

INTRON 2 SPLICE MUTATION AT CYP21 GENE IN PATIENTS WITH CONGENITAL ADRENAL HYPERPLASIA IN THE REPUBLIC OF MACEDONIA

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ABSTRACT

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder. In 90-95% of cases it results from mutations in the gene for 21-hydroxylase (CYP21, also termed CYP21A2 and P450c21). The IVS-II-656 (C/A>G) mutation leaves ~2.0% enzyme activity, and comprises 25% of the classic CYP21 deficiency alleles and 51% of alleles in the salt-wasting form.

We performed direct molecular diagnosis of the IVS-II mutation in 41 Macedonian patients with different clinical forms of CAH and 55 of their healthy parents and siblings from 37 unrelated families, using the differential polymerase chain reaction/amplification created restriction site method (PCR/ACRS). The IVS-II mutation was detected in 41.5% patients (29.3% were homozygotes and 12.2% were heterozygotes). All homozygotes had a severe classical CAH phenotype (of which 91.7% were salt-wasting and 8.3% were simple virilizing). Three of the heterozygotes had a salt-wasting (SW) phenotype and were compound heterozygotes. The IVS-II mutation was also found in 30.9% of the family members (18.2% were homozygous

and 12.7% were heterozygous) and none had any clinical manifestation. The frequency of the IVS-II mutation (41.5%) in these subjects was similar to that reported elsewhere.

Key words: Congenital adrenal hyperplasia (CAH), 21-hydroxylase deficiency, CYP21 gene, IVS-II-655 (C/ A>G) mutation.

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is one of the most common autosomal recessive disorders of adrenal steroidogenesis and characterized by impaired activity of an enzyme required for cortisol biosynthesis [1]. Congenital adrenal hyperplasia due to 21-hydroxylase (CYP21) deficiency is traditionally classed as a classic form with severe enzyme deficiency and prenatal onset of virilization; females present ambiguous genitalia at birth and in both sexes virilization continues postnatally, and a non classical form with mild enzyme deficiency and postnatal onset of premature adrenarche and pubarche in children, virilization in young women, and variable symptoms in young men. The classic form is further divided into the salt-wasting form (SW) with a severe defect in aldosterone biosynthesis, which leads to renal inability to conserve sodium and a simple virilizing (SV) form with apparently normal aldosterone biosynthesis [2-5]. The severe classical form occurs in one in 10,000

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to 15,000 Caucasians [6]. The milder non classical CAH occurs in ~1 in 1,700 in the general population [5]. Based on newborn screening data, the carrier frequency of CAH in the general population is estimated to be 1 in 55 [7].

More than 95% of cases of CAH are caused by steroid 21-hydroxylase deficiency [6]. The structural gene, CYP21, for the adrenal cytochrome P450 specific for steroid 21-hydroxylation (P450c21) is 3.2 kb long and contains 10 exons. A CYP21 gene's 2 kb transcript encodes a polypeptide of 494 or 495 amino acids. This variation results from the polymorphism length in exon 1, where a tandem repeat number may comprise (CTG)₄ or (CTG)₅ [8]. The CYP21 and a pseudogene (CYP21P, CYP21A1P) are located at the 3' terminus of each of the two genes encoding the fourth component of complements C4A and C4B within the HLA genes, in a DNA segment ~30 long, in which a tandem duplication probably occurred during evolution [9]. Because of the high homology and tandem-repeat organization, this gene cluster is subject to a high frequency of recombination events, which can lead either to unequal crossover during meiosis, which can produce a wide variety of rearrangements depending on the breakpoint, or large or small gene conversion events that transfer deleterious mutations in the CYP21P gene to the CYP21 gene [10-13]. However, apart from gene deletions and large gene conversions, nine such pseudogene-derived mutations account for about 95% of all affected CYP21 alleles in different ethnic groups, while ~5% are *de novo* mutations which did not arise in the pseudogene [6]. There is a good relationship between genotype and clinical disease presentation but a combination of CYP21 mutations can cause different phenotypes [14-16]. The IVS-II-656 (C/A>G), the 8 bp frameshift deletion at codon 111-113, the thymidine insertion at codon 306 (*p.Phe306+t*), the nonsense mutation at codon 318 (*p.Gln318X*), and the single base substitution at codon 356 (*p.Arg356Trp*), result in complete inactivation of CYP21, and are found in the severe classical SW form of CAH [17-19]. Here, we present results of direct molecular detection of a CYP21 IVS-II mutation, in which the normal polymorphic C or A at nucleotide 655 has been converted to G, in 41 Macedonian patients with different clinical forms of CAH.

