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ORIGINAL INVESTIGATION

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Characterization of mutations on the rare duplicated C4/CYP21 haplotype in steroid 21-hydroxylase deficiency

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Abstract We have defined the mutations causing congenital adrenal hyperplasia in three Swedish patients carrying a rare haplotype containing two mutated steroid 21-hydroxylase genes (CYP21) in addition to one pseudogene (CYP21P). The presence of such haplotypes complicates genetic diagnosis and screening of mutations in 21-hydroxylase deficiency, and we show how these genotypes can be resolved by amplification and analysis of each gene separately. In all cases, the rare haplotype carried the same combination of disease-causing mutations; one of the genes had the splice mutation at base 659 in intron 2, and the other had the nonsense mutation at base 1999 in exon 8 (CAG to TAG). We have thus characterized the most common haplotype containing duplicated CYP21 genes. The frequency of this haplotype is low, and if additional such haplotypes are present, they are rare in this population.

Introduction

Lesions in the gene encoding steroid 21-hydroxylase (CYP21) disrupt adrenal steroid hormone synthesis, the severe forms being known as congenital adrenal hyperplasia. The symptoms of this recessive syndrome are caused by a deficiency of aldosterone and cortisol, together with an excess of androgens, and include electrolyte disturbances, virilization of external genitalia, and growth acceleration in children. The steroid 21-hydroxylase locus has a complicated structure. Most often, one CYP21 gene and one highly homologous pseudogene, CYP21P, are present 3′ of each of the two genes encoding the fourth component of complement, C4A and C4B, forming tandemly repeated units in the HLA class III gene region on chromosome 6p21.3. However, there is a high degree of variability between haplotypes in the number and structure of these C4/21-hydroxylase (21OH) repeat units (Collier et al. 1989; Haglund-Stengler et al. 1991). The active steroid 21-hydroxylase gene and its pseudogene are highly homologous. Several sequences, including sequences that inactivate the putative protein, have been assigned as pseudogene-specific. However, sequences are occasionally transferred between CYP21 and CYP21P, further complicating analysis of this locus. The complete absence of CYP21 and sixteen sequence aberrations have been implicated in the enzyme deficiency (White et al. 1985; Amor et al. 1988; Globerman et al. 1988; Higashi et al. 1988; Speiser et al. 1988; Chiou et al. 1990; Mornet et al. 1991; Tusie-Luna et al. 1991; Helmberg et al. 1992; Owerbach et al. 1992; Wedell et al. 1992; Wedell and Luthman 1993a, b). Most of the mutations are normally present in CYP21P. Diagnosis of steroid 21-hydroxylase deficiency can be performed by genotyping using an allele-specific polymerase chain reaction (PCR) (Wedell and Luthman 1993b), and the degree of enzyme deficiency shows good correlation to the genotype (Speiser et al. 1992; Wedell et al. 1992). The existence of haplotypes carrying duplicated C4/CYP21 repeat units complicates genotyping. This has been found in three 21-hydroxylase deficiency patients in Sweden. Here we show how genotypes can be resolved in these patients and in cases when parents are not available for examining the segregation of mutations.

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Materials and methods

Subjects

The gross structure of C4/21OH haplotypes in families segregating 21-hydroxylase deficiency was determined by Southern blotting and densitometry scanning (Haglund-Stengler et al. 1991). A total of 87 disease-causing and 34 normal haplotypes were analyzed. Three 21-hydroxylase deficiency patients viz., B58, B88, and B98, were further studied, since they carried a rare C4/21OH haplotype assigned as no. 9 (Haglund-Stengler et al. 1991). This is the only

