PCR BASED DIAGNOSIS OF 21-HYDROXYLASE GENE DEFECTS IN SLOVAK PATIENTS WITH CONGENITAL ADRENAL HYPERPLASIA

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Objective. To analyse 21-hydroxylase gene for 8 most common mutations in patients with salt-wasting type of congenital adrenal hyperplasia.

Methods. Allele specific PCR performed on 8 salt-wasting CAH patients and their 23 healthy relatives.

Results. Two patients were homozygous for 8 bp deletion in exon 3, while 6 patients were homozygous for intron 2 splice mutation. Mutant allele for splice mutation was find also in both parents of patients with this type of mutation.

Conclusions. These preliminary results show that only two mutations, 8 bp deletion in exon 3 and splice mutation in intron 2, were present in this group of Slovak patients with salt-wasting type of congenital adrenal hyperplasia.

Key words: Congenital adrenal hyperplasia – Allele specific PCR – 21-hydroxylase – CYP21 gene – Slovak population

Congenital adrenal hyperplasia (CAH) is a group of inherited autosomal recessive diseases in which the impaired enzymatic activities result in insufficient cortisol production and subsequently increased pituitary adrenocorticotropic hormone (ACTH) secretion. Excessive ACTH stimulation then results in adrenocortical hyperplasia. CAH is known to be caused by 5 defects of steroidogenesis on the level of: the deficiency of StAR (steroidogenic acute regulatory) protein, 3-β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450c17 (17-α-hydroxylase), P450c21 [21-hydroxylase (21-OHase)] or P450c11/18 (11-β-hydroxylase) (Prusa and Lisa 1997). In more than 95 % of cases the CAH results from 21-OHase deficiency (Wedell 1998). The defects in 21-OHase may further result in the abnormalities of external genitalia in newborn girls and serious adrenal crisis in newborn boys as discussed in details bellow.

21-hydroxylase deficiency results in cortisol deficiency either with or without aldosterone deficiency, as well as with excess of adrenal androgen production (Pang 1994). The phenotype of 21-hydroxylase deficiency is traditionally divided into three groups: 1. simple virilizing (SV) form, with the virilization of external genitalia in newborn girls and early pseudoprecocious puberty in boys; 2. salt-wasting (SW) form, with the same symptoms but also additional neonatal aldosterone deficiency; 3. non-classical or later-onset (NC or LO) form which is associated with pseudococious puberty in girls, hirsutism, polycystic ovary syndrome and decreased fertility (Speiser et al. 1992).

The virilisation of newborn girls with SW or SV form of CAH is due to excessive androgen exposure during the embryogenesis and can vary from mild clitoromegaly alone to penile transformation of the

clitoris and urethra with complete fusion of the labia folds. While affected female newborns are externaly virilized, they do not have testes and have normal female internal genitalia. Therefore, they are capable of normal fertility after cosmetic and functional surgical repair with hormone replacement therapy to control adrenal function.

Male new-borns with SW form do not have genital abnormalities and for such reason they are usually recognised as CAH patient only after the adrenal crisis few weeks after the birth. Adrenal crisis develops as a result of inability to retain sodium and excrete potassium from the renal tubules, which leads to hyponatremia, hyperkalemia, metabolic acidosis and sometimes hypoglycemia due to cortisol deficiency.

Diagnosis of CAH due to 21-OHase deficiency is usually established by serum measurements of 17-OH progesterone (17-OHP), the metabolite immediately preceding the 21-hydroxylation step in steroidogenesis. Neonatally, the diagnosis can be made by measuring 17-OHP in dried blood spots on filter paper (Solyom 1979; Wedell 1998).

CAH patients have to be lifelong treated with glucocorticoids to replace deficient cortisol and to suppress overproduction of ACTH, thereby suppress excessive adrenal androgen production. For patients with SW form of CAH, in addition to glucocorticoid therapy, both mineralocorticoid and sodium supplement therapies are necessary to normalise electrolyte and water balance.

