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DETECTED HETEROZYGOTES DURING THE MOLECULAR ANALYSIS OF THE COMMON CYP21A2 POINT MUTATIONS IN MACEDONIAN PATIENTS WITH CONGENITAL ADRENAL HYPERPLASIA AND THEIR RELATIVES

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A b s t r a c t: *Background:* Deficiency of 21-hydroxylase is present in 90–95% cases of congenital adrenal hyperplasia (CAH), an autosomal recessive disorder. Eleven common pseudogene-derived mutations account for approximately 95% of all affected CYP21A2 alleles in all three clinical forms of the disease.

Objective: To analyse the detected heterozygotes during the molecular analysis of eleven CYP21A2 common pseudogene-derived point mutations in Macedonian CAH patients and their relatives, using the PCR-ACRS protocol.

Material and methods: We performed direct molecular detection of CYP21A2 mutations: p.P30L, IVS2-655 C/A→G, G110Δ8nt, p.I172N, p.I236N, p.V237E, p.M239K, p.F306+t, p.V281L, p.Q318X and p.R356W, in 51 CAH Macedonian patients and their 70 healthy relatives (parents and siblings), using the differential PCR-ACRS protocol.

Results: Six of the analysed mutations were detected in 29.4% (15/51) of the patients, in the heterozygous state, with the following distribution: IVS2-655 C/A→G (13.7%), p.P30L (11.8%), p.Q318X (9.8%), p.I172N (3.9%), p.R356W (3.9%) and p.V281L (1.96%). Six cases (6/15) were compound heterozygotes and nine (9/15) were simple heterozygotes. Genotype-phenotype correlation was observed in all of our detected compound heterozygote patients. Their clinical presentation was correlated with the less severely mutated allele.

Four of the analysed mutations (p.P30L 20%, IVS2-655 C/A→G 12.9%, p.Q318X 7.1% and p.R356W 2.9%) appeared in thirty healthy relatives (42.9%), in the heterozygous state.

Conclusion: Distribution of the analysed CYP21A2 mutations among CAH patients and their relatives is comparable to studies from other populations. As expected, high carrier frequency of alleles causing 21-hydroxylase deficiency was observed in relatives of CAH patients.

Key words: CAH, 21-hydroxylase deficiency, CYP21A2 gene, CYP21A2 point mutations, heterozygosity.

Introduction

Steroid 21-hydroxylase (21OH) deficiency represents about 95% of congenital adrenal hyperplasia (CAH), an inborn autosomal recessive adrenocortical steroidogenesis error [1]. The clinical presentation of CAH varies widely depending on the genetic lesion. It can present as a classical simple virilizing form (SV), causing ambiguous genitalia in females, classical salt wasting form (SW) in the neonatal period, and nonclassic late onset form (LO) causing premature adrenarche and pubarche in children, virilization in young women, and variable symptoms in young men. [1, 2]. The incidence of the severe classical form is one in 10,000 to one in 15,000 among Caucasians [3]. The prevalence of milder non-classic CAH is estimated to one in 1,700 in the general population [4]. Based on newborn screening data, the carrier frequency of CAH in the general population is estimated to be 1: 55 [5].

The wide range of CAH phenotypes is associated with multiple mutations known to affect 21-hydroxylase enzymatic activities [3]. The gene coding for 21OH is designated CYP21A2. A second duplicated inactive copy, CYP21A1P (pseudogene), shares 98% nucleotide sequence homology with CYP21A2 in the exon sequences [6]. Both the active gene and the pseudogene are located at the 3' terminus of each of the two genes encoding the fourth component of the complement, C4A and C4B, in the HLA class III gene region on the short arm of chromosome 6 [7]. The CYP21A2 and CYP21A1P are 3.2 kb long and contain 10 exons each. Because of the high homology and tandem-repeat organization, this gene cluster is subjected to a high frequency of recombination events, which can lead to two distinct situations: unequal crossing-over during meiosis, producing a wide variety of rearrangements depending on the breakpoint and large and short gene conversion events that transfer deleterious mutations present in the CYP21A1P gene to CYP21A2 [8–11]. However, apart from gene deletions and large gene conversions, nine such pseugene-derived mutations account for about 95% of all affected CYP21A2 alleles in different ethnic groups, while the remainder (5%) are new mutations not found in the pseudogene [3]. Although good genotype-phenotype correlations were demonstrated for common mutations [12–15], the combination of the CYP21A2 muta-

tions can cause different phenotypes [16–18]. Most patients are compound heterozygotes, having different CYP21A2 mutations on each allele: the clinical presentation of CAH is reported to be correlated with the less severely mutated allele [3, 19–21].