MATERIALS AND METHODS

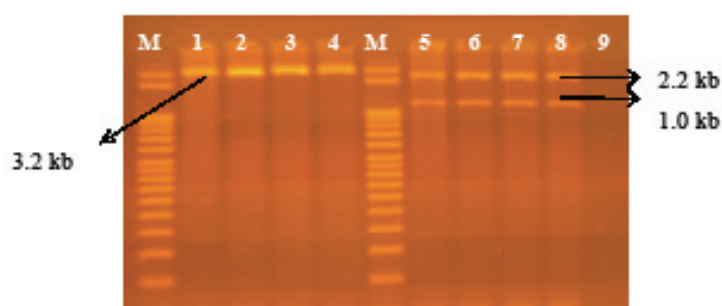
We studied 41 Macedonian patients (27 girls and 14 boys) with clinical and laboratory signs of CAH evaluated at the Department of Endocrinology and Genetics, University Children's Clinic, Skopje, Republic of Macedonia, and 55 of their healthy parents and siblings, belonging to 37 unrelated families. There was one patient in 33 families and two patients in four families. Informed consent for the genetic study was obtained from each family. The CAH patients were of mixed ethnicity (28 of Macedonian ethnicity, nine of Albanian ethnicity and four Gypsies). All patients had elevated plasma 17-hydroxyprogesterone (17-OHP) and were classified according to standard criteria [2]. Of the 41 patients, 19 (46.3%) had the SW form characterized by extremely elevated 17-OHP levels (up to 75 nmol/L) with onset of dehydration and/or shock associated with hyperkalemia and hyponatremia. Females had ambiguous genitalia. The diagnosis was made within 2 months after birth. Four patients (9.8%) with SV form at the age 2-14 years were diagnosed due to signs of androgen excess, after corticotropin stimulation. Eighteen patients (43.9%) had the LO form characterized in girls by normal external genitalia and in both sexes by precocious pubarche and elevated 17-OHP levels, 60 min. after stimulation [2]. The diagnosis was made at age 4-17 years.

Molecular Analysis. DNA samples of all subjects were obtained from peripheral blood lymphocytes by the standard proteinase K-phenol-chloroform method [20]. Direct molecular detection of the IVS-II-656 mutation was performed by the polymerase chain reaction/amplification created restriction site (PCR/ACRS) method, followed by restriction enzyme digestion [21]. Eight other common pseudogene-derived point mutations: *p.Pro30Leu*, 8 bp deletion in exon 3 (*G110Δ8nt*), *p.Ile172Asn*, exon 6 cluster (*p.Ile236Asn*, *p.Val237Glu*, *p.Met239Lys*), *p.Phe306+t*, *p.Val281-Leu*, *p.Gln318X* and *p.Arg356Trp* were also tested in all subjects (Table 1).

The primary differential PCR amplification of the active CYP21 gene, without contamination from the highly homologous pseudogene sequence, was performed with 20 pmol of each CYP21 specific primer (21BF: 5'-TCG GTG GGA GGG TAC CTG

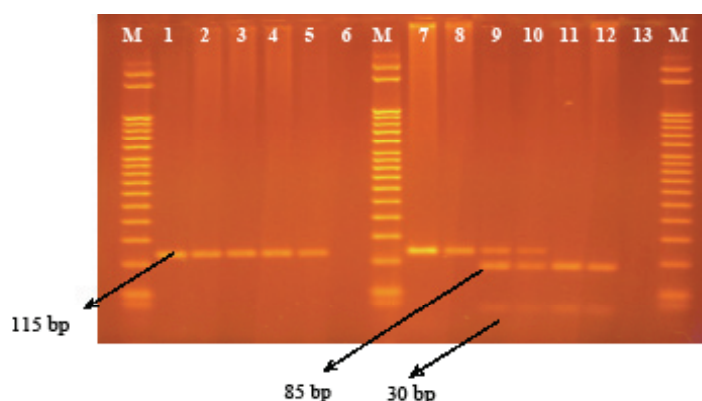
Table 1. ACRS detection of mutations in the CYP21gene.