The 21-hydroxylase locus: Gene encoding 21-hydroxylase (CYP21) is located in the middle of the major histocompatibility complex (HLA) on the short arm of chromosome 6 and it is flanked telomerically by class I HLA genes and centromerically by class II HLA genes (Strachan 1994). It consists of approximately 3 kb and contains 10 exons. Two kb transcript encodes a 21-hydroxylase peptide of 494 or 495 amino acids. This variation results from the length polymorphism in exon 1, where CTG tandem repeats varies in size from $(CTG)_4$ to $(CTG)_5$ (Strachan 1994). The gene is a part of DNA segment approximately 30 kb long which probably underwent a tandem duplication during evolution. This segment contains tandemly repeated units C4A-CYP21P and C4B-CYP21, where C4A and C4B are genes encoding complement factor 4 (Strachan 1994). CYP21P gene is 98 % homologous to CYP21 gene, but it contains a number of deleterious sequences which result in a prematurely truncated protein. Thus, CYP21P is an inactive pseudogene, whereas CYP21 encodes the active 21-OHase (Wedell 1998). There was observed a variability in number of C4-CYP21P and C4-CYP21 repeat units (Wedell 1998). If no CYP21 gene is present in the individual, it will result in salt-wasting form of CAH.

Majority of CAH causing mutations are sequences that are normally present in the pseudogene. These mutations can be transformed from CYP21P to CYP21 gene by the process of gene conversion and thus inactivate the gene (Strachan 1994). Mutations derived from the pseudogene can be divided into several groups: 1. frame shifting deletion/insertion mutations; 2. splice site mutation; 3. nonsense mutations; 4. missense mutations. These mutations account for approximately 95 % of all mutated CYP21 alleles. The remaining 5 % of mutations are population specific and are not derived from the pseudogene (Wedell 1998).

One of the mutations transferred from the pseudogene is 8 bp deletion in exon 3. Deletion of 8bp shifts the reading frame, thus producing inactive enzyme (White et al. 1994). This mutation is associated with SW phenotype (Mornet et al. 1991).

Very frequent mutation is the splice mutation in intron 2, in which an A or C at 659 nucleotide is replaced by G. This mutation alters the pre-mRNA splicing by activating another acceptor site in the splicing process and thus shifting the reading frame, creating a premature termination codon in exon 3 (Witchell et al. 1996). Splicing mutation is associated with SW and less severe SV phenotypes (Speiser et al. 1992).

Nonsense mutation derived from the pseudogene is point mutation in exon 8, where CAG is replaced by TAG at codon 318, with manifestation at the protein level as change of Gln to stop codon, thus resulting in truncated protein (Strachan 1994; Wedell 1998). This mutation is usually associated with SW phenotype.

Several point mutations derived from the CYP21P gene are missense mutations which result in the change of only one amino acid, while their functional effect is of very wide range. A cluster of mutations in exon 6 (Ile236Asn, Val237Glu, Met239Lys) abolishes enzymatic activity (White et al. 1994). Another mutation in exon 4 (Ile172Asn) changes the hydrophobic residue to a polar one in the region of the protein that normally interacts with the membrane of endoplasmatic reticulum and may disrupt