We performed molecular analysis of eleven CYP21A2 mutation in 51 CAH patients belonging to all three clinical forms of the disease, and their 70 healthy relatives, and analysed the detected heterozygotes.

Patients and methods

Patients

Fifty-one 21-hydroxylase deficiency patients from 45 unrelated families and their 70 healthy relatives (57 parents and 13 siblings), were studied. Informed consent for the molecular tests was provided. Patients were diagnosed according to standard clinical criteria [22] at the Department of Endocrinology and Genetics, University Children's Clinic, Skopje, Republic of Macedonia. Twenty patients, (39.2%) were diagnosed as salt wasters, 11 (21.6%) as simple virilizers and 20 (39.2%) as nonclassic patients.

Molecular methods

Genomic DNA was extracted from peripheral blood lymphocytes following standard phenol/chloroform protocol [23]. Combined differential PCR-ACRS (polymerase chain reaction/amplification created restriction site) protocol was used for direct molecular detection of 11 common pseudogene-derived mutations: p.P30L, IVS2-655 C/A→G, G110Δ8nt, p.I172N, p.I236N, p.V237E, p.M239K, p.F306+t, p.V281L, p.Q318X and p.R356W, accounting for approximately 95% of all affected CYP21A2 alleles, as previously described [24]. The combination of CYP21A2 specific primers (21BF/21BR) and EcoRI digestion ensured that only the active gene sequence had been amplified and analysed, without contamination from the highly homologous pseudogene sequence. The primary differential PCR amplification of the CYP21A2 gene was carried out in a final volume of 100 μl containing 1 μg of genomic DNA, 20 pmol of each specific primer (21BF: 5'-TCG GTG GGA GGG TAC CTG AAG-3' and 21BR: 5'-AAT TAA GCC TCA ATC CTC TGC AGC G-3'), 200 μM of each dNTP, 1,5 mM $Mg(OAc)_2$ and 4U r*Tth* DNA polymerase, XL (GeneAmp XL PCR kit – Applied Biosystems, Branchburg, NJ, USA). The 3.2 kb PCR products of the CYP21A2 and EcoRI digestion products (1.0 and 2.2 kb) are shown in Figure 1.

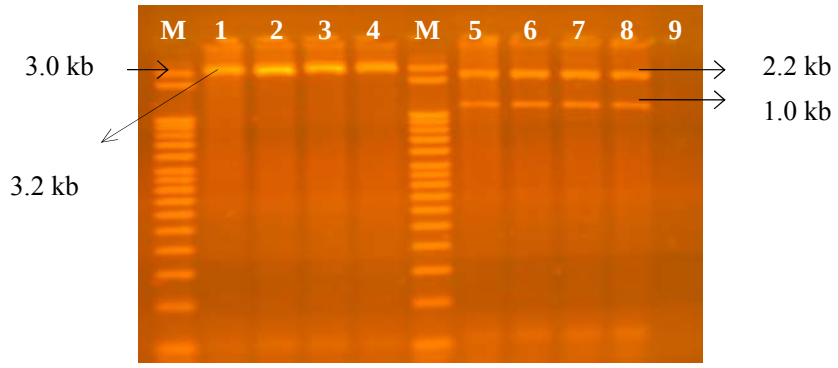

Figure 1 – A 3.2 kb PCR product of CYP21A2 gene, line 1–4 and Eco RI digestion of CYP21A2 PCR product with produced two fragments (1 kb and 2,2 kb), line 5–8; line 9-blank; M-marker (50 bp)
Слика 1 – PCR производ од ген CYP21A2, со големина од 3,2 kb, примероци од 1–4; дигестија на PCR производот од ген CYP21A2 со рестриктивната ендонуклеаза Eco RI и продукција на два фрагменти (1 kb и 2,2 kb), примероци од 5–8; примерок 9-слепа проба; М-маркер (50 bp)

The primary PCR product was then used as a template for secondary PCR amplification using ACRS, as previously described [24]. Subsequent restriction analysis allowed not only the detection but also the determination of the zygosity of the mutation analysed.

