Identification of *CYP21A2* mutant alleles in Czech patients with 21-hydroxylase deficiency

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Abstract. Congenital adrenal hyperplasia (CAH) is comprised of a group of autosomal recessive disorders caused by an enzymatic deficiency which impairs the biosynthesis of cortisol and, in most of the severe cases, also the biosynthesis of aldosterone. Approximately 90-95% of all the CAH cases are due to mutations in the steroid 21-hydroxylase gene (*CYP21A2*). In this study, the molecular genetic analysis of *CYP21A2* was performed in 267 Czech probands suspected of 21-hydroxylase deficiency (21OHD). 21OHD was confirmed in 241 probands (2 mutations were detected). In 26 probands, a mutation was found only in 1 *CYP21A2* allele. A set of 30 different mutant alleles was determined. We describe i) mutated *CYP21A2* alleles carrying novel point mutations (p.Thr168Asn, p.Ser169X and p.Pro386Arg), ii) mutated *CYP21A2* alleles carrying the novel chimeric gene designated as CH-7, which was detected in 21.4% of the mutant alleles, iii) an unusual genotype with a combination of the *CYP21A2* duplication, 2 point mutations and the *CYP21A2* large-scale gene conversion on the second allele, and (iv) a detailed analysis of the chimeric *CYP21A1P/CYP21A2* genes. In conclusion, our genotyping approach allowed for the accurate identification of the *CYP21A2* gene mutations in 21OHD patients and their families and provided some useful information on diagnosis and genetic counselling.

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Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders caused by an enzymatic deficiency which impairs the biosynthesis of cortisol and, in most of the severe cases, also the biosynthesis of aldosterone. Approximately 90-95% of all the CAH cases are due to 21-hydroxylase deficiency (21OHD), and ~5-8% cases are due to 11-ß-hydroxylase deficiency (1). In the adrenal cortex, the steroid 21-hydroxylase (21OH) converts 17-hydroxyprogesterone into 11-deoxycortisol and progesterone into 11-deoxycorticosterone. These steroids are subsequently converted into cortisol and aldosterone, respectively (1).

The steroid 21OH gene (*CYP21A2*) and its inactive pseudogene (*CYP21A1P*) are located within the HLA class III region of the major histocompatibility complex locus on chromosome 6p21.3. The *CYP21A2* gene is 98% homologous to the *CYP21A1P* pseudogene in exons and 96% in introns (2,3). Together with the neighbouring genes (serine/threonine kinase RP, complement C4 and tenascin TNX), *CYP21* forms a genetic unit termed as the RCCX module (4,5). In the RCCX bimodular haplotype (69% of chromosome 6p21.3), the orientation of genes from telomere to centromere is *RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXB*. The 3 pseudogenes, *CYP21A1P*, *TNXA* and *RP2*, located between the 2 C4 loci, do not encode functional proteins (5).

According to the severity of the disease, 3 clinical forms of CAH have been distinguished: the classical salt-wasting (SW-CAH), the classical simple virilising (SV-CAH), and the non-classical CAH (NC-CAH) (6). If the patients are not timely diagnosed (by neonatal sreening) and treated, the severe deficiency in 21OH leads to SW-CAH. In addition to having a severe cortisol deficiency, patients with SW-CAH do not synthesise enough aldosteron and therefore, are not able to maintain sodium homeostasis. Thus, affected patients usually manifest hyponatraemia, hyperkalaemia and vomiting during the first 4 weeks of life. In the female foetus, the excess of androgen causes variable degrees of external genital

virilisation, and consequently, newborn females have genital ambiguity (1,7,8). The residual activity of 21OH (aldosterone is synthetized) results in SV-CAH with external genital virilisation in females and also results in signs of precocious pseudopuberty which develop by 8 years of age in female and male patients (1,8,9). NC-CAH is associated with a moderate deficiency in 21OH and manifests in later childhood or adolescence with hirsutism and decreased fertility or precocious pseudopuberty (8). Patients with NC-CAH secrete aldosterone normally and most of the male patients diagnosed after puberty are entirely asymptomatic (1,10). In order to reduce CAH-associated morbidity and mortality through early diagnosis and treatment, the neonatal screening of CAH based on the detection of 17-hydroxyprogesterone, has been introduced since 2006 in the Czech Republic (11).

The functional *CYP21A2* gene and the *CYP21A1P* pseudogene, each containing 10 exons, are spaced over 3.4 kb (2,3). The most common source of mutations, involving ~95% of mutant alleles, is the intergenic recombination between *CYP21A2* and *CYP21A1P*, whereas the remaining 5% of mutant alleles are represented by new mutations. The main *CYP21A2* defects are comprised of *CYP21A2* deletions, large-scale gene conversions of *CYP21A2* into a structure similar to *CYP21A1P*, and *CYP21A1P/CYP21A2* chimeric genes. These mutations are generated by the unequal crossing-over during meiosis. However, the mechanism of small-scale gene conversions, that result in short-range mutations in *CYP21A2* derived from *CYP21A1P*, is not yet well understood (12,13). Unequal crossing-over can cause a 30 kb deletion, involving the 3' end of *CYP21A1P*, all of *TNXA*, *RP2*, *C4B*, and the 5' end of *CYP21A2*, which produces a non-functional chimeric gene with 5' and 3' ends corresponding to *CYP21A1P* and *CYP21A2*, respectively (14,15).

