Research Article

Complex Alleles of *CYP21A2* Are the Most Frequent Causes of Congenital Adrenal Hyperplasia in Iranian Population

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Abstract

Objectives: The purpose of this study was to investigate *CYP21A2* mutations in Iranian congenital adrenal hyperplasia (CAH) patients.

Methods: In 25 patients, the first PCR was done to distinguish the gene from the pseudogene (identification of chimeras). Then, second PCR was performed in the patients who had not chimeras, followed by complete exons and introns of *CYP21A2* sequencing to distinguish the mutations.

Results: Complex alleles comprising three compound heterozygote mutations were identified; *p.I173N*/exon 6 cluster (15.7%), *p.I173N*/exon 6 cluster/*p.V282L* (47.3%) and *IVS2-13A>C, G*/exon 6 cluster/*p.V282L* (5.2%). In order to confirm the accuracy of mutations, the parents of the patients were also analyzed. In each family, one of the parents had a heterozygote variant (*p.I173N*/*p.V282L*) and the other had exon 6 cluster heterozygote variant without any symptoms of the disease. Two different mutations in the heterozygous state, exon 6 cluster (10.5%) and exon 6 cluster/*p.V282L* (5.2%) and *p.G110Efs* the 8 nucleotide deletion in exon 3 (5.2%), *I173N* in the heterozygote state (10.5%). A high frequency of variants was found in *CYP21A2*, including: rs6477 (56%), rs6468 (8%), rs6474 (12%), rs6472 (16%), rs6473 (16%), rs6446 (16%), rs61338903 (32%) and rs193922546, rs530758070, rs11970671, rs61732108, rs778403992, rs1058152 and rs562025438 (each 4%).

Conclusions: The present study showed that the compound heterozygosity and complex alleles are the most frequent cause of congenital adrenal hyperplasia in Iranian population. Patients' clinical manifestations were correlated with mutated alleles and the residual activity of 21-hydroxylase enzyme. Based on diversity of mutations in CAH patients of this study, whole *CYP21A2* gene sequencing would be preferential approach in analyzing CAH patients in our population.

Keywords: 21-Hydroxylase Deficiency, *CYP21A1P*/*CYP21A2* Chimeras, *CYP21A2*, Congenital Adrenal Hyperplasia, Complex Alleles

1. Background

It has been estimated that about 95% of Congenital Adrenal Hyperplasias is produced by a mutation in the *CYP21A2* (cytochrome p450 family 21 subfamily A member 2) gene. The *CYP21A2* gene contains 10 exons, about 3500 bp and encode steroid 21-hydroxylase (21-OHD) protein which is a member of the cytochrome p450 super-family enzymes (1). The occurrence of the classical form in the Caucasian population is 1:10,000 - 1:15,000 (2), while the occurrence of the non-classic CAH form is significantly higher and more variant from 1:1000 to 1:2000 (3).

CYP21A2 gene is situated inside the HLA class III (human histocompatibility complex) locus on chromosome 6p21.3 region. *CYP21A2* along with its adjacent genes (*RP1, C4, TNXB*,

and their truncated pseudogenes *RP2* and *TNXA*) produce a genetic unit called the RCCX module (*RP-C4-CYP21-TNX*) which generates an extremely variable region of approximately 30 kb of DNA (4, 5).

Originally, there are two common classifications and three known forms of CAH. Individuals with the salt-wasting (SW) type, have *CYP21A2* gene mutations which resulted in a completely loss of 21-OHD enzyme function. While those with the simple virilizing (SV) type, release only a low levels of the functional enzyme. It is noticeable that about 1% to 2% of enzyme activity is enough to make some aldosterone so they do not have problems with salt wasting.

In non-classical form of CAH, the amount and also the activity of the enzyme is reduced, though the enzyme activ-

ity is more in comparison to the other types (6). The mutations which lead to milder form of the disease retain 20 to 50% of the enzyme activity (7). This form of CAH may manifest with signs and symptoms of androgen excess or may be asymptomatic (8).

In some cases, a single mutation leads to a single recognized clinical form but in many other cases multiple mutations lead to this phenotype as in CAH, where more than 70% of it are heterozygote compounds (7, 9, 10).

It is noteworthy that more than 100 mutations have been found in the *CYP21A2* gene which can cause 21-hydroxylase deficiency (2). Some of these mutations result from an exchange of genetic material between the *CYP21A2* gene and a comparable but nonfunctional piece of DNA called a *CYP21A1P* pseudogene, which is located 30kb upstream to the *CYP21A2* gene on chromosome 6. The gene and pseudogene share 98% sequence homology in exons and 96% in the non-coding region (11). The type of DNA alternation is considered as an unequal crossing over. Other mutations which are involved in 21-OHD deficiency include point mutations and large deletions or insertions of the *CYP21A2* gene (12).

