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CYP21A2 Gene Analysis in Southern Iranian CAH Patients and a Brief Review of the Mutation Spectrum

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Abstract

Background: *CYP21A2* gene mutations are responsible for more than 95% of Congenital Adrenal Hyperplasia (CAH) disorders with autosomal recessive inheritance. Most of these pathogenic mutations originate from the *CYP21A1P*, a neighboring pseudogene with 98% homology, due to unequal crossing over or gene conversion events. Mutation identification of the gene could be beneficial for accurate diagnosis and outcome prediction.

Methods: Twelve unrelated patients with CAH diagnosis were recruited for genetic counseling. To ensure distinct amplification of the *CYP21A2* gene rather than its pseudogene, the complete sequence of the gene was amplified through two overlapping fragments by specific primers. The entire sequences were screened by direct Sanger sequencing using new sequencing primers.

Results: Only two pathogenic point mutations were identified. The c.293-13C>G, also known as In2G, and the c.955C>T mutations were found in 37.5 and 33.3% of alleles, respectively. One patient showed homozygous gene deletion. We also reviewed recent reports on *CYP21A2* gene mutations in Iran.

Conclusion: Evaluating the ethnicity-specific gene mutation data is significant for populations with diverse ethnic groups including the Iranian population. Although several common mutations have been reported as causative mutations among CAH patients, identifying only two common point mutations in Fars province would help prioritize exon sequencing and reduce the cost and time of genotyping.

Keywords: Adrenal hyperplasia, Congenital, Genotyping techniques, Mutation
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Introduction

Congenital Adrenal Hyperplasia (CAH; MIM: 201910) is a group of common autosomal recessive disorders that interrupts normal steroidogenesis in the cortex of the adrenal glands [1]. These steroid hormones, including cortisol, aldosterone, and adrenal androgens, develop from cholesterol in multistep enzymatic pathways [2].

21-hydroxylase deficiency (21-OHD) caused by *CYP21A2* gene mutations, is responsible for more than 95% of CAH cases and has distinct clinical classifications [3]. The classic form is a life-threatening condition, with a prevalence of 1 in every 16,000 newborns worldwide [4]. The prevalence of the nonclassic form is 1 case per 600 persons; although it is generally asymptomatic, it may cause infertility in females [5].

In many laboratories, evaluating 17-hydroxyprogesterone (17-OHP) is the main approach to CAH diagnosis, and the accumulation of 17-OHP is interpreted as 21-OHD [6]. Some studies suggest that replacing 17-OHP with 21-deoxycortisol as the CAH biomarker can reduce false-positive test results [7]. However, based on the EMQN guideline, *CYP21A2* genotyping is the best approach for 21-OHD testing and CAH diagnoses [8].

The *CYP21A2* gene (MIM: 613815) which encodes the 21-hydroxylase enzyme and its pseudogene, *CYP21A1P*, are located approximately 30 *kb* apart in the

high-gene-density HLA III region on chromosome 6p21.3 [9]. The gene and pseudogene along with their neighbor genes are located in a genetic unit called RCCX unit [10]. There are usually two RCCX modules on each chromosome; One RCCX module includes *RP1*, *C4A*, *CYP21A1P*, and *TNX A*, and the other RCCX includes *PR2*, *C4B*, *CYP21A2*, and *TNX B* genes, (Figure 1) [11,12]. Furthermore, the *CYP21A2* gene and its pseudogene show 98% homology in their sequence [13]. These situations accelerate gene conversions and unequal crossing-over events in this region, resulting in transferring mutations of pseudogene to active *CYP21A2*, and even partial or complete deletion of the gene and neighboring regions [5].

According to previous studies, a group of nine mutations is responsible for 21-OHD in the majority of CAH patients in the world; but the frequency of each mutation is different in every ethnicity [14,15]. Thus, prioritizing mutations and related gene regions would make *CYP21A2* genetic analysis more feasible and cheaper. Moreover, the *CYP21A2* gene analysis is a valuable complement for predicting phenotype as well as confirming the diagnosis [16]. Here, we evaluate the *CYP21A2* variants in Iranian families from the south of the country. We also reviewed the prevalence and type of *CYP21A2* mutations in different parts of the country based on previous studies.

Materials and Methods

Patients and sampling

Twelve families were referred for genetic analysis by endocrinologists. CAH diagnosis was confirmed clinically according to medical examination and paraclinical findings. Patients were selected from various regions in the south of Iran. Peripheral blood samples were collected from all patients and their parents. DNA was extracted using Parstous Kit (ca.t number A101201) according to the manufacturer's instructions. The quality and quantity of DNA were assessed by the NanoDrop spectrophotometer (ND-1000) as well as agarose gel electrophoresis. The research project has been approved by the Ethics Committee of Shiraz University of Medical Sciences (Approval ID: IR.SUMS.REC.1400.378). Informed consent was obtained from all of the participants.