Results and discussion

Fifty-one 21-hydroxylase deficiency patients were studied for 11 common pseudogene-derived mutations. Homozygosity was found in 47.1% of the patients (unpablished results) for three different mutations (p.P30L, IVS2-655 C/A→G, and p.Q318X); 29.4% were heterozygotes for six mutations and 23.5% were not elucidated and might carry other rare or novel mutations. This data was very similar to theirs reported in Iranian patients by Ramazani A. *et al*, 2007 [25] and Brazilian patients by Torres N. *et al*., 2003 [26] in a similar number of CAH patients and comparable with other populations worldwide [27].

In the present study we found that six of the analysed mutations in 15/51 (29.4%) of the patients were detected in the heterozygous state, with the following distribution: IVS2-655 C/A→G (13.7%), p.P30L (11.8%), p.Q318X (9.8%), p.I172N (3.9%), p.R356W (3.9%) and p.V281L (1.96%), (table 1)

Figure 2. Among the heterozygotes, six cases (40%) were compound heterozygotes with different mutations on each chromosome and nine patients (60%) were simple heterozygotes for one mutant allele, without detection of another mutant allele between analysed CYP21A2 point mutations (potential compound heterozygotes). Several studies have confirmed that compound heterozygosity appears in 18–75% [19, 20, 25].

Table 1 – Табела 1

Genotype-phenotype correlation in CAH patients with heterozygosity for CYP21A2 mutations
Генотип-фенотип корелација кај САН пациенти, хетерозиготи за CYP21A2 мутации

	S.W.	***S.V.***	***L.O.***	***Total***
Patients (n)	**20**	**11**	**20**	**51**
P30L / IVS2		2 (18.2%)		2 (3.9%)
P30L / Q318X			1 (5%)	1 (1.96%)
IVS2 / Q318X	1 (5%)			1 (1.96%)
IVS2 / R356W	1 (5%)			1 (1.96%)
IVS2 / V281L / Q318X / R356W	1 (5%)			1 (1.96%)
P30L / ?			3 (15%)	3 (5.9%)
IVS2 / ?	1 (5%)		1 (5%)	2 (3.9%)
I172N / ?		2 (18.2%)		2 (3.9%)
Q318X / ?		1 (9.1%)	1 (5%)	2 (3.9%)
Total	**4 (20%)**	**5 (45.5%)**	**6 (30%)**	**15 (29.4%)**

Genotype-phenotype correlation was observed in all of our detected compound heterozygotes patients.

Clinically, it has been shown that the IVS2-655 C/A→G, G110Δ8nt, p.F306+t, p.Q318X and p.R356W result in a complete inactivation of 21OH and are found in severe salt wasting disease, whereas the simple virilizing form of CAH are associated with p.I172N, which abolishes 21OH activity. The p.P30L, p.P105L, p.V281L, p.P453S are associated with the milder non-classical form of CAH since the mutations result in only partial loss of the 21OH enzyme activity [24–30].