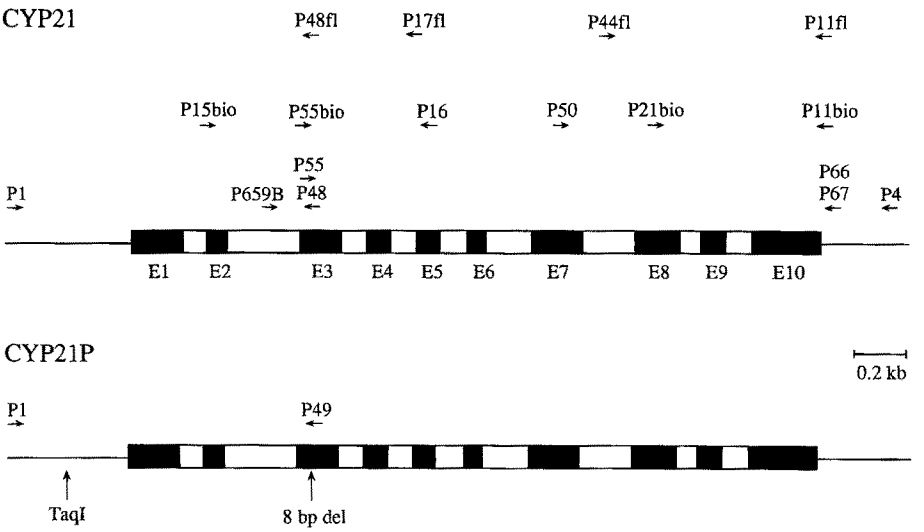

Fig. 1 Schematic illustration of the steroid 21-hydroxylase genes. *Black boxes* represent exons and *white boxes* are introns. *Arrows* indicate positions and orientations of primers for PCR and DNA sequencing (see Table 1). For CYP21P, the *TaqI* site and the 8-bp deletion used for selection are shown, together with the CYP21P specific primer P49

Table 1 Sequences and positions of primers used for PCR and sequencing of 21OH genes. Base numbering of CYP21 is identical to that reported by Rodrigues et al. (1987). Numbering of CYP21P follows CYP21, and thus lacks bases 711–718. The prefix bio indicates that the primer is biotin-labeled at the 5′ end, and fl denotes fluorescein labeling

Primer	Sequence 5′-	Position	Comment
P1	TTC AGG CGA TTC AGG AAG GC	–418–(–399)	
P4	TCT CGC ACC CCA GTA TGA CT	2911–2892	
P11bio	bio- GGA GCA ATA AAG GAG AAA CTG A	2763–2742	
P11fl	fl- GGA GCA ATA AAG GAG AAA CTG A	2763–2742	
P15bio	bio- TGG TGC TGA ACT CCA AGA GG	306– 325	
P16	GTC CAC AAT TTG GAT GGA CCA	1207–1187	
P17fl	fl- GTG GCT CCA GGT TTT TAA CAC	1186–1166	
P21bio	bio- GAG GAG CTA GAC CAC GAA CT	2002–2021	
P44fl	fl- TGT GGG CTG CTG GGG CAG	1943–1960	
P48	CAG AGC AGG GAG TAG TCT C	729– 711	Specific for CYP21
P48fl	fl- CAG AGC AGG GAG TAG TCT C	729– 711	Specific for CYP21
P49	TTT CCA GAG CAG AGA CCA AC	733– 706	Specific for CYP21P
P50	CCT CTC CTG GGC CGT GGT	1744–1761	
P55	CCT GTC CTT GGG AGA CTA CT	700– 719	Specific for CYP21
P55bio	bio- CCT GTC CTT GGG AGA CTA CT	700– 719	Specific for CYP21
P66	GTA CCC GGC TGG CAT CG	2740–2724	Allele-specific
P67	GTA CCC GGC TGG CAT CA	2740–2724	Allele-specific
P659B	CAC CCT CCA GCC CCC AA	643– 659	Allele-specific

haplotype characterized in the Swedish population with three C4/21OH repeat units in which two contain CYP21 genes and only one contains CYP21P. In total, these patients carried 3 CYP21 genes each and all were obligate carriers of disease-causing mutations. All patients were female and unrelated for at least 3 generations. B88 was diagnosed at 6 years of age because of growth acceleration and pseudoprecocious puberty. The mother stated, however, that the clitoris had been enlarged from birth. B58 and B98 were diagnosed neonatally because of severe virilization. Both showed signs of salt-wasting; B58 had sodium levels of 127 m*M* and potassium levels of 6.1 m*M*, whereas for B98, the corresponding levels were 126 m*M* and 8.1 m*M*, respectively.