PCR amplification and the sanger sequencing

To ensure specific amplification of *CYP21A2* gene rather than its pseudogene, the complete sequence of the gene was amplified through two overlapping fragments by specific primers. All coding sequences and exon-intron boundaries were screened by direct Sanger sequencing of fragments with an average size of 600 *bp*, using new sequencing primers. Table 1 shows the characteristics of primers.

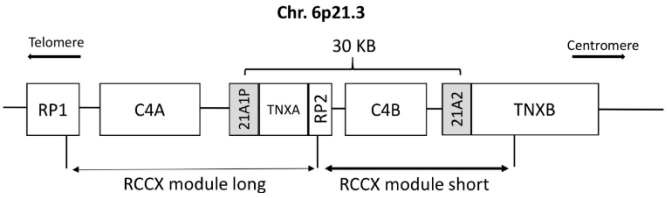

Figure 1. Bimodular form (*RP1–C4A–CYP21P–XA–RP2–C4B–CYP21–TNXB*) of the RCCX region of chromosome 6p21.3. *Cyp-21A2* gene and its pseudogene are shown in the gray boxes.

All fragments were amplified in a final reaction volume of 25 *µl* using PCR Master Mix (Ampliqon, A140303) and 0.6 *mol/L* of each primer, along with 30 *ng* of DNA. The $MgCL_2$ final concentration was 1.5 *mmol/L*. The fragment-1 PCR cycling parameters were as follows: initial denaturation of 95°*C* for 5 *min*, followed by 30 cycles of 95°*C* for 30 *s*, 70°*C* for 30 *s*, 72°*C* for 60 *s*, and a final extension of 72°*C* for 5 *min*. The fragment-2 cycling parameters were as follows: initial denaturation of 95°*C* for 5 *min*, followed by 35 cycles of 95°*C* for 30 *s*, 64°*C* for 30 *s*, 72°*C* for 90 *s*, and a final extension of 72°*C* for 7 *min*. All PCR products were controlled by Agarose gel electrophoresis (Figure 2).

Sanger Sequencing was provided by the Codon Biotech Company, Tehran, Iran. Gene deletion analysis was performed as previously described [17] by the Pishgam Biotech Company, Tehran, Iran, (www.pishgambc.com).

Data analysis

All sequences were aligned to the *CYP21A2* sequence (NM_000500.9) and *CYP21A1P* sequence (Transcript: ENST00000342991.10) to ensure the specific amplification of the *CYP21A2* gene. The potential effect and pathogenicity of observed single nucleotide variants were then evaluated using online tools including SIFT, Provean [18], PolyPhen2 [19], Mutation Taster and Combined Annotation Dependent Depletion

Table 1. Primer sequences for amplification of *CYP21A2* gene

Name	Sequence (5′ → 3′)	Primer Tm°*C*	Product size
Fragment-1-F	TGGGCGGGTCGGTGGGAGGGT	71	1535
Fragment-1-R	GCCTCAGCTGCATCTCCACGATGTGA	71	
Fragment-2-F	TACTCCCTCCTTTTCTGGCATGAC	65	2072
Fragment-2-R	TTAAGCCTCAATCCTCTGCAGCG	65	
Sequencing-EX2-R	CCCAACCCCTGCTTTCTCCCCACC	72	
Sequencing-EX3-F	GCTCTTGGGGGGGCATATCTGGTGGG	74	
Sequencing-EX9-R	GCCTGGCTCCAGGAAGCGAT	74	

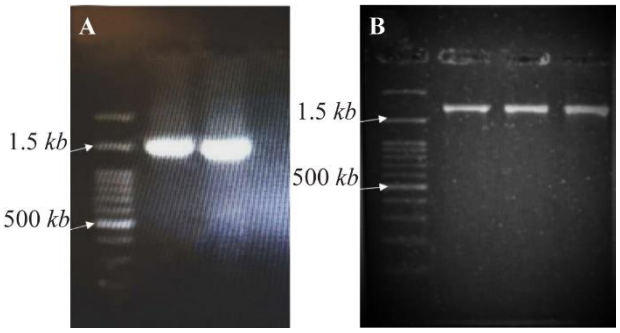

Figure 2. Gel electrophoresis results. A) PCR products of the first fragment (exons 1-6). B) PCR products of the second fragment (exons 5-10).

(CADD), as well as Clinvar and HGMD.

Furthermore, we performed a PubMed, Google Scholar, and Web of Science search to review the spectrum of reported *CYP21A2* mutations in Iran, and extracted the frequencies of mutations from studies on 244 CAH-affected families.

