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Mutational analysis of CYP21 gene in Slovak patients with 21-hydroxylase deficiency and comparison with other European populations

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Congenital adrenal hyperplasia (CAH), due to 21-hydroxylase deficiency, is an autosomal recessive disorder causing impaired secretion of cortisol and aldosteron with overproduction of adrenal androgens. To determine the mutational spectrum in Slovak CAH patients we analyzed CYP21 gene for the presence of 9 most common mutations using PCR and allele-specific PCR. Molecular analysis was performed on 27 patients, mostly with salt wasting (SW) form of CAH. Investigation of 44 unrelated alleles revealed I2 splice mutation to be the most frequent (50%) in Slovak population. Five other mutations were present in our group of patients: deletions/large gene conversions (25%), Ile172Asn (6.8%), Val281Leu (4.5%), Gln318STOP (2.3%), Leu307insT (6.8%). We observed a good correlation between genotype and phenotype. Comparison of ten European countries showed a significant difference in mutational frequencies of deletion/large gene conversion and I2 splice mutation between Middle and West European populations. This study represents the molecular analysis of CYP21 gene in Slovak patients with CAH due to 21-hydroxylase deficiency and it is thus important in molecular diagnostics of the disease.

Key words: CYP21 gene, congenital adrenal hyperplasia (CAH), 21-hydroxylase deficiency, allele-specific PCR, frequency of mutations.

Introduction

Congenital adrenal hyperplasia (CAH) is a monogenic autosomal recessive disorder resulting from deficiency of one of the five enzymes required for the synthesis of cortisol in the adrenal cortex. Deficiency of 21-hydroxylase accounts for about 90–95% of CAH cases (White & Speiser, 2000).

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Steroid 21-hydroxylase, a cytochrome P450 enzyme, catalyzes hydroxylation of 17-α-hydroxyprogesterone (17OHP) and progesterone. If the enzyme does not function properly, adrenal cortex is stimulated by corticotropin and overproduces cortisol precursors. Some of these precursors are diverted to the biosynthesis of sex hormones, which may cause signs of androgen excess, including ambiguous genitalia in newborn girls and rapid postnatal growth in both sexes. The spectrum of clinical manifestations includes a severe classical form CAH with two phenotypic categories, salt wasting (SW) with impaired synthesis of aldosterone and simple virilizing (SV) with apparently normal aldosterone biosynthesis. Both are characterized by prenatal virilization of female fetuses and postnatal virilization in both sexes. There is also a mild, non-classical form of CAH (NC) that may be asymptomatic or develops symptoms during childhood or puberty (DONOHOUE et al., 2001). The incidence of the severe forms is 1 in 15,000 among Caucasians, however, it seems to be higher for mild forms (1:100 to 1:1000) (SPEISER et al., 1988).

The structural gene (CYP21) for 21-hydroxylase is located in the HLA class III region on the short arm of chromosome 6 (6p 21.3) together with highly homologous pseudogene CYP21P. They are 98% identical in exons and are located adjacent to the genes C4A and C4B for complement system (HIGASHI et al., 1986). High sequence identity and close proximity of these two genes can cause gene recombination and gene conversion events in this region. Therefore, the mutations that account for 21-hydroxylase deficiency can be CYP21 deletions, large gene conversions or point mutations (URABE et al., 1990).

Approximately 75% represent deleterious mutations found in the pseudogene and are transferred to CYP21 during mitosis by gene conversion or "microconversion". The frequency of CYP21 large deletions and gene conversions caused by meiotic recombinations varies from 20 to 30% in several studies on Caucasian populations (WEDELL, 1998). More than 60 additional mutations account for the remaining 5% (http://archive.uwcm.ac.uk/uwcm/mg/search/120605.html).

Mutations defined by those pre-existing in the CYP21P gene can be assayed on selectively amplified CYP21 gene sequences by allele-specific amplification (WEDELL et al., 1994; FERENCZI et al., 1999), amplification-created restriction site (ACRS) method (LEE et al., 1996) or by oligonucleotide hybridisation (DOLŻAN et al., 2003).

Phenotype of the patient usually correlates with the genotype, however, many patients 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 allele (SPEISER, 2001).

The aims of this study were: (i) to establish the PCR diagnostics of Slovak CAH patients; (ii) to determine the frequency of deletions/large gene conversions and 8 most common point mutations in CYP21 gene in this group of patients; and (iii) to compare the mutation frequencies with the studies focused on the other European populations.

