Analysis of the Chimeric CYP21P/CYP21 Gene in Steroid 21-Hydroxylase Deficiency

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Background: A single nonfunctional chimeric gene with its 5' and 3' ends corresponding to CYP21P and CYP21, respectively, is caused by unequal gene crossover in the CYP21 genes during meiosis. The presence of the chimeric CYP21P/CYP21 molecule can not be detected by conventional PCR methods and therefore may be lost in PCR amplification. This leads to a false result and diagnostic discordance.

Methods: We developed a rapid and direct method to detect a chimeric CYP21P/CYP21 gene that uses a 3'-specific primer for the CYP21 gene and two different 5' primers for both CYP21 and CYP21P to amplify the wild-type CYP21 and the chimeric CYP21P/CYP21 genes. A secondary PCR that can differentiate the chimeric from the wild-type gene was also performed. The PCR product was directly analyzed on agarose gel.

Results: After careful titration, we found that earlier failure to detect the chimeric CYP21P/CYP21 gene could be caused by unequal concentrations of two independent alleles as the PCR template or by the lack of primers to amplify chimeric molecules. We successfully amplified the chimeric gene using our improved method.

Conclusions: The chimeric CYP21P/CYP21 is present in a large portion of congenital adrenal hyperplasia patients. By adding a CYP21P/CYP21-specific primer, we were able to amplify and detect both homozygous and heterozygous chimeric genes. Therefore, our new PCR-based assay is a more effective way to analyze congenital adrenal hyperplasia mutations.

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Congenital adrenal hyperplasia (CAH)4 is an inherited disorder mainly resulting from a defect in the steroid 21-hydroxylase (CYP21) gene and is one of the most common inborn errors of metabolism in humans. Six different enzymes are responsible for the synthesis of cortisol and aldosterone in the adrenal cortex-mix. Deficiency in the CYP21 gene prevents the conversion of 17-hydroxyprogesterone to 11-deoxycorticosterone, leading to an excessive production of androgen, which in turn affects several stages of growth and development (1). Multiple mutations in the CYP21 gene lead to a wide range of CAH phenotypes. Basically, two forms, classical and nonclassical, are diagnosed depending on the clinical symptoms. The classical form consists of the simple virilizing and the salt-wasting types, and the milder nonclassical form is usually late onset (1–3).

More than 90% of CAH cases are caused by mutations of the CYP21 gene (2, 4, 5). Approximately 75% of the defective CYP21 genes are generated through intergenic recombination so that CYP21 carries one or more deleterious mutations usually found in the neighboring CYP21P (6). These mutations are termed “apparent gene conversion” (7, 8). Approximately 20% of alleles have a 30-kb deletion that includes the 3' end of CYP21P, all of C4B, and the 5' end of CYP21. This allele carries a single nonfunctional chimeric gene with its 5’ and 3’ ends corresponding to CYP21P and CYP21, respectively (9). This is presumably related to unequal crossover during meiosis (9).

The CYP21 and CYP21P genes both are 3.2 kb long and share 98% homology in exons (10, 11). The mutation of the aberrant splicing site at nucleotide (nt) 656 of intron 2 (A/C→G) is the most common mutation in CAH in

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4 Nonstandard abbreviations: CAH, congenital adrenal hyperplasia; CYP21, cytochrome P450c21 gene; CYP21P, cytochrome P450c21 pseudogene; nt, nucleotide; and ACRS, amplification-created restriction site.
Taiwanese (12, 13). In addition, there is a seemingly high rate (~10–30%) of apparent gene deletions (12, 13). During molecular analysis using the differential PCR that we developed (12), we noticed that we could preferentially amplify the allele containing the intron 2 nt 656 mutation, which sometimes is also combined with an 8-bp deletion at exon 3 (codons 111–113). The unknown mutated allele, however, could not be amplified. This allele dropout may result from the formation of the chimeric CYP21P/CYP21, which lacks a specific primer for amplification. Because our primers were designed specifically for the CYP21 gene, they would not be able to amplify the chimeric mutant CYP21P/CYP21 gene. Apparently, we need another analysis method in these cases.

