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Sequence Analysis of *CYP21A1P* in a German Population to Aid in the Molecular Biological Diagnosis of Congenital Adrenal Hyperplasia

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BACKGROUND: The high homology between the *CYP21A2* (cytochrome P450, family 21, subfamily A, polypeptide 2) and *CYP21A1P* (cytochrome P450, family 21, subfamily A, polypeptide 1 pseudogene) genes is the major obstacle to risk-free genetic diagnosis of congenital adrenal hyperplasia, especially regarding the quantification of gene dosage. Because of the lack of a comprehensive study providing useful information about the detailed genetic structure of *CYP21A1P*, we used a large data set to analyze and characterize this pseudogene.

METHODS: We amplified and directly sequenced the *CYP21A1P* and *CYP21A2* genes of 200 unrelated individuals. The resulting sequence data were aligned against the manually curated transcript ENST0000448314 from Havana/Vega matching to the genebuild ENSG00000198457; all differences were documented. Copy number was measured by multiplex ligation-dependent probe amplification when necessary.

RESULTS: We found that 40 potentially variable positions in *CYP21A2* were conserved in *CYP21A1P* in all study participants. In addition, we detected 14 *CYP21A1P* variants that were not previously reported in either *CYP21A2* or *CYP21A1P*. Unlike *CYP21A2*, *CYP21A1P* possessed certain mutation haplotypes.

CONCLUSIONS: The genetic structure of *CYP21A1P* and the potential risks of false conclusions it may introduce are essential considerations in designing a PCR-based diagnosis procedure for congenital adrenal hyperplasia.

Congenital adrenal hyperplasia is one of the most common autosomal recessive diseases causing adrenal insufficiency. About 95% of cases are caused by deficiency in 21-hydroxylase, a cytochrome P450 enzyme encoded by the *CYP21A2*[3] (cytochrome P450, family 21, subfamily A, polypeptide 2) gene (MIM 201910; GeneID, 1589) *(1, 2)*. The *CYP21A2* gene is located in the HLA gene–encoding regions on the short arm of chromosome 6 (6p21.3) and is approximately 3.2 kb in length *(3)*. 21-Hydroxylase is responsible for the efficient production of 2 vital adrenal steroid hormones, cortisol and aldosterone. Deficient production of these hormones causes disruption of hormonal balance. Deficiency of 21-hydroxylase prevents the conversion of 17-hydroxyprogesterone to 11-deoxycorticosterone, leading to excessive production of androgens, which ultimately affects several stages of growth and development *(4)*.

A pseudogene [*CYP21A1P* (cytochrome P450, family 21, subfamily A, polypeptide 1 pseudogene; GeneID, 1590] occurs about 30 kb upstream of *CYP21A2*. Inactivating mutations prevent it from producing an active enzyme. In humans, these 2 genes are located very close to the 3′ end of the 2 genes encoding the fourth component of serum complement, *C4A* [complement component 4A (Rodgers blood group)] and *C4B* [complement component 4B (Chido blood group)]. The homology between *CYP21A2* and *CYP21A1P* is up to 98% in exons and 96% in introns *(5)*. *CYP21A2* and *CYP21A1P* interact in 2 ways to produce 21-hydroxylase deficiency: unequal crossing-over during meiosis, which causes complete deletion of *C4B* and produces a fusion gene with a 5′ end similar to *CYP21A1P* and a 3′ end similar to *CYP21A2* (known as the "30-kb deletion"), and gene-conversion events during mitosis that transfer *CYP21A1P* inactivating mutations to *CYP21A2 (6)*.

Investigators have developed a large number of methods that can detect mutations and single-nucleotide polymorphisms in *CYP21A2*, such as the amplification-created restriction site approach *(7)*,

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[3] Human genes: *CYP21A2*, cytochrome P450, family 21, subfamily A, polypeptide 2; *CYP21A1P*, cytochrome P450, family 21, subfamily A, polypeptide 1 pseudogene; *C4A*, complement component 4A (Rodgers blood group); *C4B*, complement component 4B (Chido blood group).

Table 1. Specific primers used for *CYP21A2* and *CYP21A1P* amplification.

