Review

An Overview of Molecular Diagnosis of Steroid 21-Hydroxylase Deficiency

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Steroid 21-hydroxylase deficiency is the most frequent cause of congenital adrenal hyperplasia (CAH), a group of inborn errors of adrenal steroid biosynthesis.¹ Approximately 1 in 14,000 newborns is affected with the classic form of the disease. A milder, late-onset or non-classic form that is clinically evident in approximately 1 in 1000 females also exists. Both forms are the result of mutations at the same locus and are inherited as autosomal recessive traits. Very rarely, 21-hydroxylase deficiency can result from uniparental disomy.² The primary laboratory means of diagnosis of this disease is by measurement of serum 17-hydroxyprogesterone, which is elevated in subjects with deficiency of this enzyme. This testing is also used in newborn screening programs that screen for 21-hydroxylase deficiency. Although biochemical testing remains the mainstay of diagnosis, molecular methods have acquired an essential role for identification of mutations and for assessment of at-risk pregnancies. This article provides an overview of published analytical methods for molecular diagnosis of steroid 21-hydroxylase deficiency.

Biochemical Abnormalities in CAH

Approximately 90% of CAH result from deficiency of adrenal steroid 21-hydroxylase. Owing to a deficiency of this enzyme activity, there is a reduction in the ability of the adrenal cortices to produce physiological quantities of adrenal glucocorticoids, primarily cortisol. Synthesis of mineralocorticoids is also reduced, but because of the relatively smaller physiological requirement for mineralocorticoids, primarily aldosterone, impairment of enzyme function that does not abolish all activity may not be manifest as overt salt-wasting. Salt-wasting results from mineralocorticoid insufficiency at the distal renal tubule where sodium is reabsorbed from the glomerular filtrate and potassium and hydrogen ions are excreted. This syndrome results in loss of body sodium and water, metabolic acidosis, and the hyponatremia and hyperkalemia characteristic of adrenal insufficiency. Clinically, the dis-

order is characterized by life-threatening dehydration, vomiting, and diarrhea in the first few weeks of life. Approximately one-third of patients with classic CAH are able to produce enough mineralocorticoid to prevent salt-wasting. The other major biochemical feature of CAH is increased production of adrenal androgens. These compounds, which do not require 21-hydroxylase activity for their synthesis, provide the outlet for steroid synthesis in the adrenal gland. Owing to the lack of negative feedback that cortisol normally provides to the pituitary and hypothalamus, ACTH levels are elevated, which, in turn, stimulates the adrenal cortices. This stimulation causes hyperplasia of the glands and, combined with the metabolic block, results in high levels of production of adrenal androgens. This effect occurs *in utero* and results in variable degrees of virilization of the external genitalia of affected female fetuses and newborns. Affected males generally appear normal at birth.

The mainstay of therapy is replacement of the missing steroids, electrolytes, and fluids. Surgical correction of the external genital abnormalities is commonly required in affected females.

Molecular Genetics of Steroid 21-Hydroxylase

The functional *CYP21* gene and the non-functional pseudogene, *CYP21P*, are located on the short arm of chromosome 6 in the class III region of the major histocompatibility complex (MHC).^{3,4} The functional gene is a member of the cytochrome P450 family that specifically hydroxylates C21 on steroid precursors in the adrenal cortex. Both genes are normally in tandem arrangement with the genes encoding complement proteins 4A and 4B (Figure 1). It is generally believed that this pattern results from an ancient gene duplication event. Similar arrangements of 21-hydroxylase and *C4* genes are found in several other mammalian species, suggesting that the duplication event preceded the radiation of mammals in

Accepted for publication March 2, 2001.

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Figure 1. Arrangement of the 21-hydroxylase and *C4* genes in the class III region of the MHC. *CYP21* is the functional steroid 21-hydroxylase, *CYP21P* is a pseudogene. Both *C4A* and *C4B* can encode functional complement 4 proteins.

evolution and implying that it occurred at least 60 million years ago.