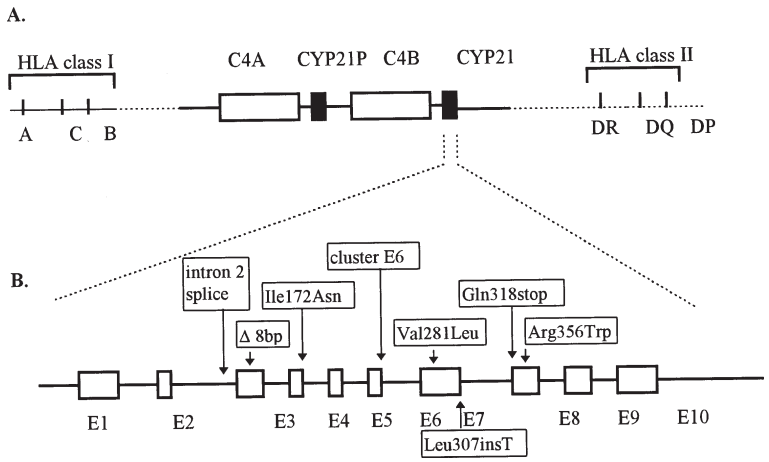

Fig. 1 Location of 21-hydroxylase gene on chromosome 6 and its structure. A. 21-hydroxylase gene (CYP21) is located on chromosome 6 in the HLA region, between HLA class I and class II genes. Gene for 21-hydroxylase has 2 copies, CYP21P pseudogene and CYP21 active gene, tandemly repeated together with gene C4 for complement 4. B. CYP21 gene contains 10 exons (represented by empty boxes). The most common mutations and their positions are marked by arrows.

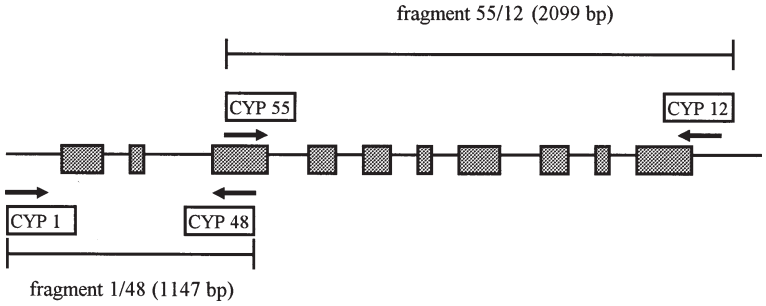

Fig. 2 Location of primers CYP1 and CYP48 which are used for the amplification of the first part of the gene CYP21 (fragment 1/48) and primers CYP12 and CYP55 which are used for amplification of the second part of this gene (fragment 55/12). Exons of the gene CYP21 are marked with grey boxes.

such an interaction. The mutant enzyme is apparently not properly localized in microsomes and its activity is about 1 % of normal (White et al. 1994). Mutation Val281Leu in exon 7 may affect secondary structure of the enzyme. Such enzyme is not normally localized in the endoplasmatic reticulum and also binds heme poorly. Mutation Arg356Trp destroys all enzymatic activity probably affecting the interaction with substrate (White et al. 1994).

In addition to the mentioned mutations many other mutations were found on the gene for 21-OHase, but they are much less frequent (Wedell et al. 1992; Wedell and Luthman 1993).

The aim of this study was to analyse DNA samples of Slovak patients suffering from CAH and their relatives for 8 most common above mentioned mutations in 21-hydroxylase gene.

Materials and Methods

Patients. This study comprises 8 patients (3 girls and 5 boys) aged 2-17 yr with diagnosis of CAH due to 21-OHase deficiency and 23 relatives of these patients. The diagnosis was based on clinical signs and laboratory findings (elevated 17-OHP in blood or ketosteroids in urine). All patients had the SW

Table 1
The name, localization and oligonucleotide sequences of primers used in PCRs for all examinated mutations.

