

CLINICAL STUDY

Mutational spectrum of steroid 21-hydroxylase and the genotype-phenotype association in Middle European patients with congenital adrenal hyperplasia

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Abstract

Objective: To analyze the mutational spectrum of steroid 21-hydroxylase (CYP21) and the genotype–phenotype correlation in patients with congenital adrenal hyperplasia (CAH) registered in the Middle European Society for Pediatric Endocrinology CAH database, and to design a reliable and rational approach for CYP21 mutation detection in Middle European populations.

Design and methods: Molecular analysis of the CYP21 gene was performed in 432 CAH patients and 298 family members. Low-resolution genotyping was performed to detect the eight most common point mutations. High-resolution genotyping, including Southern blotting and sequencing was performed to detect CYP21 gene deletions, conversions, point mutations or other sequence changes.

Results: CYP21 gene deletion and In2 and Ile172Asn mutation accounted for 72.7% of the affected alleles in the whole study group. A good genotype–phenotype correlation was observed, with the exception of Ile172Asn and Pro30Leu mutations. In 37% of patients low resolution genotyping could not identify the causative mutation or distinguish homozygosity from hemizygoty. Using high-resolution genotyping, the causative mutations could be identified in 341 out of 348 analyzed patients. A novel mutation Gln315Stop was found in one simple virilising CAH (SV-CAH) patient from Austria. In the remaining seven patients polymorphisms were identified as the leading sequence alteration. The presence of elevated basal and ACTH-stimulated 17-hydroxyprogesterone, premature pubarche, advanced bone age and clitoral hypertrophy directly implicated Asn493Ser polymorphism in the manifestation of nonclassical- (NC) and even SV-CAH.

Conclusions: By genotyping for the most common point mutations, CYP21 gene deletion/conversion and the 8 bp deletion in exon 3, it should be possible to identify the mutation in 94–99% of the diseased alleles in any investigated Middle European population. In patients with a mild form of the disease and no detectable mutation CYP21 gene polymorphisms should be considered as a plausible disease-causing mutation.

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Introduction

Congenital adrenal hyperplasia (CAH) due to steroid 21-hydroxylase deficiency is one of the most common inborn endocrine disorders and is inherited in an autosomal recessive manner (1). Defects of the steroid 21-hydroxylase gene (CYP21) coding for the steroid 21-hydroxylase (21-OH) lead to various degrees of impaired cortisol and aldosterone synthesis and to androgen excess (1, 2). Deficiency of both cortisol and aldosterone synthesis results in salt wasting CAH (SW-CAH), usually manifesting early after birth as a salt wast-

ing crisis, with ambiguous external genitalia in girls. In simple virilising CAH (SV-CAH) manifesting with early precocious pubarche and different degree of virilisation of external genitalia in girls, only cortisol synthesis is deficient. In nonclassical CAH (NC-CAH) the disease is mild and might not be detected until later in life (2, 3). However, the spectrum of disease manifestations represents rather a continuum with no clear limits. The most likely mutations in the CYP21 gene originate from the more than 98% homologous CYP21P pseudogene (4, 5) and can be easily assayed on selectively amplified CYP21 gene sequences by allele-specific

amplification or by oligonucleotide hybridisation (6). However, the occurrence of alleles with changed gene copy number, the high frequency of compound heterozygotes having inherited a different mutation in each CYP21 allele, and alleles carrying multiple mutations complicate molecular genetic diagnostics (7). The severity of the disease is determined by the activity of the less severely affected of the two alleles but different phenotypes can be associated with the same mutation and the severity of clinical manifestations may vary within the genotype (8–10).

The Middle European Society for Pediatric Endocrinology (MESPE) has created a large database since 1998 containing information on 598 children with CAH from Austria, the Czech Republic, Hungary, Slovakia and Slovenia in order to study the endocrinological parameters, the efficiency of clinical diagnostics and the treatment and genetics of CAH in Middle European countries (11–13). The database is described in detail elsewhere (11).