Results

According to family history 10 (83%) of marriages were consanguineous, among them 70% were first-cousin. There was a positive history of CAH in only one pedigree. Patients comprised six girls and seven boys ranging from 1 to 10 years old from the south of the country. They had ambiguous genitalia (simple virilizing) at the delivery time or Salt-Wasting problems in the initial weeks or suffered from precocious puberty in childhood (Table 2).

Mutation analysis was performed for all individuals. Direct sequencing of the entire region of *CYP21A2* gene by overlapping specific primers identified causative mutations in 17 out of 24 alleles. One patient (family 6P) showed whole gene deletion in a homozygous state. In one patient only a single mutation was identified, and in the two remaining families no mutation was detected. Figure 3 shows the chromatograms of identified mutations in each family. The c.293-13C>G -SNP code rs6467, also known as In2G- in the second intron was the most frequent photogenic variant with a frequency of 37.5%. After that, the c.955C>T -SNP code rs7755898, also known as Gln318*- in the 8th exon was the only pathogenic variant among the studied patients (Table 2). A total of 22 polymorphisms were also observed through the analysis.

According to previous studies on 244 families with CAH disease in Iran, eight different DNA mutations as well as gene deletions and chimeras were responsible for CAH pathogenesis (Table 3). Most of the families were from Tehran; the capital city in the center of the country and consisted of different ethnicities.

Discussion

Herein, we evaluated the entire *CYP21A2* gene in 12 unrelated families from south of Iran and found two deleterious mutations, *In2G* and *Q318X* as the most frequent pathogenic mutations in the studied families. Each of these mutations has its specific mutagenesis mechanism and origin, as well as different distributions in any population. Although several common mutations have been reported as causative mutations among CAH patients, identifying only two common point mutations in Fars province is interesting. The patients were ethnically Persian and Lur. This could help prioritize exon sequencing and reduce the cost and time of genotyping.

Until 2022 several studies have performed genotyping approaches to investigate *CYP21A2* alleles in Iranian CAH patients. According to previous studies reviewed in table 3, *In2G* and *Q318X* are the most reported mutations in Iranian families [20-27]. Our findings coincide with previous studies. Besides, *I172N* and In2G are among the most prevalent mutations which cause the classic form of CAH worldwide, and *V281L*

Table 2. clinical features and genotyping results of studied patients

	Sex	Age	SW	SV	PP	Consanguineous marriage	Pathogenic variant 1	Pathogenic variant 2
1P	F	9	✓	-	✓	No	c.955C>T	c.955C>T
2P	M	5	✓	-	-	Yes	c.293-13C>G	c.293-13C>G
3P	F	3	✓	✓	-	Yes	c.293-13C>G	c.293-13C>G
4P	F	4	✓	✓	-	Yes	c.293-13C>G	c.293-13C>G
5P	M	3	✓	-	-	Yes	c.955C>T	c.955C>T
6P	M	2	✓	-	-	Yes	Gene deletion	Gene deletion
7P	M	3	✓	-	-	Yes	c.293-13C>G	c.955C>T
8P	F	1	✓	✓	-	Yes	c.955C>T	c.955C>T
9P	M	2.5	-	-	✓	Yes	-	-
10P	M	9	✓	-	-	Yes	c.293-13C>G	c.293-13C>G
11P	F*	3	-	✓	-	Yes	-	-
12P**	M	1	✓	-	-	No	c.955C>T	?

P= Proband, F= Female, M= Male, SW= Salt-Wasting, SV= Simple virilizing, PP= Precocious puberty.
* Identical female twins.
** Adrenal Crisis was diagnosed.

