CLINICAL STUDY

Mutational spectrum of congenital adrenal hyperplasia in Slovenian patients: a novel Ala15Thr mutation and Pro30Leu within a larger gene conversion associated with a severe form of the disease

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Abstract

Objective: To analyse the mutational spectrum, the associated haplotypes and the genotype–phenotype correlation, and to design a reliable and rational approach for CYP21 mutation detection in Slovenian congenital adrenal hyperplasia (CAH) patients.

Design: Molecular analysis of the CYP21 gene was performed in 36 CAH patients and 79 family members.

Methods: Southern blotting, sequence-specific PCR amplification (PCR-SSP), sequence-specific oligonucleotide hybridisation (PCR-SSO) and sequencing were used to detect CYP21 gene deletions, conversions and point mutations.

Results: CYP21 gene deletion was the most frequent mutation (36.4%). Large gene conversions detectable only by Southern blotting represented 12.1%, and gene conversions involving the promoter region represented 7.6% of the mutated alleles. The most frequent point mutations were: intron 2 splice mutation 16.7%, Ile172Asn mutation 7.6%, Gln318Stop 7.5% and Pro30Leu 12.2% of alleles. A correlation between the genotype and the clinical phenotype similar to those described for large populations was observed. The finding of Pro30Leu mutation linked to a gene conversion could explain the simple virilising (SV) phenotype in compound heterozygotes for the Pro30Leu and a severe mutation. In two siblings with a salt wasting form of CAH (SW-CAH), a novel mutation Ala15Thr was found on the allele characterised by Pro30Leu mutation and gene conversion involving the promoter region.

Conclusions: Our genotyping approach allowed reliable diagnosis of CAH in the Slovenian population. The high frequency of CYP21 gene aberrations on Pro30Leu positive alleles justified systematic searching for a gene conversion in the promoter region using the PCR-SSP reaction.

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Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders caused by defective adrenal steroidogenesis. More than 90% of the cases are due to mutations in the cytochrome P450 21-hydroxylase gene (CYP21) coding for the steroid 21-hydroxylase (21-OH) (1). Inherited defects of this enzyme, required for conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol, lead to various degrees of impaired cortisol and aldosterone synthesis and to androgen excess (1, 2). If 21-OH is absent or completely inactive the disease manifests as a salt wasting form (SW-CAH) deficient in cortisol and aldosterone

synthesis. The symptoms of SW-CAH manifest early after birth - in girls with ambiguous external genitalia, in boys often as a salt-losing crisis. In the simple virilising form of CAH (SV-CAH) aldosterone synthesis is sufficient. Different extents of virilisation of the external genitalia are present in girls, while in boys early precocious puberty is usually the first sign of disease. In non-classical CAH (NC-CAH) the disease is mild and usually not detected until later in life (2, 3).

The extent of enzyme impairment and the clinical phenotype of the disease is determined by the severity of the genetic defect in the CYP21 gene (3). The active gene, CYP21, is 98% homologous to the pseudo gene, CYP21P, which is non-functional due to

several mutations (4, 5). Both genes are located within the HLA class III region on chromosome 6p21.3 and form two C4/CYP21 units with adjacent complement C4A and C4B genes (6). High sequence identity and close proximity of the two genes facilitate gene recombination and gene conversion events in this region, resulting in gene deletions, duplications or large gene conversions (7). Random point mutations may occur but are rare in CYP21 (8). The most likely mutations defined by those pre-existing in the CYP21P pseudo gene can easily be assayed on selectively amplified CYP21 gene sequences by allele-specific amplification or by oligonucleotide hybridisation. However, the frequent recombination events in this region resulting in alleles with changed gene copy number or in alleles carrying multiple mutations complicate molecular genetic diagnosis. Additionally, many patients with CAH are compound heterozygotes, having inherited a different mutation in each CYP21 allele. The severity of disease is determined by the activity of the less severely affected of the two alleles; however more than one clinical phenotype can be associated with some of the mutations (4, 9–11).

A study on the incidence of 21-OH deficiency in Slovenian hyper androgenic women pointed out that the mutational spectrum of the CYP21 gene in the Slovenian population might differ from the surrounding populations (12). The observed frequency of carriers of CYP21 gene conversion/deletion was much higher, but the frequency of the Val281Leu mutation was much lower than in a comparable group of Italian hyper-androgenic women (13).