The incidence of classical CAH in Central Europe is 1/10,000, with a gender ratio of 40% boys and 60% girls, and the carrier rate is 1/50 (7,16). The milder, non-classical form of CAH is much more common, with a prevalence of 1/100 in the general population (8). As *CYP21A2* analysis has been made available in many countries, studies on *CYP21A2* mutations in large national patient series have provided an insight into specific mutation distributions. In this study, we analyzed *CYP21A2* mutations and their frequencies in 21OHD patients in the Czech population, and compared the results with previous studies from other countries.

Patients and methods

Subjects. The patients were sent to our laboratory for the molecular analysis of the *CYP21A2* gene after endocrine and clinical evaluation. DNA samples were obtained from peripheral blood leukocytes and/or amniotic fluid by the standard salting-out method. All the studies were approved by the Ethics Board of the University Hospital Brno, and all the patients and their family members gave their written, informed consent for DNA analysis.

Detection of CYP21A2 point mutations derived from CYP21A1P and chimeric CYP21A1P/CYP21A2 genes. The direct screening of *CYP21A2* mutations required the specific amplification of the *CYP21A2* gene. This step was performed by PCR with primers specific for *CYP21A2*, which did not allow for the concomitant amplification of *CYP21A1P* (17). The product of this step was used as the DNA template for the nested amplifications of the *CYP21A2* gene fragments carrying the most frequent point mutations. The specific amplification of *CYP21A2* was performed by the Expand Long Template System kit (Roche Diagnostics, GmbH), and the nested amplifications were performed by Taq DNA Polymerase (Fermentas, GmbH). The PCR primers for the nested PCRs are shown in Table I. The detection of the most common point mutations was performed by long-template PCR and nested PCRs using restriction fragment length polymorphisms (RFLP) (p.Pro30Leu, p.Ser97fsX12, p.Gly110ValfsX21, p.Ile172Asn, p.Val237Glu, p.Val281Leu, p.Gln318X and p.Arg356Trp) and DNA sequencing (p.Leu307PhefsX6) (17). Long-template PCR was also used for the amplification of the chimeric *CYP21A1P/CYP21A2* gene. This step was performed using the 5' primer specific for *CYP21A1P*, and the 3' primer specific for *CYP21A2* (17). The nested PCRs and RFLPs were applied analogously to the detection of the most common point mutations in order to detect the type of chimeric *CYP21A1P/CYP21A2* gene (Fig. 1) (17).

Multiplex ligation-dependent probe amplification (MLPA). MLPA is a method used for the detection of large gene deletions and duplications. For the CAH diagnostics, we used the SALSA MLPA kit P050B CAH (MRS Holland, The Netherlands). This kit contains probes and primers for the analysis of 5 *CYP21A2* gene fragments located in the promoter region and exons 3, 4, 6 and 8. The established MLPA method detects deletions and duplications of the *CYP21A2* gene, as well as particular *CYP21A2* point mutations, provided that these mutations are located at probe binding sites (p.Gly110ValfsX21, p.Thr168Asn, p.Ser169X, p.Ile172Asn, p.Val237Glu, p.Arg316X and p.Gln318X). Additionally, the MLPA kit contains 28 *CYP21A2* non-specific probes that are the control standards and sets for the amplification of adjacent genes (*CREBL*, *C4B* and *TNXB*) and pseudogenes (*C4A* and *TNXA*). The assay was performed according to the manufacturer's recommendations. Genomic DNA (200 ng) was denatured at 98˚C for 5 min, hybridised overnight at 60˚C with the SALSA probemix, and treated with the Ligase-65 enzyme at 54˚C for 15 min. The reactions were stopped by incubation at 98˚C for 5 min. Subsequently, PCRs were performed with the specific SALSA PCR primers (cycling conditions were 35 cycles at 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 60 sec). The amplification products were run on the CEQ 8000 Genetic Analyzer (Beckman Coulter). Two healthy men were included in every analysis as the controls. The peak height of each analysed fragment was normalised to the peak height of the control sample. Relative peak heights were reduced to 40-60% with deletions and increased by 30-50% with duplications.

The results obtained by MLPA were correlated with the results obtained using long-template PCR, nested PCRs, RFLP and/or DNA sequencing. Identified mutations of the *CYP21A2* gene were also confirmed by the segregation of the parents' alleles. Detected *CYP21A2* deletions can also pose *CYP21A2* large-scale conversions into a structure similar to

Table I. Primers used for the amplification of the *CYP21A2* gene.