2. Objectives

The purpose of our study was to describe a comprehensive *CYP21A2* mutation analysis in a cohort of CAH patients and determine the frequency of genetic variations that complicate the accuracy of routine genotyping of patients of our country. Also, for the first time we report the different polymorphisms of the *CYP21A2* gene in the Iranian population.

3. Methods

3.1. Patients

The current study was conducted on 25 Iranian CAH patients (6 females and 19 males) with twenty-two unrelated families whose ages ranged from 6 months to 21 years. Fourteen families had consanguineous marriage. The informed consent for participation in the study was obtained from patients or their parents. Blood samples were collected of patients referred to Ali-Asghar Hospital in Tehran. All patients were clinically diagnosed as CAH. They originated from different regions of Iran. This is an experimental study.

3.2. PCR Amplification

Genomic DNA was isolated from peripheral blood using Yekta Tajhiz Azma (YTA) kit (Iran). *CYP21A2/CYP21A1P* chimeras, resulted from the unequal crossing over were analyzed using two PCRs, by sequence- specific primers (2-7, 9-13).

Initially, to detect the normal gene from pseudogene, PCR was performed by employing two specific sets (Table 1). To recognize the presence of a recombination site, a fragment of 1327 bp spanning from exon 1-6 was amplified using a *CYP21*-specific forward (F1) and *CYP21*-specific reverse (R1) primer. *CYP21P*-specific forward primer (F1/P1) with the same reverse primer of the gene (R1) was used to amplify the pseudogene. Similarly, a second PCR reaction was set up. A *CYP21P*-specific (F2) or *CYP21*-specific (F2/P2) forward primer was used in combination with *CYP21*-specific reverse primer (R2) to detect either the allele with a recombination in this region or the normal allele (exon 6-10) respectively, and a fragment of size 1800 bp was amplified (Table 1). The PCR program exists in Appendix 1 in Supplementary File.

After separating gene from pseudogene, in a second step, PCR performed on 19 patients, which followed by sequencing of gene to distinguish the mutations in main gene. In order to analyze the 1 - 7 exons of *CYP21A2*, we used three different primers which were unable to amplify pseudogene. Primer sequences and PCR amplification are described in supplementary file appendix 1 (13). Furthermore, the PCR products of exon 6 - 10 (if did not have chimeras) were sequenced with set2 primers (Table 1).

Sequencing was carried out by MACROGEN company in South-Korea, using the classic Sanger method with ABI. Data analysis was performed by Sequence Scanner v1.0 and Chromas software.

4. Results

In the initial scanning, the data indicated that 6 (24%) probands had unequal crossing over (*CYP21A1P/CYP21A2* chimeras). The remaining 19 (76%) patients had a point or compound heterozygous mutations or deletion in the *CYP21A2* gene (Table 2).

Overall among 25 recognized patients, 16 (64%) possessed the SV-CAH form and 9 (36%) had the SW-CAH form.

In our study, *p.I173N/exon 6 cluster/p.V282L* compound heterozygous mutations were the most frequent (47.3%) ones (Figure 1).

To analyze genotype-phenotype correlations, disease-causing mutations and polymorphisms were divided into four groups according to Finkielstain et al. (14). Our patients were phenotypically placed in the same group as their patients which confirms the existence of genotype-phenotype correlations. In summary, Group 0, included patients with null mutations (gene deletions, *p.G110Efs*; exon 6 cluster) on both alleles (no enzymatic activity). Group A, included patients with compound heterozygous

Table 1. Specific Primers Used for Detection of *CYP21A2/CYP21A1P*

PCR Set/Primers	Position	Sequence (5' → 3')	Product Size, bp
PCR set1			1327
F1 (forward)	EXON1, Normal gene	5'-TCCGGAGCCTCCACCTCCC-3'	
F1/P1 (forward)	EXON1, pseudogene	5'-TCCGGAGCCTCCACCTCCT-3'	
R1 (reverse)	EXON 6, Normal gene	5'-AGCTGCATCTCCACGATGTGA-3'	
PCR set2			1800
F2 (forward)	EXON 6, Normal gene	5'-GCCATAGAGAAGAGGGATCACAT-3'	
F2/P2 (forward)	EXON 6, pseudogene	5'-GCCATAGAGAAGAGGGACCACAA-3'	
R2 (reverse)	EXON10, Normal gene	5'-TTAAGCCTCAATCCTCTGCAGCG-3'	