The aims of this study were to analyse systematically the mutational spectrum and the associated haplotypes, as well as the genotype–phenotype correlation, in CAH patients and their family members registered in Slovenia, and to design a reliable and rational approach for CYP21 mutation detection needed for the purpose of pre-, peri- and postnatal diagnosis and for genetic counselling in our population.

Patients and methods

Since 1971, 41 patients with CAH have been treated and followed up at the University Children's Hospital in Ljubljana. Among them, 33 families with 36 patients and 79 first degree relatives (16 siblings and 63 parents), in total 115 subjects, agreed to participate in the study. Written informed consent was obtained from all participants. The study design was approved by the Slovene Medical Ethics Committee (#18-4-95).

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 with inadequate steroid hormone synthesis were defined as having the SW form when clinical and laboratory signs of renal

salt wasting were present in the first months of life, the SV form if virilisation appeared before the age of 4 years and the late onset or non-classical (NC) form when the signs and symptoms of androgen excess became evident after the age of 4 years.

Molecular analysis of CYP21 gene conversions/deletions

DNA was isolated from peripheral blood leukocytes by the salting out method (15). CYP21 and C4 gene deletions, duplications and large gene conversions were analysed by Southern blot hybridisation of TaqI restricted genomic DNA with CYP21 and C4 gene-specific cDNA probes as previously described (12). Because only TaqI digests were analysed, gene conversions involving the TaqI restriction site were not distinguishable from deletions of the CYP21 gene. Deletions/conversions of CYP21 were identified by the decreased intensity of the 3.7 kb TaqI fragment as compared with the 3.2 kb TaqI fragment (Fig. 1) (16).

To further characterise CYP21 gene rearrangements, two PCR reactions with sequence-specific primers (PCR-SSP) were used (Fig. 2). In the PCR-SSP-A reaction the presence of chimeric CYP21 gene originating from a 'classical' deletion of 30 kb including the 5' part of CYP21 with a breakpoint between exons 7 and 8, as well as large gene conversions involving

Figure 1 Southern blot hybridisation of TaqI digested DNA hybridised with CYP21 and C4 probes. On the left side, the size of the fragments of radio-labelled standards (S) are shown in kilo bases (kb). On the right side, the fragment lengths in kb and their association with the corresponding gene are shown. N1, N2 - typical results of patients with two CYP21 genes; N1 has two 6 kb C4B fragments while N2 has one 6 kb and one 5.4 kb C4B fragment. P1 - a SW patient. The absence of 3.7 kb TaqI band indicates a CYP21 gene conversion/deletion, decreased intensity of the 6 kb TaqI fragment as compared to the 7 kb band indicates that this conversion/deletion also encompasses the C4B gene. P2 - a SW patient. The absence of the 3.7 kb TaqI band indicates a CYP21 gene conversion/deletion which does not involve the C4B gene as indicated by the equal intensity of 6 kb C4B and 7 kb C4A fragments. F - obligate carrier (father of P1). The decreased intensities of the 3.7 kb CYP21 fragment as compared to the 3.2 kb CYP21P fragment indicate a conversion/deletion of one CYP21 allele.

Figure 2 PCR-SSP reactions differentiating between CYP21 gene deletion and gene conversion in the promoter region. (A) PCR-SSP-A. 1, amplification with CYP21-specific forward and CYP21-specific reverse primers was used to amplify the normal sequence. 2, amplification with CYP21P-specific forward and CYP21-specific reverse primers. The presence of 1800 bp PCR product indicates the presence of a 'classical' deletion of 30 kb including the 5' part of CYP21 with a break point between exons 7 and 8, or a large gene conversion involving exon 6 as described in the methods section. S, 1 kb ladder; F, father; M, mother; P1, patient 13; P2, patient 14. (B) Similarly, a PCR-SSP-B reaction was set up to detect CYP21P/CYP21 recombinations in the region between –302 and exon 3 in order to establish whether the presence of the TaqI restriction site in CYP21 is due to a gene deletion or a conversion event in this region, as described in the Patients and methods section. 1, amplification with CYP21-specific forward and CYP21-specific reverse primers was used to amplify the normal sequence. 2, amplification with CYP21P-specific forward and CYP21-specific reverse primers was used to detect the allele with a recombination in the promoter region. S, 1 kb ladder; F, father; M, mother; P1, patient 13; P2, patient 14.