The *CYP21P* pseudogene usually contains a number of deleterious mutations that render it non-functional. These include a premature splice mutation in intron 2, a 8-bp deletion in exon 3, a series of point mutations that cause several missense mutations in exon 6, and a nonsense mutation in exon 8. Mutations that are commonly found in *CYP21* are shown in Table 1. The majority of mutations that appear in *CYP21* are either gene conversions of deleterious sequences normally found in *CYP21P*, or they are deletions. The severity of the phenotype generally depends on the mutations present on both alleles, but the phenotype is not entirely consistent among unrelated patients with the same mutations, or even among siblings of the same genotype. In general, gene deletions, the 8-bp deletion in exon 3, the exon 6 cluster of mutations, and Arg356Trp are associated with salt-wasting. The intron 2 mutation can be found in either salt-wasting or simple virilizing disease. Ile172Asn is associated with simple virilization, whereas Pro30Leu and Val281Leu are commonly seen in patients with non-classic disease. Based on consideration of the expected enzyme activity of the less severely impaired allele, it is possible to predict the phenotype with reasonable, but not complete, certainty.⁵

Linkage

The initial report of linkage of human leukocyte antigen (HLA) serotypes with 21-hydroxylase deficiency indicated the approximate position of the locus.⁶ Linkage-based diagnostic methods have been reported using various markers in the MHC. HLA serotyping⁷ provided a mechanism to track mutant alleles within families but

Table 2. Expected RFLP Sizes of *CYP21* and *CYP21P* after Restriction Digestion

Enzyme	<i>CYP21</i>	<i>CYP21P</i>
<i>TaqI</i>	3.7 kb	3.2 kb
<i>BglII</i>	10 kb	12 kb

could not be easily applied to prenatal samples obtained by amniocentesis. However, serotyping revealed the existence of important linkage disequilibrium between certain HLA haplotypes and 21-hydroxylase deficiency. One of these that involves the HLA haplotype A3, B47, DR7, is associated with a 30-kb deletion that has removed most of the *CYP21* functional gene and the adjacent *C4B* gene, and left a non-functional hybrid gene composed of 5' sequence from *CYP21P* and 3' sequence from *CYP21*.⁸

Molecular approaches to performing HLA genotyping signaled the first DNA methods suitable for linkage-based prenatal diagnosis. These methods involved the use of various class I DNA probes, a HLA-B specific probe, and HLA class II probes.⁹⁻¹² These probes were used to test restriction fragment length polymorphisms (RFLPs) on Southern blots and took at least several days to perform. As with any linkage-based diagnostic strategy, errors can arise from recombination events between the marker locus and the disease locus, non-paternity, new mutation or back mutation, or mistaken clinical diagnosis in the proband. In addition, linkage-based approaches are only applicable to families in which an informative polymorphism can be identified. More recently, diagnostic approaches based on linkage have used polymorphic microsatellite markers in the region that can be typed by polymerase chain reaction (PCR).¹³⁻¹⁵ The principal advantage of testing these is the ability to type the marker more rapidly than is possible by using Southern blot analysis for RFLPs.

Our laboratory has identified several intragenic single nucleotide polymorphisms (SNPs) in intron 2 and in intron 6 of *CYP21* and *CYP21P*.^{16,17} These can provide information on the inheritance of alleles in families and are also useful in identifying deletions that appear as apparent non-transmission of an allele by a parent.

Southern Blots and Deletions

The most widely used method to detect the relatively common deletion of *CYP21* in affected families is Southern blot analysis. The use of restriction enzymes that generate separate fragments that contain *CYP21* and *CYP21P* allows for estimation of the relative dosage of *CYP21*. Standard restriction enzymes used to digest genomic DNA include *TaqI* and *BglII*. The expected sizes of restriction fragments generated from these digestions are shown in Table 2. It is also useful to probe Southern blots for the neighboring *C4* genes because deletions of *CYP21* frequently include *C4B*. *TaqI*-restricted DNA can be simultaneously probed for both the *C4* genes and the 21-hydroxylase genes because of the non-overlapping RFLPs generated. DNA probes for Southern blot analysis

Table 1. Frequent Mutations in *CYP21* in Classic CAH

Mutation	Location	Approximate frequency (%)
Deletion	<i>CYP21</i>	25-30
Pro30Leu	Exon 1	5-10
A(C)656G	Intron 2	20-25
Δ8bp	Exon 3	5-10
Ile172Asn	Exon 4	5-10
Exon 6 cluster*	Exon 6	5-10
Val281Leu	Exon 7	5-10
1757+T	Exon 7	<5
Gln318Stop	Exon 8	5-10
Arg356Trp	Exon 8	10

*Exon 6 cluster includes Ile236Asn, Val237Glu, and Met239Lys. Data are based on frequencies reported in Reference 36.

for the 21-hydroxylase and *C4* genes are available from the American Type Culture Collection (Manassas, VA).

