Characterisation of CAH alleles with non-radioactive DNA single strand conformation polymorphism analysis of the CYP21 gene

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Abstract

The major cause of congenital adrenal hyperplasia (CAH), a common recessive genetic disease, is the deficiency of steroid 21-hydroxylase (21OH), a microsomal enzyme encoded by the CYP21 gene. Although several CAH causing mutations have been identified in the CYP21 gene of patients with 21OH deficiency, genotyping of the 21OH locus is quite complex because of the high frequency of gene conversion and the presence of multiple mutations on single CAH alleles. In order to perform the complete characterisation of the CYP21 gene coding region more simply, we developed a highly sensitive, non-radioactive method allowing DNA single strand conformation polymorphism (DNA-SSCP) analysis. This method was applied to the characterisation of all the exons and intron-exon junctions of the CYP21 gene in five patients affected by the simple virilising form and one affected by the salt wasting form. In all samples showing SSCP signals, direct sequence analysis showed the presence of more than one single sequence variant. In particular, four mutations which are already known to cause the disease, 16 polymorphisms, and one newly identified C to T transition at position 849 were detected. A random sequence analysis, performed on 31 out of 81 exons showing a normal SSCP pattern, shows the method to be highly sensitive: no sequence variant was detected, thus confirming the validity of this non-radioactive DNA-SSCP analysis in characterising the CYP21 gene in patients with steroid 21OH deficiency. Notwithstanding the complete characterisation of all exons and exon/intron junctions of the CYP21 gene, no complete genotype/phenotype correlation was found in the panel of patients analysed, thus suggesting that characterisation of CAH alleles must be extended to outside the coding region of the CYP21 gene, most probably into the promoter region.

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cause is steroid 21-hydroxylase (21OH) deficiency. It is a heterogeneous trait both at the clinical and the biochemical level with the clinical phenotype being divided into three forms: salt wasting (SW), simple virilising (SV), and non-classical (NC). An incidence of severe forms of 1:5000 to 1:15 000 in most white populations^{2 3} and 1:100 to 1:1000 persons for mild forms⁴ has been reported.

Steroid 21-hydroxylase (P450c21, E.C.1.14.99.10) is a microsomal enzyme responsible for conversion of progesterone and 17 α -hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol respectively. There are two genes encoding 21-hydroxylase, CYP21P and CYP21, which are located within the HLA class III gene region on the short arm of chromosome 6 in 3' position of each of the two genes encoding the fourth component of complement, C4A and C4B. CYP21P and CYP21 genes are both 3.4 kb long and split into 10 exons. The complete nucleotide sequence of both genes has been reported.^{2 3 5} Three mutations in the CYP21P gene prevent synthesis of an active protein, which means that this is a "pseudogene" with no evident function. The tandemly duplicated C4 and CYP21 genes allow for possible misalignment during meiotic metaphase and unequal crossing over between sister chromatids, resulting in a chromosome containing one or three sets of C4 and CYP21 genes.^{6 7} Moreover, small exchanges of sequences between homologous genes, named gene conversion, could create many of the mutated alleles by transferring some of the deleterious mutations from the pseudogene to the CYP21 gene.⁸⁻¹¹

To date, a high level of heterogeneity has been found for CAH causing mutations in the CYP21 gene. Although deletion of the CYP21 gene and several sequence aberrations have so far been reported to result in steroid 21-hydroxylase deficiency,¹²⁻²⁰ there is growing evidence that there is no clear correlation between clinical expression of endocrine disease and mutations of the gene's primary structure.^{21 22} In the light of this, direct sequencing of the entire gene seems to be the best way to characterise fully a 21OH deficiency patient. Alternatively, the radioactive DNA-SSCP method has been used to analyse only part of the gene.²³

In an attempt to perform a fast and complete characterisation of the CYP21 gene coding region, we devised a rapid, non-radioactive,