Primers	Localization	Sequence direction (5'- 3')
30F	Promoter	CAGTCTACACAGCAGGAGGGATGGC
30R	Exon 1	AGCAAGTGCAAGAAGCCCGGGGCAAGCTG
97/110F	Intron 2	ATCAGTTCCCACCCTCCAGCCCCGA
97/110R	Exon 3	AGGGCTGAGCGGGTGAGCTTC
172F	Exon 4	GAGGAATTCTCTCTCCTCACCTGCAGCATTA
172R	Intron 4	AGTTGTCGTCCTGCCAGAAAAGGA
236/237/239F	Exon 6	AGCAGGCCATAGAGAAGAGGGATCACATCG
236/237/239R	Intron 6	ATGCAAAAGAACCCGCCTCATAGC
281F	Exon 7	TGCAGGAGAGCCTCGTGGCAGG
281R	Exon 7	GACGCACCTCAGGGTGGTGAAG
318/356F	Intron 7	GCTGGGGCAGGACTCCACCCGA
318/356R	Exon 8	GTGGGGCAAGGCTAAGGGCACAACTGGC
1R*	Intron 1	AAGCAGCGTCAGCGGAGAGGG
2F*	Intron 1	TTGAGGCTGAGGTGGGAGGA
2R*	Intron 2	GCGGAGGTGACGGAGAGGGT
3F*	Intron 2	AAGCTCTTGGGGGGCATATC
3R*	Intron 3	GGCTACTGTGAGAGGCGAGG
4F*	Intron 3	GTCAGCCTCGCCTCTCACAG
4R*	Intron 4	CAGTTCAGGACAAGGAGAGGCT
5F*	Intron 4	AGCCCCTCCCTGAGCCTCTC
5R*	Intron 5	AGCCTCTCCCTCCACCCCAG
6F*	Intron 5	TGGGTTGTAGGGGAGAGGCT
6R*	Intron 6	TAGCAATGCTGAGGCCGGTA
7F*	Intron 6	TGCCACTCTGTACTCCTCTC
7R*	Intron 7	ACAGTGCTCAGAGCTGAGTG
8F*	Intron 7	CTCACCGGCACTCAGGCTCA
8R*	Intron 8	AAGGGGGCTGGAGTTAGAGGCT
9F*	Intron 8	AGTGAGGAAAGCCCGAGCCC
9R*	Intron 9	GTGGGTGGGGAGGCGTTCAG
10F*	Intron 9	AAAATGTGGTGGAGGCTGGT
10R*	3'UTR	ACGGGAGCAATAAAGGAGAAAC
RM1F	Promoter	TTCAGGCGATTCAGGAAGGC
RM1R	Exon 3	CTTTCCAGAGCAGGGAGT
RM2F	Exon 3	CGGACCTGTCCTTGGGAGACTAC
RM2R	3'UTR	TTTCAGCCCCACAGTGTAACAGG

F, forward primer; R, reverse primer. *Primers used for PCR-sequencing. No (*) primers used for PCR-RFLP (the names of the primers are consistent with the no. of codons carrying an analysed mutation, and primer 30F was also used as the forward primer for the sequencing of exon 1). The RM primers were used for the specific amplification of *CYP21A2* in order to perform the sequencing analysis of individual exons. For the cDNA reference sequence go to, http://www.ncbi.nlm.nih.gov/nuccore/187895.

CYP21A1P. This type of *CYP21A2* deletion was verified by PCR with primers specific to the *TNXB/TNXA* hybrid gene. (see below) (16).

Detection of CYP21A2 large-scale conversions into CYP21A1P associated with the TNXB/TNXA hybrid gene. The *TNXB/TNXA* hybrid gene was detected using PCR with the 5' primer specific to *TNXB* and the 3' primer specific to the both genes, *TNXB* and *TNXA* (16). In the presence of the *TNXB/TNXA* hybrid gene, the PCR product sized 2688 bp was generated in contrast to the PCR product sized 2808 pb resulting from the *TNXB* gene (for the schematic presentation see ref.16).

Detection of rare mutations in the CYP21A2 gene. DNA sequencing was applied for the detection of rare *CYP21A2* mutations in patients with a mutation identified only in 1 *CYP21A2* allele after basic DNA diagnosis, which included the detection of i) 9 point mutations derived from *CYP21A1P*, ii) chimeric genes, and iii) *CYP21A2* deletions and duplications. As 90-95% of the mutant alleles carried ≥1 discrete mutation, the samples carrying none of these mutations were presumed to be unaffected with >99% confidence (1). As a primary template for this analysis, we did not use long-template PCR product (see the detection of *CYP21A2* point mutations derived from *CYP21A1P* and chimeric *CYP21A1P/CYP21A2* genes) as various polymorphisms were identified