The clinical expression of CAH is reported to be correlated with the less severely mutated allele, and consequently with the residual activity of 21-hydroxylase [21] that was detected in all of our compound heterozygotes, also. The patient who was compound heterozygote for p.P30L and p.Q318X presented a non-classical phenotype and two other compound heterozygotes for p.P30L and

IVS2 had SV phenotype. Patients with the p.P30L allele, although still categorized as non-classical, tend to have pronounced evidence of androgen excess [31]. The compound heterozygotes for severe mutations (IVS2/p.Q318X; IVS2/p.R356W; IVS2/p.V281L/p.Q318X/p.R356W) presented a SW classical

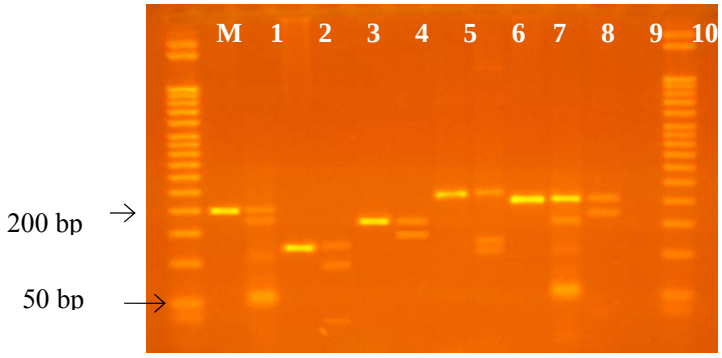

Figure 2 – ACRS/PCR analysis in the CAH patients with detected CYP21A2 mutations in heterozygous state; PCR II products of exon 1 (195 bp), intron 2 (115 bp), exon 4 (159 bp), exon 7 (213 bp) and exon 8 (197 bp) – lines 1,3,5,7,9 respectively; Detected heterozygosity for CYP21A2 mutations with the restrictive enzymes digestions: Pst I (fragments -195, 164+31 bp), Sac I (115 bp, 85+30 bp), Mse I (159 bp, 130+29 bp), Apal I (213 bp, 116+101 bp), Pst I (197 bp, 146+51 bp) and Msc I (197 bp, 167+30 bp) – lines 2,4,6,8,10,11 respectively; 12 – blank; M – marker (50 bp)

Слика 2 – ACRS/PCR анализа кај САН пациенти со детектирани CYP21A2 мутации во хетерозиготна состојба; PCR II продукти од ексон 1 (195 bp), интрон 2 (115 bp), ексон 4 (159 bp), ексон 7 (213 bp) и ексон 8 (197 bp) – примероци 1,3,5,7,9 соодветно; Детектирани CYP21A2 мутации во хетерозиготна состојба по дигестија со рестриктивни ензими, претставени со следните фрагменти: Pst I (фрагменти -195, 164+31 bp), Sac I (115 bp, 85+30 bp), Mse I (159 bp, 130+29 bp), Apal I (213 bp, 116+101 bp), Pst I (197 bp, 146+51 bp) и Msc I (197 bp, 167+30 bp) – примероци 2,4,6,8,10,11 соодветно; 12 – слепа проба; M – маркер (50 bp)

phenotype (table 1). Although p.V281L is generally associated with the non-classical form of the disease [32], the association with severe mutations produced the classical form of the disease [26]. Thus, the compound heterozygote patient for p.V281L and other three severe mutations (IVS2, p.Q318X and p.R356W) had a classical phenotype.

In nine patients only one mutation was detected. A second mutation in those patients with clear clinical presentations (Table 1) was not detected between the analysed CYP21A2 point mutations with the PCR/ACRS method. This might be a result of the novel mutations, rare mutations or gene deletions.

Four of the analysed mutations, with the following distribution: p.P30L 20%, IVS2-655 C/A→G 12.9%, p.Q318X 7.1% and p.R356W 2.9%, appeared in thirty healthy relatives (42.9%), in the heterozygous state. Twenty-six of the parents (45.6%) and four of the siblings (30.8%) were carriers of detected mutations (table 2). It is worth mentioning that 7/57 (12.3%) of the parents and 3/13 (23.1%) of the siblings were homozygous for IVS2-655 C/A→G splice mutation. An unusually high frequency of ,"asymptomatic homozygotes'' for a mutation expected to severely compromise 21-hydroxylase function was described previously [17, 33).