PCR

Genomic DNA was prepared from peripheral leukocytes; 100 ng DNA were used for each 50-µl amplification. Primers P48 and P55 correspond to the region around the 8-bp sequence that is deleted in CYP21P (Fig. 1, Table 1), and were used to select against pseudogenes. A first round of PCR was performed using primer P1 together with P48, and P55 together with P4 (Fig. 1, Table 1), at concentrations of 0.3 µ*M*. An aliquot of 1 µl of the PCR products was used for a second PCR, utilizing one unlabeled and one biotin-labeled primer at concentrations of 0.1 µ*M*. These primer pairs were P15bio/P48, P55bio/P16, P50/P11bio, and P21bio/P4 (Fig. 1, Table 1). All amplifications were performed for thirty cycles at 96° C for 1 min, 56° C for 30 s, and 72° C for 3 min, in a buffer containing 1.0 m*M* $MgCl_2$, 0.2 m*M* dNTP, 10 m*M* TRIS-HCl pH 8.4 at 70° C, 0.1% Tween 20, and 0.2 U *Taq* polymerase. A concentration of 20 m*M* KCl was used for the P1/P48 fragment, since this gives more equal amplification of the different alleles of heterozygotes in this region. In all other amplifications, 45 m*M* KCl was used. Allele-specific amplification of full-length CYP21 genes was performed with primers P1/P66 and P1/P67 after removing CYP21P by *TaqI* cleavage. P66 and P67 discriminate between alleles polymorphic at position 2724, 12 bases downstream of exon 10 (Figs. 1, 4, Table 1). To confirm the usefulness of the *TaqI* site at –210 as a marker for CYP21P in these patients, DNA was amplified with primers P1/P48 and P1/P49, and the produced fragments were submitted to *TaqI* cleavage. Primer P49 covers the pseudogene sequence corresponding to P48 of CYP21 (Fig. 1). Allele-specific amplification of CYP21 genes with the wild-type sequence at position 659 was performed with primers P659B/P4 (Figs. 1, 4, Table 1). Conditions for allele-specific amplification were as above, except that annealing was performed at 59° C for

P66, 57°C for P67, and 61°C when primer P659B was used. The extension time for the P1/P66 and P1/P67 fragments was 15 min; a shorter extension time has previously failed to generate the desired full-length 3.3-kb CYP21 gene reproducibly (Wedell et al. 1992).

Direct sequencing

The biotinylated PCR fragments were immobilized by binding to streptavidin-coated magnetic beads (Dynabeads, Dynal, Oslo, Norway), and the non-biotinylated strands were removed by incubating in 50 µl 0.15 *M* NaOH for 5 min at room temperature (Hultman et al. 1989). The remaining DNA was rinsed 3 times, suspended in 13 µl distilled water, and sequenced by the chain-termination method, using the Auto-Read Sequencing kit (Pharmacia, Sweden). Sequencing was performed with fluorescence-labeled primers P48fl, P17fl, P44fl, and P11fl (Fig. 1, Table 1), and the products were analyzed using the Pharmacia A.L.F. DNA Sequencer. All primers were synthesized on a Gene Assembler Plus (Pharmacia, Sweden). Labeling of primers with biotin or fluorescein was performed by incorporating BioDite or FluorePrime phosphoamidites (Pharmacia, Sweden), respectively, during synthesis. Fluorescence-labeled primers were purified by reversed phase chromatography on a PepRPC column (Pharmacia FPLC System, Pharmacia, Sweden).