Table 2. Frequency of Allelic CYP21A2 Mutations by Phenotype in 19 Patients with 21-OHD

Genotype	Phenotype	Number of Patients	Frequency, %
p.I173N/exon 6 cluster/ p.V282L	SV	9	47.3
IVS2-13A>C,G/ exon 6 cluster/p.V282L	SW	1	5.2
p.I173N/exon 6 cluster	SV	3	15.7
p.G110Efs/ p.G110Efs	SW	1	5.2
p.I173N/-	SV	2	10.5
Exon 6 cluster/-	SV	2	10.5
Exon 6 cluster/ p.V282L	SV	1	5.2
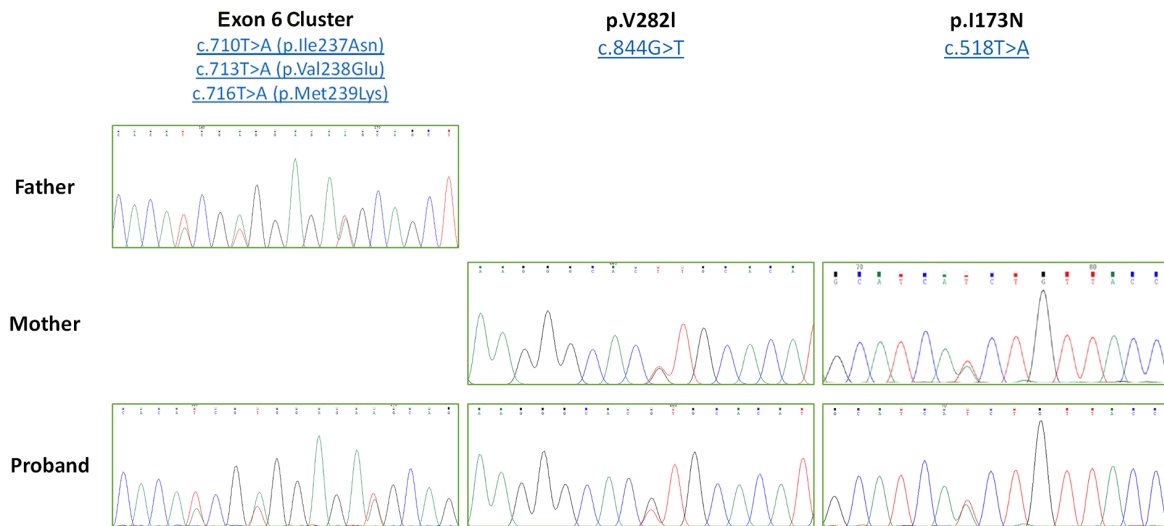
Figure 1. Electropherogram of compound heterozygous mutation (exon 6 cluster/*p.V282L/p.I173N*). There is exon 6 cluster in father and *p.V282L/p.I173N* variant in mother as heterozygote form. In proband graph, all three variants (exon 6 cluster, *p.V282L* and *p.I173N*) exist together causing pathogenic compound heterozygote form. The 8 nucleotide deletions in exon 3 and *IVS2-13A>C, G*/exon 6 cluster/*p.V282L* and *Exon 6 cluster/p.V282L* as compound heterozygous mutations were less frequent (5.2%). The frequency of the mutated alleles in patients is shown in Table 2.

for *IVS2-13A/C>G. IVS2-13A/C>G*, which is known to have minimal residual enzymatic activity. Group B, included patients with *p.I173N* (2% residual enzymatic activity) and any other

homozygous or compound heterozygous mutations that exist in groups 0, or A. Group C, included patients with *p.V282L* mutations (20% - 60% residual enzymatic activity)

compound heterozygous with groups 0, and A or B (Table 3).

The results were evaluated by considering the international genetic databases "dbSNP" and "The Human Gene Mutation Database" HGMD". Variations were found in 14 patients as follows: The variants rs6477 [c.747C>G(p.Leu249=)], rs6474 [c.118C>T(p.Leu40=)], rs6446 [c.1473G>A(p.Pro491=)], rs1058152, rs562025438 [c.*39G>A], rs11970671 [c.822T>C(p.Ser274=)], rs530758070, rs6468 [c.118C>T(p.Leu40=)], rs6472 [c.806G>C(p.Ser269Thr)], rs6473 [c.1481G>A(p.Ser494Asn)] and rs193922546 [c.342C>T (p.Ser114=)] found in this study were reported as "polymorphisms" in the dbSNP and HGMD (Table 4).