In *TaqI*-restricted genomic DNA, *CYP21* and *CYP21P* are located on 3.7-kb and 3.2-kb restriction fragments, respectively. Subjects with heterozygous deletions of *CYP21* can be identified by decreased intensity of the 3.7-kb fragment relative to that of the 3.2-kb fragment. Subjects with homozygous *CYP21* deletion show absence of the 3.7-kb fragment.

Difficulties with interpretation of Southern blots can arise from some of the duplication and deletion patterns that can be found at the 21-hydroxylase loci. Deletions of *CYP21P* are fairly common, being present in 9 to 14% of Caucasian chromosomes.^{17,18} Because the dosage of *CYP21* is determined by comparing its signal strength to that of *CYP21P* on Southern blots, it follows that a subject with deletions of both *CYP21* and *CYP21P* could be mistakenly thought to have a normal gene dosage.¹³ Subjects with a duplication of either 21-hydroxylase gene might also give unexpected results on gene dosage studies by Southern blot analysis. These issues emphasize the need for careful family studies when evaluating a subject for gene deletions.¹ Pulsed-field gel electrophoresis may provide additional information on gene dosage in unusual cases.¹⁹

PCR

Considerations in PCR Amplification of *CYP21*

As with many genetic diseases, PCR has been widely adopted for amplification of the locus before specific mutation identification. Owing to the presence of the highly homologous pseudogene, amplification of *CYP21* is not straightforward. Primers must be selected that recognize sequences in *CYP21* that are not present in *CYP21P*. These primer binding sites must be sufficiently specific for *CYP21* such that *CYP21P* cannot be amplified in the PCR through promiscuous priming. These requirements severely limit the number of *CYP21*-specific primer targets. Most authors have exploited two regions in which *CYP21* differs from *CYP21P*. These are the 8-bp sequence in exon 3 that is present in *CYP21* but deleted in *CYP21P*, and the exon 6 cluster of mutations that is composed of four nucleotide differences between the functional gene and pseudogene. Using this approach, *CYP21* can be amplified in two overlapping fragments as shown in Figure 2. After amplification, a variety of methods to detect the mutations present can be used.

Several caveats involving PCR amplification of *CYP21* need to be considered. First, several studies have reported the inability to amplify both *CYP21* alleles, particularly around intron 2, the site of the important premature splice mutation, A655G or C655G. This can result in subjects who are heterozygous for this mutation appearing to be homozygous.²⁰ A report indicates that this artifact may be overcome through the use of a blend of DNA polymerases that includes both *Taq* polymerase and *Pwo* polymerase.²¹ Careful examination of the mutational status of family members and of the segregation of

Figure 2. Typical PCR method for amplification of *CYP21* in two overlapping fragments. The numbered boxes represent exons in *CYP21*. The lines below represent the two PCR fragments, which are approximately 1.3 kb and 2.2 kb in length, respectively. Approximate primer locations and 5'–3' orientations of the primers are shown with arrows at the end of the fragments. To provide specificity for amplification of *CYP21*, the primer that hybridizes to sequences in exon 6 (right end of the top PCR fragment) recognizes a cluster of nucleotides that differ from the corresponding nucleotides in *CYP21P*. The primer that hybridizes to exon 3 (left end of the bottom PCR fragment) recognizes the 8 bp present in that exon, but which are absent from *CYP21P*. The other primers can hybridize to either gene.

linked microsatellites may be useful in identifying this artifact.