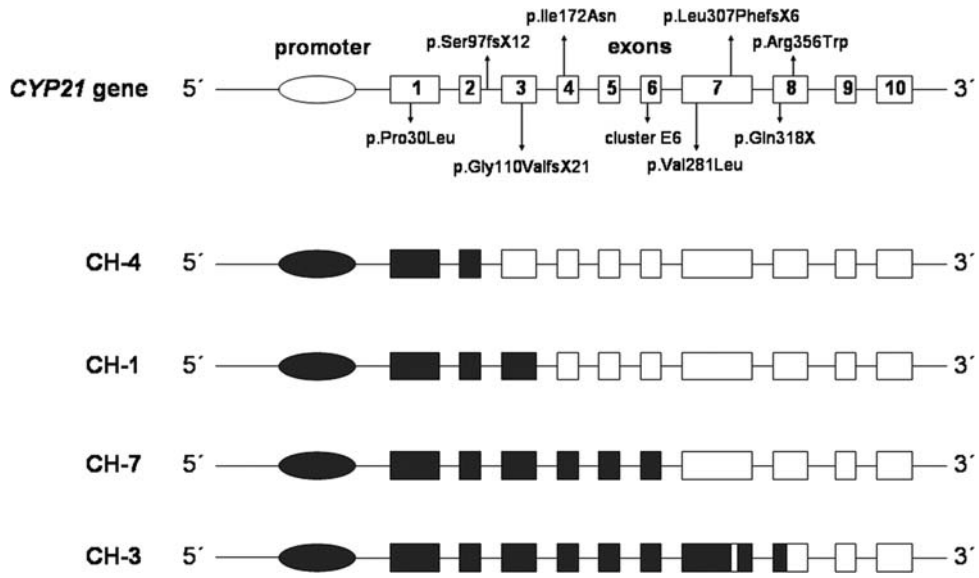

Figure 1. Types of chimeric *CYP21A1P*/*CYP21A2* genes identified in the Czech 21OHD patients. White boxes, structure of the functional *CYP21A2* gene; black boxes, non-functional *CYP21A1P* pseudogene; arrows, mutations existing in *CYP21A1P*. From *CYP21A1P*, the chimeric gene, CH-4, harbours the promoter, exon 1 (p.Pro30Leu) and exon 2. The chimeric gene, CH-1, bears exon 1, exon 2 (p.Ser97fsX12) and exon 3 (p.Gly110ValfsX21). The chimeric gene, CH-7, carries the promoter, exons 1-3, exon 4 (p.Ile172Asn), exon 5 and exon 6 (p.Ile236Asn, p.Val237Glu, Met239Lys). The chimeric gene, CH-3, harbours the promoter, exons 1-6, exon 7 (p.Leu307PhefsX6, but not Val281Leu) and exon 8 (p.Gln318X).

in the annealing sequences of the *CYP21A2* specific primers in some patients. For this reason, the detection of rare mutations was performed with 2 primary PCRs (primers are marked RM1 and RM2, Table I). The first amplified fragment was comprised of a region from the promoter to exon 3, and the second one of a region from exon 3 to 3'UTR. Both PCRs used *CYP21A2* specific primers which were complementary to an 8-bp segment in exon 3 deleted in *CYP21A1P* (18). PCR products were used as DNA templates for nested amplifications using sequencing primers (Table I). For the localization of primers for primary PCRs inside exon 3, DNA sequencing of this exon was performed using PCR product from long-template PCR (17). All the amplifications were performed by Taq DNA Polymerase (Fermentas). The purified PCR products were subsequently sequenced by the BigDye Terminator kit (Applied Biosystems) and analysed on an ABI PRISM 310 sequencer (Applied Biosystems).

Results

The molecular genetic analysis of the *CYP21A2* gene was performed in 267 Czech probands suspected of 21OHD. The diagnosis was confirmed in 241 of them (2 *CYP21A2* mutations were found). In 26 probands, a mutation was determined in only 1 *CYP21A2* allele (7 patients had SW-CAH, 8 had SV-CAH and 11 had NC-CAH). In the set of 21OHD probands, we determined 30 different mutant alleles (Table II).

CYP21A1P/CYP21A2 chimeric genes. The most frequent mutation, the chimeric *CYP21A1P/CYP21A2* gene, was found in 33.7% of mutant alleles. Four types of chimeric *CYP21A1P/CYP21A2* genes were detected in the Czech patients: CH-1, CH-3, CH-4 and CH-7 (Fig. 1). All the hybrid genes had a different extent of the *CYP21A1P* sequence attached to the 3' part of *CYP21A2*. The types CH-1, CH-3 and CH-4, have been previously described (12,19). The CH-4 type was found in 2.6% of the mutant alleles. This hybrid molecule differs from the functional *CYP21A2* gene by the *CYP21A1P* promoter sequence and the p.Pro30Leu mutation in exon 1. The CH-1 type is identical to the *CYP21A1P* gene in the promoter and exons 1-3, and the mutation p.Ile172Asn is not present in exon 4. This type of chimeric gene was detected in 9.3% of the mutant alleles. In addition, 1% of the patients had an atypical mutant allele with CH-1 and the p.Arg356Trp mutation in exon 8. Mutant alleles carrying CH-1 and p.Arg356Trp were confirmed by the segregation of the parents' alleles. The most common type of the chimeric gene was the newly characterized chimeric gene, CH-7 (21.4% of the mutant alleles). This chimeric gene involved the *CYP21A1P* sequence from the promoter up to exon 6, and the p.Val281Leu mutation was not present in exon 7. The least frequent chimeric gene, CH-3 (0.4% of mutant alleles) was identical to the *CYP21A1P* pseudogene from the promoter up to exon 8, and the p.Gln318X mutation was present in the chimeric gene in contrast to the p.Arg356Gln mutation. In all the cases, the mutant CH-3 allele did not contain the p.Val281Leu mutation.