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Table 1 Primer sequences

Primers	Sequence 5'—3'	Position	Amplified exon	T annealing
Yfor	TTCaggCgATTCCaggAAggC	(-406-(-387))		59 °C
Yrev	AgCagggAg T Ag TCTCCCA AggA	(722-700)		
Efor	gTgTgAgCCACCTT T ggggC	(510-529)		65 °C
Hrev	TTgTCgTCCTgCCAgAAA Agg Ag	(1126-1104)		
Zfor	CCTgTCCTTggg AgACT ACT	(696-715)		58 °C
Zrev	TCTCgCACC CC AgTATgACT	(2905-2886)		
Afor	TCTACACAgCAggAgggATg	(-74-(-55))	Exon 1	65 °C
Brev	ggAgACAgCCAAAgCAgCgT	(294-275)		
Cfor	ACggAgAgggTCCTCTCTCC	(251-270)	Exon 2	64 °C
Drev	CCCAAgAgCTTCCAgggA	(561-544)		
Ffor	AAggTCAggCCCTCAgCTgCCTT CA	(606-630)	Exon 3	71 °C
Frev	CTgTgAgAggCgAggCTgAC	(871-852)		
Gfor	ggCTgggCTCCTgAggCCAC	(827-846)	Exon 4	71 °C
Hrev	TTgTCgTCCTgCCAgAAA Agg Ag	(1126-1104)		
Ifor	CTCACAgCCCCTC Ag CCCA	(1035-1054)	Exon 5	65 °C
Lrev	CAggAAgCATgAgAA T gCAg	(1315-1296)		
Mfor	CTgggTTgTAggggAgAggC	(1246-1265)	Exon 6	65 °C
Nrev	CggCTggCCTggggAgAggT	(1530-1549)		
Ofor	CCCCAgTTATgggCCTgTTg	(1499-1518)	Exon 7	65 °C
Prev	TgAggCAAgCACAgCCCCAg	(1866-1885)		
Qfor	TgggCACCTCACTCAgCTCTgA	(1832-1854)	Exon 8	71 °C
Rrev	AAgggCAgCCgTgCACg g TCCTT	(2075-2051)		
Sfor	AggAggAgCTAgACCACgAACTg	(1995-2017)	Exon 8	71 °C
Trev	gggCTggAgTTAgAggCTgg	(2214-2233)		
Ufor	TTggggATgAgTgAggAAAg	(2172-2191)	Exon 9	65 °C
Vrev	ggCgggTggACAgGTgggT	(2417-2435)		
Wfor	TCCCCgCTgCCgCTgAA C gC	(2391-2410)	Exon 10	67 °C
Xrev	ACCCggCTggCATCg g TCCT	(2713-2732)		

Primers Yrev, Efor, Hrev, Zfor, and Ffor are specific sequences for the CYP21 gene. Specific nucleotides for the gene are showed by bold letters and are underlined. Position indicates the nucleotide number following the sequence reported by Higashi *et al.*⁵ The DNA sequence of primers Yfor, Zfor, Zrev,¹⁹ and Yrev, Hrev, Ffor, Qfor, Rrev, Sfor²³ have already been reported.

modified DNA-SSCP based analysis of all exons and exon/intron junctions followed by direct sequencing of conformational variants. This study was carried out on six patients affected by 21OH deficiency showing different clinical phenotypes, with analysis also extended to their parents in three cases.

Materials and methods

PATIENTS AND FAMILIES

The Italian families and patients enrolled in this study were unrelated and had no known consanguinity. Patients were diagnosed on the basis of increased plasma 17α-hydroxyprogesterone (17OHP). All patients examined had high values of both baseline and ACTH stimulated 17OHP serum concentration, they aggregate in the upper part of the nomogram reported in Wilson *et al.*²² and were classified as having one of the three clinical forms of CAH. Family 1 has two affected children (probands 1 and 2) who were diagnosed as having the SV form. In particular, proband 1 was phenotypically and karyotypically male with very precocious pseudopuberty; proband 2 was diagnosed in the neonatal period by means of a very high basal level of 17OHP and began therapy during the first few days of life. In family 2 the affected child (proband 3) was a female diagnosed as SV at the age of 5 because of genital ambiguity. In family 3, proband 4 had the SW form characterised by onset of hyperkalaemia, hyponatraemia, dehydration, and shock which required treatment with both mineralcorticoids and glucocorticoids during the neonatal period. Patients 5 and 6 had male phenotypes with a 46,XX karyotype and were diagnosed as having the SV form. A subject not showing any CAH phenotype was also enrolled in this study as a control.

DNA-SSCP ANALYSIS

Genomic DNA was prepared from peripheral blood leucocytes by standard procedures. Oligonucleotides were purchased from Pharmacia (Sweden) and numbered following the sequence reported by Higashi *et al*⁵ (table 1). PCR amplifications were performed by using about 100 ng of genomic DNA in the presence of 200 μmol/l of each dNTP, 1 mmol/l MgCl₂, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 50 pmol of each primer, and 2.0 U Amplitaq (Perkin Elmer) in a volume of 100 μl. In the initial phase the CYP21 gene was specifically amplified with three pairs of primers which allow discrimination between gene and pseudogene, since at least one primer was specific for the CYP21 sequence. Thus, the entire gene sequence was covered by three overlapping fragments of 1128, 616, and 2209 bp, starting from the 5' non-coding region and amplified respectively with primer pairs Yfor/Yrev, Efor/Hrev, and Zfor/Zrev. PCR conditions were as follows: denaturation for one minute at 94°C, annealing for one minute at 59°C or 58°C, and extension for three or four minutes at 72°C, respectively in the case of the 1128 bp or the 2209 bp fragments. Thirty cycles of denaturation at 94°C for 45 seconds, annealing at 65°C for 45 seconds, and extension at 72°C for 45 seconds were carried out to amplify the 616 bp fragment.

