patients has been identified that is compound heterozygous for the A304E mutation or for two other mutations, where at least one mutation is present that does not eliminate MCAD activity. These patients are much less likely to experience metabolic decompensation; however, even mildly symptomatic patients should avoid circumstances that could precipitate a metabolic crisis,

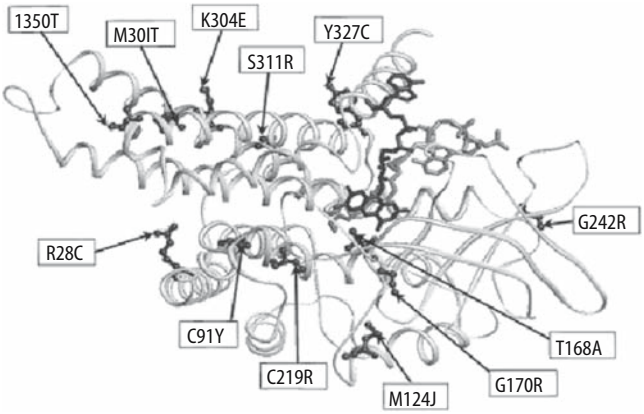

Figure 8-2. Schematic representation of a human MCAD monomer based on the crystal structure with cofactor flavin adenine dinucleotide (FAD) (black) and bound C8-CoA substrate (darker gray). The side chains for residues in which missense mutations have been published are shown in ball-and-stick representation. Only one of these mutations (T168A) is located in close proximity to the active site, forming a hydrogen bond to the flavin N(5) of FAD. (Reprinted from Gregersen N, Andresen BS, Corydon MJ, et al. Mutation analysis in mitochondrial fatty acid oxidation defects: exemplified by acyl-CoA dehydrogenase deficiencies, with special focus on genotype-phenotype relationship. *Human Mutation* 18(3):169–189, by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc., © 2001.)

since there is variability in age of onset even with classic MCAD deficiency.

PEROXISOMAL DISORDERS (X-LINKED ADRENOLEUKODYSTROPHY)

Genetic disorders of peroxisomal biogenesis and function have severe phenotypic consequences that often result in death in early childhood. A number of important metabolic processes, including β-oxidation of long- and very-long-chain fatty acids and the degradation of H_2O_2, take place in the unique microenvironment of the single-membrane-bound matrix of the peroxisome. Whereas the symptoms of most metabolic diseases manifest after birth, disorders of peroxisome biogenesis, such as Zellweger syndrome, are associated with multiple congenital anomalies (for review, see Reference 25). In this section, X-linked adrenoleukodystrophy (X-ALD) is highlighted, as it is the most common of the peroxisomal disorders and one of the few for which clinical molecular genetic testing is available.

Molecular Basis of the Disease

X-ALD is a severe, often fatal disease that manifests in a progressive demyelination of the central nervous system, dysfunction of the adrenal cortex, and testicular dysfunction in hemizygous males. The most common form has an early onset that typically appears at 4 to 8 years of age and results in a progressive irreversible dementia and often death. Less severe presentations of the disorder include adrenomyeloneuropathy (AMN), which has a later age of onset, often adrenal insufficiency, and neurological complications that are limited to the spinal cord and peripheral nerves.[26] Although the disease is inherited in an X-linked recessive manner, up to 20% of carrier females manifest late onset neurological symptoms similar to AMN. More than 93% of X-ALD patients inherit mutations from their mothers, while the remaining 7% carry de novo mutations. The primary biochemical defect is an impaired peroxisomal β-oxidation with the subsequent accumulation of very-long-chain fatty acids (VLCFAs), most notably C26, in the plasma and tissues. Therefore, X-ALD is not a disorder of peroxisomal biogenesis, but rather a specific defect of peroxisomal function. The accumulation of VLCFAs and the accompanying inflammatory response are thought to mediate the severity of the disease phenotype. In the great majority of hemizygous males (99%) and approximately 85% of carrier females, the plasma concentration of VLCFAs is elevated, a measurement that can be used as a diagnostic marker for the disease.[27]

Defects in the peroxisomal membrane protein, ALDP, a member of the ATP-binding cassette family of molecular transporters, cause the severe juvenile form of X-ALD and its milder associated forms. The X-ALD gene, *ABCD1*, is located on Xq28, spans 19 kb, contains 10 exons, and encodes a protein of 745 amino acids. The overall incidence of X-ALD and all variant forms is 1 in 15,000, making it the most common genetic determinant of peroxisomal disease. More than 400 different mutations have been found in the *ABCD1* gene, with the vast majority being point mutations, although deletions and duplications also have been identified (http://www.x-ald.nl/). In addition, mutations in all 10 exons have been reported. No genotype-phenotype correlations are apparent, and wide phenotypic variation has been reported within families.