exon 6 were detected using the approach described by Chu *et al.* (17). Upon a presence of a recombination site a fragment of 1800 bp spanning from exon 6 to 3' untranslated region was amplified using a CYP21P-specific forward (ex6PF: 5'-GCCATAGAGAAG-AGGGACCACAA) and CYP21-specific reverse (ex10R: 5'-TTAAGCCTCAATCCTCTGCAGCG) primer. An homologous, but CYP21-specific forward primer (ex6NF: 5'-GCCATAGAGAAGAGGGATCACAT) was used in a control reaction to amplify the normal sequence (Fig. 2A). PCR conditions were as described previously (17).

Similarly, a PCR-SSP-B reaction was set up. Two pairs of PCR primers were used to detect CYP21P/CYP21 recombinations in the region between –302 and exon 3 in order to establish whether the presence of a TaqI restriction site in CYP21 is due to a gene deletion or a conversion event in this region. A CYP21P-specific (CAH-302P: 5'-GCGGACACCCTTGCC-TGCAG) or CYP21-specific (CAH-302N: 5'-GCGGAC-ACTATTGCCTGCACA) forward primer was used in combination with a CYP21-specific reverse primer (CAH-P2: 5'-AGCAGGGAGTAGTCTCCCAAG) to detect either the allele with a recombination in this region or the normal allele respectively (Fig. 2B). Additionally, allele-specific PCR was used to analyse for 8 bp deletion in exon 3 (18).

Point mutations analysis by sequence-specific oligonucleotide hybridisation (PCR-SSO)

To screen for point mutations in CYP21 gene exons 1-3 and exons 3-10 were selectively amplified in all participants with two sets of primers using the 8 bp deletion in exon 3 to distinguish between the functional CYP21 gene and the CYP21P pseudo-gene (3). Dot blot hybridisation analysis of the PCR product with 5' biotinylated oligonucleotides was used to screen for the presence of normal or mutated sequences at the following sites of CYP21 gene: Pro30Leu (exon 1), A/C656G (in2) (–13 intron 2), Ile172Asn (exon 4), Ile-Val-Glu-Met236-239Asn-Glu-Glu-Lys (exon 6), Val281Leu (exon 7), insT307 (exon 7), Gln318Stop (exon 8) and Arg356Trp (exon 8) (12). Oligonucleotide sequences were essentially as previously described (3) only they were shorter at both ends by 2-3 nucleotides. An additional nine oligonucleotides were utilised to screen for the presence of GT1769CT splice site mutation (intron 7), Arg339His (exon 8), Pro453Ser (exon 10) and Arg/Pro484Ser (exon 10) (19).

Sequencing analysis

CYP21 gene and its proximal promoter region were first specifically amplified in two large fragments (12). Amplified fragments were purified on QIAquick PCR purification columns (Quiagen, Hilden, Germany) and sequenced with forward and reverse primers using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, Cheshire, UK) on an Abi Prism 310 Genetic Analyser. Sequencing primers were as previously described (12).

Results

In the present study we systematically genotyped 36 Slovenian CAH patients from 33 families, as well as 63 parents and 16 apparently healthy siblings. The clinical characteristics and genotyping results of the patients (proband) are shown in Table 1. Among 36 patients, 21 presented with SW-CAH (seven boys and 14 girls), nine had SV-CAH (four boys and five girls) and six had NC-CAH (two boys and four girls). The affected siblings with identical genotypes presented with a similar phenotype (patients 10-11, 13-14 and 26-27 in Table 1). Among 16 clinically normal siblings, nine were identified as carriers for CYP21 gene mutations (data not shown). Among parents, two mothers were found to be compound heterozygotes for mutations characteristic for NC-CAH (in2/Val281-Leu and Pro30Leu/del respectively), while among four fathers two were homozygous for the in2 mutation, one had the in2/Gln318Stop and one the Val281Leu/del genotype. Interestingly, CAH was not recognised in any of these affected parents during their childhood,

Table 1 Clinical characteristics and complete CYP21 genotypes of Slovenian CAH patients.