Results

Patient B88

The C4/21OH haplotypes of this family have been described (Haglund-Stengler et al. 1991) and are presented schematically in Fig. 2, together with relevant sequence information. Direct sequencing of CYP21 from B88 revealed the splice mutation at base 659, 13 bases upstream of exon 3 (Higashi et al. 1988, 1991), the 173 Ile to Asn mutation at base 1004 (T to A) in exon 4 (Amor et al. 1988), and the nonsense mutation at base 1999 (C to T), causing a stop codon at Gln 319 in exon 8 (Globerman et al. 1988). Three heterozygous polymorphisms, including (C/T) at position 2724, were also present. Sequencing of the father revealed the mutations at 659 and 1999, and the three polymorphisms, thus assigned to his haplotype no. 9. The mutation at base 1004 was traced to the mother. All other known disease-causing mutations were excluded by allele-specific PCR (Wedell and Luthman 1993b).

Patient B98

Hybridization patterns of the C4/21OH gene region in the patient and her parents were explained by C4/21OH haplotypes of type 9 and 1 in the father, two haplotypes of type 2 in the mother, and type 9 and 2 in the affected child (unpublished) (Fig. 3). When sequencing, the splice mutation at base 659, the nonsense mutation at base 1999, and the C/T polymorphism at base 2724 were found in the patient. The three CYP21 sequences of B98 were separated by allele-specific PCR and sequenced (Fig. 4). This demonstrated that one CYP21 gene had a T at position 2724 and the splice mutation at position 659, whereas two

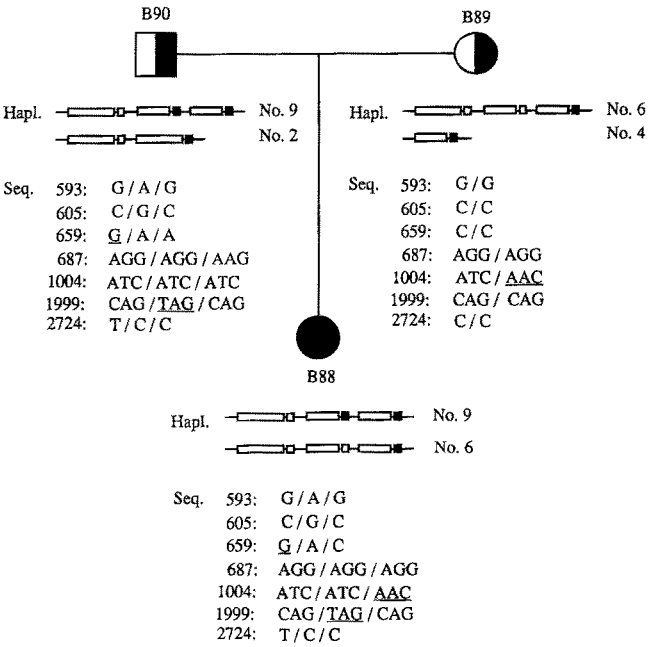

Fig. 2 Pedigree of the family of patient B88. *Hapl.* denotes C4/21OH haplotypes for the different individuals, determined as described (Haglund-Stengler et al. 1991). In short, *large white boxes* represent C4 genes (long or short variants), *small white boxes* are CYP21P genes, and *small black boxes* are CYP21 genes. The relative order of C4/21OH repeat units on each haplotype has not been determined. *Seq.* denotes the DNA sequence at various polymorphic nucleotides positions in CYP21. For positions involving amino acid changes, the whole codon is given (AGG/AAG at position 687 represents a neutral polymorphism). The sequences representing disease-causing mutations are *underlined*