Primer Pair	Mutation Allele	Restriction Site		Fragment Size (bp)	
		Natural	Created	Normal	Mutant
C1N/C1	p.Pro30Leu	–	<i>Pst</i> I	195	164+31
C3B/C4A	IVS-II-656	–	<i>Sac</i> I	115	85+30
C3B/C4A	8 bp deletion	–	<i>Rsa</i> I	115	89+26
C5/C6	p.Ile172Asn	–	<i>Mse</i> I	148	118+30
C7D1/C8	Exon 6 cluster (p.Ile236Asn)	–	<i>Mbo</i> I	114+26	140
C7E/C8	Exon 6 cluster (p.Val237Glu)	–	<i>Taq</i> I	140	116+24
C7C1/C8	Exon 6 cluster (p.Met239Lys)	–	<i>Mse</i> I	140	110+30
C9/C10-1	p.Val281Leu	<i>Apa</i> L1	–	116+101	213
C9A/C9B	p.Phe306+t	–	<i>Mwo</i> I	123+34	157
C11/C12	p.Gln318X	<i>Pst</i> I	–	146+51	197
C11/C12	p.Arg356Trp	–	<i>Msc</i> I	197	167+30

**Figure 1.** Lanes 1-4: a 3.2 kb PCR product of the CYP21 gene; lanes 5-8: an *Eco*RI digestion of the CYP21 PCR product which produced two fragments (1.0 and 2.2 kb); lane 9: blank; M-marker (50 bp).

AAG-3' and 21BR: 5'-AAT TAA GCC TCA ATC CTC TGC AGC G-3') under the PCR conditions described in Gene Amp XL (Extra Long) PCR Kit (Applied Biosystems, Branchburg, NJ, USA). The *Eco*RI digestion of the 3.2 kb PCR product from the CYP21 gene results in two fragments (1.0 and 2.2 kb), whereas the pseudogene digested similarly results in three fragments (0.5, 0.6, and 2.2 kb). This ensured that only the active gene sequence had been amplified and analyzed (Figure1).

The primary PCR product was used as a template for a secondary PCR amplification using the ACRS method with primers specific for detection of the IVS-II mutation (C3B: 5'-TTC ATC AGT TCC CAC CC TCC AGC CCC gA-3' and C4A: 5'-CTT CTT GTG GGC TTT CCA GAG CAG GtA-3'). The secondary ACRS PCR was carried out in a final volume of 50 μ L containing primary PCR product, 50 pmol of each primer, 200 μ M of each dNTP, 1,5 mM $MgCl_2$ and 1.25 U Taq DNA polymerase (Ampli Taq Gold; Applied Biosystems) for 30 cycles at 95°C for 1 min., 62°C for 1 min. and 72°C for 2.5 min. A 115 bp PCR product was obtained (Figure 2). The amplified fragments were digested with 5-10 U of a *Sac*I restriction enzyme at 37°C overnight and the digestion products analyzed on 2% high resolution agarose gel detected by ethidium bromide staining on UV radiation. The mutant allele provides a recognition site for *Sac*I, so that digestion with this enzyme produces two fragments of 85 and

**Figure 2.** Lanes 1-5: a 115 bp intron 2 PCR product of the CYP21 gene; lanes 7 and 8: (digestion with *Sac*I restriction enzyme) normal (115bp); lanes 9 and 10: heterozygous (115 bp/85+30bp); lanes 11 and 12: homozygous (85+30bp); lanes 6 and 13: blank; M-marker (50 bp).

30bp. The normal allele contains no restriction site for *SacI* (Figure 2). The digestion with the restriction enzyme allowed not only the mutation detection but also determination of the zygosity of the analyzed mutation in an individual.

RESULTS AND DISCUSSION

The IVS-II mutation was detected in 17 (41.5%) of the CAH patients. Twelve (29.3%) were homozygous and five (12.2%) were heterozygous. All homozygous patients had severe classical CAH phenotype with 91.7% having the SW phenotype. Although this phenotype is usual in the homozygous state for this mutation [22], one of our homozygous patients displayed the SV phenotype.

Three heterozygous patients with the SW phenotype, were compound heterozygotes having another of the tested mutations (*p.Pro30Leu*; 8 bp deletion in exon 3 (*G110Δ8n*); *p.Ile172Asn*; exon 6 cluster (*p.Ile236Asn*, *p.Val237 Glu*, *p.Met239Lys*); *p.Phe306+t*; *p.Val281Leu*; *p.Gln 318X*; *p.Arg356-Trp*). One of the compound heterozygotes had four different mutations: IVS-II, *p.Val281Leu*, *p.Gln 318X* and *p.Arg356Trp*. These complex alleles may have resulted from small or large gene

conversions or from multiple mutation events. The second and the third compound heterozygote each had a mutation on another allele, *p.Gln318X* and *p.Arg356Trp*, respectively. The genotypes of the three compound heterozygotes and of their parents are presented in Table 2. In two other heterozygotes, one with SW and another with the LO phenotype, no second mutant allele was detected (Table 3).