Mutation	Primers		Localization	Sequences 5'→3'
8 bp deletion	sense	CYP5	307 – 325	GGT GCT GAA CTC CAA GAG G
in Exon 3	antis.	CYP16	1187 – 1207	GTC CAC AAT TTG GATGGACCA
	sense	CYP55	700 – 719B	CCT GTC CTT GGG AGA CTA CT
Intron 2 splice	sense	CYP 659H (WT A)	644 – 659	ACC CTC CAG CCC CCA A
	sense	CYP 659I (WT C)	644 – 659	ACC CTC CAG CCC CCA C
	sense	CYP 659G (MUT)	644 – 659	ACC CTC CAG CCC CCA G
	antis.	CYP 48	711 – 729B	CAG AGC AGG GAG TAG TCT C
	sense	CYP 5	307 – 325	GGT GCT GAA CTC CAA GAG G
Ile172Asn	antis.	CYP 1004D (WT)	1004 – 1022	CCG AAG GTG AGG TAA CAG A
	antis.	CYP 1004H (MUT)	1004 – 1021	CG AAG GTG AGG TAA CAG T
	antis.	CYP 19	2066 – 2084	TTG AGC AAG GGC AGC CGT G
	sense	CYP 55	700 – 719B	CCT GTC CTT GGG AGA CTA CT
Cluster in	antis.	CYP 1388D (WT)	1388 – 1405	GCC TCA GCT GCA TCT CCA
exon 6	antis.	CYP 1388E (MUT)	1388 – 1405	GCC TCA GCT GCT TCT CCT
	antis.	CYP 19	2066 – 2084	TTG AGC AAG GGC AGC CGT G
	sense	CYP 55	700 – 719B	CCT GTC CTT GGG AGA CTA CT
Val281Leu	antis.	CYP 1688G (WT)	1688 – 1703	CT GCA GCC ATG TGC AC
	antis.	CYP 1688F (MUT)	1688 – 1704	ACT GCA GCC ATG TGC AA
	antis.	CYP 19	2066 – 2084	TTG AGC AAG GGC AGC CGT G
	sense	CYP 55	700 – 719B	CCT GTC CTT GGG AGA CTA CT
Leu307insT	antis.	CYP 1768I (WT)	1760 – 1776	TG GTG AAG CAA AAA AAC
	antis.	CYP 1768H (MUT)	1760 – 1777	GTG GTG AAC CAA AAA AAA
	sense	CYP 55	700 – 719B	CCT GTC CTT GGG AGA CTA CT
	antis.	CYP 11	2742 – 2763	GGA GCA ATA AAG GAG AAA TG A
Gln318Stop	antis.	CYP 1999F (WT)	1999 – 2015	TGG TCT AGC TCC TCC TG
	antis.	CYP 1999E (MUT)	1999 – 2015	TGG TCT AGC TCC TCC TA
	sense	CYP 55	700 – 719B	CCT GTC CTT GGG AGA CTA CT
	antis.	CYP 11	2742 – 2763	GGA GCA ATA AAG GAG AAA CTG A
Arg356Trp	antis.	CYP 2113D (WT)	2113 – 2127	G GGC ACA ACG GGC CG
	anti.	CYP 2113C (MUT)	2113 – 2129	AAG GGC ACA ACG GGC CA
	sense	CYP 55	700 – 719B	CCT GTC CTT GGG AGA CTA CT
	antis.	CYP 11	2742 – 2763	GGA GCA ATA AAG GAG AAA CTG A

form of CAH, which presented by salt crisis in neonatal age and were treated with hydrocortisone and 9α–fludrocortisone at pediatric endocrinology clinics in different regions of Slovakia.

Blood samples were collected and DNA isolation was performed in the Centre of Clinical Genetics, University Hospital in Bratislava. DNA was isolated from peripheral leukocytes by "Wizard Genomic DNA Purification Kit" (Promega).

Allele specific PCR for detection of CYP21 gene mutations was performed in the Laboratory of Molecular Biology, 2nd Department of Pediatrics, Semmelweis University of Medicine, Budapest, Hungary.