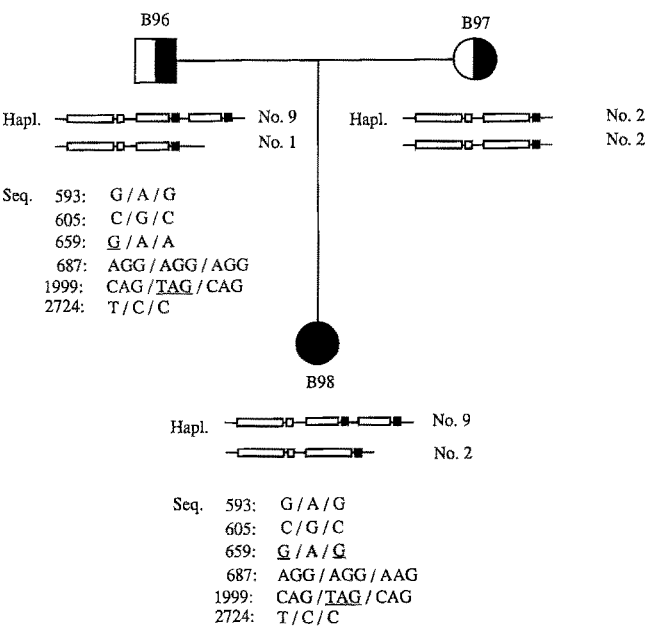

Fig. 3 Pedigree of the family of patient B98. For details, see legend to Fig. 2

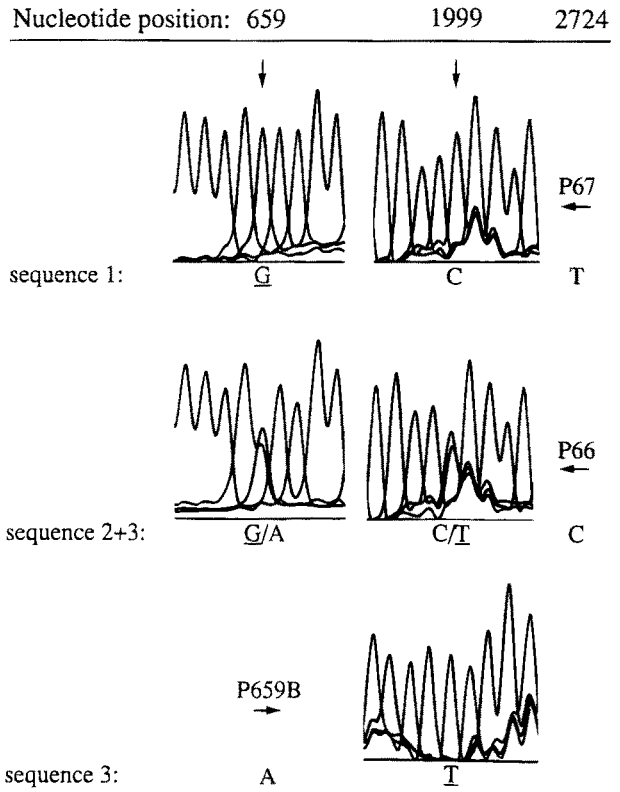

Fig. 4 Outline of the procedure for separation of the three CYP21 gene sequences of patients B58 and B98 using allele-specific PCR and direct DNA sequencing. *Horizontal arrows* represent PCR primers amplifying specific alleles; some DNA sequencing results, around the positions of the mutations, are shown. P67 is a PCR primer amplifying only alleles with a T at position 2724. As indicated, one such sequence was present in the two patients, carrying the splice mutation at position 659 *(G)* and wild-type sequence at position 1999 (C) *(sequence 1)*. P66 amplifies alleles with a C at position 2724. Two different DNA sequences were generated, heterozygous at position 659 (*G*/A) and at position 1999 (C/*T*) (sequences 2 + 3). Final separation was achieved using primer P659B, specifically amplifying the allele with the wild-type sequence at position 659 (A) *(sequence 3)*. Sequencing verified that this allele carried the nonsense mutation at position 1999 *(T)*