Patient	Status	Sex	Age at diagnosis	Clinical phenotype	Virilisation in females	CYP21 haplotypes	
						Paternal	Maternal
1	P	F	1 d	SW	SU	large conv	del
2	P	M	14 d	SW		del	del
3	P	M	20 d	SW		del	del
4	P	M	20 d	SW		del	del
5	P	F	16 d	SW	SU	del	del
6	P	F	10 d	SW	SU	del	del
7	P	F	3 d	SW	SU	del	del
8	P	F	7 d	SW	SU	del <i>de novo</i>	large conv
9	P	F	14 d	SW	SU	InsT307+Gln318Stop	del
10	P	M	10 d	SW		InsT307+Gln318Stop	In2+Ex6+Val281Leu+Gln318Stop
11	AS	M	9 d	SW		InsT307+Gln318Stop	In2+Ex6+Val281Leu+Gln318Stop
12	P	F	5 y	SV	CM	InsT307	Gln318Stop
13	P	F	4 m	SW	CM	del	conv+Pro30Leu+Ala15Thr*
14	AS	F	21 d	SW	CM	del	conv+Pro30Leu+Ala15Thr*
15	P	F	14 d	SW	SU	Pro30Leu+In2+conv	large conv
16	P	F	12.3 y	NC	CM	large conv	conv+Pro30Leu
17	P	M	3.5 y	SV		conv+Pro30Leu	large conv
18	P	F	1.1 y	SV	SU	conv+Pro30Leu	Gln318Stop
19	P	M	12 d	SW		del	In2
20	P	F	9 d	SW	SU	In2	large conv
21	P	F	8 d	SW	SU	del	In2
22	P	F	5 d	SW	SU	In2	del
23	P	M	24 d	SW		In2	del
24	P	F	10 d	SW	SU	In2	In2
25	P	F	20 d	SW	SU	In2	In2
26	P	F	0.8 y	SV	SU	del	Ile172Asn
27	AS	M	4 y	SV		del	Ile172Asn
28	P	F	8 d	SV	SU	Ile172Asn	del
29	P	M	7.2 y	SV		Ile172Asn	In2
30	P	F	2 y	SV	CM	Ile172Asn	Ile172Asn
31	P	M	1 m	NC		Pro453Ser	del
32	P	M	6.8 y	NC/SV		Pro30Leu	In2
33	P	F	12 y	NC		Pro30Leu	del
34	P	M	7.2 y	NC		Pro30Leu	Val281Leu
35	P	F	6 y	NC		Val281Leu	del
36	P	F	1.3 y	NC	SU	large conv	N

Status: P, proband; AS, affected sibling. Sex: F, female; M, male. Age at diagnosis: d, days; m, months; y, years. Clinical phenotype: SW, salt wasting; SV, simple virilising; NC, non-classical. Virilisation in females: CM, clitoromegaly; SU, sinus urogenitalis. Haplotypes: del, CYP21 gene deletion; large conv, large CYP21 gene conversion; conv, CYP21 gene conversion involving the promoter region; In2, intron 2 splice mutation; N, normal; * novel mutation.

although some clinical signs (short stature and increased virilisation) were present in their adulthood.

Using sequence-specific oligonucleotide hybridisation (PCR-SSO), sequence-specific PCR amplification (PCR-SSP), Southern blotting and sequencing we could identify the causative mutation on 65 out of 66 (98.5%) unrelated disease alleles (Table 2). The one allele that could not be identified represents 1.5% of all unrelated alleles and belonged to an NC-CAH patient having a CYP21 gene deletion on the second allele.

