



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

Danial Zangene¹, Hossein Moravej², Homa Ilkhanipoor³, Anis Amirhakimi³, Zhila Afshar³ and Mona Entezam^{1*}

1. Department of Medical Genetics, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

2. Neonatal Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

3. Department of Pediatric Endocrinology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

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

To cite this article: Zangene D, Moravej H, Ilkhanipoor H, Amirhakimi A, Afshar Z, Entezam M. *CYP21A2* Gene Analysis in Southern Iranian CAH Patients and a Brief Review of the Mutation Spectrum. *Avicenna J Med Biotech* 2024;16(2):130-135.

* Corresponding author:

Mona Entezam, Ph.D.,
Department of Medical Genetics,
School of Medicine, Shiraz
University of Medical Sciences,
Shiraz, Iran

Tel: +98 71 32349610

Fax: +98 71 32349610

E-mail:
entezam@sums.ac.ir

Received: 12 Jun 2023

Accepted: 18 Sept 2023

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 glands¹. These steroid hormones, including cortisol, aldosterone, and adrenal androgens, develop from cholesterol in multistep enzymatic pathways².

21-hydroxylase deficiency (21-OHD) caused by *CYP21A2* gene mutations, is responsible for more than 95% of CAH cases and has distinct clinical classifications³. The classic form is a life-threatening condition, with a prevalence of 1 in every 16,000 newborns worldwide⁴. The prevalence of the nonclassic form is

1 case per 600 persons; although it is generally asymptomatic, it may cause infertility in females⁵.

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-OHD⁶. Some studies suggest that replacing 17-OHP with 21-deoxycortisol as the CAH biomarker can reduce false-positive test results⁷. However, based on the EMQN guideline, *CYP21A2* genotyping is the best approach for 21-OHD testing and CAH diagnoses⁸.

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 6p21.3⁹. The gene and pseudogene along with their neighbor genes are located in a genetic unit called RCCX unit¹⁰. There are usually two RCCX modules on each chromosome; One RCCX module includes *RP1*, *C4A*, *CYP21A1P*, and *TNX A*, and the other RCCX includes *RP2*, *C4B*, *CYP21A2*, and *TNX B* genes, (Figure 1)^{11,12}. Furthermore, the *CYP21A2* gene and its pseudogene show 98% homology in their sequence¹³. 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 regions⁵.

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 diagnosis¹⁶. 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 para-clinical 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

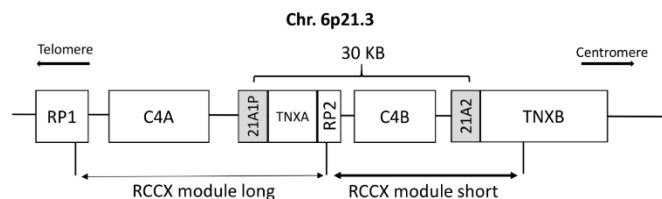


Figure 1. Bimodular form (*RP1–C4A–CYP21P–XA–RP2–C4B–CYP21–TNXB*) of the RCCX region of chromosome 6p21.3. *Cyp-21A2* gene and its pseudogene are shown in the gray boxes.

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.

All fragments were amplified in a final reaction volume of 25 μ l 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 described¹⁷ by the Pishgam Biotech Company, Tehran, Iran, (www.pishgam-bc.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, Provean¹⁸, PolyPhen2¹⁹, 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	CCCAACCCTGCTTTCTCCCCACC	72	
Sequencing-EX3-F	GCTCTTGGGGGGGCATATCTGGTGGG	74	
Sequencing-EX9-R	GCCTGGCTCCAGGAAGCGAT	74	

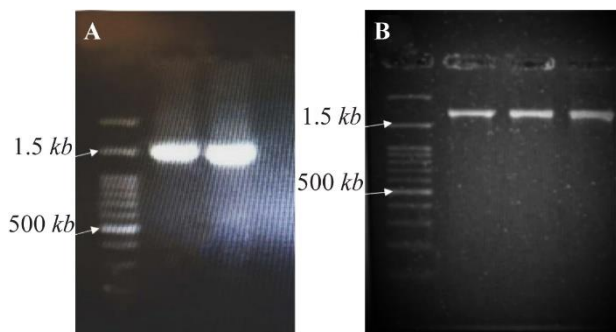


Figure 2. Gel electrophoresis results. A) PCR products of the first fragment (exons 1-6). B) PCR products of the second fragment (exons 5-10).