PCR detecting 8 bp deletion in exon 3. PCR detecting 8 bp deletion in exon 3 of the CYP 21 gene requires two sense (CYP5 and CYP55) and 1 antisense primers (CYP16). The positions and sequences of the primers are indicated in Tab. 1. CYP5 (located in exon 2) and (CYP16 located in exon 5), are defining 900 bp long PCR product – the control fragment. CYP55 corresponds to the region in exon 3, which, in pseudogene, contains the 8 bp deletion. PCR with CYP55 and CYP16 amplifies 507 bp long fragment only in the absence of the deletion. If the deletion is present in exon 3, the 507 bp fragment is not amplified because of no annealing of primer

Table 2

Primers used for amplification of control and specific fragments for mutations of CYP21 gene. For each mutation the greatness of the control, mutant specific and wildtype specific PCR fragments are mentioned as well as primers which were used for amplification of the concrete fragments.

Mutation	Fragments	bp	Primers
Intron 2 splice	control	352	CYP5, CYP48
	wildtype specific	85	CYP48, CYP659H
	wildtype specific	85	CYP48, CYP659I
	mutant specific	85	CYP48, CYP659G
Ile172Asn	control	1384	CYP55, CYP19
Exon 4	wildtype specific	322	CYP55, CYP1004D
	mutant specific	322	CYP55, CYP1004H
Cluster in Exon 6	control	1384	CYP55, CYP19
	wildtype specific	705	CYP55, CYP1388D
	mutant specific	705	CYP55, CYP1388H
Val281Leu	control	1384	CYP55, CYP19
Exon 7	wildtype specific	1004	CYP55, CYP1688G
	mutant specific	1004	CYP55, CYP1688F
Leu307insT	control	2063	CYP55, CYP11
Exon 7	wildtype specific	1077	CYP55, CYP1768I
	mutant specific	1077	CYP55, CYP1768H
Gln318Stop	control	2063	CYP55, CYP11
Exon 8	wildtype specific	1315	CYP55, CYP1999F
	mutant specific	1315	CYP55, CYP1999E
Arg356Trp	control	2063	CYP55, CYP11
Exon 8	wildtype specific	1429	CYP55, CYP2113D
	mutant specific	1429	CYP55, CYP2113C

CYP55. Thus the result of the PCR amplification in patients without deletion is represented by two bands on agarose gel, while patients with the deletion have only one band – the control fragment.

Allele specific PCR. If 8bp mutation in exon 3 is not present, allele specific PCR follows. Allele specific PCR is used for the determination of the splice mutation in intron 2 and for other 6 point mutations mentioned above (Tab. 1).

Analysis of CYP21 gene, without contamination by CYP21P pseudogene sequences, is performed by using PCR primers discriminating between these two genes. For this purpose 8bp deletion in exon 3 of CYP21P can be used. CYP21 is amplified in two parts, from the beginning of the gene to exon 3 and from exon 3 to the end of the gene. Primers CYP1 and CYP48 were used for the amplification of the first part of the gene (fragment 1/48) and CYP12 and CYP55 for the second part of the gene (fragment 12/55). Primers CYP48 and CYP55 are situated into the region of 8 bp deletion (Fig. 2).

Fragments 1/48 and 12/55 are then used in subsequent allele specific PCR. Each allele specific PCR produce one control and one specific fragment. Two separate reactions are performed for each mutation, one with the wildtype-specific and other with the mutant-specific primer. Oligonucleotide sequences for both primers are nearly homologous, they differ in one nucleotide at 3' end of the primer. For splice mutation in intron 2, three reactions are necessary, because two wild-type alleles are common. Primers used for amplification of control and specific fragments for each mutation, as well as the length of these fragments are indicated in Tab. 2.