CYP21 gene deletions/conversions were found to be the most frequent mutation in Slovenian CAH patients. In order to characterise these alleles better PCR-SSP-A and PCR-SSP-B amplifications were performed in addition to Southern blotting. The parental origin of the converted/deleted alleles found in patients was determined by PCR-SSP analysis of the parents. Among 60 parents analysed by Southern blotting, 33 had decreased intensity of the 3.7 kb/TaqI band

when compared with the 3.2 kb/TaqI band. In 21 out of these 33 parents this could be attributed to classical CYP21 gene deletion or large gene conversion with a breakpoint between exon 6 and exon 10 based on the positive result of the PCR-SSP-A reaction. These alleles were designated as CYP21 gene deletion. In seven out of these 33 parents both the PCR-SSP-A and PCR-SSP-B reactions amplified only normal alleles, suggesting that the absence of the 3.7 kb/TaqI band might be due to a gene conversion with a breakpoint before exon 6 or to a gene conversion involving other regions. These alleles were designated as large gene conversion (large conv). In five out of these 33 parents the decreased intensity of the 3.7 kb/TaqI band could be attributed to a gene conversion in the promoter region based on the positive result of the PCR-SSP-B reaction. Both PCR-SSO hybridisation analysis and the sequencing indicated that these conversions always encompassed codon 30, therefore they were designated as

Table 2 Allele frequency of mutations detected in unrelated Slovenian CAH patients.

Allele	Number	% of alleles
del	24	36.4
large conv	8	12.1
In2+conv ex6-ex8	1	1.5
conv+Pro30Leu	3	4.6
conv+Pro30Leu+Ala15Thr	1	1.5
conv+Pro30Leu+In2	1	1.5
Pro30Leu	3	4.6
In2, splice	11	16.7
Ile172Asn	5	7.6
Val281Leu	2	3.0
Ins307T	1	1.5
Gln318Stop	2	3.0
Ins307T+Gln318Stop	2	3.0
Pro453Ser	1	1.5
N	1	1.5
Total	66	100.0

del, CYP21 gene deletion; large conv, large CYP21 gene conversion; conv, CYP21 gene conversion involving the promoter region; conv ex6-ex8, cluster of mutations in exon 6+Val281Leu+Gln318Stop; In2, intron 2 splice mutation. N, normal.

conv+Pro30Leu. We also observed that the PCR-SSP-A reaction aimed to detect the classical CYP21 gene deletion amplified a specific product in all individuals positive for Val281Leu mutation, a finding which we explained by the presence of a duplicated CYP21P gene.

The frequencies of CYP21 gene mutations in Slovenian CAH patients were calculated based on systematic genotyping of 66 unrelated chromosomes. As shown in Table 2, CYP21 gene deletion was the most common cause of CAH and was found on 36.4% of unrelated alleles, large gene conversions detectable only by Southern blotting represented 12.1% and gene conversions involving the promoter region represented 7.6% of the mutated alleles. The most frequent point mutations were intron 2 splice mutation found on 16.7% of the alleles and Ile172Asn mutation present

on 7.6% of the alleles. Other point mutations detected were Gln318Stop on 7.5% of alleles and Pro30Leu on 12.2% of alleles. Both were frequently found on chromosomes carrying additional point mutations or gene conversions involving the promoter region in the latter case. Gln318Stop was found as a single point mutation on three unrelated alleles and transferred *en bloc* with insT307 on two alleles. Alleles with multiple mutations were found in two siblings (patients 10 and 11 in Table 1). Val281Leu mutation was found on two unrelated alleles, while isolated insT307 and Pro453Ser mutations represented only single unrelated alleles.

As shown in Table 1, genotype–phenotype correlations observed in Slovenian CAH patients were similar to those reported from large populations (9). The finding of Pro30Leu mutation linked to a gene conversion or to an additional mutation could explain the SW or SV phenotype in patients who are compound heterozygotes for the Pro30Leu and a severe mutation. As shown in Table 3, nine compound heterozygotes for Pro30Leu were identified. Among them, Pro30Leu was transmitted as a single point mutation in only three patients, two of which presented with NC-CAH and the third with NC/SV-CAH. In three patients Pro30Leu was transmitted *en bloc* with a gene conversion in the promoter region. Two of these patients presented with SV-CAH, while the third, a female with NC-CAH, presented with clitoromegaly. In three patients, all manifesting with SW-CAH, additional point mutations on the chromosome characterised by Pro30Leu mutation and gene conversion in the promoter region were identified – one patient had the intron 2 splice mutation while two siblings were hemizygous for a novel mutation Ala15Thr (Fig. 3) which has not been described to date in CAH patients. A schematic presentation of mutations detected in Slovenian CAH patients is shown in Fig. 4.