CYP21A2 deletions and duplications. Total deletions of the *CYP21A2* gene were detected in 4.9% of the *CYP21A2* mutant alleles. The *CYP21A2* large-scale conversions into *CYP21A1P* generating the *TNXB/TNXA* hybrid gene represent the majority part of these (3.1% of the mutant alleles). Koppens *et al* described that *CYP21A2* large-scale conversions generating the *TNXA/TNXB* hybrid gene are associated with a presence of 2 *CYP21A1P* pseudogenes on the involved chromosome (13). The presence of 2 copies of the *CYP21A1P* pseudogenes on 1 chromosome was identified in all the

Table II. Types of *CYP21A2* mutant alleles and their frequency in Czech 21OHD probands.

Mutant allele	Mutation at cDNA level	Mutation at protein level	Localization of mutation	Phenotype	No. of alleles	Frequency (%)
1	Chimeric *CYP21A1P*/ *CYP21A2* gene (CH-4)		Promoter - exon 2	NC, SV	13	2,6
2	Chimeric *CYP21A1P*/ *CYP21A2* gene (CH-1)		Promoter - exon 3	SW	42	8,3
3	Chimeric *CYP21A1P*/ *CYP21A2* gene (CH-1) + p.Arg356Trp		Promoter - exon 3, exon 8	SW	5	1,0
4	Chimeric *CYP21A1P*/ *CYP21A2* gene (CH-7)		Promoter - exon 6	SW	109	21,4
5	Chimeric *CYP21A1P*/ *CYP21A2* gene (CH-3)		Promoter - exon 8	SW	2	0,4
6	*CYP21A2* deletion		*CYP21A2* gene	SW	9	1,8
7	*CYP21A2* large-scale gene conversion		*CYP21A2* gene	SW	16	3,1
8	*CYP21A2* duplication c.290-13A/C>G, c.952C>T	*CYP21A2* duplication p.Ser97fsX12, p.Gln318X	*CYP21A2* gene Intron 2, exon 8	SW	2	0,4
9	c.89C>T	p.Pro30Leu	Exon 1	NC	19	3,5
10	c.290-13A/C>G	p.Ser97fsX12	Intron 2	SW	122	24,00
11	c.329_336del8	p.Gly110ValfsX21	Exon 3	SW	6	1,2
12	**c.503C>A***	**p.Thr168Asn***	**Exon 4**	**NC**	**1**	**0,2**
13	**c.507C>A***	**p.Ser169X***	**Exon 4**	**SW**	**1**	**0,2**
14	c.515T>A	p.Ile172Asn	Exon 4	SV	57	11,2
15	c. 841G>T	p.Val281Leu	Exon 7	NC	47	9,3
16	c.918_919insT	p.Leu307PhefsX6	Exon 7	SW	1	0,2
17	**c.946C>T**	**p.Arg316X**	**Exon 8**	**SW**	**1**	**0,2**
18	c.952C>T	p.Gln318X	Exon 8	SW	18	3,5
19	c.1066C>T	p.Arg356Trp	Exon 8	SW	20	3,9
20	**c.1067G>A**	**p.Arg356Gln**	**Exon 8**	**SV**	**2**	**0,4**
21	**c.1157C>G***	**p.Pro386Arg***	**Exon 9**	**SW**	**1**	**0,2**
22	**c.1357C>T**	**p.Pro453Ser**	**Exon 10**	**NC**	**3**	**0,6**
23	**c.1375C>T**	**p.Pro459Ser**	**Exon 10**	**SV**	**1**	**0,2**
24	**c.1448G>C**	**p.Arg483Pro**	**Exon10**	**SV**	**2**	**0,4**
25	c.290_13A/C>G, c.329_336del8	p. Ser97fsX12, p.Gly110ValfsX21	Intron 2, exon 3	SW	1	0,2
26	c.290_13A/C>G, c.329_336del8, c.515T>A	p.Ser97fsX12, p.Gly110ValfsX21, p.Ile172Asn	Intron 2, exons 3 and 4	SW	1	0,2
27	c.707T>A, c.710 T>A, c.716T>A	p.Ile236Asn, p.Val237Glu, p.Met239Lys	Exon 6	SW	3	0,6
28	c.841G>T, c.952C>T	p.Val281Leu, p.Gln318X,	Exons 7 and 8	SW	1	0,2
29	c.841G>T, c.920_921insT, c.952C>T, c.1066C>T	p.Val281Leu, p.Leu307PhefsX6, p.Gln318X, p.Arg356Trp	Exons 7 and 8	SW	1	0,2
30	c.952C>T, c.1066C>T	p.Gln318X, p.Arg356Trp	Exon 8	SW	1	0,2

Rare *CYP21A2* mutations (not derived from *CYP21A1P*) are shown in bold letters. *These are novel mutations, and have not been described previously.

Table III. Correlation between genotype and phenotype in 142 Czech 21OHD patients.