Second, it is clear that *CYP21* sequences can be back-converted to *CYP21P*.²² This brings sequences from *CYP21* into the pseudogene. If *CYP21* primer binding sequences were introduced into *CYP21P*, such conversion could conceivably lead to unintended PCR priming of *CYP21P* and mistaken identification of mutations that could be assumed to represent gene conversion events in *CYP21*. Additional useful information may be obtained by analysis of overlapping PCR products and by probing Southern blots with gene-specific oligonucleotide probes for the primer-binding regions.²³

Detection of Mutations after PCR

Following gene-specific amplification of *CYP21*, some method for detection of mutations is required. Various schemes have been used, all of which have been used for mutation detection in other genetic disorders. These include allele-specific oligonucleotide hybridization, restriction fragment length polymorphism analysis, ligase chain reaction, single-strand conformational polymorphism (SSCP), cleavage fragment length polymorphism analysis, and heteroduplex analysis. Some of these techniques can indicate only the presence of a sequence variant and must be followed by DNA sequencing for identification of a mutation.

Allele-Specific Oligonucleotide Probing

Allele-specific oligonucleotides have been used to probe PCR-amplified DNA in a dot blot format.²⁴ In this approach, overlapping fragments of *CYP21* are amplified, immobilized on a membrane and probed with labeled probes for 9 common mutations. This approach was used to identify mutations in 24 at-risk pregnancies using DNA obtained by chorionic villous biopsy.²⁵ Mutations were identified in 95% of these cases, and the molecular diagnosis was clinically confirmed in 96%. Southern blot analysis was performed to detect gene deletions.

Allele-Specific PCR

The use of allele-specific PCR to detect *CYP21* mutations has been reported.²⁶ In this approach, primers that are

specific for eight common mutations and the corresponding primers for the wild-type sequences are used to perform PCR. Gene specificity is conferred by the use of primers for the exon 3 or exon 6 cluster of nucleotides that are characteristic of *CYP21*. Of 160 alleles typed using this method, mutations could be identified in 148. Deletions were detected by use of Southern blot analysis. Because absence of amplification is a possible result, it is good laboratory practice to include primers for an unrelated target to confirm the presence of both amplifiable DNA and the reagents necessary for amplification.

PCR with Restriction Enzyme Digestion

In this approach, *CYP21* is amplified with gene-specific PCR primers. Nested PCR reactions are performed using mutagenic primers to introduce restriction sites as needed.^{27,28} Restriction of the resulting PCR products is used to detect the presence of mutations. This approach has advantages and disadvantages. The use of nested PCR reactions requires additional steps and, more importantly, increases the possibility of PCR contamination because of the additional manipulations required. An advantage of this technique is that if additional restriction sites are present in the amplified product, they can serve as important internal controls of restriction enzyme activity.

Ligase Chain Reaction (LCR)

A few papers have reported the use of LCR for detection of *CYP21* mutations. The strategy used involves initial PCR using the exon 3, 8-bp, and exon 6 cluster positions to provide targets for gene-specific priming.^{15,22} A total of 4 PCR products are generated from *CYP21* in overlapping segments. These PCR products are subjected to multiplex LCR using oligonucleotides specific for the mutations to be tested. By adding poly(A) tails to the LCR oligonucleotides, LCR products of different length for each mutation can be generated. Size separation of these allows for identification of the mutation present. The advantages of this approach are that much of the analysis can be multiplexed and, by using fluorescent labels on the oligonucleotides, the LCR products can be rapidly analyzed using sequencing-type apparatus.

Screening for Mutations

Cleavage Fragment Length Polymorphism

Cleavage fragment length polymorphism analysis was used to detect the presence of several frequent polymorphisms in intron 2 of *CYP21*.¹⁶ Subsequently, the utility of this technique for screening for common salt-wasting mutations in *CYP21* was reported.²⁹ These included the intron 2 splice mutation, exon 3 8-bp deletion, exon 6 cluster, and R356W. A conclusion of the referenced study was that although the technique was able to detect the mutations, a clear distinction between homozygosity and

heterozygosity was not always possible, which limits the usefulness of the method for clinical diagnostic work.

SSCP

SSCP analysis has been used to detect mutations in *CYP21* after gene-specific PCR.^{30,31} Unlike most methods, SSCP does not identify the specific mutation, and subsequent characterization is required. If samples containing known mutations are run in parallel with an unknown sample, it may be possible to infer the presence of the known mutation in the unknown sample if the SSCP conformers have identical mobility. Given the technical complexities of running SSCP gels that require rigorously controlled temperatures and possibly multiple gels involving the use of glycerol, the advantages of SSCP are not obvious, and the technique is not widely used in practice.