The aim of this study was to systematically analyze the mutational spectrum and the genotype–phenotype correlation in CAH patients and their family members registered in the MESPE-CAH database. Furthermore, we aimed to design a rational approach for CYP21 mutation detection needed for reliable pre-, peri- and postnatal diagnostics and for genetic counseling in the Middle European countries and in ethnic groups originating from the region.

Subjects and methods

Study participants

Patients from the MESPE-CAH database and their family members were invited to participate in the study and written informed consent was obtained from all the participants. The respective national ethics committees for research in medicine approved the study design. The clinical and laboratory data on the patients were available on the database. The clinical diagnosis of different types of 21-OH deficiency was made by paediatric endocrinologists based on the history, physical examination, electrolyte and hormonal data (1, 14). Patients were considered to have the SW form when clinical and laboratory signs of renal salt wasting were present in the first month of life; the SV form was assigned if clinical symptoms of CAH without SW were present before the age of puberty onset, i.e. 8 years, and the NC form when symptoms of androgen excess became evident during or after puberty (11). Patients without reliable clinical information were excluded from the study.

Methods

National coordinators were asked to provide peripheral blood or genomic DNA samples of patients and their first-degree relatives for genotyping. Out of 598 CAH

patients from the five Middle European countries included in the MESPE-CAH database peripheral blood or genomic DNA samples were available from 476 patients (79.6%). Sequence analysis was used to detect CYP21 gene mutations in Austrian patients using the approach previously described (15). For patients from other countries a two-step genotyping approach was employed. Low-resolution genotyping for 8 common point mutations was performed using allele-specific polymerase chain reaction (PCR-SSP) and/or PCR and sequence specific oligonucleotide hybridisation (PCR-SSO) by participating centers (16–18). High resolution genotyping by PCR-SSP, PCR-SSO, Southern blotting and sequencing was performed in Ljubljana to detect CYP21 gene deletions, conversions, point mutations or other sequence changes when mutation could not be identified on one or both alleles by low resolution genotyping or when homozygosity could not be distinguished from hemizyosity. The genotyping approach is described in detail elsewhere (17, 18). The reference sequence reported by White *et al.* (4) was used for numbering of nucleotides and amino acids.

Results

In the present study we systematically genotyped 476 CAH patients from five Middle European countries. Additionally, 298 first-degree relatives (236 parents and 62 siblings) were genotyped to confirm allele segregation. The diagnosis of CAH could not be confirmed in 34 patients and 10 patients had 11 β -hydroxylase deficiency, therefore these patients were excluded from further study.

Using low-resolution genotyping mutations could not be identified or homozygosity could not be distinguished from hemizyosity in 160 out of 432 patients (37%). From 84 of these patients DNA was not available for further analysis. Using high-resolution genotyping, Southern blotting and sequencing the causative mutations could be identified in 341 out of 348 (98.0%) patients while in the remaining seven patients polymorphisms were identified as the leading sequence alteration.

A novel mutation Gln315Stop (Fig. 1) resulting in a premature stop codon which has not been described to date in CAH patients, was found in a patient from Austria. The girl was diagnosed at age 4.4 and was clinically SV. The sequencing analysis of both parents confirmed the inheritance of a Gln315Stop mutation from her mother and of a Pro30Leu mutation from her father.

Complete genotypes and clinical phenotypes of MESPE-CAH patients are shown in Table 1. Among 348 patients 221 (63.5%) presented with SW-CAH (92 boys and 129 girls), 92 (26.4%) had SV-CAH (35 boys and 57 girls) and 35 (10.1%) had NC-CAH (12 boys and 23 girls). One third of the patients were compound