(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 identi-

fied, 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 pathogenic 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 families²⁰⁻²⁷. 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.

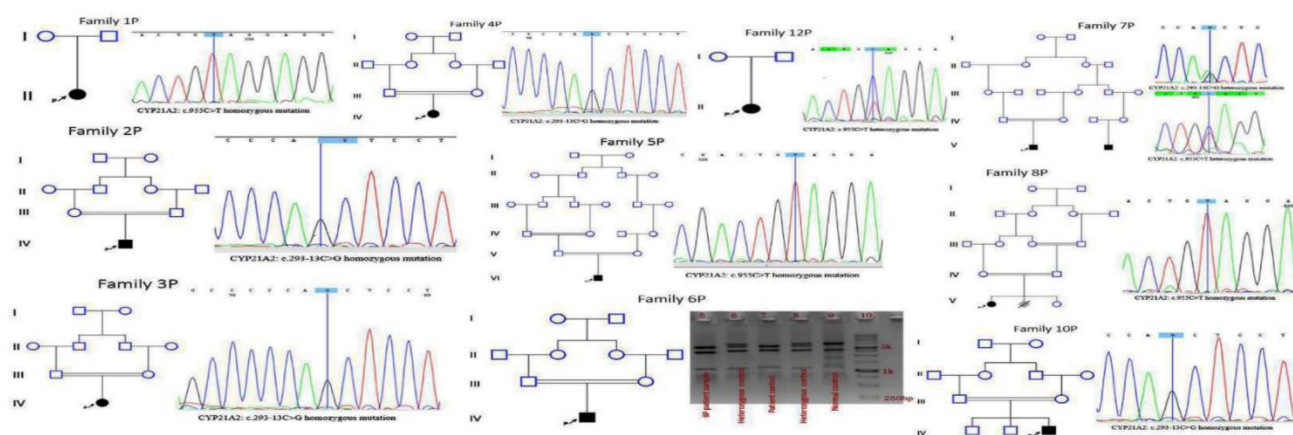


Figure 3. Families' pedigrees and images of founding mutations.

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%
	rs1554299737		c.710T>A	p.Ile237Asn			
Cluster E6	rs12530380	Exone 6	c.713T>A	p.Val238Glu	26	8.3 %	2.1%
	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.

Acknowledgement

This manuscript was supported by a grant from Shiraz University of Medical Sciences under the Agreement No. 22941. The ethical code of the research project by the Ethics Committee of Shiraz University of Medical Sciences was: IR. SUMS.REC.1400.378. We would like to express our deepest gratitude to Dr. Saman Nahid and Farzanegan Laboratory for doing biochemical tests and blood sampling as well as Pishgam Biothech Company for confirming deleted alleles. We are also, very thankful to all the patients and their families for their kind collaboration.

Conflict of Interest

The authors declare no conflict of interest for this article.