PCR reaction was performed in total volume of 20 µl. Reaction mixture contained 1 U of *Taq* polymerase (Pharmacia) and 10 mM Tris-HCl buffer, pH 9 (Pharmacia) (buffer contains: 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM of each dNTP, 5% glycerol, 10 pmol of re-

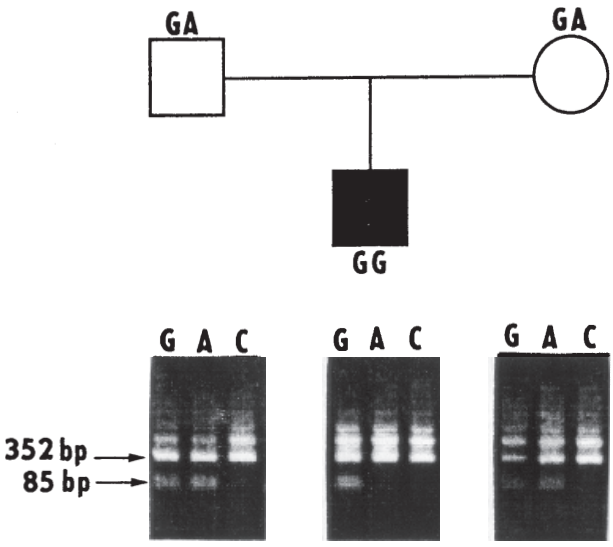

Fig. 3 Typical family with splice mutation in intron 2. Mother and father are heterozygous for mutant allele G, with allele A and child is homozygous for mutant allele G. Results of the agarose gel electrophoresis of the PCR products are represented below the pedigree. The upper arrow indicates 352 bp long control fragment and lower arrow indicates 85 bp long specific fragment.

spective primer (3 pmol in case of CYP5, 5 pmol in case of CYP19 and 15 pmol in case of CYP1004 H/D). 180 ng of DNA is added to the reaction mixture when amplifying 55/12 and 1/48 fragments and specific fragment for the deletion in exon 3. For the amplification of fragments specific for intron 2 splice mutation 0.5 μl of 1/48 fragment was used in the reaction mixture and for the amplification of specific fragments of the rest of the mutations 0.5 μl of 55/12 fragment is added instead of the genomic DNA. Samples were subjected to denaturation at 94 °C for 1 min followed by 35 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 2 min (for amplification of 55/12, 1/48 and deletion specific fragments) or 35 cycles of 94 °C for 30 sec, 64 °C for 30 sec, 72 °C for 2 min (for amplification of intron 2 splice mutation specific fragments) or 30 cycles of 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 2 min (for specific fragments amplification of the rest of the mutations, with except of the Leu308 insT mutation where the annealing temperature was 48 °C) ending by extension at 72 °C for 8 min. The reactions were then directly electrophoresed on 1 % agarose gel in the presence of ethidium bromide stain.

Two lines on agarose gel (three for the splice mutation) are necessary for each mutation, one line for the wild-type primer PCR products (two lines for splice mutation) and second for the mutant-specific primer PCR products. To avoid false negative results, the control fragment have to be present in all lines. The specific fragment is present only in the line where primers respecting genotype are used for amplification. That mean, specific fragment is present in both lines when patient is heterozygous for the mutation and it is present only in one line when the patient is homozygous for mutation or wildtype allele.

Results

DNA samples of eight patients suffering from CAH and 23 healthy parents and siblings of these patients were analysed for the presence of 8 most common mutations in gene for 21-hydroxylase. For this purpose allele specific PCR was performed, where two separate PCR reactions with primers specific for the mutant or wild-type allele were used for the detection of each mutation.

Six of eight patients were homozygous for splice mutation in intron 2. In all cases where this mutation was found in patients, the same mutation in paternal and maternal samples was also present, suggesting this mutation in patients did not arise *de novo*. Fig. 3 shows typical family with splice mutation in intron 2, where both parents are heterozygous for mutant allele G, with wild-type allele A and the child is homozygous for mutant allele G. The 352 bp band is the control fragment indicating the reliability of the PCR assay. The 85 bp band is the fragment determining the type of allele (mutant allele G, wild-type allele A and C).

Two of eight patients were homozygous for the 8 bp deletion in exon 3 (Fig. 4). As shown by electrophoresis, PCR amplification in these patients resulted in one band, the control 900 bp fragment, while in the patients without deletion it resulted in two bands, the 900 bp control and 507 bp specific band which presence indicates the absence of 8 bp in exon 3.