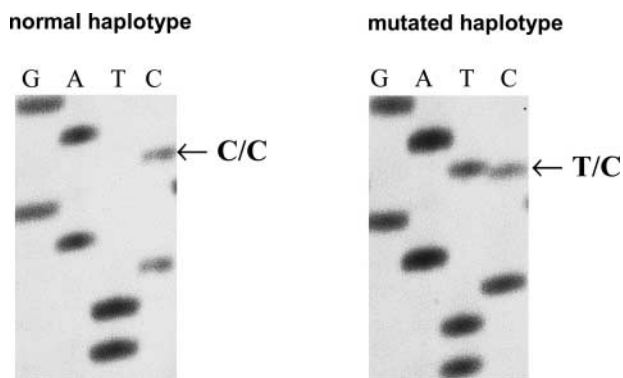


Figure 1 Sequence analysis of the Gln315 locus in exon 8 of the CYP21 gene. Arrowheads indicate the heterozygous nucleotide substitution (C → T) from the functionally normal CYP21 haplotype CAG to TAC, resulting in the change of Glutamine (Gln) to a premature stop codon.

heterozygotes, having inherited a different mutation in each CYP21 allele, 43.8% of all patients were homozygous and 22.9% were hemizygous for the mutation identified. The most common mutations found in SW-CAH were In2 (49.8%) and CYP21 gene deletion/conversion (29.9%). Ile172Asn was the most common mutation found in SV-CAH (59.8%), however it was also observed in 6.8% of SW-CAH as well as in 8.3% of NC-CAH patients. Other common mutations found in SV-CAH were Pro30Leu in 16.3%, a promoter conversion associated with Pro30Leu in 8.7% and In2 in 7.6% of SV patients. Together with Ile172Asn these mutations accounted for more than 90% of the SV form. The most common NC-CAH mutations were Val281Leu (41.7%), Pro30Leu (22.2%) and the Asn493Ser polymorphism (16.7%) together accounting for 80% of the NC-CAH. The Val281Leu mutation also represented the least severe mutation in 4.4% of SV-CAH patients.

To analyze the genotype–phenotype correlation, CYP21 gene mutations were categorized into four groups based on *in vitro* established enzyme activity as described previously (3, 8, 10). Alleles with multiple mutations were grouped according to the most severe mutation of the haplotype (Table 2). Although the impairment of enzyme activity for CYP21 gene conversion involving the promoter region alone or combined with Pro30Leu substitution has not been established *in vitro* these mutations were categorized in group B based on the previous reports of clinical phenotypes more severe than predicted in patients carrying these mutations (15, 18). As summarized in Table 2 a good genotype–phenotype correlation was observed in patients with severe mutations categorised in mutation groups 0 and A. Greater diversity of clinical phenotypes was observed in patients with less severe mutations, especially in carriers of Ile172Asn and Pro30Leu mutations, categorized in mutation groups B and C, respectively. In most patients the observed phenotype matched the severity of the less affected of the two alleles,

Table 1 Genotypes and clinical phenotypes in 348 MESPE patients with 21-hydroxylase deficiency.

CYP21 haplotype (allele 1/allele 2)	CAH phenotype		
	SW	SV	NC
Del/del or conv	61	0	1
Del/del 8ntG110 (ex3)	1	0	0
Del/InsT307	1	0	0
Del/Gln318Stop	1	0	0
Del/Arg356Trp	1	0	0
Del/Arg483GG → C	2	0	0
Small conv/conv	4	0	0
Del 8ntG110 (ex3)/del 8ntG110 (ex3)	3	0	0
Cluster ex6/cluster ex6	1	0	0
InsT307/small conv	1	0	0
InsT307/InsT307	2	0	0
InsT307/Gln318Stop	1	1	0
Gln318Stop/Gln318Stop	1	0	0
Arg356Trp/small conv	1	0	0
Arg356Trp/Arg356Trp	4	0	0
Arg483Pro/Arg483Pro	1	0	0
In2/del or conv	36	1	1
In2/In2	62	5	0
In2/InsT307	3	0	0
In2/Gln318Stop	4	1	0
In2/Arg483Pro	1	0	0
In2/Arg483GG + C	1	0	0
Prom conv + Pro30Leu/del or conv	1	5	2
Prom conv + Pro30Leu/del 8ntG110 (ex3)	1	0	0
Prom conv + Pro30Leu/In2	0	1	0
Prom conv + Pro30Leu/Ile172Asn	0	1	0
Prom conv + Pro30Leu/Gln318Stop	0	1	0
Ile172Asn/del or conv	6	16	1
Ile172Asn/In2	2	17	0
Ile172Asn/del 8 bp	0	1	0
Ile172Asn/Ile172Asn	4	14	1
Ile172Asn/Gln318Stop	2	3	1
Ile172Asn/Arg356Trp	1	3	0
Ile172Asn/Arg483GG + C	0	1	0
Pro30Leu/del or conv	1	4	2
Pro30Leu/Pro30Leu	0	1	1
Pro30Leu/In2	0	6	0
Pro30Leu/Ile172Asn	0	3	3
Pro30Leu/Val281Leu	0	0	2
Pro30Leu/Gln315Stop	0	1	0
Val281Leu/del or conv	1	2	6
Val281Leu/In2	0	2	3
Val281Leu/Val281Leu	1	0	1
Val281Leu/InsT307	0	0	2
Val281Leu/Gln318Stop	0	0	1
Val281Leu/Arg356Trp	1	0	0
Pro453Ser/del	1	0	1
Pro453Ser/In2	3	0	0
Prom conv/del or conv	3	1	0
Asn493Ser/del	0	1	1
Asn493Ser/Arg356Trp	0	0	2
Asn493Ser/Asn493Ser	0	0	2
Asn493Ser/Asn493Ser + T-107C	0	0	1
Total	221	92	35