References

- Carvalho B, Marques CJ, Santos-Silva R, Fontoura M, Carvalho D, Carvalho F. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency: An update on genetic analysis of CYP21A2 gene. *Exp Clin Endocrinol Diabetes* 2021 Jul;129(7):477-81.
- Kanczkowski W, Sue M, Bornstein SR. The adrenal gland microenvironment in health, disease and during regeneration. *Hormones (Athens)* 2017;16(3):251-65.
- Espinosa Reyes TM, Collazo Mesa T, Lantigua Cruz PA, Agramonte Machado A, Domínguez Alonso E, Falhammar H. Molecular diagnosis of patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *BMC Endocr Disord*. 2020;20(1):165.
- Turcu AF, Auchus RJ. The next 150 years of congenital adrenal hyperplasia. *J Steroid Biochem Mol Biol Sep* 1; 153:63-71.
- Dumić M. [Congenital adrenal hyperplasia due to 21-hydroxylase enzyme deficiency]. *Lijec Vjesn* 1996 Mar; 118 Suppl 1:13-6. Croatian.
- Held PK, Bird IM, Heather NL. Newborn screening for congenital adrenal hyperplasia: Review of factors affecting screening accuracy. *Int J Neonatal Screen* 2020;6(3):67.
- Miller WL. Congenital adrenal hyperplasia: Time to replace 17OHP with 21-deoxycortisol. *Horm Res Paediatr* 2019;91(6):416-20.
- Baumgartner-Parzer S, Witsch-Baumgartner M, Hoepfner W. EMQN best practice guidelines for molecular genetic testing and reporting of 21-hydroxylase deficiency. *Eur J Hum Genet* 2020;28(10):1341-67.
- Xu Z, Chen W, Merke DP, McDonnell NB. Comprehensive mutation analysis of the CYP21A2 gene: An efficient multistep approach to the molecular diagnosis of congenital adrenal hyperplasia. *J Mol Diagn* 2013 Nov; 15(6):745-53.
- Gitelman SE, Bristow J, Miller WL. Mechanism and consequences of the duplication of the human C4/P450c21/gene X locus. *Mol Cell Biol* 1992;12(5):2124-34.
- Yang Z, Mendoza AR, Welch TR, Zipf WB, Yung Yu C. Modular variations of the human major histocompatibility complex class III genes for serine/threonine kinase RP, complement component C4, steroid 21-hydroxylase CYP21, and tenascin TNX (the RCCX module): A mechanism for gene deletions and disease associat. *J Biol Chem* 1999;274(17):12147-56.
- Lee HH. The chimeric CYP21P/CYP21 gene and 21-hydroxylase deficiency. *J Hum Genet* 2004;49(2):65-72.
- Higashi Y, Yoshioka H, Yamane M, Gotoht O, Fujm-Kuriyama Y. Complete nucleotide sequence of two steroid 21-hydroxylase genes tandemly arranged in human chromosome: A pseudogene and a genuine gene. *Proc Natl Acad Sci USA* 1986 May;83(9):2841-5.
- Falhammar H, Nordenström A. Nonclassic congenital adrenal hyperplasia due to 21-hydroxylase deficiency: clinical presentation, diagnosis, treatment, and outcome. *Endocrine* 2015 Sep;50(1):32-50.
- Ghizzoni L, Cappa M, Chrousos G, Loche S, Maghnie M. Molecular Genetics of 21-Hydroxylase Deficiency. Vol. 20, *Endocr Dev*. Basel, Karger. 2011. P. 80-7.
- Speiser PW, Arlt W, Auchus RJ, Baskin LS, Conway GS, Merke DP, et al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: An endocrine society* clinical practice guideline. *J Clin Endocrinol Metab* 2018 Nov 1;103(11):4043-88.
- Lee HH, Lee YJ, Lin CY. PCR-based detection of the CYP21 deletion and TNXA/TNXB hybrid in the RCCX module. *Genomics* 2004 May 1;83(5):944-50.
- Choi Y, Sims GE, Murphy S, Miller JR, Chan AP. Predicting the functional effect of amino acid substitutions and indels. *PLoS One* 2012 Oct 8;7(10):e46688.
- Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods* 2010 Apr;7(4):248-9.
- Vakili R, Baradaran-Heravi A, Barid-Fatehi B, Gholamin M, Ghaemi N, Abbaszadegan MR. Molecular analysis of the CYP21 gene and prenatal diagnosis in families with 21-hydroxylase deficiency in Northeastern Iran. *Horm Res* 2005 Apr;63(3):119-24.