Material and methods

Patients

We studied 27 Slovak patients (12 males and 15 females) with steroid 21-hydroxylase deficiency and their parents, when available, registered in the Center of Medical Genetics, University Hospital (Bratislava, Slovakia). In the case of patients No. 23 and 24 (Table 2), the parents were not available and in the case of patients No. 25, 26 and 27 only one parent was analyzed. The clinical diagnosis of different types of CAH was made by pediatric endocrinologists based on the physical examination, electrolyte and hormonal data. The levels of 17-α-hydroxyprogesterone in blood at the time of diagnosis ranged between 150–2092 nM. Some patients were diagnosed according to the levels of ketosteroids in urine. Patients with the Na-levels below 130 mM and K-levels over 7 mM were diagnosed as salt wasters.

The probands included in this study were born between the years 1971 and 2003.

Human DNA preparation

DNA was isolated from peripheral blood leukocytes by the salting out method (MILLER et al., 1988) with slight modifications or using the Flexi Gene DNA Isolation Kit (Qiagen).

PCR analysis

PCR detecting the presence of GAGACTAC sequence at the position 707-714 of exon 3. PCR reaction for detection of 707-714delGAGACTAC (8 bp deletion in exon 3) was performed in total volume of 20 μL, containing 1 U of Taq polymerase (Fermentas), 2 μL of buffer (Fermentas), 1.5 mM $MgCl_2$(Fermentas), 200 μM of each dNTP, 10 pmol of primers CYP55, CYP16 and 3 pmol of primer CYP5 and 180 ng of genomic DNA (WEDELL et al., 1994; FERENCZI et al., 1999; PINTÉROVÁ et al., 2000). Cycling conditions were as follows: initial denaturation at 94 °C for 1 min; 35 cycles at 94 °C for 30 sec, 56 °C for 30 sec, 72 °C for 2 min; and a final extension at 72 °C for 7 min.

PCR amplification of CYP21 gene. To selectively amplify CYP21 gene without the presence of CYP21P pseudogene, CYP21 sequence specific primer CYP21-F (5'-CGGGTCGGTGGGAGGGTA-3') and CYP21-R (5'-GCGATCTCGCAGCACTGTGT-3') were used

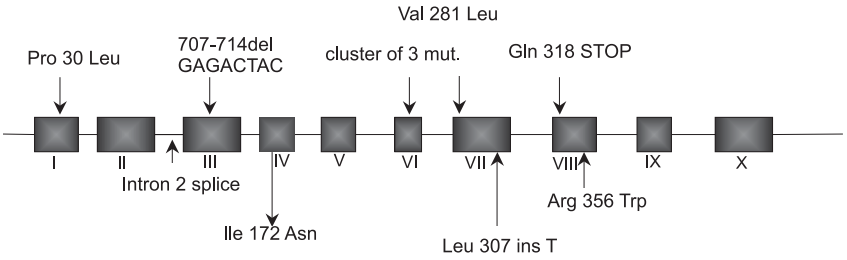

Fig. 1. Schematic localization of 9 CYP21 gene mutations analysed in Slovak patients.

Table 1. Primers and cycling conditions of PCR reactions for detection of 8 point mutation in CYP21 gene.

Mutation	Primers	Cycling conditions
I2 splice	CYP5, CYP48, CYP659H (wt), CYP659I (wt), CYP659G (M)	94 ℃ for 1 min 35 cycles: 94 ℃ for 30 s 64 ℃ for 30 s 72 ℃ for 2 min 72 ℃ for 7 min
rare mutations: Ile172Asn Exon 6 cluster Val281Leu Leu307insT Gln318STOP Arg356Trp	 CYP55, CYP19, CYP1004D (wt), CYP1004H (M) CYP55, CYP19, CYP 1388D (wt), CYP1388E (M) CYP55, CYP19, CYP1688G (wt), CYP1688F (M) CYP55, CYP11, CYP1768I (wt), CYP1768H (M) CYP55, CYP11, CYP1999F (wt), CYP1999E (M) CYP55, CYP11, CYP2113D (wt), CYP2113C (M)	 94 ℃ for 1 min 30 cycles: 94 ℃ for 30 s 56.5 ℃ for 30 s 72 ℃ for 2 min 72 ℃ for 8 min
Pro30Leu	CYP5, CYP48, CYP92D (wt), CYP92E (M)	94 ℃ for 2 min. 30 cycles: 94 ℃ for 30 s 59.7 ℃ for 30 s 72 ℃ for 2 min 72 ℃ for 7 min

(Krone et al., 2002). The PCR was performed using puRe *Taq*™ Ready-To-Go™ PCR Beads (Amersham Biosciences) and reaction was carried out in total volume of 25 μL containing 10 pmol of each primer and 200 ng of genomic DNA. Cycling conditions of PCR were as follows: initial denaturation at 95 ℃ for 5 min; 30 cycles at 95 ℃ for 1 min, 65 ℃ for 1 min, 72 ℃ for 3 min; and a final extension at 72 ℃ for 10 min.