Del, CYP21 gene deletion; large conv, large gene conversion; prom conv, gene conversion in promoter region; small conv, multiple mutations present on the same allele, conversion not detectable by PCR-SSP or Southern blot analysis; conv, large or small conversion; Ins, insertion; in2, intron 2 mutation (A,C656G); del 8ntG110, 8 bp deletion in exon 3.

Table 2 Genotype-phenotype correlation in MESPE-CAH patients.

Mutation group		Predicted phenotype	Observed phenotype		
Allele 1	Allele 2		SW	SV	NC
0	0	SW	81	1	1
A	0	SW	44	1	1
A	A	SW	64	5	0
B	0	SV	15	29	4
B	A	SV	4	21	0
B	B	SV	5	15	1
C	0	NC	4	4	12
C	A	NC	3	9	3
C	B	NC	0	4	3
C	C	NC	1	1	4
Not classified					
Gln315Stop	C		0	1	0
Asn493Ser	0		0	1	2
Asn493Ser	Asn493Ser		0	0	3
Lys102Arg Ser268Thr	0		0	0	1
Total			221	92	35

Mutation group 0, CYP21 gene deletion, large gene conversion, 8 bp deletion in exon 3, cluster mutation in exon 6, InsT307, Gln318Stop, Arg356Trp, Arg483Pro, Arg483GG → C, and multiple mutations alleles containing any of these mutations; mutation group A, intron 2 mutation; mutation group B, Ile172Asn, promoter conversion + Pro30Leu, promoter conversion; mutation group C, Pro30Leu, Val28Leu, Pro453Ser.

however the presence of a group 0 or A mutation on the second allele frequently resulted in a more severe clinical phenotype.

The allele frequencies of CYP21 gene mutations in MESPE-CAH patients were calculated based on systematic genotyping of 696 unrelated chromosomes. As shown in Table 3 only three mutations: CYP21 gene deletion, In2 and Ile172Asn were found to occur with a frequency higher than 10% each. Together they represented almost three quarters (72.7%) of the affected alleles in all the populations studied. Country-specific differences were observed in the frequencies of a particular mutation. The frequency of CYP21 gene deletion was 27.1% in the whole study group and ranged from 37.2% in Slovenian patients to 13.9% in Slovak patients. The In 2 mutation with the allele frequency of 31.1% in the whole study group was the most prevalent mutation in Slovak (50.0%) and in Hungarian patients (35.9%) and it was the lowest in Slovenian patients (16.7%). Similarly, Ile172Asn had the lowest allele frequency in Slovenian patients (7.7%) while this mutation's frequency was the highest in Austrian patients (22.5%).