Heteroduplex Analysis

Heteroduplex analysis has not been extensively applied to diagnosis of 21-hydroxylase deficiency, although it is a very widely used technique for mutation screening in other disorders. Heteroduplex analysis has been used to detect the T insertion mutation³² and the value of heteroduplex analysis in distinguishing 2 mutations in *cis* versus in *trans* has been reported.³³ As with SSCP analysis, heteroduplex analysis does not provide characterization of the mutation present; for this, sequencing or some other mutation identification scheme is necessary.

Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis of restricted genomic DNA followed by Southern blot analysis using a 21-hydroxylase probe demonstrated a striking degree of polymorphism of *CYP21* among normal individuals.³⁴ These polymorphisms were shown to be inherited as Mendelian traits and could be used to track mutant *CYP21* alleles in families affected by CAH. Presumably these polymorphisms observed on denaturing gradient gel electrophoresis are due to the known polymorphisms in the exons and introns of *CYP21* and *CYP21P*; however, a complete characterization of multiple alleles from normal individuals may reveal additional polymorphic sites.

Prenatal Screening

Steroid 21-hydroxylase deficiency can be treated *in utero*, and several papers have reported a benefit of prenatal hormonal therapy on the severity of virilization in affected newborn females.¹ The usual situation in which prenatal diagnosis is considered is that of a family with a previously affected child. Prenatal diagnosis of 21-hydroxylase deficiency has been available for several decades based on analysis of 17-hydroxyprogesterone in amniotic fluid. This approach is of limited value because virilization of affected females begins much earlier in

pregnancy, probably around the middle of the first trimester. Prenatal hormonal therapy, which consists of administration of dexamethasone to the mother, needs to be initiated as soon as pregnancy is detected and this should not await the results of genetic testing.²⁶ Genetic testing based on DNA analysis can be performed on chorionic villi or on amniocytes. It is very helpful to have DNA available from a previously affected child, any other children, and both parents for both mutation identification and for testing chromosomal inheritance using microsatellites. The expected fraction of fetuses that can be expected to benefit from prenatal therapy is 1/8 (ie, affected females). This treatment is not without possible risks to the mother or the 7 of every 8 fetuses who will not benefit from *in utero* exposure to dexamethasone.¹ Therapy can be stopped if genetic testing indicates the fetus is a male, or is an unaffected female.³⁵

Newborn Screening

In many countries and States, congenital adrenal hyperplasia is on the schedule of diseases that are tested in newborn screening programs. Interest in laboratory identification of the disease is therefore substantially greater than might be expected for a relatively uncommon disease. DNA based diagnosis has not replaced routine biochemical screening using 17-hydroxyprogesterone as the test analyte, and is unlikely to do so in the near future. Reasons for this include the relative cost of DNA testing compared with immunoassay, the complexity of DNA testing for multiple mutations, particularly the common gene deletions, and locus heterogeneity (approximately 10% of cases of CAH are due to mutations in other genes, most often steroid 11 β -hydroxylase. Moreover, since ~2% of the population are carriers, it is unclear what counseling or further testing should be offered to parents of carrier newborns, who should provide this advice, and what such interventions might involve in terms of personnel and financial costs. Of course, these considerations are not unique to CAH but are applicable to a number of other inborn errors of metabolism.

In most cases, DNA analysis is performed for prenatal assessment of at-risk pregnancies or for mutation identification in affected families. In the laboratory evaluation of families with CAH, several different techniques are necessary. Southern analysis remains the most reliable method to detect gene deletions. PCR followed by one of the specific mutation detection schemes listed above is needed to identify non-deletional mutations. The choice of which mutation detection system is used depends on the laboratory; no method is clearly superior, and no PCR method is free from the potentially confounding effects of gene conversion at primer binding sites. Occasionally, DNA sequencing is needed. Amplification of overlapping regions of *CYP21* is the standard method to amplify this gene. PCR of closely-linked microsatellite markers still has a role in family studies.

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