21. Ramazani A, Kahrizi K, Razaghiazar M, Mahdieh N, Koppens P. The frequency of eight common point mutations in CYP21 gene in Iranian patients with congenital adrenal hyperplasia. *IBJ* 2008;12(1): 49-53.
22. Rabbani B, Akbari MT, Mahdieh N, Zaridust E, Ashtiani MTH, Lee HH, et al. Homozygous complete deletion of CYP21A2 causes a simple virilizing phenotype in an Azeri child. *Asian Biomedicine* 2011 Dec;5(6):889-92.
23. Rabbani B, Mahdieh N, Ashtiani MTH, Larijani B, Akbari MT, New M, et al. Mutation analysis of the CYP-21A2 gene in the Iranian population. *Genet Test Mol Biomarkers* 2012 Feb 1;16(2):82-90.
24. Forouzanfar K, Seifi M, Hashemi-Gorji F, Karimi N, Estiar MA, Karimoei M, et al. Mutation analysis of the CYP21A2 gene in congenital adrenal hyperplasia. *Cell Mol Biol* 2015;61(4):51-5.
25. Kolahdouz M, Hashemipour M, Khanahmad H, Rabbani B, Salehi M, Rabbani A, et al. Mutation detection of CYP21A2 gene in nonclassical congenital adrenal hyperplasia patients with premature pubarche. *Adv Biomed Res* 2016;5(1):33.
26. Kollahi NA, Rohani F, Baghbani-Arani F, Shojaei A. Complex alleles of cyp21a2 are the most frequent causes of congenital adrenal hyperplasia in Iranian population. *Iran J Pediatr* 2019 Dec 1;29(6).
27. Soveizi M, Mahdieh N, Setoodeh A, Sayarifard F, Abbasi F, Bose HS, et al. P.Gln318X and p.Val281Leu as the Major Variants of CYP21A2 Gene in Children with Idiopathic Premature Pubarche. *Int J Endocrinol* 2020; 2020:4329791.
28. Claahsen - van der Grinten HL, Speiser PW, Ahmed SF, Arlt W, Auchus RJ, Falhammar H, et al. Congenital adrenal hyperplasia-current insights in pathophysiology, diagnostics, and management. *Endocr Rev* 2022 Jan 12;43 (1):91-159.
29. Narasimhan ML, Khattab A. Genetics of congenital adrenal hyperplasia and genotype-phenotype correlation. *Fertil Steril* 2019;111(1):24-9.
30. Witchel SF, Azziz R. Nonclassic congenital adrenal hyperplasia. *Int J Pediatr Endocrinol* 2010;2010:625105.
31. Higashi Y, Tanaet 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* 1988 Oct;85(20):7486-90.
32. Lee HH, Chang SF, Tsai FJ, Tsai LP, Lin CY. Mutation of IVS2-12A/C>G in combination with 707-714del-GAGACTAC in the CYP21 gene is caused by deletion of the C4-CYP21 repeat module with steroid 21-hydroxylase deficiency. *J Clin Endocrinol Metab* 2003 Jun;88 (6):2726-9.
33. Riedl S, Röhl FW, Bonfig W, Brämwig J, Richter-Unruh A, Fricke-Otto S, et al. Genotype/phenotype correlations in 538 congenital adrenal hyperplasia patients from Germany and Austria: Discordances in milder genotypes and in screened versus prescreening patients. *Endocr Connect* 2019;8(2):86-94.
34. Tajima T, Fujieda K, Fujii-Kuriyama Y. de novo mutation causes steroid 21-hydroxylase deficiency in one family of HLA-identical affected and unaffected siblings. *J Clin Endocrinol Metab* 1993 Jul;77(1):86-9.
35. Ezquieta B, Cueva E, Oyarzábal M, Oliver A, Varela JM, Jariego C. Gene conversion (655G splicing mutation) and the founder effect (Gln318Stop) contribute to the most frequent severe point mutations in congenital adrenal hyperplasia (21-hydroxylase deficiency) in the Spanish population. *Clin Genet* 2002 Aug;62(2):181-8.
36. Globberman H, Amor M, Parker KL, New MI, White PC. Nonsense mutation causing steroid 21-hydroxylase deficiency. *J Clin Invest* 1988;82(1):139-44.
37. Kleinle S, Lang R, Fischer GF, Vierhapper H, Waldhauser F, Födinger M, et al. Duplications of the functional CYP21A2 gene are primarily restricted to Q318X alleles: Evidence for a founder effect. *J Clin Endocrinol Metab* 2009;94(10):3954-8.
38. Prado MJ, de Castro SM, Kopacek C, de Mello MP, Rispoli T, Grandi T, et al. Development of CYP21A2 Genotyping Assay for the Diagnosis of Congenital Adrenal Hyperplasia. *Mol Diagn Ther* 2017;21(6):663-75.
39. Saadat M, Ansari-Lari M, Farhud DD. Consanguineous marriage in Iran. *Ann Hum Biol* 2004;31(2):263-9.
40. Koohiyan M, Azadegan-Dehkordi F, Koohian F, Hashemzadeh-Chaleshtori M. Genetics of hearing loss in north Iran population: An update of spectrum and frequency of GJB2 mutations *J Audiol Otol* 2019 Oct;23(4): 175-180.