The three gene specific fragments were used as a template for the amplification of the 10 exons and all the exon/intron junctions of the CYP21 gene. The relevant primer pairs are listed in table 1, together with the annealing temperature used for the amplification of each exon in 25 polymerisation cycles (30 seconds at 94°C, 30 seconds annealing, 30 seconds at 72°C). A total of 11 amplified fragments were obtained since exon 8 was split into two fragments. Subsequently, 1 μl of the amplification mixture of each exon and exon/intron junction was mixed with 9 μl of a denaturing solution containing 95% (v/v) formamide, 0.05% w/v of both bromophenol blue and xylencianol, 0.1 N NaOH, 20 mmol/l EDTA, pH 8.0, and underwent SSCP analysis.²⁴ After 10 minutes incubation at 96°C, the samples were cooled in a 4°C ice bath and electrophoresed in TBE buffer at 10 mA for three to four hours. In order to analyse SSCP patterns, minigels containing different percentages of acrylamide, bisacrylamide, and glycerol were used. Gels were stained using standard silver nitrate staining (Bio-Rad Lab).

All the PCR products showing positive SSCP pattern were purified with the Quiaquick kit (Quiagen) and directly sequenced using the Sequenase version 2.0 kit (Amersham).

Results

Specific amplification of the CYP21 gene was achieved by using gene specific primer pairs, as already reported.^{23 25} Three overlapping gene specific fragments of 1128, 616, and 2209 bp in length, starting from the 5' end of the gene, were obtained and used as templates for the subsequent amplification of all exons and exon/intron junctions. The primers used for

Figure 1 DNA-SSCP analysis of the CYP21 gene with abnormal patterns indicated by an asterisk. (A) SSCP gel results for exon 1. Lanes a-d: family 1, father, mother, probands 2 and 1; lanes e-g: family 2, father, mother, proband 3; lanes i-m: family 3, father, mother, proband 4; lane n: patient 5; lane o: patient 6; lanes h and p: controls. (B) SSCP gel results for the 5' half of exon 8. Lanes a-d: family 1, father, mother, probands 1 and 2; lanes f-h: family 2, father, mother, proband 3; lanes i-m: family 3, father, mother, proband 4; lane n: patient 5; lane o: patient 6; lanes e and p: controls.

this purpose, including 18 newly designed oligonucleotides, are shown in table 1, with the respective amplification fragments ranging in size from 238 to 386 bp.

To perform DNA-SSCP analysis, different electrophoretic conditions were set up for each one of the amplified DNA fragments. Thus, to detect abnormal SSCP patterns in exons 1, 2, 7, 9, 10, and in the 5' portion of exon 8, minigels containing 10% acrylamide with 2.0% crosslinking and 5% glycerol were used and the electrophoretic run was performed at room temperature. For analysis of exons 3 and 6, the gel electrophoresis was run under the same conditions reported above but without glycerol and with 12% and 10% acrylamide, respectively, while in the case of exon 4, a minigel with 7% acrylamide, 2.5% crosslinking, and 5% glycerol was run at 4°C. Electrophoretic band shift, owing to single strand conformation polymorphism, was detected in all but exon 5 and the 3' portion of exon 8, for which no difference in the mobility of the single strand was detected after running gels with either 2.0 or 2.5% crosslinking both in the presence or absence of glycerol.

Typical SSCP patterns obtained are shown in fig 1. Panel A shows results obtained from analysis of the fragment which contains exon 1, amplified with primer pair Afor/Brev. Abnormal patterns were observed in proband 1 (lane d) and his father (lane a), in proband 3 (lane g) and her parents (lanes e,f), and in proband 4 (lane m) and his father (lane i). Patients 5 and 6 (lanes n and o) behave like the normal control (lanes h and p).

Panel B shows SSCP analysis of the fragment amplified with primers Qfor/Rrev which corresponds to the 5' portion of exon 8, from nucleotide 1832 to 2075. Only lanes h

and f, corresponding respectively to proband 3 and her father, showed a different migration as compared to the other samples (lanes a-d, g, i-o) and to the normal control (lanes e and p).