Clinical Utility of Testing

Molecular testing is most useful for determining the carrier status of at-risk women and for prenatal diagnosis, since 15% of carrier females will not have elevated VLCFA levels and therefore will have a false-negative result by biochemical methods.

Available Assays

Molecular genetic testing of the *ABCD1* gene is available clinically from a few laboratories. Because many of the mutations identified are private mutations specific to a particular family, PCR amplification and SSCP or direct sequencing of all 10 exons has been used successfully to identify mutations in the majority of cases,[28] whereas Southern blotting can be used to assess deletion and duplication status as long as the rearrangement is small enough to be detected with gene-specific probes. Complications can arise during PCR amplification due to the presence of paralogous gene segments of *ABCD1* spanning exons 7 to 10 on chromosomes 2p11, 10p11, 16p11, and 22q11, but can be overcome by choice of primers that avoids amplification from the other chromosomes.[28] Although mutations have been identified throughout the entire *ABCD1* gene, a 2 bp AG deletion in exon 5 was found in 10.3% of families with X-ALD and is therefore the most common mutation identified in the *ABCD1* gene.[29] Interestingly, this mutation, which is associated with all X-ALD phenotypes, does not represent a founder allele and is therefore a mutation hotspot within the *ABCD1* gene.

Interpretation of Test Results

The majority of mutations in *ABCD1* are missense mutations (58.4%), with frameshifts and nonsense mutations accounting for 23.9% and 9.1% of mutation alleles, respectively, and amino acid insertions and deletions and whole exon deletions accounting for 4.6% and 4%, respectively. A recent study of the effects of missense mutations on ALDP stability demonstrated that approximately 70% resulted in absent or reduced ALDP, indicating that most mutations in *ABCD1* result in complete loss of protein function (Figure 8-3).[29] These findings are consistent with the observed lack of genotype-phenotype correlation and lend support to the existence of additional genetic and environmental factors that modify the X-ALD phenotype.

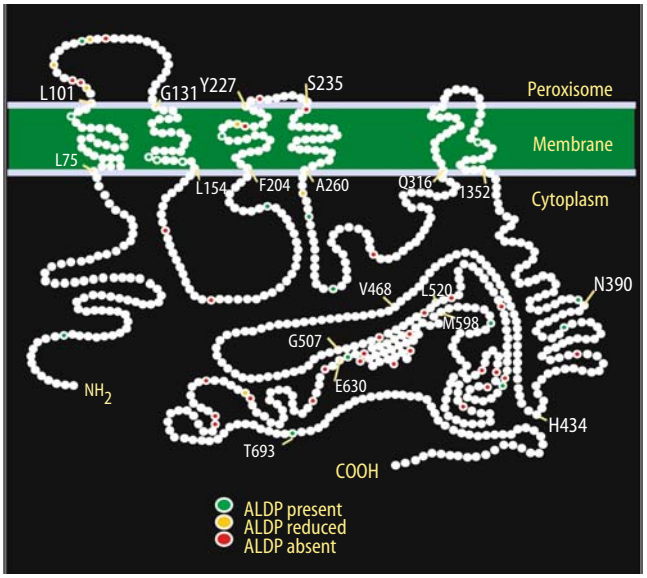

Figure 8-3. A hypothetical model of ALDP is shown. Individual amino acids are represented by circles. Missense mutations may affect the stability of ALDP. The effect of missense mutations on the stability of ALDP has been investigated for 52 independent missense mutations. Of these 52 amino acid substitutions, 31% do not affect ALDP stability and the cells have normal ALDP levels (green circles). Eleven percent of missense mutations result in reduced amounts of ALDP (orange circles). However, at 2 of these 6 positions, missense mutations have been reported that destabilize ALDP and are marked in red. Fifty eight percent of missense mutations result in no detectable ALDP (red circles). Overall, 69% of all missense mutations result in reduced or absent ALDP activity.[29] All other mutations, including in-frame amino acid deletions and truncations near the carboxy terminus, result in the absence of detectable levels of ALDP. (Reproduced from the Web site http://www.x-ald.nl/, with permission © J. Berger and S. Kemp.)