5. Discussion

In CAH, mutations are usually categorized in compound heterozygous status, which possess diverse 21-OHD enzymatic activity. Therefore, there is a high degree of phenotypic overlapping between moderate and mild forms of CAH, reflected by the wide and heterogeneous spectrum of clinical manifestations. The *CYP1A2* gene variations determined in the patients, supported the clinical signs of the disease ([8], [12], [15], [16]).

In previous reports, about 65% - 75% of CAH patients that had different mutations of the *CYP21A2* gene were in compound heterozygous state. This study sequenced the whole gene including introns and the data showed that majority (68%) of patients were compound heterozygotes which is compatible with other reports around the world ([17], [18]). In a previous study of CAH in Iran, by Forouzanfar et al., 2015, 13 of 21 (61%) patients had compound heterozygote mutations which is close to our study, although in Rabbani et al. 2008 study, 26 cases of 44 (59%) patients had compound heterozygote mutations ([19]), a rate was less than ours (68%).

Our study showed 6 (24%) patients had chimeric gene mutation. The most frequent (31.8%) mutations detected by Rabbani et al. in 2008, were gene deletions and chimera ([10], [20]) which is close to our results. It is stated that pseudogenes could be exposed to crossing over and chimeric mutation ([21]).

Some studies declare more than one mutation in some alleles of *CYP21A2* gene ([14], [19], [20], [22-24]). Finkielstain et al. ([14]) found some cases with classic form of CAH which had complex alleles comprising 3 mutations (*p.I173/N+ other mutations: exon 6 cluster, p.V282L, p.Leu307fs, p.Q318X*). Similarly, we found three allelic compound heterozygote mutations in 8 patients with SV-CAH form. However, in one case, a sixteen-year old girl with a multiplex compound heterozygous pathogenic mutation (*I2G/exon 6 cluster, p.V282L*)

we found it in SW form. These findings are consistent with the studies mentioned above.

Compound heterozygote mutations of *exon 6 cluster* (*I236N-V237E-M239K*)/*p.V282L*/ *p.I173N* were found in 9 patients. In order to confirm the accuracy of mutations, their parents were also analyzed separately. In each family, one of the parents had a heterozygote variant (*p.I173N*/*p.V282L*) and other one had exon 6 cluster heterozygote variant without any symptoms of the disease. Of course, existence of these three variants (exon 6 cluster, *p.V282L* and *p.I173N*) in the heterozygous form in each individual are pathogenic but it seems that in our population these two variants (*p.I173N*/*p.V282L*) and existence of *exon 6 cluster* by itself is not considered as pathogenic. It should be considered that each of these variants by themselves are pathogenic in the homozygote form ([25]).

Association between exon 6 cluster and *p.V282L*, seems not pathogenic in our study as parents and their children had both mutations in heterozygote form, and we did not find this compound heterozygote in reviewed papers as pathogenic, but in one study of Bas et al. ([23], [26], [27]) *p.I173N*/exon 6 cluster compound heterozygote mutation was seen in three patients. This mutation was previously reported in the study of Finkielstain et al. as compound heterozygote with SV form. Our patients showed the same clinical symptoms ([14]).

G110Δ8nt mutation is an 8 bp deletion in exon3 that creates SW-CAH form and was identified in one of our patients. The frequency of this mutation was previously reported as 4.5% in Iran ([20]) and 4.8%, 4.3% and 3.2% in USA ([28]), Netherlands ([29]) and Iraq ([30]), respectively. Our finding (4%) was compatible with that of other researches.

The more interesting finding of our study is submission the percent of variants in *CYP21A2* gene, for instance: rs6477 (56%), rs6468 (8%), rs6474 (12%), rs6472 (16%), rs6473 (16%), rs6446 (16%) andrs193922546, rs530758070, rs11970671, rs61732108, rs778403992, rs1058152 and rs562025438 each (4%). The current study is the first report which gives distribution of the variants in Iranian population; however it was reported in other countries of the world ([31], [32]). Additionally, our study indicated correlation between some of these variants, which is shown in Table 4. A study conducted in 2015 by Gürkan et al. confirms this correlation ([31]).