Primer name	Sequence (5′ → 3′)[a]	Position[b]
Cyp21A2_Fs	CCTTGCTTCT**T**GATGGGT-G-A-T-**C**	c.1−220 → c.1−199
Cyp21A1P_Fs	CCTTGCTTCT**C**GATGGGT-G-A-T-**T**	c.1−220 → c.1−199
Cyp21A2_Rs	GCCTCAATCCTCTGC-**A**-G-C-**G**	c.1486+440 → c.1486+458
Cyp21A1P_Rs	GCCTCAATCCTCTGC-**G**-G-C-**A**	c.1486+440 → c.1486+458

[a] Bases that are different in *CYP21A2* and *CYP21A1P* are in boldface. Hyphens indicate thiophosphate bonds.
[b] Position numbers are assigned according to the Ensembl transcript ENST0000448314. The adenine of the start codon is referenced as base no. 1 (c.1).

multiplex minisequencing *(8)*, and direct gene sequencing *(9, 10)*. In addition, functional and expression studies have been used in in vitro assays to search for novel mutations *(11–14)*. The identification of large chromosomal rearrangements and gene dosage analyses have mostly been performed with Southern blot techniques *(15–17)*, quantitative real-time PCR methods *(18–20)*, and, recently, multiplex ligation-dependent probe amplification (MLPA) *(21)*.

The very high homology between *CYP21A2* and *CYP21A1P* is the biggest source of risk in both *CYP21A2* mutation screening and quantification experiments. To overcome this complication requires that gene variants and their frequencies be known for both *CYP21A1P* and *CYP21A2*. With this requirement in mind, we analyzed both the active and inactive genes from unrelated 200 individuals in the study, including 198 bp upstream of the 5′ promoter region and 441 bp downstream of the 3′ end.

Materials and Methods

BLOOD SAMPLES

We used 200 anonymized and unrelated blood samples from individuals of the German population (not necessarily of German ethnicity). Following doctor referral, all individuals agreed to a genetic test for *CYP21A2* at the Bioglobe molecular genetics laboratory. Genomic DNA was isolated from blood samples with the QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's manual.

CYP21A2 AND *CYP21A1P* AMPLIFICATION

We used long-range PCR for separate and specific amplification of *CYP21A2* and *CYP21A1P*. For this purpose, we designed gene-specific forward primers. Because the 3′ ends of the 2 genes are subject to mutation, we implemented a reverse primer mix (Table 1). This strategy also ensured the amplification of possible hybrid genes. We increased PCR specificity by modifying the last 5 bases of the 3′ end with thiophosphate bonds, which prevent miscorrection by the proofreading activity of the DNA polymerase. All oligonucleotides were obtained from Metabion. The 50-μL PCR mix consisted of the buffer provided in the Platinum *Taq* DNA Polymerase High Fidelity kit (1× final concentration; Invitrogen), 0.2 mmol/L of each deoxynucleoside triphosphate (Roche Diagnostics), 0.75 μmol/L of one of the forward primers (Cyp21A2_Fs or Cyp21A1P_Fs), 0.5 μmol/L each of both reverse primers (Cyp21A2_Rs and Cyp21A1P_Rs), 2 mmol/L $MgSO_4$ (Invitrogen), 0.5 μL DMSO (Merck), 0.75 U Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen), 0.8 g/L BSA (New England Biolabs), and 2.5 μL genomic DNA (approximately 100 ng). The PCR thermocycling program was 2 min at 94 °C; 35 cycles of 35 s at 94 °C, 45 s at 62 °C, and 4 min at 68 °C; and 10 min at 68 °C. PCR yield was analyzed visually on a 10 g/L agarose gel.

PCR PURIFICATION AND BigDye™ TERMINATION CYCLING

The product of the long-range PCR was purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's manual. We used 2 μL of the purified product as template in BigDye termination cycle sequencing reaction in a 15-μL total volume. Universal primers for *CYP21A2* and *CYP21A1P* were used in this second cycling step (Table 2). The 15-μL reaction mix contained Sequencing Buffer (GE Healthcare) (0.5× final concentration), 1.2 μmol/L primer (forward or reverse), 3 μL BigDye Termination Mix (GE Healthcare), and 2 μL purified long-range PCR product. The thermocycling program consisted of an initial denaturation step of 2 min at 96 °C followed by 35 cycles of 10 s at 96 °C, 10 s at 53 °C, and 4 min at 60 °C. The products of BigDye termination cycling were purified with the illustra AutoSeq 96 G-50 for MegaBACE Analyzers (GE Healthcare).