Table 2 – Табела 2

Detected CYP21A2 mutations in heterozygous state in CAH patients' relatives
Детектирани CYP21A2 мутации во хетерозиготна состојба кај роднини на пациенти со CAH

Mutation	***Parents***	***Siblings***	***Total relatives***
p. P30L	12/57 (21.1%)	2/13 (15.4%)	14/70 (20%)
p. IVS2	7/57 (12.3%)	2/13 (15.4%)	9/70 (12.9%)
p. Q318X	5/57 (8.8%)	/	5/70 (7.1%)
p. R356W	2/57 (3.5%)	/	2/70 (2.9%)
Total	**26/57 (45.6%)**	**4/13 (30.8%)**	**30/70 42.9%**

Carriers of CYP21A2 mutations are relatively common in the general healthy population 1: 55 [5]. It is more frequent in middle European populations (1: 10), in Yugoslav (9.5%) and non-Yugoslav individuals (8.6%), [34].

There are no data for CAH carrier frequency in the healthy Macedonian population. Thus, further analysis for CYP21A2 mutation frequency in the Macedonian population is warranted.

Conclusion

In the present study we found that six out of eleven analysed mutations were detected in the heterozygous state in 29.4% patients. High carrier frequency of alleles causing 21-hydroxylase deficiency (42.9%) was observed in relatives of CAH patients.

Genotype-phenotype correlation was observed in all of our detected compound heterozygote patients. Their clinical expression was correlated with the less severely mutated allele and consequently, with the residual activity of 21-hydroxylase.

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Резиме

ДЕТЕКТИРАНИ ХЕТЕРОЗИГОТИ ВО ТЕКОТ НА МОЛЕКУЛАРНАТА АНАЛИЗА НА ЧЕСТИ CYP21A2 ТОЧКЕСТИ МУТАЦИИ КАЈ МАКЕДОНСКИ ПАЦИЕНТИ СО КОНГЕНИТАЛНА АДРЕНАЛНА ХИПЕРПЛАЗИЈА И НИВНИ РОДНИНИ

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Конгенитална адренална хиперплазија (CAH) е автосомно рецесивна болест, која во 90–95% од случаите е последица од дефицит на ензимот 21-хидроксилаза, кодиран од CYP21A2 генот. Единаесет точкести мутации во CYP21A2 генот, по потекло од псевдогенот, се причина за околу 95% од афектираните CYP21A2 алели, кај сите три клинички форми на болеста.

Цел: Да се анализираат хетерозиготи детектирани во текот на молекуларната анализа на 11 чести точкести мутации во CYP21A2 генот, кај пациенти со CAH и членови на нивните фамилии во Р. Македонија.

Материјал и методи: Анализирани се 51 пациент и 70 членови на нивните фамилии за присуство на следните CYP21A2 мутации: p.P30L, IVS2-655 C/A→G, G110Δ8nt, p.I172N, p.I236N, p.V237E, p.M239K, p.F306+t, p.V281L, p.Q318X и p.R356W, со користење на молекуларна PCR-ACRS метода.

Резултати: Кај 15/51 (29.4%) пациенти е присутна хетерозиготност за една од вкупно шест детектирани мутации: IVS2-655 C/A→G (13.7%), p.P30L (11.8%), p.Q318X (9.8%), p.I172N (3.9%), p.R356W (3.9%) и p.V281L (1.96%). Кај шест од хетерозиготите (6/15) е најдена мутација на двата алела (compound хетерозиготи), додека кај девет хетерозиготи (9/15) е детектирана мутација само на едната алела. Корелација помеѓу генотипот и фенотипот е констатирана кај сите compound хетерозиготи.

Меѓу членовите на нивните фамилии констатирана е хетерозиготност кај 30/70 (42.9%), со следната дистрибуција: p.P30L 20%, IVS2-655 C/A→G 12.9%, p.Q318X 7.1% и p.R356W 2.9%.

Заклучок: Дистрибуцијата на анализираните CYP21A2 мутации кај пациентите со CAH и членовите на нивните фамилии е слична со таа констатирана кај други популации. Детектирана е очекувана висока фреквенција на носители на мутантни CYP21A2 алели кај членови на фамилии на CAH пациентите.

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Клучни зборови: CAH, дефицит на 21-хидроксилаза, CYP21A2 ген, CYP21A2 точкести мутации, хетерозиготност.

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