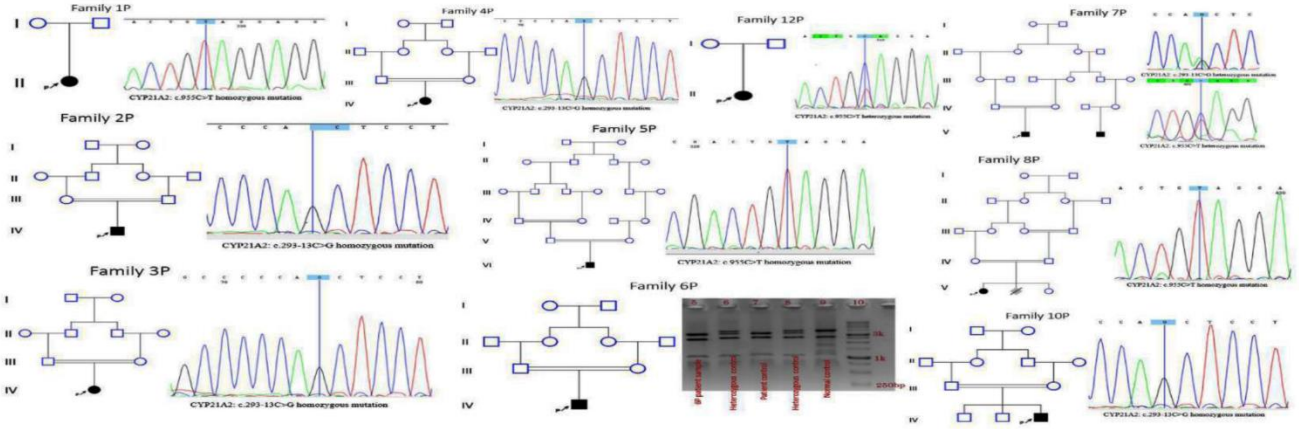

Figure 3. Families' pedigrees and images of founding mutations.

Table 3. characteristics of various mutations reported in previous studies

Mutation common name	SNP ID	Location	mRNA position	Protein position	Number	Frequency in Iran %	Frequency in the world % (18)
del-CYP21A2	-	-	-	-	30	9.5 %	21.5%
Chimera	-	-	-	-	26	8.3 %	
P30L	rs9378251	Exone 1	c.92C>T	p.Pro31Leu	0	0 %	1.8%
In2G	rs6467	Intron 2	c.293-13C>G	-	53	16.8 %	25.3%
G110Δ8nt	rs387906510	Exone 3	c.332_339del	p.Gly111fs	22	7 %	1.8%
I172N	rs1776095671	Exone 4	c.515T>A	p.Ile172Asn	35	11.1 %	11.4%
Cluster E6	rs1554299737	Exone 6	c.710T>A	p.Ile237Asn	26	8.3 %	2.1%
	rs12530380		c.713T>A	p.Val238Glu			
	rs6476		c.719T>A	p.Met240Lys			
V281L	rs6471	Exone 7	c.844G>T	p.Val282Leu	27	8.5 %	15.7%
Q318X	rs7755898	Exone 8	c.955C>T	p.Gln319Ter	59	18.7 %	4.2%
R356W	rs7769409	Exone 8	c.1069C>T	p.Arg357Trp	22	7 %	4.5%
Complex alleles *	-	-	-	-	15	4.8 %	-
Total					315 alleles	100 %	7101 alleles

* Each complex allele has a couple of mutations. These alleles are: I172N+V281L, I172N+I2G, I2G+V281L, I2G+G110Δ8nt, I2G+Q318X, V281L+Q318X, G110Δ8nt+Q318X, Cluster E6+Q318X, and R356X+P453S.

mutation is the most common mutation causing the nonclassic form [20,28,30].

The c.293-13A/C>G (*In2G*) mutation makes an adverse splicing site and disrupts the CYP21A2 pre-mRNA [31,32]. In this case, the patient 21-OH enzyme will have only 0-1% residual function, and the classic form of CAH disease will emerge [33]. Overall, the *In2G* allele is the world's most frequent pathogenic point mutation in the *CYP21A2* gene. Studies on the origin of this mutation suggest that the *In2G* mutagenesis can spontaneously occur due to conversion events; so the frequency of this mutation is nearly the same in every population [34,35]. We found *In2G* mutation in 9/24 studied *CYP21A2* alleles (37%).

The c.955C>T (Q318X) nonsense substitution turns the glutamine codon (CAG) into a termination codon (TAG). This mutation leads to a completely nonfunctional 21-OH enzyme, and the patient manifests the classic symptoms of the CAH disease [36]. HLA haplotyping and segregation studies reveal the old origin of this mutation. So, the frequency of this mutation can be very diverse in every population. Moreover, it is suggested as a founder mutation in some populations [35,37]. Nevertheless, this pathogenic variant is rare in many countries. In the USA and middle Europe, the frequency of this mutation is only 2.6 and 3.7%, respectively among all *CYP21A2* pathogenic variants [38]. In Iran however, the *Q318X* is one of the main reasons for the *CYP21A2* gene corruption and CAH disease. We found the *Q318X* mutation in 7/24 studied CYP21A2 alleles (29%).

Iran had a specific geographical location throughout

history and comprises various ethnic groups, suggesting high genetic heterogeneity. However, founder mutations and other factors mainly consanguineous marriage result in homogeneity in some genetic loci and mutations [39,40]. Albeit, more studies are needed to better clarify the mutation origin.

Conclusion

Evaluating the ethnicity-specific gene mutation data is significant for populations with diverse ethnic groups including the Iranian population. Although the study is limited by the small number of patients, according to our findings we recommended to sequence exons 3 and 8 (*In2G* and *Q318X* mutations) as the first step for *CYP21A2* genotyping.

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Conflict of Interest

The authors declare no conflict of interest for this article.

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