Group	Mutant allele 1	Mutant allele 2	Phenotype	No. of probands	Frequency (%)
A	Chimeric gene	Chimeric gene	SW	18	12.7
	CH-4	Chimeric gene	SW	1	0.7
	Chimeric gene	*CYP21A2* deletion	SW	2	1.4
	CH-4	*CYP21A2* deletion	SW	3	2.1
	Chimeric gene	p.Ser97fsX12	SW	13	9.2
	Chimeric gene	p.Gly110ValfsX21	SW	2	1.4
	Chimeric gene	p.Val237Glu	SW	2	1.4
	Chimeric gene	p.Val281Leu*	SW	1	0.7
	Chimeric gene	p.Gln318X	SW	4	2.8
	CYP21A2 deletion	*CYP21A2* deletion	SW	3	2.1
	CYP21A2 deletion	dup[p.Ser97fsX12; p.Gln318X]	SW	1	0.7
	CYP21A2 deletion	p.Ser97fsX12	SW	6	4.2
	p.Pro30Leu*	p.Arg356Trp	SW	1	0.7
	p.Ser97fsX12	dup[p.Ser97fsX12; p.Gln318X]	SW	1	0.7
	p.Ser97fsX12	p.Ser97fsX12	SW	4	2.8
	p.Ser97fsX12	p.Ser169X	SW	1	0.7
	p.Ser97fsX12	p.Gln318X	SW	3	2.1
	p.Ser97fsX12	p.Arg356Trp	SW	1	0.7
	p.Ser97fsX12	p.Pro386Arg	SW	1	0.7
	p.Ser97fsX12, p.Gly110ValfsX21	p.Gly110ValfsX21	SW	1	0.7
	p.Val281Leu, p.Leu307PhefsX6, p.Gln318X, p.Arg356Trp	p.Gln318X	SW	1	0.7
	p.Arg356Trp	p.Arg356Trp	SW	2	1.4
B	Chimeric gene	p.Ser97fsX12	SW/SV	4	2.8
	CYP21A2 deletion	p.Ser97fsX12	SW/SV	1	0.7
	p.Ser97fsX12	p.Ser97fsX12	SW/SV	5	3.5
	p.Ser97fsX12	p.Arg356Trp	SW/SV	3	2.1
C	CH-4	Chimeric gene	SV	6	4.2
	CH-4	p.Ser97fsX12	SV	1	0.7
	Chimeric gene	p.Ile172Asn	SV	11	7.7
	Chimeric gene	p.Pro459Ser	SV	1	0.7
	CYP21A2 deletion	p.Ile172Asn	SV	2	1.4
	p.Ser97fsX12	p.Ile172Asn	SV	6	4.2
	p.Ile172Asn	p.Ile172Asn	SV	4	2.8
	p.Ile172Asn	p.Val237Glu	SV	1	0.7
	p.Ile172Asn	p.Val281Leu	SV	1	0.7
	p.Ile172Asn	p.Arg316X	SV	1	0.7
	p.Ile172Asn	p.Gln318X	SV	1	0.7
	p.Ile172Asn	p.Arg356Trp	SV	2	1.4
D	CH-4	CH-4	NC	1	0.7
	Chimeric gene	p.Pro30Leu	NC	2	1.4
	Chimeric gene	p.Thr168Asn	NC	1	0.7
	CH-4	p.Val281Leu	NC	1	0.7
	p.Pro30Leu	p.Pro30Leu	NC	1	0.7
	p.Pro30Leu	p.Ser97fsX12	NC	1	0.7
	p.Pro30Leu	p.Val281Leu	NC	1	0.7
	p.Ser97fsX12	p.Val281Leu	NC	3	2.1
	p.Ile172Asn	p.Val281Leu	NC	2	1.4
	p.Val281Leu	p.Val281Leu	NC	4	2.8
	p.Val281Leu	p.Gln318X	NC	1	0.7
	p.Val281Leu	p.Arg356Trp	NC	2	1.4

Group A, patients with SW-CAH; group B, patients with indefinite diagnosis, SV or SW-CAH; group C, patients with SV-CAH; group D, patients with NC-CAH. Chimeric gene, the *CYP21A1P/CYP21A2* chimeric gene (CH-1, CH-3 and CH-7 forms are not distinguished here).
*Mutation which does not correspond to the patient's phenotype.

Table IV. *CYP21A2* allele frequencies in different European populations.

P	TA	Del/ con	p.Pro30 Leu	p.Ser97 fsX12	p.Gly 110Valf sX21	p.Ile 172 Asn	p.Val 281Leu	p.Gln 318X	p.Arg 356Trp	T (%)
Czech Republic	508	38.6	3.5	24.0	1.2	11.2	9.3	3.5	3.9	95.2
Central Europe (20)	696	34.1	3.7	31.2	1.0	14.5	3.4	2.6	2.4	92.9
Austria (21)	158	35.4	3.2	22.8	0.0	15.8	12.0	2.5	3.2	94.9
Hungary (20)	270	27.1	3.0	35.9	0.0	14.1	5.6	1.9	3.0	90.6
Southern Germany (22)	310	29.0	2.6	30.3	1.6	19.7	2.9	4.8	4.5	95.4
The Netherlands (23)	370	31.9	0.3	28.1	4.3	12.4	2.2	3.5	8.4	91.1
Spain (24)	266	5.6	1.5	6.0	1.1	2.3	63.2	2.3	0.8	82.7

P, population; TA, total no. of alleles; del, deletion; con, conversion; T, total frequency of given mutations.