To circumvent the above problem, and as a step toward accurate clinical diagnosis, we describe here a mixed-primer PCR that allows the amplification of both the wild-type CYP21 and the chimeric CYP21P/CYP21 genes. This approach was the first attempt to detect gene fusion in steroid 21-hydroxylase in Chinese CAH patients.

Materials and Methods

Subjects

We studied 94 CAH families during the past 5 years from hospitals in Taiwan as described previously (13). The Institute Review Board of Veterans General Hospital-Taipei approved the protocol, and the study strictly followed their guideline (14). Each CAH family had at least one affected child. Eighteen healthy parents, from 94 unrelated CAH families, suspected of having one gene deletion were studied. Most of these families have at least one affected child with a mutation at nt 656 of intron 2 and no other mutation. All patients had increased 17-hydroxyprogesterone concentrations at the time of admission, and the Na⁺ and K⁺ of most of these patients had been analyzed, as described previously (13).

PCR and molecular analysis of the CYP21 gene

For CYP21 gene amplification, a differential PCR was used as described previously (12). To amplify both the chimeric CYP21P/CYP21 and CYP21 molecules, primers BF1, AF1, and 21BR (Table 1) (12) were mixed in a 50-μL reaction. A secondary PCR was then performed as described previously (12). The secondary PCR products were routinely run in 1.5% agarose gels, and if necessary, 2.5% Metaphor (FMC BioProducts) agarose gels were used. To detect the three most common mutations in Chinese at codons 172 and 356 of intron 2, we first amplified the CYP21 gene (primers BF1/21BR) (12), followed by the amplification-created restriction site (ACRS) method (12). If no mutation was found, single-strand conformation polymorphism analysis using 11 pairs of primers designed for quick screening of the CYP21 gene, including 10 exons and the 5′-untranslated region, was applied (13).

Primers

The primers used for detecting the chimeric CYP21P/CYP21 molecule and secondary PCR for determining the extent of gene conversion and crossover by size-differential amplification are listed in Table 1. The forward (C3B) and the reverse (C4) primers for ACRS detection of intron 2 nt 656 are also listed in Table 1.

Results

Allele dropout as an artifact generated by two-step PCR

A PCR is based on the knowledge of the nucleic acid sequence for a region that, for diagnostic applications, can be analyzed repeatedly from different individuals. A critical aspect of PCR is the specificity of oligonucleotide primers. Decreased amounts of PCR products often arise when PCR primers do not match templates perfectly. This effect can be amplified in the secondary PCR reactions. To test this phenomenon, we used unequal amounts of templates in PCR reactions to simulate uneven primary PCR.

A site at nt 656 of intron 2 (A/C→G) of the CYP21 gene was tested using the specific primers C3B and C4 (Table 1) (12). A 132-bp (Fig. 1, lane 1) or a 124-bp (Fig. 1, lane 2)
PCR product was derived from the CYP21 and CYP21P genes, respectively, of a healthy individual. The mutant CYP21P (124 bp) was digested into a 93-bp and a small 31-bp fragment by SacI, whereas the 132-bp wild-type CYP21 gene (Fig. 1, lane 6) could not be digested (Fig. 1, lane 7). To demonstrate whether template concentration during the first PCR affects amplification in the second round, equal concentrations of the 3.3-kb primary PCR products from both CYP21 and CYP21P were mixed and subjected to a secondary PCR amplification. The same amounts of the 132- and 124-bp PCR products were obtained (Fig. 1, lane 8) when the template ratio was changed to 5:1 for CYP21 and CYP21P, the mutant secondary PCR product was present as a faint band compared with the wild-type band (Fig. 1, lane 4). This was better seen when secondary PCR products were digested with SacI (Fig. 1, lane 9). Conversely, amplification of a 5× concentration of CYP21P to a 1× concentration of CYP21 (Fig. 1, lane 5) followed by SacI digestion produced a clear mutant band (93 bp) and a faint wild-type one (132 bp; Fig. 1, lane 10). This lack of amplification attributable to the low concentration of the template was because PCR amplification is a competitive reaction. The unequal amplification during the primary PCR reaction led to failure to detect the intron 2 mutation. In addition, the same phenomenon was also observed for other common mutations such as at codon 172 (Ile→Asn) and codon 356 (Arg→Trp; data not shown). The analysis indicates that allele dropout might occur when amplification of both alleles in the primary PCR is unequal.