Many diverse alleles with multiple mutations were found: 4.3% of all the alleles carried two mutations and 1.4% of alleles carried more than two mutations. The frequency of alleles with multiple mutations was highest among Slovenian patients (15.4% of all alleles), where the haplotypes were confirmed by analyzing the segregation of mutations in the families. The most common mutation occurring on the same haplotype with other mutations was In2 (2.1% of all alleles). Among 43 alleles with the Pro30Leu mutation only 26 (60.5%) represented a single nucleotide

substitution, while Pro30Leu and promoter conversion alone or with a third mutation occurred on 17 separate alleles (39.5%) and represented 2.4% of all alleles.

In seven patients polymorphisms were identified to be the leading sequence alteration as the direct sequencing of all exons, introns and the proximal promoter region of CYP21 gene did not reveal any putative new mutation. Among these patients three were homo- or hemizygous for Asn493Ser and three were compound heterozygotes for this substitution. The clinical characteristics of these patients are given in Table 4. Most of the patients with Asn493Ser presented with elevated baseline or ACTH-stimulated 17OHP levels, premature pubarche or advanced bone age and one girl had clitoral hypertrophy. Elevated basal 17OHP levels were also observed in the NC patient hemizygous for the Lys102Arg Ser268Thr polymorphism, who also presented with clitoral hypertrophy.

Discussion

Systematic analysis of the mutational spectrum of the CYP21 gene and the associated haplotypes was performed in 348 patients with CAH registered in the MESPE-CAH database. Among them 221 presented with SW-CAH, 92 with SV-CAH and 35 with NC-CAH. In a particular clinical form two to three different mutations were found in more than 80% of all haplotypes: In2 and CYP21 gene deletion/conversion in SW-CAH, Ile172Asn and Pro30Leu with or without promoter conversion in SV-CAH and Val281Leu, Pro30Leu and Asn493Ser in NC-CAH.

Table 3 CYP21 allele frequencies in different populations within MESPE-CAH study.

CYP21 alleles	Austrian		Czech		Hungarian		Slovak		Slovenian		Total	
	N	%	N	%	N	%	N	%	N	%	N	%
Del	40	33.3	55	28.4	60	22.2	5	13.9	29	38.2	189	28.4
Large conv			5	2.6	5	1.9			5	6.6	15	2.2
Prom conv			3	1.5	1	0.4	3	8.3			7	1.0
Pro30Leu	11	9.2	4	2.1	8	3.0			3	3.9	26	3.7
In2	26	21.7	63	32.5	97	35.9	18	50.0	13	17.1	217	31.2
Del 8ntG110 (ex3)			3	1.5			4	11.1			7	1.0
Ile172Asn	27	22.5	27	13.9	38	14.1	3	8.3	6	7.9	101	14.5
Cluster ex6			2	1.0							2	0.3
Val281Leu	4	3.3	3	1.5	15	5.6			2	2.6	24	3.4
InsT307					10	3.7			1	1.3	11	1.6
Gln315Stop	1	0.8									1	0.1
Gln318Stop	4	3.3	6	3.1	5	1.9	1	2.8	2	2.6	18	2.6
Arg356Trp	5	4.2	4	2.1	8	3.0					17	2.4
Pro453Ser	1	0.8			3	1.1			1	1.3	5	0.7
Arg483Pro	1	0.8									1	0.1
Arg483GG → C			6	3.1							6	0.9
Asn493Ser			6	3.1	2	0.7			2	2.6	10	1.4
Small conversions												
Prom conv + Pro30Leu			4	2.1	7	2.6			3	3.9	14	2.0
Prom conv + Pro30Leu + In2									1	1.3	1	0.1
Prom conv + Pro30Leu + Ala15Thr									2	2.6	2	0.3
Prom conv + del 8 bp							1	2.8			1	0.1
In2 + Ile172Asn			2	1.0	3	1.1					5	0.7
In2 + Val281Leu					2	0.7					2	0.3
In2 + Val281Leu + Gln318Stop					1	0.4	1	2.8			2	0.3
In2 + Arg356Trp			1	0.5	1	0.4					2	0.3
In2 + Ex6 + Val281Leu + Gln318Stop									2	2.6	2	0.3
In2 + Val281Leu + Gln318Stop + Arg356Trp					1	0.4					1	0.1
Ex 4-ex8 conversion					1	0.4					1	0.1
Ile172Asn + ex6 + Pro453Ser					1	0.4			1	1.3	1	0.1
Val281Leu + InsT307 + Gln318Stop + Arg356Trp					1	0.4					1	0.1
InsT307 + Gln318Stop									3	3.9	3	0.4
InsT307 + Arg356Trp					1	0.4					1	0.1
Total small conversion alleles			7	3.6	18	6.7	2	5.6	12	15.6	39	5.3
Total alleles	120	100.0	194	100.0	270	100.0	36	100.0	76	100.0	696	100.0