Differential PCR amplification of CYP21 gene is not necessary but for further allele-specific amplification suitable.

Allele-specific PCR. In the case that the homozygous 707-714delGAGACTAC was not present, allele-specific PCR was performed for detection of 8-point mutations (Fig. 1). As a template, 100-fold diluted PCR product of differential PCR reaction was used.

PCR reaction was carried out in total volume of 20 μL, containing 1 U of Taq polymerase (Fermentas), 2 μL of buffer (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 200 μM of each dNTP, 10 pmol of respective primer (3 pmol in case of CYP5, 5 pmol in case of CYP 19 and CYP48, and 15 pmol in the case of CYP 1004H/D), and 0.5 μL of 100-fold diluted PCR product. Table 1 shows appropriate primers and the cycling conditions for individual mutations.

Statistical analysis

Mutation frequencies of CYP21 gene reported in studies from different European countries were compared using Fisher's F-test and statistical significance was assigned to a value of $P < 0.01$.

Results

DNA samples of 27 Slovak patients carrying 21-hydroxylase deficiency (54 unrelated chromosomes) and their parents, when available, were analyzed for the presence of 9 most common mutations in CYP21 gene. Of 27 unrelated patients,

Table 2. Clinical characteristics and CYP21 genotypes of Slovak CAH patients.

Patient[a]	Sex	Age at diagnosis	Clinical phenotype[c]	CYP21 genotype[d]
1	F	10 days	SW	Del;conv/Del;conv
2	F	n[b]	SW	Del;conv/Del;conv
3	M	n	SW	Del;conv/Del;conv
4	M	n	SW	Del;conv/Del;conv
5	M	1 month	SW	Del;conv/Del;conv
6	M	17 days	SW	Del;conv/I2splice
7	M	29 days	SW	I2splice/I2splice
8	M	23 days	SW	I2splice/I2splice
9	M	1 month	SW	I2splice/I2splice
10	F	8 days	SW	I2splice/I2splice
11	F	1 month	SW	I2splice/I2splice
12	F	n	n	I2splice/I2splice
13	F	11 days	SW	I2splice/I2splice
14	M	1,5 month	SW	I2splice/I2splice
15	F	8 days	SW	I2splice/Gln318STOP
16	F	n	n	I2splice/Ile172Asn
17	F	17 days	SW	I2splice/Leu307insT
18	M	7 days	SW	I2splice/N[e]
19	M	n	n	I2splice/N
20	F	1 day	SW	Leu307insT/Leu307insT
21	F	10 days	SV	Ile172Asn/Ile172Asn
22	F	15 years	NC	Val281Leu/Val281Leu
*23	F	6 days	SW	I2splice/I2splice
24	M	18 days	SW	I2splice/I2splice
25	F	n	SW	I2splice/I2splice
26	M	14 days	SW	I2splice/I2splice
27	F	n	SV	Ile172Asn/Ile172Asn

[a] * – additional data of patients without genotyping results of both parents.
[b] n – data not available.
[c] clinical phenotype: SW – salt wasting, SV – simple virilising, NC – non-classic.
[d] Del;conv – large deletion or 5' end conversion.
[e] N – mutation not detected.

21 (77%) were classified as suffering from the SW form and only 2 and 1 patients suffered from SV and NC forms, respectively. The clinical characteristics and genotyping results of probands are shown in Table 2. In the case of patients No. 23 and 24, and the patients No. 25, 26 and 27, none or only one parent was available, respectively. The parents of patients No. 25, 26 and 27 carried heterozygous I2 splice mutation and Ile172Asn substitution, respectively. Since without the genotyping of both parents the results of these patients may be misinterpreted, therefore they are not included in calculation of mutation frequencies.

In 72.7% of patients (16/22) a homozygous genotype was detected, while only 27.3% of the patients (6/22) were compound heterozygotes. I2 splice mutation in homozygous form always associated with severe SW form of CAH.