Discussion

Taking in account the size of our patient group and the uniformity of their phenotype, it is not surprising that we found only two types of mutations.

Fig. 4 PCR analysis of 8 bp mutation in exon 3. First two lanes represent the patients with deletion in exon 3 in which PCR amplify only the control fragment (900 bp) and last three lanes belong to patients without this mutation that amplify both, control and specific fragment (507 bp).

Splice mutation in intron 2 is the most frequent mutation of CYP21 in SW patients, the frequency of which in the studies including the patients with all types of CAH phenotypes was 19 to 42 % (Owerbach et al. 1990; Mornet et al. 1991; Speiser et al. 1992; Carrera et al. 1996; Wedell 1998; Fardella et al. 1998). Our results are similar to those by Owerbach et al. (1992) demonstrating that 75 % of their SW patient were homozygous or hemizygous for this splice mutation. The frequency of the allele with 8 bp deletion vary between 1 and 10 % in classic patients (Amor et al. 1988; Higashi et al. 1988; Owerbach et al. 1990; Mornet et al. 1991). The frequency of this mutation in our group is higher (25 %), in our study all patients have the same form of CAH (SW CAH) which is contrasting to previous studies where all classic patients were included.

Although both of these mutations in homozygous form are usually associated with SW phenotype, some patients homozygous for intron 2 splice mutation display even SV phenotype (Wedell 1998). Indeed, in the majority of cases the relationship between CYP21 genotype and CAH phenotype can be find. The mutations that completely inactivate the gene are associated with the most severe salt-wasting form of CAH. This group contains following mutations: 8 bp deletion in exon 3, cluster in exon 6, Leu307insT, Gln318stop, Arg356Trp (Wedell 1998). The Ile172Asn is associated with prenatal virilization without salt wasting. On the other hand, the Val281Leu and Pro453Ser mutations are associated with non classic CAH and the Pro30Leu mutation is associated with mild prenatal clitoromegaly (Wedell 1998). The association between the genotype and phenotype can help to predict the disease outcome in CAH patients.

The possibility to predict the disease outcome in CAH patients by mutation analyses might help adjusting the dose of steroid substitution. Genotype identification of CAH is of a basic importance for successful prenatal treatment. Prenatal diagnosis is possible in the first trimester or in second trimester from DNA isolated from chorionic villus samples or amniotic fluid samples, respectively. Prenatal treatment is based on dexamethasone administration to pregnant women (Pang 1994). Dexamethasone crosses the placenta and is able to decrease androgen levels in foetus. The goal of prenatal treatment of CAH is to prevent the virilization of the external genitalia of affected female foetus, thereby eliminating the need of postnatal corrective genital surgery. In a case of following pregnancy in the families which already have CAH patient, dexamethasone treatment should be initiated as early as possible. After the confirmation or exclusion of CAH by prenatal diagnosis, dexamethasone treatment should be withdrawn in all male foetuses and healthy female foetuses, but in CAH female foetuses the treatment with dexamethasone should continue until the end of pregnancy. To minimise the maternal side effect, the dose can be decreased after the period of sexual differentiation. Although there were no apparent teratogenic effects of dexamethasone therapy, prenatal treatment is still at experimental level, since the long-term effects of dexamethasone treatment still have to be definitely evaluated (Pang 1994).

In summary, our preliminary results with Slovak CAH patients refer to the presence of only two mutations in CYP21 gene, 8 bp deletion in exon 3 and intron 2 splice mutation. It is surprissing that we found only two types of mutations, however it can result from small number of patients in our group and of fact that all patients had the same form of CAH, the salt wasting form. These results are the first data about the mutations in CYP 21 gene in Slovak CAH patients and serve as a pilot study of complete ongoing evalution of Slovak population of CAH patients.