Del, CYP21 gene deletion; large conv, large gene conversion; prom conv, gene conversion in promoter region; In2, intron 2 splice site mutation (A, C656G); del 8ntG110, 8 bp deletion in exon 3; ex6, cluster mutation in exon6; Ex4-ex8 conversion, Ile172Asn + ex6 + Val281Leu + InsT307 + Gln318Stop + Arg356Trp.

We found a Gln315Stop substitution in one SV-CAH patient from Austria. This mutation has not been described to date in CAH patients and results in a premature stop codon. Although a functional assay has to be performed to determine the residual enzyme activity of this mutation *in vitro* we assume that such a mutation would completely abolish enzyme activity, similarly to the nearby Gln318Stop mutation. This would also explain a SV phenotype in this patient carrying Pro30Leu mutation on the less affected haplotype.

The large number of patients included in this study enabled us to analyze the genotype–phenotype correlation. In general a good genotype–phenotype correlation was observed in patients with severe mutations, however carriers of the Ile172Asn and Pro30Leu mutations on the less affected allele displayed a greater variability of clinical phenotypes. This phenotypic variability of Ile172Asn mutation has also been

reported in other European populations showing an otherwise good correlation between the genotype and phenotype (3, 10, 19, 20). Similarly, many investigators have reported phenotypes more severe than expected in carriers of the Pro30Leu mutation (9, 10, 15, 18, 21). The finding of the Pro30Leu mutation linked to a gene conversion in the promoter region or with an additional mutation, Ala15Thr, could explain the increased severity of phenotypes in Slovenian patients (18). The *in vitro* expression analysis did not demonstrate the impairment of enzyme activity by Ala15Thr mutation alone (22) but it is not excluded that this mutation has a synergistic effect on 21-hydroxylase activity when combined with the Pro30Leu mutation and/or gene conversion in the promoter region (23).

The relatively slight differences in the distribution of CYP21 mutations observed between individual countries within the Middle European region suggest

Table 4 Clinical characteristics of patients carrying polymorphic alleles.

No.	Sex	Year of birth	Genotype	Clinical phenotype	Age at dg (years)	Pubarche age (years)	Virilization of external genitalia	17OHP 0 > min (nmol/l)	17OHP 60 min (nmol/l)	Bone age (years)
CZ8	M	1980	Del/Asn493Ser	SV	2.1			*		+4
H56	F	1978	R356W/Asn493Ser	NC	8.1	6.5	CH	51	159	+1.5
H63	M	1979	R356W/Asn493Ser	NC	7.3	5		34	351	+2
CZ6	M	1986	Asn493Ser/Lys102Arg	NC	8.9	8		3.9	36.4	+1.6
SLO37	F	1995	T-107C Asn493Ser/T-107C	NC	5	5	None	10.7	45	+2
CZ13	F	1965	Asn493Ser/Asn493Ser	NC	ND	ND	None	ND	ND	ND
CZ10	F	1982	Del/C-4T, Lys102Arg, Ser268Thr	NC	15.5	#	CH	66	ND	ND