DNA SEQUENCING AND MLPA

Sequence determination was performed on a MegaBACE 1000 DNA Analysis System (GE Healthcare) under the following conditions: injection voltage, 2

Table 2. Universal forward and reverse sequencing primers for *CYP21A2* and *CYP21A1P*.

Name	Sequence (5′ → 3′)	Position
Ex1F	GGGATGGCTGGGGCTCTTG	c.1−60 → c.1−42
Ex2F	GCTGCAAGGTGAGAGGCTGAT	c.192 → c.199+13
Ex3F	GCCCAGGCTGGTCTTAAATTC	c.289+69 → c.289+89
Ex4F	AAGCCCACAAGAAGCTCACC	c.350 → c.369
Ex5/6F	GATCAAGGTGCCTCACAGCC	c.540 → c.546+13
Ex7F	AGGCAGCACAAGGTGGGGAC	c.724 → c.735+8
Ex8F	TTTTTTTGCTTCACCACCCTG	c.914 → c.934
Ex9F	CACCACACGGCCCAGCAGG	c.1098 → c.1115+1
Ex10F	CCTGCCGTGAAAATGTGGTGG	c.1219+20 → c.1219+40
Ex1R	GAGGACCCTCTCCGTCACC	c.200−33 → c.199+47
Ex2R	CTTGAGGCTGAGGTGGGAG	c.289+124 → c.289+106
Ex3R	AGCCCAGCCTTACCTCAC	c.444+13 → c.440
Ex4R	CAGGACAAGGAGAGGCTCAG	c.547−31 → c.546+39
Ex5/6R	GCAATGCTGAGGCCGGTAGC	c.735+67 → c.735+48
Ex7R	GCCAGGTTGCTGGGAAGGAG	c.936+45 → c.936+26
Ex8R	GCTGGAGTTAGAGGCTGGC	c.1116−10 → c.1116−28
Ex9R	GGTGGGTGGGGAGGCGTTC	c.1120−18 → c.1120−36
Ex10R	GCGATCTCGCAGCACTGTGT	c.1486+100 → c.1486+81

kV; injection time, 75 s; run voltage, 8 kV; run time, 110 min. Data were aligned against the manually curated Ensembl transcript ENST0000448314 from Havana/Vega matching to the Ensembl genebuild ENSG00000198457, and identified variations were documented. The results were analyzed with Sequence Analyzer 4.0 (GE Healthcare) and Sequencher 4.5 (Gene Codes Corporation) software. We used SALSA MLPA Kit P050-B2 CAH (MRC-Holland) for quantitative analysis when necessary. Probe hybridization and MLPA PCR were carried out according to the manufacturer's manual. Results were analyzed with the free software Gene Marker 1.6.

Results

The *CYP21A2* and *CYP21A1P* genes of 200 unrelated individuals were specifically amplified by long-range PCR. The following crosswise primer combinations were used to ensure that possible hybrid genes also were amplified: Cyp21A2_Fs with Cyp21A2_Rs and Cyp21A1P_Rs; Cyp21A1P_Fs with Cyp21A2_Rs and Cyp21A1P_Rs.

MLPA was performed for samples that produced no amplification of *CYP21A2* and/or *CYP21A1P*, and for samples that possessed a hybrid gene. One sample produced no amplification in the long-range PCR of *CYP21A2*. MLPA in this case produced no signals for *CYP21A2*-specific probes from exon 1 to exon 8. MLPA analysis of this sample indicated that 2 *CYP21A1P* copies were present. *CYP21A1P* amplification produced no products for 18 samples, 16 of which had a complete *CYP21A1P* deletion; MLPA analysis showed that the remaining 2 samples exhibited deletion of the 5′ end of *CYP21A1P*.