Using PCR and allele-specific PCR we determined intron 2 splice mutation to be the most frequent (50%) in our group of patients. Eleven of 44 affected alleles (25%) carried large deletion or 5' end conversion, including 707-714delGAGACTAC. Absence of GAGACTAC sequence in exon 3 is consistent with a homozygous state of 8 bp deletion/large gene deletions/5'-end conversion. PCR method using CYP21 specific primer from exon 3, which is almost always included in large deletions and 5' end conversions, has limitations in determination of these mutations in heterozygous state and is unable to distinguish them in homozygous form. Apparent gene conversions and deletions were not distinguished from each other and are included in the same group. Mutational analysis of parents was performed, that could partially suggest heterozygous

Table 3. Frequency of mutations (%) of CYP21 gene in Slovak patients with CAH compared with other European populations.

Country	No. of alleles	Percentage frequencies of CYP21 gene mutations									References
		Del/gene conversion	I2 splice	P30L	I172N	V281L	Q318X	Exon 6 cluster	F307insT	R356W	ND*	
Slovakia	44	25	50	0	6.8	4.5	2.3	0	6.8	0	4.5	Present study
Czech Rep.	152	15.8	46.7	5.9	12.5	6.5	5.3	0	3.9	3.3	–	(a)
Hungary	306	35	43	–	23	9	7	1	7	8	14	(b)
Austria	158	31	22.8	0	15.8	12	2.5	1.9	0	3.2	0.6	(c)
Germany	310	30.6	30.3	1.6	19.7	2.9	4.8	1	0.3	4.5	1.3	(d)
France	258	27.7	20.5	2.7	8.9	16.7	3.9	5	1.1	–	11.2	(e)
Slovenia	66	48.5	16.7	–	7.6	3	3	0	1.5	0	1.5	(f)
Sweden	400	33.5	26.6	1.3	19.8	5.7	2.4	1.1	0.5	3	3.8	(g)
Spain	58	31	26	2	2	17	3	–	–	3	11	(h)
Italy	114	19.7	21	2.6	8	24.5	4.4	1.7	0	2.6	6.1	(i)

[a] References: (a) Kotaška et al. (2003); (b) Ferenczi et al. (1999); (c) Baumgartner-Parzer et al. (2001); (d) Krone et al. (2000); (e) Barbat et al. (1995); (f) Dolžan et al. (2003); (g) Wedell (1998); (h) Ezquieta et al. (1995); (i) Balsamo et al. (2000). * ND – not determined.

large deletion/5'-end conversion in probands. In patient No. 6 homozygous I2 splice mutation was determined. Further analysis of parent's genotypes showed, that the father was a carrier for I2 splice mutation, while the mother carried only wild type allele 659(A). Absence of mutation at this locus suggests a possible deletion/5'-end conversion on the allele inherited from the mother. Patient No. 6 was then interpreted as heterozygous for I2 splice mutation and deletion/5'-end conversion.

Substitutions Ile172Asn, Leu307insT, Val281Leu and Gln318STOP were present rarely, 6.8%, 6.8%, 4.5% and 2.3% of affected alleles, respectively. None of the studied patients carried Pro30Leu, exon 6 cluster or Arg356Trp mutations.

One patient (No. 18) with apparent SW phenotype and one (No. 19) with unknown phenotype carried I2 splice mutation on one allele but none of 8 point mutations on the other allele.

In order to compare allele percentage distribution in our group with other populations, we chose three Central European, three West European, one North European and two South European populations. The data are shown in Table 3.

Discussion

The aim of the study was to determine the genotype distribution of the Slovak CAH patients with 21-hydroxylase deficiency. This is the first larger study of the molecular diagnostics of CAH in Slovakia. In 2000, eight Slovak patients and their relatives were genotyped in the Laboratory of Molecular Biology and Genetics, 2nd Department of Pediatrics, Semmelweis University of Medicine, Budapest, Hungary (Pintérová et al., 2000).

Our group of patients consists of 27 Slovak CAH patients. If we consider the average incidence 1:15,000 of CAH cases worldwide (Donohoue et al., 2001), 3–4 patients would be expected to be born every year in Slovakia. Patients included in this study cover 30-year period, so we should expect 110 CAH patients. Thus, this study involves only about 25% of possible CAH cases in our country. We found 44% males and 56% females in our group of patients. Several studies showed significantly fewer males than females diagnosed especially during the first year of life. Newborn males are therefore more likely exposed to life threatening salt crisis (Frisch et al., 2002). Individuals with SV or NC form, especially boys, often remain undiagnosed. The unbalanced sex ratio requires hormonal screening for CAH in Slovak newborns (Kovács et al., 2001).

PCR analysis revealed the predominance of I2 splice mutation (50%) in our population. High frequency of this mutation was reported in many studies, but it varies from 12–47% (Barbat et al., 1995; Levo & Partanen, 1997; Krone et al., 2000; Kotaška et al., 2003). If we consider only SW form of CAH, I2 splice mutation was present in 71.4% (15/21) of SW patients, which is similar to the study of Owerbach et al. (1992), which demonstrated that 75% of their SW patients are homozygous or hemizygous for this splice mutation. The only mutation associated with SV form was the Ile172Asn, and Val281Leu substitution was detected in one patient with NC form of CAH.