The frequency of the IVS-II mutation allele in our subjects is similar to that reported for other populations. This mutation has a high frequency in many parts of the world, except in some groups of patients in South Europe, and may be a hot-spot [23-25].

The IVS-II mutation was also found in 17 (30.9%) family members, of whom 10 (18.2%) were homozygotes and seven (12.7%) were heterozygotes. None had any clinical manifestation.

The IVS-II mutation alters pre-mRNA splicing by activating another acceptor site for the splicing process and thus shifting the reading frame to create premature termination of translation [26]. Comparison of the phenotypic features with the IVS-II genotypes, shows phenotypic heterogeneity extending from classical SW CYP21 deficiency to asymptomatic carrier [26,27].

Table 2. Detected genotypes in the compound CAH heterozygotes and their parents.

No	Compound Heterozygote Patient	Mother's Allele	Father's Allele
1	IVS-II/ <i>p.Val281Leu</i> / <i>p.Gln318X</i> / <i>p.Arg356Trp</i>	<i>p.Val281Leu</i> / <i>p.Val281Leu</i> / <i>p.Gln318X</i> / <i>p.Gln318X</i> / <i>p.Arg356Trp</i>	IVS-II/
2	IVS-II/ <i>p.Gln318X</i>	IVS-II/IVS-II	<i>p.Gln318X</i> /
3	IVS-II/ <i>p.Arg356Trp</i>	IVS-II/IVS-II	<i>p.Arg356Trp</i> /

Table 3. Genotype-phenotype correlation in CAH patients with the detected IVS-II mutation.

Mutation	<i>n</i>	Salt-Wasting (<i>n</i> =19)	Simple Virilizing (<i>n</i> =4)	Late Onset (<i>n</i> =18)
IVS-II/IVS-II	12	11	1	—
IVS-II/ <i>p.Val281Leu</i> / <i>p.Gln318X</i> / <i>p.Arg356Trp</i>	1	1	—	—
IVS-II/ <i>p.Gln318X</i>	1	1	—	—
IVS-II/ <i>p.Arg356Trp</i>	1	1	—	—
IVS-II/?	2	1	—	1
Total	17	15	1	1

An unusually high frequency of “asymptomatic homozygotes” for a mutation expected to severely compromise CYP21 function has been described [28]. That an apparent homozygosity for the IVS-II mutation may result from unequal amplification (“allele dropout”) of CYP21 alleles which leads to obscured normal alleles and represent a common diagnostic pitfall of methods employing PCR. For prenatal diagnosis, microsatellite typing could be used as a supplement to CYP21 genotyping so as to resolve ambiguities at nucleotide 656 on intron 2 [28,29]. However, asymptomatic homozygotes for the IVS-II mutation, may be polymorphic for unidentified splice regulatory factors such that the cryptic splice becomes ignored, permitting correct splicing of CYP21 pre-mRNA [28,30]. Since a severe IVS-II mutation almost totally abolishes CYP21 activity, homozygosity for splicing mutation in family members, could be attributed to the tendency of cytochrome P450 enzymes to be “promiscuous” enzymes that bind many different substrates and catalyze a wide variety of hydroxylations, so that the expression of such an enzyme in the adrenal gland could account for the cryptic CYP21 activity [31].

The observed discordance between genotype and phenotype may result from either the postulated extra adrenal hydroxylase activity or from other factors that modify steroid CYP21 transcription, translation and action. Although some promoter elements important for a CYP21 gene transcription are known, and regulatory proteins that affect CYP21 expression have been proposed [32,33], much remains unexplained. Moreover, different receptor numbers or binding affinity for androgens, cortisol or aldosterone may contribute to the phenotypic variability. Also, the activity of transcription factors and the expression of transport proteins may be individually regulated [34].

Strong genotype-phenotype correlation was observed in all Macedonian CAH patients with the detected IVS-II mutation. However, our results in the other family members confirm that the genotype cannot be completely predictive of phenotype [22,35].

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