We detected 2 samples possessing a hybrid gene produced by the aforementioned 30-kb deletion. This gene had a *CYP21A1P*-like 5′ end and a *CYP21A2*-like 3′ end. In both samples, the breakpoint was in exon 3, between the 8-bp deletion and c.515T>A (p.Ile172Asn). Both samples had 1 *CYP21A2* copy in addition to the hybrid gene.

Pseudogene-specific variations, which are known to have accumulated during evolution, were confirmed for all samples in this study. Table 3 lists the *CYP21A1P* variants with a frequency of 1.0. Complete frequency data are presented in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue3. In addition to conserved genotypes, we detected base changes not previously reported for *CYP21A2* or *CYP21A1P*. Table 4 lists these variants, together with their locations and frequencies.

CYP21A1P had 5 mutation haplotypes in our sample set. Genotypes of 3 positions in 1 case and genotypes of 2 positions in 4 cases were in linkage disequilibrium and formed a haplotype structure. These positions are located as far as 2484 bp apart. Table 5

Table 3. Bases conserved in *CYP21A1P* with a frequency of 1.0.[a]

Region	Position	Variation[b]	Region	Position	Variation[b]
5′ promoter	c.1−210	T>C	Intron 2	c.290−123	C>A
5′ promoter	c.1−199	C>T	Intron 2	c.290−96_94	GGT>TCA
5′ promoter	c.1−190	->insT	Intron 2	c.290−91	G>A
5′ promoter	c.1−126	C>T	Intron 2	c.290−88_87	GA>AG
5′ promoter	c.1−113	G>A	Intron 2	c.290−79	G>T
5′ promoter	c.1−110	T>C	Intron 2	c.290−74	G>A
5′ promoter	c.1−103	A>G	Intron 2	c.290−67	G/C>A
Exon 1	c.115	T>C	Intron 2	c.290−48	A>G
Exon 1	c.135	A>C	Intron 2	c.290−44	G>T
Intron 2	c.289+45	insTGT	Intron 2	c.290−39_38	CA>GG
Intron 2	c.289+46	A>T	Intron 2	c.290−13	A/C>G
Intron 2	c.289+56	T>G	Intron 4	c.547−15	C>A
Intron 2	c.289+84	A>G	Intron 4	c.547−8	T>C
Intron 2	c.289+92	A>G	Exon 5	c.549	C>G
Intron 2	c.289+100	A>G	Exon 6	c.702	T>C
Intron 2	c.289+127	T>G	Exon 6	c.707	T>A
Intron 2	c.289+138	T>C	Exon 6	c.716	T>A
Intron 2	c.289+139	->G	Intron 6	c.735+12/13	AC>GT
Intron 2	c.290−139	A>T	Exon 7	c.920	insT
Intron 2	c.290−129	insTCC	Intron 7	c.936+11	G>C

[a] Hybrid genes are excluded.
[b] Genotypes are shown as changes from *CYP21A2* to *CYP21A1P* (i.e., *CYP21A2* genotype>*CYP21A1P* genotype).

Table 4. New variants detected in *CYP21A1P*.

Region	Position	Variation	Frequency in *CYP21A1P*
Intron 2	c.290−136	C>T	0.036
Intron 2	c.290−130	C>T	0.019
Intron 2	c.290−115	C>G	0.006
Exon 4	c.507	C>T	0.003
Intron 5	c.649−5	C>T	0.008
Exon 6	c.709_711	delGTG	0.030
Exon 7	c.874	G>A	0.011
Intron 9	c.1219+22	T>C	0.006
Intron 9	c.1220−21	C>T	0.019
Exon 10	c.1317	C>T	0.003
Exon 10	c.1394	C>G	0.006
3′ UTR[a]	c.1484+88	G>A	0.003
3′ UTR	c.1484+114	T>C	0.006
3′ UTR	c.1484+119	C>T	0.006

[a] UTR, untranslated region.

lists 5 *CYP21A1P* mutation haplotypes, together with their frequencies and locations. No similar linkage disequilibrium was observed in *CYP21A2*.