LEUKODYSTROPHIES (CANAVAN DISEASE)

The leukodystrophies are a group of degenerative metabolic diseases that involve the white matter of the brain, resulting predominately from disruption in the synthesis, transport, or catabolism of myelin. Examples include Krabbe disease and metachromatic leukodystrophy (MLD), which affect lysosomal function and degradation of myelin, and Pelizaeus-Merzbacher disease, which is due to the abnormal synthesis of proteolipid protein. Another example, discussed in detail below, is Canavan disease, characterized by loss of axonal myelin sheaths and spongiform degeneration of the brain. Neurological deterioration in most leukodystrophies occurs after a period of normal development, and therapy is usually limited to the alleviation of symptoms.

Molecular Basis of the Disease

Canavan disease (CD) is an autosomal recessive disorder found mainly in Ashkenazi Jewish families and is caused by deficiency in the activity of the enzyme aspartoacylase (for review, see Reference 30). The pathophysiologic relationship between the loss of this enzymatic activity and the development of CD remains to be elucidated. Diagnosis usually is established by the demonstration of increased levels of the substrate N-acetylaspartic acid in urine because enzymatic studies have been shown to be quite variable. Clinical symptoms associated with CD include macrocephaly, hypotonia, severe developmental delay, optic atrophy, poor head control, and death in childhood.

The gene encoding aspartoacylase (*ASPA*), located on the short arm of chromosome 17, is relatively small, with 6 exons spanning 30 kb of genomic sequence. Two point mutations, E285A and Y231X, are responsible for more than 97% of mutant alleles in Ashkenazi Jews. Mutations in non-Jewish individuals are more heterogeneous; however, a panethnic mutation, A305E, accounts for approximately 40% to 48% of non-Jewish European alleles.[31]

Clinical Utility of Testing

The genes responsible for many of the leukodystrophies have been cloned and characterized. However, in most instances the mutations in these genes are diverse. Therefore, biochemical diagnoses are still widely used, although molecular testing may be performed for carrier and subsequent prenatal testing. CD, however, occurs at increased frequency in the Ashkenazi Jewish population and screening for a limited number of mutations is feasible.

CD population screening of Ashkenazi Jewish individuals has demonstrated a carrier frequency of 1 in 40 to 59 (References 32 and 33 and our unpublished data). The carrier frequency for non-Jewish individuals has not been adequately determined, but it is far lower than that seen in the Ashkenazi Jewish population. As the carrier frequency is so high in the Ashkenazi population and the sensitivity of the assay is well above 90%, ACOG and the ACMG recommended in 1998 that carrier screening for CD be performed preconceptually on couples with Ashkenazi Jewish ancestry.

Available Assays

A number of laboratories test for the two common Ashkenazi Jewish mutations, while some also test for A305E. A few laboratories also test for the less-frequent non-Jewish mutation, 433–2A→G. Testing methodologies commonly used for the detection of these mutations include PCR followed by ASO hybridization or restriction enzyme digestion, or allele-specific amplification. Miami Children's Hospital Research Institute holds a patent on the CD gene and testing and requires laboratories to obtain a license, with a royalty fee for each test performed.

Interpretation of Test Results

If carrier screening indicates that both partners are carriers, prenatal testing should be offered. The preferred method of testing is DNA analysis of known mutations.

For couples in which one partner is shown to be a carrier and the other partner is negative by targeted molecular testing, particularly if they are not Jewish, biochemical testing by measurement of the substrate in amniotic fluid is possible when the fetus has been shown to carry the one identified parental mutation.

Laboratory Issues

As discussed in this chapter, molecular genetic testing for metabolic disorders is used primarily as a follow-up to biochemical analyses for confirmation of findings, prognosis, carrier screening, or prenatal testing. Readers interested in learning which laboratories perform individual tests should refer to the GeneClinics Web site (http://www.geneclinics.org), which includes a listing of laboratories certified by the Clinical Laboratory Improvement Amendments (CLIA). As testing for many of these disorders is performed in only a few laboratories, commercial test kits and proficiency testing is limited. Proficiency testing for TSD is available through the California Tay-Sachs Disease Prevention Program. For other disorders, interlaboratory exchange of samples is common practice. Several companies have developed assays for a panel of Ashkenazi Jewish disorders that include TSD, CD, and most likely GD among others. As mutation scanning methods become more routine, molecular testing for metabolic disorders may become more commonplace.

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