All samples showing SSCP signals were purified from agarose gels and directly sequenced. As expected, the presence of a number of sequence variants was shown (fig 2). On the other hand, 31 out of 81 amplified fragments showing normal SSCP patterns were purified from agarose and directly sequenced. These samples were randomly chosen from among the subjects and analysed in such a way that for each exon about 50% of the negative signals were sequenced. This strategy was also applied in the case of exon 5 and the 3' end of exon 8, the only two exons showing no SSCP signals in the experimental conditions used. Sequence analysis of these negative SSCP samples did not show any nucleotide variations as compared to the sequence of the normal allele,⁵ thus confirming that no sequence variant was lost in our procedure and showing the high sensitivity of the method.

The complete genotypes of the CYP21 alleles in the three families and two single patients analysed according to this method are shown in table 2. For each patient at least one of the known disease causing mutations was identified together with a number of other sequence variants. In particular, the four mutations I2 splice, I172N, cluster E6, and Q318X were found. They have been previously described as causing the disease on the basis of in vitro mutagenesis and expression studies to predict the degree of enzyme deficiency for each mutation.^{8 15 26} A T→C transition at base 395 in intron 2, which has not been excluded as affecting gene expression,²⁵ was also identified. Six silent mutations, L39L, P45P, D234D,

Table 2 Allelic segregation of CYP21 gene mutations in CAH families

Mutations	Family 1				Family 2			Family 3				
	Father	Mother	Proband 1	Proband 2	Father	Mother	Proband 3	Father	Mother	Proband 4	Patient 5	Patient 6
-4c→t	+-	--	+/0	-/-	--	--	-/-	--	--	-/-	--	--
delLeu10	+-	++	-/0	+/+	--	--	-/-	--	++	-/+	++	++
L39L	--	--	-/0	-/-	--	++	-/+	+-	--	-/-	--	--
P45P	--	--	-/0	-/-	++	++	+/+	+-	--	-/-	--	--
t→c395	--	--	-/0	-/-	--	--	-/-	+-	--	+/+	--	--
c→a419	+-	--	+/0	-/-	--	--	-/-	++	--	+/+	--	--
t→c453	+-	--	+/0	-/-	--	--	-/-	++	--	+/+	--	--
I2 splice	--	--	-/0	-/-	--	--	-/-	--	++	+/+	++	++
K102R	+-	--	+/0	-/-	++	++	+/+	++	++	+/+	--	--
c→t849	--	--	-/0	-/-	+-	--	-/-	--	--	-/-	--	--
c→t860	--	--	-/0	-/-	+-	--	+/+	+-	--	+/+	--	--
I172N	+-	--	+/0	+/+	--	+-	-/+	--	--	-/-	+-	--
D234D	--	--	-/0	-/-	--	--	-/-	+-	--	+/+	--	--
Cluster E6	--	--	-/0	-/-	--	--	-/-	+-	--	+/+	--	--
a→g1420	--	--	-/0	-/-	+-	--	+/+	--	+-	-/+	--	--
c→t1421	--	--	-/0	-/-	+-	--	+/+	--	+-	-/+	--	--
L248L	--	++	-/0	-/+	--	--	-/-	--	--	-/-	--	--
Q318X	--	--	-/0	-/-	+-	--	+/+	--	--	-/-	--	--
S374S	--	++	-/0	-/+	--	--	-/-	--	--	-/-	+-	+-
P490P	+-	--	+/-	+/+	+-	++	+/+	++	--	+/+	--	--
N493S	+-	--	+/0	+/+	+-	++	+/+	++	--	+/+	--	--

+, presence of the mutation; -, absence of the mutation; 0, chromosome deletion. / indicates that inherited chromosomes were determined by a family study with the paternal chromosome reported on the left and the maternal chromosome on the right of the slash. *, de novo mutation in the paternally transmitted chromosome. The C insertion at position 887, G insertion at position 2356, and the substitution of proline-426 with arginine are not included in the table because they are referred to as normal sequence by White *et al*² and Rodrigues *et al*.³

S374S, L248L, and P490P, which apparently do not cause changes in amino acid composition were also found. Other nucleotides that varied among the CYP21 alleles analysed include a C→T transition four bases upstream of the initiation codon, deletion of Leu10, five intron substitutions (c→a 419, t→c 453, c→t 860, a→g 1420, and c→t 1421), and two sequence variations leading to amino acid changes (K102R and N493S). All these nucleotide variations have already been reported and considered neutral with regard to 21OH deficiency, since they have been found in normal alleles.^{3 15 19} A C insertion at position 887, a G insertion at position 2356, and a substitution of proline-426 with arginine were also found in all the subjects analysed, but not included in table 2. These are referred to as normal sequence by White *et al*² and Rodrigues *et al*.³ A new C→T transition at base 849 has been also identified.