Discussion

We analyzed the *CYP21A2* and *CYP21A1P* genes of 200 individuals to better understand this complicated chromosomal region. Our study has demonstrated that simultaneous *CYP21A1P* analysis is also necessary for a better quantitative analysis of *CYP21A2* analysis and to minimize the potential for false diagnosis.

Depending on the method of gene amplification and the *CYP21A1P* variants present, one could obtain results that do not reflect the real situation. Sample 84 in our set, for example, had the 8-bp deletion in only one of its *CYP21A1P* alleles. If we had used the technique of amplification of overlapping fragments, in which the forward primer for the second fragment binds to the 8-bp sequence, the *CYP21A1P* allele possessing this 8-bp sequence would be amplified along with the *CYP21A2* allele. This would, of course, produce the wrong genotype.

Table 5. Mutation haplotypes found in linkage disequilibrium in *CYP21A1P*.

Position	Genotype	Cases, n
c.289+116/c.708/c.803	G-G/C-C/G-G	149
c.289+116/c.708/c.803	G-A/C-G/G-C	26
c.289+116/c.708/c.803	A-A/G-G/C-C	7
c.444+39/c.631	A-A/A-A	17
c.444+39/c.631	A-G/A-G	40
c.444+39/c.631	G-G/G-G	125
c.648+30/c.819	A-A/C-C	2
c.648+30/c.819	A-G/C-T	7
c.648+30/c.819	G-G/T-T	173
c.185/c.1448	T-T/C-C	10
c.185/c.1448	A-T/G-C	22
c.185/c.1448	A-A/G-G	150
c.290−130/c.444+38	T-T/T-T	1
c.290−130/c.444+38	C-T/C-T	5
c.290−130/c.444+38	C-C/C-C	176

In our study, we discovered 5 mutation haplotypes in *CYP21A1P* with specific genotype patterns but observed no such haplotype formation in *CYP21A2*. The linkage disequilibria were not exceptional despite the large distances between the bases. The distance between the involved genotypes was 264 bp in the closest linkage disequilibrium and 2484 bp in the farthest. Because we studied the pseudogene, we cannot assess the clinical impact of these mutation groups. It would, however, be worth using computational-modeling approaches to investigate how these mutation haplotypes would alter protein structure and, ultimately, enzyme concentration if they were transferred to *CYP21A2* simultaneously.

Concolino et al. used MLPA and evaluated its performance for *CYP21A2* and *CYP21A1P* quantification *(21)*. Concordantly, we determined that *CYP21A1P* genotypes interfere with quantitative analysis of *CYP21A2* by MLPA to a certain extent. For samples with the wild-type allele in homozygous or heterozygous form at position c.515T>A (p.Ile172Asn) in *CYP21A1P*, 3 *CYP21A2* copies were reported. The frequency of the absence of p.Ile172Asn in *CYP21A1P* was 0.036 in our sample set. Without a parallel *CYP21A1P* analysis, one would falsely infer a heterozygous duplication of *CYP21A2* with a risk of 3.6%. Yet another unreliable position is in exon 8, where the gene copy number is determined with the mutation c.952C>T (p.Gln318X) as the ligation site. In our sample set, the frequency of the wild-type allele at this position in