Table 3 Clinical characteristics and genotype–phenotype correlations in patients positive for Pro30Leu mutation.

Patient	Sex	Age	Clinical phenotype	Virilisation of females	Patient's genotype		PCR-SSP-B – 302–ex3	PCR-SSP-A ex6–ex10
					Allele 1	Allele 2		
13	F	4 m	SW	CM	conv+Ala15Thr+Pro30Leu	del	P	P
14	F	21 d	SW	CM	conv+Ala15Thr+Pro30Leu	del	P	P
15	F	14 d	SW	SU	conv+Pro30Leu+In2	large conv	P	N
17	M	3.5 y	SV		conv+Pro30Leu	large conv	N/P	N
18	F	1.1 y	SV	SU	conv+Pro30Leu	Gln318Stop	N/P	N
16	F	12.3 y	NC	CM	conv+Pro30Leu	large conv	N/P	N
32	M	6.8 y	NC/SV		Pro30Leu	In2	N	N
33	F	12 y	NC		Pro30Leu	del	N	N/P
34	M	7.2 y	NC		Pro30Leu	Val281Leu	N	N/P

Sex: F, female, M, male. Age at diagnosis: d, days, m, months, y, years. Clinical phenotype: SW, salt wasting, SV, simple virilising, NC, non-classical. Virilisation of females: CM, clitoromegaly, SU, sinus urogenitalis. Patient's genotype: del, CYP21 gene deletion; large conv, large CYP21 gene conversion; conv, CYP21 gene conversion involving the promoter region. PCR-SSP-B sequence specific PCR for detection of CYP21 gene conversion in the promoter region (– 302–ex3); PCR-SSP-A sequence specific PCR for detection of CYP21 gene deletion (ex6–ex10): N, CYP21-specific sequence amplified, P, CYP21P specific-sequence amplified.

Figure 3 DNA sequence of part of exon 1 of the CYP21 gene in patient 13 (see Table 1). The arrow is pointing at the G to A transition at nucleotide 43, which results in the hemizygous Ala to Thr mutation at codon 15. The paternal allele carried a CYP21 gene deletion and was not amplified by the PCR-SSP-B reaction. The (maternal) allele with a gene conversion in the promoter region was specifically amplified in the PCR-SSP-B reaction and sequenced with exon 1 forward sequencing primer (12). Further downstream, at nucleotide 89, the G to A transition is present on the same allele, leading to the Pro to Leu mutation at codon 30 (not shown).

Figure 4 Schematic presentation of mutations detected in the Slovenian population of CAH patients, including the putative novel Ala15Thr point mutation.

Discussion

Systematic analysis of the mutational spectrum and the associated haplotypes, as well as the genotype–phenotype correlation, was performed in 36 Slovenian CAH patients and 79 family members. These patients are representative for 21-OH deficiency alleles in the Slovenian population with 41 patients registered since 1971 at the University Children's Hospital in Ljubljana where the CAH diagnosis and management is centralised.

In total, 115 individuals were analysed for the presence of 12 point mutations, the deletion of 8 bp in exon 3 and for large rearrangements in the CYP21 gene. Southern blotting and the family segregation analysis allowed us to distinguish homozygosity from hemizygosity for mutations.

The first interesting finding was the very high frequency of CYP21 gene deletions and large gene conversions, representing 36.4% and 12.1% of unrelated disease alleles respectively. Gene conversions involving the promoter region were found on 7.6% of unrelated disease alleles. The only population reported so far with a similar frequency of CYP21 gene deletions/conversions is British (45%) (20), while in all the surrounding populations these frequencies are lower:

38.3% in Italian (21), 31.0% in Austrian (22) and 27.4% in German CAH patients (11). Due to the high frequency of CYP21 gene deletions/conversions we employed PCR-SSP amplification that enabled us to characterise these alleles better and which can be used for rapid screening and prenatal diagnosis as well as in cases where not enough genetic material is available for Southern blotting. The latter technique can distinguish between CYP21 gene deletion and large conversion when at least two digestion reactions are used for restriction fragment length polymorphism (16) but a lot of genetic material is required and the method is labour intensive and time consuming. Using two PCR-SSP reactions we could assign the missing 3.7 kb/TaqI band to a classical CYP21 gene deletion or to a gene conversion in the promoter region in 65.6% (21/32) and 15.6% (5/32) of parental alleles respectively. However 21.9% (7/32) of parental alleles with one missing 3.7 kb/TaqI band could not be assigned and they most probably represent a large gene conversion with a breakpoint before exon 6. Employing only PCR-SSP analysis these gene conversions would be missed. Regarding the fact that the breakpoint area may vary and that conversion from CYP21 back to CYP21P may occur, a single PCR method may not be sufficient to substitute for Southern blotting, although many attempts have been made lately to speed up and simplify the analysis (23–25).

The second important feature of 21-hydroxylase deficiency in the Slovenian population was the great diversity of clinical phenotypes in compound heterozygotes for Pro30Leu and a severe mutation. Already the first report on Pro30Leu emphasises that affected females frequently present with clitoromegaly (26). Many authors later reported observations of more severe clinical phenotype than predicted by genotyping in carriers of Pro30Leu mutation (10, 11, 22). In the Slovenian population only one-third of CAH alleles positive for Pro30Leu mutation actually represent a single base substitution and result in NC-CAH. In the second third of Pro30Leu-positive alleles this mutation is a part of a gene conversion encompassing the promoter region and exon 1. The existence of such alleles has previously been described in patients with the classical form of the disease (22, 24). However, on one-third of Pro30Leu-positive alleles we detected an additional point mutation besides gene conversion. This was the intron 2 mutation in one SW patient while in two siblings presenting with SW-CAH a substitution, Ala15Thr, was detected. This mutation has not been described to date in CAH patients and results in the change of a non-polar amino acid residue for a polar one and represents a putative novel mutation which leads to a similar clinical phenotype as the additional intron 2 mutation. A functional assay is needed to evaluate the relative contribution of Ala15Thr to the SW phenotype observed in these siblings. The high frequency of other CYP21 gene aberrations on

Pro30Leu-positive alleles emphasised the importance of systematic searching for a gene conversion in the promoter region using the PCR-SSP reaction in patients positive for Pro30Leu, especially when a prenatal diagnosis and treatment are planned.

Another interesting finding was the low frequency of NC-CAH and related mutations in the Slovenian population in comparison with surrounding populations in which a high incidence of NC-CAH has been reported: for example 1/330 in the Italian and 1/63 in the Croatian population (27). Only six out of 36 patients (two males, four females) presented with NC-CAH (16.7%), which implied a very low prevalence of NC-CAH in the population. One possible explanation is that the majority of NC-CAH patients may not be detected until later in life, or may remain asymptomatic, especially males. Among the unrelated disease alleles non-classical mutations Val281Leu, the isolated Pro30Leu and Pro453Ser represented 3.0%, 4.6% and 1.5% respectively. These findings are in agreement with the finding of much lower frequency of Val281Leu mutation in Slovenian hyper-androgenic women than in a comparable group of Italian patients (13). In the Slovenian population, in contrast to the Croatian NC-CAH patients and similar to the Italian patients, the association between Val281Leu mutation and HLA-B14, a marker characteristic for NC-CAH, was confirmed (12, 28, 29).

The good correlation between the genotype and the clinical phenotype observed in our study can be attributed to systematic genotyping and the family segregation studies. The unbalanced sex ratio observed in SW-CAH patients (six males, 13 females) emphasises the need for biochemical/hormonal screening for CAH in Slovenian newborns (30). Genotyping for mutations in CYP21 gene has been proven to be a valuable diagnostic tool in cases when slightly elevated 17-OHP levels were detected during the neonatal screening (31).

In conclusion, the results of this study have brought new insights into the molecular pathology of the CYP21 gene in the Slovenian population, and the finding of a novel Ala15Thr mutation within a gene conversion involving the promoter region and Pro30Leu associated with a severe form of the disease has been described. Our genotyping approach allowed accurate and sensitive identification of CYP21 gene mutations in CAH patients and their families and represents a cornerstone for a reliable pre-, peri- and postnatal diagnosis and for adequate genetic counselling in our population.

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