CHIMERIC CYP21P/CYP21 GENE LEADS TO AMBIGUOUS MUTATION IDENTIFICATION

The CYP21 and CYP21P genes have 90% (31 of 284 nt) sequence homology in the intron 2 region, and in particular, there is almost complete sequence identity for exons 4–10 (10, 11). The region between intron 2 and the 3' end of exon 3 is considered the hotspot of recombination. This is where microconversion frequently occurs (15). In screening 94 CAH families with 21-hydroxylase deficiency, we detected 15 individuals with intron 2 mutations in one allele; however, the mutation in the other allele was not detected by the ACRS method. In addition, single-strand conformation polymorphism analysis in this region failed to obtain a precise result. Therefore, it is possible that a chimeric CYP21P/CYP21 gene, which cannot be detected by the original ACRS method, may be present. The strategy for verifying the existence of a chimeric CYP21P/CYP21 gene is described in Fig. 2A. We used a 3'-specific primer (primer 21BR) for the CYP21 gene and two different 5'-primers for both the wild-type CYP21 (primer BF1) and the CYP21P (primer AF1) in the chimeric CYP21P/CYP21 genes during the primary PCR amplification (Table 1). A 3.3-kb PCR product was produced from both the CYP21 and the chimeric CYP21P/
CYP21 genes. To distinguish between wild-type and chimeric genes, we performed a secondary PCR reaction using a common 3' primer (IN3R) and two specific 5' primers for the wild-type CYP21 gene (B1) and the chimeric gene (2HP), respectively (Fig. 2B). Secondary PCR products of 307 bp (B1/IN3R) and 281 bp (2HP/IN3R) could be amplified simultaneously (Table 1).

One example of this analysis is shown in Fig. 3. The conventional PCR amplification for the primary PCR product by BF1/21BR primers (12) followed by SacI digestion of the secondary PCR product amplified with primers B1/21BR was analyzed in A and B. The primary PCR product amplified by primed primers B1/AF1/21BR was analyzed in C and D. Lane M, mother; lane F, father; lane P, proband; lane mk, 100-bp ladder used as molecular marker. A 2.5% MetaPhor gel was used for the analysis of the size-differential PCR product in B and D.

Analysis of another CAH patient showed similar results (Fig. 4A). A single 103-bp band representing the ACRS mutant in the proband (Fig. 4A, lane P) appeared when a pair of primers (BF1/21BR) were used. In this case, the father (Fig. 4A, lane F) had both the wild-type (132 bp) and mutant (103 bp, without the 8-bp deletion) bands, whereas the mother (Fig. 4A, lane M) had an intense wild-type band (132 bp) and a weak mutant band (93 bp, with the 8-bp deletion). The presence of the weak mutant band probably is attributable to mispriming of the primary PCR by the supposedly CYP21-specific BF1/21BR primer pair. When three primers were used for primary PCR, the 93-bp defective allele from the mother became apparent (Fig. 4B). It indicated that the proband (Fig. 4B, lane P) carried two mutant bands (103 and 93 bp), one of which was inherited from the father (103-bp; Fig. 4B, lane F) and one from the mother (93-bp; Fig. 4B, lane M), who had a chimeric gene in one allele. When we used size-differential amplification (B1/2HP/IN3R) of the primary PCR product amplified with two primers (BF1/21BR), both the parents (Fig. 4C, lanes M and F) and the proband (Fig. 4C, lane P) had only a 307-bp wild-type band. Size-differential amplification using the primary PCR product with mixed-primer amplification (BF1/AF1/21BR) indicated that the proband (Fig. 4D, lane P) had not only a 307-bp wild-type band but also carried a 281-bp chimeric molecule, which was inherited from the mother (Fig. 4D, lane M). The father had one 307-bp defective allele (Fig. 3A, lane P). Similarly, secondary amplification with the B1/2HP/IN3R primers detected only the wild-type 307-bp allele from the parents (Fig. 3B, lanes M and F). The same procedure detected a single 281-bp allele from the proband (Fig. 3B, lane P). When the same family members were analyzed by primary amplification with mixed primers (BFI/AF1/21BR), both parents had the wild-type 132-bp and the defective 93-bp bands (Fig. 3C, lanes M and F). Similarly, the analysis of size-differential amplification by mixed primers (B1/2HP/IN3R) showed the parents (Fig. 3D, lanes M and F) had both the 281-p and 307-bp fragments. The proband (Fig. 3D, lane P) had only a 281-bp fragment. The results clearly demonstrated that both parents had the chimeric CYP21P/CYP21 gene in one of their chromosomes.
wild-type band. It indicated that there was no chimeric gene in the father’s alleles. Our analysis established that the father carries the mutation nt 656G on one chromosome and that the other is unaffected. The mother has one wild-type chromosome, and the other is a chimera containing the intron 2 nt 656G mutation plus the 8-bp deletion. This analysis for the chimeric molecule of CYP21P/CYP21 in CYP21 deficiency demonstrated that in this case allele dropout from the analysis of the chimeric CYP21P/CYP21 gene was attributable to the lack of specific primers for this molecule in PCR amplification.