genes had a C at position 2724. In these two genes, the splice mutation at position 659 and the nonsense mutation at position 1999 were found in heterozygous form. The gene carrying the wild-type sequence at position 659 was further shown to carry the nonsense mutation at position 1999. Thus, patient B98 carried the three mutations 659, 1999, and 659, unambiguously assigned to each of her three CYP21 alleles. All other known disease-causing mutations were excluded by allele-specific PCR (Wedell and Luthman 1993b). Sequencing of the father verified that he carried the 659 mutation and the 1999 mutation on his haplotype no. 9. The mother was only characterized regarding her C4/21OH haplotypes, but is an obligate carrier of the 659 mutation.

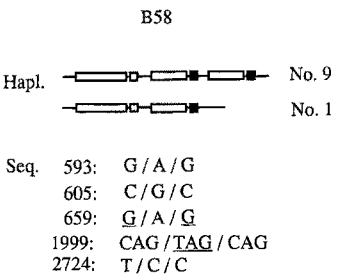

Fig. 5 C4/21OH haplotypes and CYP21 DNA sequence information for patient B58. Symbols are identical to those in Figs. 2, 3

Patient B58

The parents of this patient were not available for analysis. Hybridization patterns, however, can only be explained by the presence of haplotypes 9 and 1 (unpublished) (Fig. 5). The splice mutation at base 659 and the nonsense mutation at base 1999 were found, together with the heterozygous polymorphism (C/T) at position 2724. The same procedures for allele-specific PCR were performed as for patient B98 (Fig. 4), and patient B58 was shown to carry the mutations 659/1999/659, unambiguously assigned to each of three CYP21 alleles without the need of segregating chromosomes from the parents. All other known disease-causing mutations were excluded.

Discussion

The existence of chromosomes carrying two CYP21 genes with different sequences complicates genotyping in 21-hydroxylase deficiency. We have characterized the only haplotype that has been shown to carry duplicated CYP21 genes in the Swedish population. The sequence of this haplotype was conserved in all three cases found. The risk of encountering patients with more than two CYP21 genes, involving combinations of mutations other than the one described, is thus small. We have screened for CYP21 mutations among 186 unrelated chromosomes from patients affected by 21-hydroxylase deficiency. Only patients B58, B88, and B98 carried the described combination of mutations. Consequently, this haplotype accounts for less than 2% of the disease-causing chromosomes in this population.

Obviously, the correct prediction of clinical outcome by genotyping is dependent on the total combination of mutations in this recessive condition. The nonsense mutation at base 1999 is an obvious cause of complete enzyme inactivation, and the splice mutation at position 659 is associated with the most severe salt-wasting form of the disease in the majority of cases. Accordingly, the 659/1999/659 combination of mutations resulted in the severe salt-wasting form of the disease in patients B58 and B98. The 173 Ile to Asn missense mutation at position 1004 is associated with a less severe phenotype, with neonatal virilization, but usually without salt-wasting.

This is in agreement with the findings in patient B88 carrying the 659/1999/1004 combination of mutations.

The splice mutation at base 659 is one of the most common gene lesions causing 21-hydroxylase deficiency. In all three cases here described, however, the allele carrying the splice mutation on the rare haplotype no. 9 also contained a polymorphism downstream of exon 10, a polymorphism that has not previously been encountered. The nonsense mutation at base 1999 is only responsible for approximately 2% of all defective CYP21 alleles in Sweden (unpublished). Thus, both CYP21 genes present on this rare haplotype represent rare sequences. It is hard to explain why certain haplotypes carry conserved sequences, whereas others seem to undergo a more rapid diversification. One possible explanation is that the three chromosomes described have the same ancestry, and that they have been maintained because the structure with three C4/21OH repeat units has suppressed further genetic recombination. This would fit with the finding that the Val 282 to Leu mutation has, so far, been found exclusively associated with two other triplicated haplotypes, carrying two CYP21P and one CYP21 gene (Wedell et al. 1992).

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