It has been shown that rs6467 (HGMD: CS880069, rs6472HGMD: CM994664 and rs6473 have been associated with classical CAH (21-hydroxylase deficiency) and rs6445 has been associated with CAH (21-hydroxylase deficiency) non-classical type. rs6474, rs6477 and rs61338903(*c.29-31delCTG*) belong to a non-pathogenic variant and are without effects on the 21-OHD activity according to HGMD and dbSNP ([31]). Also, in our findings, this association between

Table 3. Genotype and Phenotype in 19 Patients with CAH Due to 21-OHD

Patient	Genotype Group	Gender	Pathogenic Mutations	Polymorphisms	Phenotype
1	B	Male	p.I173N/exon 6 cluster/p.V282L	Rs6468	SV
2	A	Female	IVS2-13A/C>G /p.I173N/exon 6 cluster	-	SW
3	B	Male	Exon 6 cluster/p.I173N	Rs6474-rs6477, Rs6472-rs778403992, c.29-31delCTG	SV
4	B	Male	p.I173N /exon 6 cluster/p.V282L	Rs6477	SV
5	B	Male	p.I173N/-	Rs530758070-rs6477, c.29-31delCTG	SV
6	B	Male	Exon 6 cluster/p.I173N	Rs530758070-rs6477	SV
7	B	Female	Exon 6 cluster/p.I173N	Rs6474-rs6446-rs6473-rs562025438	SV
8	B	Male	p.I173N/exon 6 cluster/p.V282L	Rs11970671	SV
9	B	Female	p.I173N/exon 6 cluster/p.V282L	Rs6477	SV
10	0	Male	Exon 6 cluster/-	RS6477	SV
11	B	Male	p.I173N/-	-	SV
12	B	Male	p.I173N /exon 6 cluster/p.V282L	c.29-31delCTG	SV
13	B	Female	p.I173N /exon 6 cluster/p.V282L	Rs6477	SV
14	0	Male	Exon 6 cluster/-	Rs6472-c.29-31delCTG	SW
15	B	Male	p.I173N /exon 6 cluster/p.V282L	-	SV
16	0	Male	G110△8nt/ G110△8nt	-	SW
17	B	Female	p.I173N /exon 6 cluster/p.V282L	-	SV
18	C	Male	Exon 6 cluster/ p.V282L	Rs6472-Rs6473-rs6446-c.29-31delCTG-Rs11970671	SV
19	B	Male	p.I173N /exon 6 cluster/p.V282L	Rs6473-rs6446-rs6477-c.29-31delCTG	SV

Table 4. Polymorphisms, Their Localization in CYP21A2 Gene and Genotypes

Number of SNP	Exon	Protein	Patient Genotype (No.)			SNP code
1	Exon 1	p.Leu40=	C/T (2)	C/C (0)	T/T (0)	rs6468
2	Exon 3	p.Arg103Lys	A/G (4)	A/A (0)	G/G (0)	rs6474
3	Exon 3	p.Ser114=	C/T (1)	C/C (0)	T/T (0)	rs193922546
5	Exon 7	p.Leu249=	C/G (13)	C/C (0)	G/G (1)	rs6477
6	Exon 7	p.Ser269Thr	C/G (4)	G/G (0)	C/C (0)	rs6472
7	Exon 7	p.Ser274=	C/T (1)	C/C (0)	T/T (0)	rs11970671
8	Exon 10	p.Pro491=	A/G (4)	A/A (0)	G/G (0)	rs6446
9	Exon 10	p.Ser494Asn	A/G (4)	A/A (0)	G/G (0)	rs6473
10	3'UTR	unknown	C/T (1)	C/C (0)	T/T (0)	rs1058152
11	3'UTR	c.*39G>A	C/T (1)	C/C (0)	T/T (0)	rs562025438

variants and phenotypes is consistent with HGMD and db-SNP (Table 4).

Gurgov et al. examined the association between *CYP21A2* mutations and virilization, and showed either one of the following mutations: (A) deletion, (B) *I2G*, and (C) *I173N* combined, demonstrate ambiguous genitalia in 94% of case. This confirms high reliability of predicting the degree of virilization based on the genotype in these groups. In our study, when *I173N* was combined with any other mu-

tation in compound heterozygote form, virilization was seen, too (25).

Indeed, we reported a heterozygote variant *c.29-31delCTG* (rs61338903) that was detected in 8 patients and there was a deletion of a CTG repeat in tandem repeats of 5 CTGs. Rodrigues et al. have reported an insertion in this position, but this insertion had no effect on the enzymatic activity (33).

5.1. Conclusions

In conclusion, a great diversity of compound heterozygosity was found in our population with large spectrum of mutated alleles. Therefore, The PCR-sequencing of the whole gene (*CYP21A2*) seems a preferred method, although the first step approach could be analyzing CAH patients based on the prevalence of the mutations of this gene in case that there is limitations of funding. Our patient's clinical manifestations were correlated with mutated alleles and the residual activity of 21-hydroxylase enzyme. This study could help genetic counseling of these patients in our population and contribute to better understanding of the molecular landscape of CAH in Iran.

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Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

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Footnotes

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