M, male; F, female; SV, simple virilising; NC, non-classical; CH, clitoral hypertrophy; 17OHP 0 min, basal serum 17-hydroxyprogesterone; 17OHP 60 min, serum 17-hydroxyprogesterone 60 min after ACTH stimulation; *, urinary 17-ketosteroids; 19.47 µmol/l; #, primary amenorrhoea; ND, no data.

that, despite differences in ethnic origins, similar genetic mechanisms were probably involved in the transmission of the disease. A possible explanation for this may be a high degree of ethnic mixing over the centuries in some populations (16). The observed frequencies of the CYP21 gene deletion (range 13.9–37.2%) and In2 mutation (range 16.7–50.0%) were comparable to the frequencies observed in most of the other European countries. In CAH patients from the Middle European populations studied, the frequencies of the Val281Leu mutation (range 1.5–5.6%) were lower than in Italy, France and Spain (range 11.0–16.7%), but comparable to other European countries (range 2.2–5.7%). The frequencies of Pro30Leu in Middle European populations (range 2.1–9.2%) were higher, particularly in Austrian CAH patients than in other European populations (range 0.3–2.7%) (8, 10, 20, 24–26).

A high frequency of alleles with multiple mutations was found: 5.6% of the alleles in the whole study group and 15.4% of all the alleles in Slovenian patients carried two or more mutations. The clustering of point mutations on one allele has been previously reported in 1.9% of unrelated alleles in Dutch patients (20). The relatively high frequency of alleles with multiple mutations in Middle European patients compared to some other single point mutations considered to be common in CAH, such as Gln318Stop (2.6%) and Arg356Trp (2.4%), demonstrates the importance of including the parents of the index case in the molecular analysis if segregation of alleles needs further determination. If families are not available, mutations can be allocated to separate alleles by allele-specific PCR amplifications (8).

By low-resolution genotyping for the eight most common CYP21 point mutations, mutations could not be identified or homozygosity could not be distinguished from hemizyosity in 37% of patients. Using high-resolution genotyping, PCR-SSP amplification and Southern blotting for the detection of CYP21 gene deletion and large gene conversions only 2% of alleles remained unidentified. Among these, direct sequencing of CYP21 identified Asn493Ser substitution as the leading sequence change in six patients. Some authors describe this substitution as a naturally occurring polymorphism (27) and some as a disease causing mutation (28) but its influence on residual enzyme activity has never been analyzed *in vitro*. In Mexican CAH patients a higher proportion of homozygosity for the Asn493Ser substitution was observed than in a healthy population (29). It was proposed that a synergistic effect between two mutations could lead to decreased enzymatic activity in CAH patients homozygous for Ser268Thr and Asn493Ser (30, 31), but, except in one patient with a T-107C substitution and one patient with concomitant Lys102Arg on one haplotype, the sequencing of all the exons, introns and 340 nucleotides of the proximal promoter region

revealed no other sequence alteration on alleles characterised by Asn493Ser. The presence of elevated basal and ACTH-stimulated 17OHP, premature pubarche, advanced bone age in all patients and also clitoral hypertrophy in one girl implicates a direct role of this substitution in the disease manifestation of NC and even SV-CAH.

Lys102Arg and Ser268Thr mutations were also reported to be normal polymorphisms, however, a synergistic effect resulting in a decreased enzymatic activity was observed when both mutations were transmitted on the same haplotype (30, 31). This could explain the finding of clitoral hypertrophy and elevated basal 17OHP levels in one NC patient hemizygous for Lys102Arg Ser268Thr substitutions.

In conclusion, our genotyping approach allowed accurate and sensitive identification of CYP21 gene mutations in CAH patients and their families and offered reliable information needed for diagnostics and for adequate genetic counselling in the Middle European countries and in ethnic groups originating from the region. A novel Gln315Stop mutation was identified. By genotyping for the most common point mutations, CYP21 gene deletion/conversion and the 8 bp deletion in exon 3, it should be possible to identify the responsible mutation in 94–99% of the diseased alleles in any investigated population. In patients with a mild form of the disease and a normal genotype at all positions, CYP21 gene polymorphisms such as Asn493Ser should be considered as a plausible disease causing mutation.

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