Discussion

The majority of mutations (>90%) that cause CYP21 deficiency are attributable to recombination between CYP21 and its neighboring homologous CYP21P pseudogene. An aberrant splicing site at nt 656 of intron 2 is the most common mutation in CYP21 deficiency. It leads to the formation of a frame-shift mutation and causes a premature stop codon at amino acid 102 (16, 17). Many methods have been devised to detect the CYP21 gene in CAH, including PCR/single-strand conformation polymorphism analysis (18), allele-specific oligonucleotide typing (19), PCR/ligase detection (20), and solid-phase minisequencing (21). Recently, a two-step amplification of the CYP21 gene has gained popularity (22, 23). Many artifacts, however, can be created during this process. One common problem is the preferential amplification of one allele and lack of amplification of another. We were able to simulate this situation by titrating template concentrations using CYP21 genomic DNA (Fig. 1). It demonstrated that when unequal amounts of primary PCR products are used as a template for secondary amplification, allele dropout occurs. This shows that a smaller amount of product from one allele cannot compete with abundant product from another allele. This phenomenon is similar to the amplification of trisomy 21 by homologous gene quantitative PCR (24). The amplification of an unequal copy of chromosome 21 leads to an uneven amplification product.

In analyzing 94 unrelated CAH families, we detected 15 CAH patients carrying a mutation at nt 656 of intron 2 (A/C→G) without a combined 8-bp deletion at codons 111–113 in one allele. The cause of mutation in the other allele was uncertain with differential PCR combined with ACRS (12). Using the improved method, in which three primers were added, we showed the presence of a chimeric CYP21P/CYP21 gene in 9 CAH families. Only 6 of the 15 patients had one gene deletion. The presence of a chimeric CYP21P/CYP21 gene often is mistaken for gene deletion. Thus, the frequency of gene deletion in CYP21 deficiency may not be as high as reported (16, 17). As described earlier (20, 22), allele dropout was attributable to preferential amplification of DNA segments and was an artifact caused by polymerase (25). This led to an excessively high frequency of apparent homozygosity and genotyping discordance between parents and probands. Our cases showed that the allele dropout was related to the presence of chimeric molecules. The PCR product for the CYP21 gene would be the major product, and because of the PCR competition reaction, there is very little chimeric CYP21P/CYP21 product. This study indicated that the small amount of chimeric CYP21P/CYP21 primary PCR product cannot compete with the CYP21 PCR product if its concentration is only one-fifth that of the more abundant product (Fig. 1). This leads to the failure of the ACRS method to detect the intron 2 mutation in the secondary amplification. The improved method that uses three primers for primary PCR amplification could successfully detect the chimeric gene in the detection of the intron 2 mutation. Therefore, we suggest that both unequal concentrations of two independent alleles as a template for PCR amplification and the lack of a primer that targets the chimeric molecule may lead to allele dropout in the detection of CAH mutations.

We have successfully amplified two independent chromosomes that contain one of the chimeric genes. Combined with size differentiation, this leads to accurate genotyping. Our ability to demonstrate CAH allele dropout was based on the methodology of molecular techniques and intensive multiple-mutation typing and clinical information about this disease. When screening for other genetic traits containing homologous genes such as CYP11B1 and CYP11B2 hydroxylases, mixed primer amplification combined with size differentiation would help ensure that both chromosomes amplify.

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References