Czech patients carrying a *CYP21A2* large-scale conversion into *CYP21A1P*.

Duplications of *CYP21A2* associated with a mutation on both gene copies, were found in 2 probands (0.4% of the mutated alleles). In both cases, the *CYP21A2* genes localized on 1 chromosome carried the p.Ser97fsX12 mutation, as well as the p.Gln318X mutation, respectively. The association between the *CYP21A2* duplication and the mentioned point mutations was confirmed by the segregation of the parents' alleles.

CYP21A2 point mutations. Small DNA rearrangements of the *CYP21A2* gene, derived and non-derived from *CYP21A1P*, were present in 56.8 and 2.4% of the mutant alleles, respectively. The most frequent point mutations were p.Ser97fsX12, p.Ile172Asn and p.Val281Leu. We also detected 7 types of alleles with ≥2 mutations in 1 *CYP21A2* gene.

DNA sequencing of the *CYP21A2* exons and adjacent intron regions was performed in 38 probands with 1 identified *CYP21A2* mutant allele. Point mutations non-derived from *CYP21A1P* were found in 12 patients (Table II). Three of the identified point mutations have not been described previously (p.Thr168Asn, p.Ser169X and p.Pro386Arg). Female patient 1 (NC-CAH) is a compound heterozygote for the p.Thr168Asn mutation (the paternal allele), and the chimeric gene CH-7 (the maternal allele). Female patients 2 and 3 carried a phenotype associated with SW-CAH and both sufferred from genital virilisation and a life-threatening salt-wasting crisis in the neonatal phase. Patient 2 carried the p.Ser169X mutation, and the p.Ser97fsX12 mutation (DNA of parents was unavailable for analysis). Patient 3 carried the p.Pro386Arg mutation (the paternal allele) and the p.Ser97fsX12 mutation (the maternal allele).

Phenotype-genotype correlations. The correlation between genotype and phenotype was presented for 142 patients (Table III) in which sufficient clinical and biochemical data were available. Seventy-two patients suffered from the classic SW-CAH form (Group A), 13 had an indefinite phenotype, in that it was impossible to distinguish the SW and SV-CAH form (Group B), 37 suffered from the classic SV-CAH form (Group C), and 20 had the NC-CAH form (Group D). We observed a good correlation between genotype and phenotype. Discrepancies between genotype and phenotype were found in 2 patients with the SW-CAH phenotype (both patients had a mutation associated with the more moderate phenotype). The first patient is a male compound heterozygote for the p.Val281Leu mutation and the chimeric gene, CH-7. The second one is a female compound heterozygote carrying the p.Pro30Leu and the p.Arg356Trp mutations.

Discussion

The gene encoding the steroid 21OH enzyme, *CYP21A2*, is considered to be one of the most polymorphic human genes. Point mutations and copy number variations, such as deletions and duplications, have been described in many populations (20-24). Using the analysis of the *CYP21A2* gene, we confirmed the referral diagnosis in 241 Czech unrelated patients suspected of 21OHD. *CYP21A2* mutations can be predicted to cause a certain phenotype (SW, SV and NC), on the basis of the reduction of 21OH enzymatic activity. As 21OHD is an autosomal recessive disease and most 21OHD patients are compound heterozygotes, the phenotype of a patient should reflect a mutation that is predicted to cause the least severe impairment of enzymatic activity. This approach to phenotype prediction has been shown to be correct, although slight deviations to this correlation exist (1,23,25). We checked for correlations between the genotypes and phenotypes in 142 probands with 21OHD. A discrepancy was observed in 2 patients with SW-CAH. The first one was a male proband carrying the p.Val281Leu mutation (associated with NC-

CAH) (1) and the CH-7 chimeric gene. The second one was a female proband carrying the p.Pro30Leu mutation (associated with NC-CAH) and the p.Arg356Trp mutation (associated with SV-CAH) (1). Both patients suffered from a salt wasting crisis in the neonatal period and were treated with fludrocortisone and hydrocortisone.