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References

Amor M, Parker KL, Globerman H, New MI, White PC: A mutation in the CYP21B gene (ILE172 to ASN172) causes steroid 21-hydroxylase deficiency. Proc Natl Acad Sci USA **85**,1600-1604,1988

Carrera P, Bordone L, Azzani T, Brunelli V, Garancini MP, Chiumello G, Ferrari M: Point mutations in Italian patients with classic, non-classic, and cryptic forms of steroid 21-hydroxylase deficiency. Hum Genet **98**,662-665,1996

Fardella CE, Poggi H, Pineda P, Soto J, Torrealba I, Cattani A, Oestreicher E, Foradori A: Salt-wasting congenital adrenal hyperplasia: detection of mutations in CYP21B gene in a Chilean population. J Clin Endocrinol Metab **83**,3357-3360,1998

Higashi Y, Tanae A, Inoue H, Hiromasa T, Fujii-Kuriyama Y: Aberrant splicing and missense mutations cause steroid 21-hydroxylase [P-450(C21)] deficiency in humans: possible gene conversion products. Proc Natl Acad Sci USA **85**,7486-7490,1988

Mornet E, Crete P, Kutten F, Raux-Demay M-C, Boue J, Whitep C, Boue A: Distribution of deletions and seven point mutations on CYP21B genes in three clinical forms of steroid 21-hydroxylase deficiency. Am J Hum Genet **48**,79-88,1991

Owerbach D, Crawford YM, Drazin MB: Direct analysis of CYP21B genes in 21-hydroxylase deficiency using polymerase chain reaction amplification. Mol Endocrinol **4**,125-131,1990

Owerbach D, Ballard A-L, Draznin MB: Salt-wasting congenital adrenal hyperplasia: detection and characterization of mutations in steroid 21-hydroxylase gene, CYP21, using the polymerase chain reaction. J Clin Endocrinol Metab **74**,553-558,1992

Pang S: Congenital adrenal hyperplasia. Curr Ther Endocrinol Metab **5**,157-166,1994

Pruša R, Lisa L: Molecular analysis of congenital adrenal hyperplasia (in Czech). Cs Pediatr **52**,51-55,1997

Speiser PW, Dupont J, Zhu D, Serrat J, Buegeleisen M, Tusie-Luna M-T, Lesser M, New MI, White PC: Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Clin Invest **90**,584-585,1992

Soylom J, Hammond GL, Vihko R: A method for identification and follow-up of patients with a steroid-21-hydroxylase deficiency. Clin Chim Acta **92**,117-124,1979

Strachan T: Molecular patology of 21-hydroxylase deficiency. J Inher Metab Dis **17**,430-441,1994

Wedell A, Ritzen EM, Haglund-Stengler B, Luthman H: Steroid 21- hydroxylase deficiency: Three additional mutated alleles and establishment of phenotype-genotype relatioships of commmon mutations. Proc Natl Acad Sci USA **89**,7232-7236,1992

Wedell A, Luthman H: Steroid 21-hydroxylase (P450c21): a new allele and spread of mutations through the pseudogene. Hum Genet **91**,236-240,1993

Wedell A: Molecular genetics of congenital adrenal hyperplasia (21- hydroxylase deficiency): implication for diagnosis, prognosis and treatment. Acta Paediatr **87**,159-164,1998

Witchel SF, Bhamidipati DK, Hoffman EP, Cohen JB: Phenotypic heterogeneity associated with splicing mutation in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Clin Endocrinol Metab **81**,4081-4088,1996

White PC, Vitek A, Dupont B, New Mi: Characterization of frequent deletions causing steroid 21-hydroxylase deficiency. Proc Natl Acad Sci USA **85**,4436-4440,1988

White PC, Tusie-Luna M-T, New MI, Speiser PW: Mutation in steroid 21-hydroxylase (CYP21). Hum Mut **3**,373-378,1994

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