For each proband following the segregation of each of the sequence variants described it was possible to identify both paternal and maternal alleles (table 2). Analysis of the segregation of mutations in family 1 showed that proband 1 is hemizygous owing to deletion of the maternal chromosome. Apparently the paternal chromosome of proband 2 is different from that of proband 1. In this family paternity has been assessed by the analysis of a polymorphic minisatellite system of the phenylalanine hydroxylase gene, according to Goltsov *et al*²⁷ (data not

shown), thus suggesting a recombination event in one of the two sibs.

Discussion

To date a complete characterisation of the CYP21 gene in patients with 21OH deficiency by direct sequencing has been considered as the only totally inclusive strategy for analysing the CYP21 gene. In this paper, we describe an SSCP approach in which use is made of minigel separation together with non-radioactive detection of the single strands. One hundred percent detection of CYP21 gene sequence changes in our panel of patients and their parents was achieved through the analysis of PCR products ranging between 238 and 386 bp, by slightly modifying some of the electrophoretic conditions. In single 21OH deficient alleles one or two disease causing mutations were identified together with several polymorphisms with up to as many as 13 different variants detected per allele in one patient. If we exclude a duplication event, which accounts for only about 6% of Italian CAH chromosomes,²⁸ this method proved to be sufficiently efficient to characterise the CYP21 gene completely. This may be a more suitable method for use in clinical laboratories, since it is faster and reduces the number of samples to be sequenced, in comparison with total direct sequencing.

To the best of our knowledge, the C→T transition at base 849 has not been described previously. This variant does not segregate with

Figure 2 Schematic representation of the mutations and polymorphisms identified in the human CYP21 gene. Exons are numbered and represented by boxes, introns by a line.

the disease in the family in which it has been identified, but since it apparently abolishes a second splicing site located 30 bp downstream of the first splicing site, the functional significance of this finding needs further investigation.

The complete genotyping of mutant CYP21 alleles obtained by this approach was used to attempt a genotype/phenotype correlation. In particular, proband 1 is hemizygous for the I172N mutation, which was shown by several groups to exhibit extremely low in vitro residual enzymatic activity.^{15 26} Proband 3 was a compound heterozygote for the I172N and Q318X mutations, with a truncated protein generated in the latter case.²⁶ In both cases, the observed genotypes are compatible with the SV form of disease shown by the patients. Proband 4, who is affected by the SW form, was found to be homozygous for the I2 splice mutation and heterozygous for cluster E6 mutations, with no residual enzymatic activity detected in either case after in vitro transfection.⁸ Patients 5 and 6, who developed an SV phenotype, proved to be homozygous for the splicing mutation in intron 2, with patient 5 also heterozygous for the I172N mutation.

On the other hand, there were two subjects for whom it was difficult to find any clear genotype/phenotype correlation. Proband 2 in family 1 is particularly interesting; although he was diagnosed as a simple viriliser from the genotype analysis performed in this study he was found to be only heterozygous for the I172N mutation. Moreover, the mother in family 3 was found to be homozygous for the I2 splice mutation. We could not perform the ACTH test since she would not give her consent. However, she is clinically asymptomatic, shows mild hirsutism, and has no history of infertility. In this case the existence of silent splicing sites within intron 2 could only partially explain the presence of a normal phenotype.²⁹ On the other hand, in both proband 2 and in the mother of family 3, some modifications may have occurred in the regulatory regions, thereby accounting for the clinical outcome.

Although the DNA-SSCP method described here proved to be an efficient system for characterising the CYP21 gene in patients affected by 21OH deficiency, it seems that there was insufficient genotyping of CYP21 mutant alleles to make a clear genotype/phenotype correlation. It therefore seems worthwhile to extend the genotyping to the promoter region also. Indeed it was recently reported that one or more of the 35 base differences between the upstream regions of the gene and those of a pseudogene could modify a recognition site which is fundamental for transcription activation.^{30 31} A cryptic CYP21 promoter element has also been proposed to be located in intron 35 of the human C4A gene.³² The DNA-SSCP method could be useful for screening the regulatory region of CAH alleles. Work on this regulatory region is at present in progress in our laboratory.

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