Duplications of *CYP21A2* associated with a mutation on both gene copies were found in 2 probands. In both cases, the *CYP21A2* duplication was associated with the mutations, p.Ser97fsX12 and p.Gln318X (the same mutant allele has been previously described) (24). Both probands with the *CYP21A2* duplication, p.Ser97fsX12 and p.Gln318X on 1 allele have SW-CAH phenotypes. The complete genotypes of these patients were dup[p.Ser97fsX12; p.Gln318X]/p.Ser97fsX12 and dup[p.Ser97fsX12; p.Gln318X]/*CYP21A2* deletion. Determination of the genotype, dup[p.Ser97fsX12; p.Gln318X]/*CYP21A2* deletion, was quite difficult. Using long-template PCR, nested PCRs, and PCR-RFLP, the mutations, p.Ser97fsX12 and p.Gln318X, were detected and this result was also confirmed by MLPA. MLPA showed 1 copy of exon 8 (the p.Gln318X mutation was located in the probe binding site), and other *CYP21A2* fragments detected by MLPA had normal peak sizes corresponding to 2 *CYP21A2* copies. Thus, we presumed that this patient's genotype was p.Ser97fsX12/p.Gln318X. Using long-template amplification with primers specific to the *TNX* genes, we discovered the presence of the *TNXB*/*TNXA* hybrid gene and thus the presence of the *CYP21A2* large-scale conversion into structure similar to *CYP21A1P*. In this patient, a rare genotype combination was characterized, which has not been described so far. For the verification and diagnostic accuracy of our DNA, we also performed DNA analyses on the patients' family members. The mutant allele carrying the *CYP21A2* duplication with p.Ser97fsX12 and p.Gln318X was inherited from the mother and the mutant allele with the *CYP21A2* deletion was inherited from the father. We then offered the prenatal diagnosis to this family. After DNA analysis of the amniotic fluid, we detected the *CYP21A2* duplication and the point mutations, p.Ser97fsX12 and p.Gln318X, (using PCR-RFLP and MLPA), and the *CYP21A2* large-scale gene conversion.

The most frequent mutations detected in the Czech 21OHD patients were the chimeric *CYP21A1P*/*CYP21A2* genes. To date, 6 different chimeric *CYP21A1P*/*CYP21A2* genes have been characterized (12,14,19,26) The CH-1, CH-3 and CH-4 chimeric genes were detected in the Czech population and in addition, 1 novel type (CH-7) was determined (Fig. 1). The 5' untranslated region of the *CYP21A2* gene responsible for the transcriptional activity, is located in the first 167 nucleotides upstream of the ATG codon and contains binding sites for the specificity protein, Sp-1, and adrenal-specific protein transcription factors (27,28). In this fragment, the pseudogene promoter differs from the *CYP21A2* promoter in 4 nucleotides, located at the -126, -113, -110 and -103 positions. These differences cause a lower affinity of the pseudogene promoter to the transcription factors and, consequently, reduce its transcriptional activity to 20% when compared to the *CYP21A2* gene (15,29). The mutation p.Pro30Leu is associated with NC-CAH, and reduces the 21OH activity to 30-40% of the normal enzyme. In the chimeric gene CH-4, the mutation p.Pro30Leu, in synergism with the promoter sequence substitutions, can decrease enzyme activity to 4-10% (30). In the set of our 21OHD probands, we detected 13 patients with at least 1 CH-4 chimeric gene. Three probands with the genotype CH-4/*CYP21A2* deletion, and 1 proband with the genotype CH-4/CH-7 suffered from SW-CAH, 7 probands suffered from SV-CAH and their genotypes were CH-4/CH-1, CH-4/CH-7 and CH-4/p.Ser97fsX12, and 2 probands were clinically diagnosed as NC-CAH and had the genotypes CH-4/p.Val281Leu and CH-4 in the homozygous state.

Furthermore, we detected 6 types of alleles with ≥2 mutations in 1 *CYP21A2* gene. In the case of mutant alleles 25, 26, 27, 29, and 30 (Table II), mutations present in *CYP21A1P* were probably transferred to *CYP21A2* as 1 recombination event. In the case of alleles 3 and 28, we presume that 2 recombination events took place (*CYP21A1P* mutations lying between transferred mutations are missing in these alleles).

In the set of our 21OHD patients, we detected 1 novel non-sense mutation (p.Ser169X) and 2 novel missense (p.Thr168Asn and p.Pro386Arg) mutations. The p.Thr168Asn mutation is associated with NC-CAH, and the p.Pro386Arg mutation with SW-CAH. A conservation of amino acid residues was determined by Robins *et al*, based on the comparison of multiple species-specific sequence variants (pig, dog, cow, sheep, mouse, rat, eel, and puffer fish) homologous to the human *CYP21A2* (31). Based on this result, both missense mutations (p.Thr168Asn and p.Pro386Arg) are located in a highly conserved region. The p.Thr168Asn mutation is situated in the central part of helix E and p.Pro386Arg in a loop between the ß-sheet 1-3 and helix K' (31). In addition, the p.Pro386Arg is located in the heme-binding site (31).

CYP21A2 mutations were detected in 508 alleles of unrelated patients suspected of 21OHD. A comparison with mutation frequencies in other European countries showed similar results in general (Table IV), especially with central European countries. In addition, we described i) mutated *CYP21A2* alleles carrying novel point mutations (p.Thr168Asn, p.Ser169X and p.Pro386Arg), ii) mutated *CYP21A2* alleles carrying the novel chimeric gene designated as CH-7, iii) an unusual genotype with a combination of the *CYP21A2* duplication and the *CYP21A2* large-scale gene conversion on the second allele, and iv) a detailed analysis of the chimeric *CYP21A1P*/*CYP21A2* genes. In conclusion, our genotyping approach allowed for the accurate identification of *CYP21A2* gene mutations in 21OHD patients and their families and provided some useful information on diagnosis and genetic counselling.

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