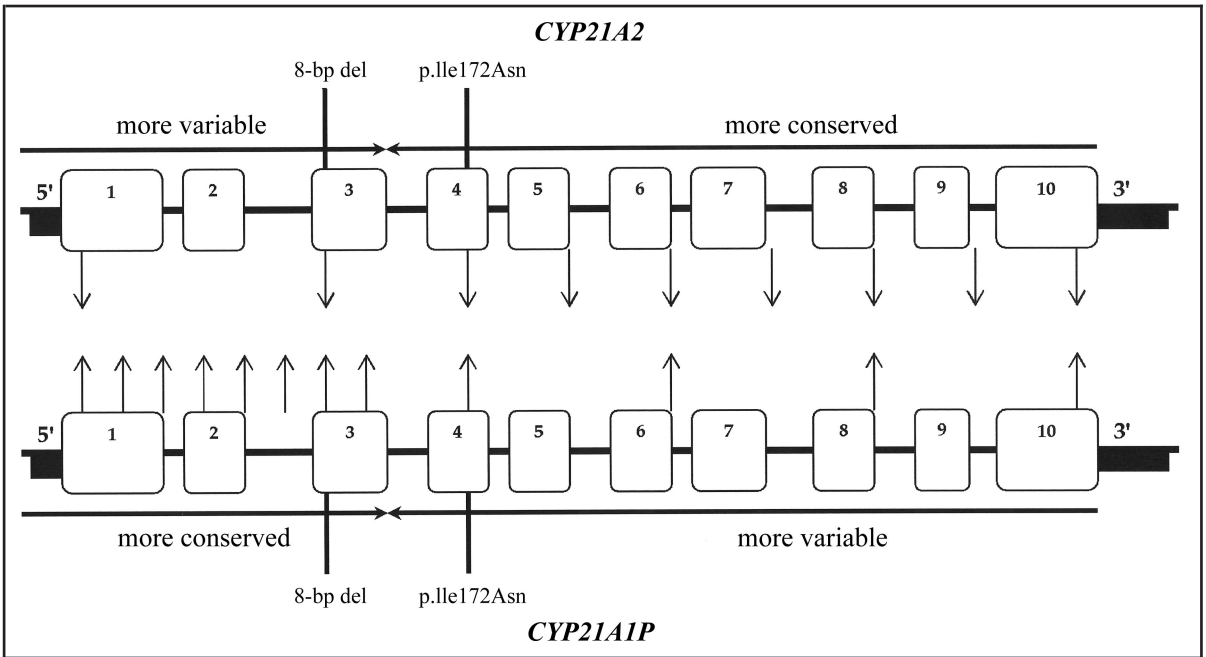

Fig. 1. Schematic representation of *CYP21A2* and *CYP21A1P*.

Exons are numbered rectangles; introns are shown as horizontal bars. 5′- and 3′-untranslated regions are indicated by filled rectangles under introns. The 8-bp deletion and p.Ile172Asn are indicated with vertical bars. Arrows are placed to represent the direction and frequency of base transfer in the region. Arrow density and location have no meaning regarding scaling or absolute frequency.

CYP21A1P was 0.085. This result indicates that, owing to a lack of *CYP21A1P* genotype data, one would falsely infer a duplication of the *CYP21A2* gene in approximately 8.5% of the cases.

A consensus sequence for *CYP21A1P* for the German population was developed by calculating allele frequencies for the 200 samples. We recommend that these data be used by researchers who are interested in further investigating the *CYP21A1P* gene (see Table 2 in the online Data Supplement).

We observed the pseudogene to be more conserved upstream of the breakpoint (between the 8-bp deletion and p.Ile172Asn) until the position where the 30-kb deletion usually occurs. On the other hand, the active gene was more conserved in the sequence downstream of the breakpoint (Fig. 1). This observation is in agreement with the mechanism of chimeric gene formation that has been proposed for this region *(5)*. Because of recombinations during meiosis, a 30-kb segment that encompasses the 3′ end of *CYP21A1P*, all of the adjacent *C4B* gene, and the 5′ end of *CYP21A2* can be completely deleted or duplicated. This region, where both genes are not conserved, contains most of the polymorphisms and mutations. For instance, the polymorphism rs6463 (c.289+33C>A) is never detected in *CYP21A1P*; however, its frequency in *CYP21A2* is 0.69. The polymorphism rs12525076 (c.648+35G>A), on the other hand, is present at a frequency of 0.04 in *CYP21A2* and 0.88 in *CYP21A1P*.

We observed different rates of transfer of the variants between the 2 genes. The introduction of variations can often occur via transfer from one gene to the other, but transfer in the opposite direction seldom occurs or not at all. Throughout our sample, we found genetic transfer between *CYP21A2* and *CYP21A1P* to occur more frequently via previously established directions, e.g., from *CYP21A1P* to *CYP21A2*, rather than from *CYP21A2* to *CYP21A1P*. The reason why one direction is preferred over the other remains unknown.

In conclusion, our results show that to have a complete and accurate *CYP21A2* analysis, *CYP21A1P* results, as mentioned above, should be taken into consideration when designing any kind of assay for sequencing, genotyping, and, especially, gene copy number evaluation.

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