These two mutations are considered to be the most frequent mutations for SV and NC forms, respectively (White & Speiser, 2000).

However, PCR analysis used in our study is not able to discriminate among 707-714delGAGACTAC, large deletions and 5' end conversions. Absence of GAGACTAC sequence in exon 3 prevents any amplification using primers specific to this sequence and thus giving false homozygosity for other mutations. The other reason for high homozygosity of I2 splice mutation was reported in the study of Day et al. (1996) as a consequence of "allele dropout" phenomenon, possible loss of amplification of one allele in heterozygous patients at nucleotide position 659. Therefore genotypes of patients (No. 23, 24, 25, 26, 27), whose parents were not analysed, could give inaccurate results. For further investigation and discrimination between deletions and large gene conversions in homozygous or heterozygous state, additional methods based on Southern blot analysis (Dolžan et al., 2003) or semiquantitative polymerase chain reaction/enzyme digestion (Tukel et al., 2003) should be used.

Our results showed a frequency of homozygosity 72.7%, which seems to be extremely high in comparison with other populations where this frequency usually does not exceed 30% (Grigorescu-Sido et al., 2002; Stikkelbroeck et al., 2003). In spite of the fact that no consanquinity among families included in this study was registered, Slovak population showed discrepancy between the mutation frequencies and the diversion of actual numbers of different genotypes from the expected numbers for homozygous and compound heterozygous individuals in a randomly mating population. Further studies on larger patient group involving more SV and NC CAH patients are therefore needed to obtain heterogenous statistical group of individuals.

Only two alleles (4.5%) remained undetermined suggesting, according to the literature, that these 9 mutations account for more than 90% of all CYP21 gene mutations (Wedell et al., 1994). To reveal the complete genotypes of patients No. 18 and 19, sequence analysis is being performed. However, several other genetic or environmental factors other than 21-hydroxylase activity may influence the CAH phenotype (Speiser, 2001).

Statistical analysis of CYP21 gene mutation frequencies among 10 European countries showed significant differences in distribution of deletions/large gene conversions and I2 splice mutation. I2 splice mutation is the most frequent mutation in the Middle European countries – Slovakia, Czech Republic and Hungary – however, it differs in Slovenian population where deletions and large gene conversions are predominant. Large deletions and gene conversions are the most frequent mutations in West European countries - Austria, Germany, France and in Sweden and Italy (Barbat et al., 1995; Wedell, 1998; Ferenczi et al., 1999; Balsamo et al., 2000; Krone et al., 2000; Baumgartner-Parzer et al., 2001; Dolžan et al., 2003; Kotaška et al., 2003). No significant difference was observed for frequencies of rare mutations. Some differences between our and other populations may arise from different methods used for genotype analyses, small number of patients available for this study or from real ethnic differences.

Whereas most of the patients were homozygous for CYP21 gene mutations and none of the alleles analysed by PCR carried a double mutation, we observed good genotype-phenotype correlation in our CAH patients. We should mention that I2 splice mutation in homozygous form was always associated with severe mineralocorticoid deficiency. Kotaška et al. (2003) reported I2 splice mutation to be the most frequent also in SV and NC form of CAH. Clinical manifestation of I2 splice mutation is usually severe SW or SV form, but in rare cases mild NC or asymptomatic phenotypes were confirmed (Witchel et al., 1996; Krone et al., 2000).

For accurate estimation of genotype-phenotype correlation, levels of 17-OHP and serum levels of Na/K are needed. The hormonal data of our patients are often inconsistent and for many cases not available. Additional measurement of 17-OHP is often influenced by previous hormonal treatment and the data do not reflect the real hormonal profile of the patient.

The present study is the first larger molecular study of CYP21 gene in Slovakia bringing the information about mutation frequencies in our population. In spite of some limitations, allele-specific amplification was proven to be effective for rapid identification of most common mutations in CYP21 gene. However, additional investigation on larger patient group and establishment of other molecular methods (e.g. sequencing analysis, Southern blotting) are required to estimate the real frequencies of mutations and incidence of the disease in our population. For better understanding of genotype-phenotype correlation exact hormonal data are necessary. In order to increase the number of genotyped patients with SV and NC form of congenital adrenal hyperplasia, it is important to improve

cooperation among pediatric endocrinologists and genetists.

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