

# Mutation detection of *CYP21A2* gene in nonclassical congenital adrenal hyperplasia patients with premature pubarche

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## Abstract

**Background:** Congenital adrenal hyperplasia (CAH) due to mutations in the gene encoding 21-hydroxylase is one of common disease with an autosomal recessive form. In this study, our aim is to detect the prevalence of eight common mutations in nonclassical congenital adrenal hyperplasia (NCAH).

**Materials and Methods:** A total of 30 patients with clinical and laboratory evidence of NCAH was selected. Gene-specific polymerase chain reaction (PCR) without contamination of pseudogene was carried out, and PCR product of this step was used to amplification-refractory mutation system PCR on eight common mutations in *CYP21A2* gene.

**Results:** Two heterozygote patients for I2G mutation and six heterozygote patients for Q318X mutation is reported in our study. These mutations associated with the classic form of CAH, and heterozygotes presented with NC symptom, including premature pubarche and hirsutism.

**Conclusion:** There are some data about the association of the mutation with the clinical form of CAH including classic (salt-wasting and simple virilizing) and NC form. I2G and Q318X mutations were reported in classic form in homozygote state, but the heterozygote form associated with NC form. CAH diagnosis with NC symptom and with measurement of 17-hydroxyprogesterone as NCAH is not a trusted assessment and require to molecular analysis for accurate diagnosis.

**Key Words:** 21-hydroxylase deficiency, amplification-refractory mutation system polymerase chain reaction, *CYP21A2* gene, nonclassic congenital adrenal hyperplasia

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## INTRODUCTION

Congenital adrenal hyperplasia (CAH) is one of the most common inborn endocrine disorders<sup>[1]</sup> with the autosomal recessive pattern because of 21-hydroxylase

deficiency (21-OHD).<sup>[2]</sup> 21-OHD included about 95% of CAH cases and were the most common cause of ambiguous genitalia in girls.<sup>[3]</sup> About 5–8% cases are due to 11-βhydroxylase deficiency.<sup>[4]</sup> 21-hydroxylase

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is an enzyme of cytochrome P450, encoded by the *CYP21A2* gene.<sup>[5]</sup> The *CYP21A2* is located on the short arm of chromosome 6 (6p21.3) adjacent to its inactive pseudogene (*CYP21A1P*) within the HLA region.<sup>[6]</sup> The *CYP21A2* and *CYP21A1P* have 98% homology in exons and 96% in introns.<sup>[3]</sup> Each gene and pseudogene contain 10 exons with 3.1 kb in length.<sup>[7]</sup>

The pseudogene is situated about 30 kb upstream of *CYP21A2*. In humans, these two genes are located immediately next to the 3' end of the *C4A* and *C4B* genes encoding the fourth component of serum complement.<sup>[5]</sup> Due to a high degree of sequence homology between gene and pseudogene, unequal crossover, and gene conversion events usually occur in *CYP21A2* gene and, therefore, generate a mutation in the gene.<sup>[1]</sup>

Enzyme 21-hydroxylase catalyze the conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycorticosterone. Therefore deficiency of 21-hydroxylase decreases the rate of this conversion and leading to androgen excess and also disruption the balance of cortisol and aldosterone.<sup>[5]</sup> Disruption of cortisol synthesis induces adrenocorticotrophic hormone (ACTH) secretion by the hypophysis.<sup>[8]</sup> Two main clinical forms of the disease include: (1) Classic CAH which is clinically categorized in two groups, the simple virilizing (SV) and salt-wasting (SW) and (2) nonclassic (NC) form.<sup>[9]</sup> The frequency of classic form is about 1 of 10,000 live births. Approximately, 30% of classic form show SV features and about 70% show additional SW. SW form, a fatal inability to preserve dietary sodium, has shown a severe defect in both cortisol and aldosterone synthesis.<sup>[10]</sup> Ambiguous external genitalia in female newborn is seen in the SW form. Immediate diagnosis and treatment with mineralocorticoid and salt supplementation after birth result in reduced mortality and morbidity.<sup>[11]</sup> All patients with classical or NC disease can be treated with glucocorticoids.<sup>[12]</sup> Impairment of cortisol synthesis is recognized in SV, whereas aldosterone synthesis is in normal range. The masculinization of external genitalia represents in the female. In the NC or late-onset form, a delicate defect in cortisol synthesis can typically solely be detected throughout stimulation with corticotropin.<sup>[13]</sup> Female NC 21-OHD patients do not exhibit genital ambiguity at birth and may reveal some signs of androgen excess in late childhood or early adulthood.<sup>[14]</sup> The most common symptoms are premature pubarche in children, severe acne, hirsutism and decreased fertility.<sup>[15]</sup> Precocious pseudopuberty can happen in both sexes, but it is comparatively rare. The prevalence for the NC form is 1:53 for Hispanics, 1:27 for Ashkenazi Jews, 1:333 for Italians and 1:1000 for other Caucasians.<sup>[16]</sup>

The molecular genetic basis of CAH has been entirely investigated, and different mutations were classified based on 21-hydroxylase activity to predict the related phenotype of the affected individuals.<sup>[17]</sup>

Approximately, 65–70% of *CYP21A2* mutations are deleterious and due to microconversion derived from pseudogene *CYP21A1P*<sup>[18]</sup> including eight common mutations (p.P30L, c. 293-13A/C >G in intron 2 splice site, 8-bp deletion in exon 3, p.I172L, exon 6 cluster [p.I236N, p.V237E, p.M239K], p.V281L, p.Q318X, p.R356W).<sup>[19]</sup> About 25–30% are caused by unequal meiotic crossovers (or deletions).<sup>[18]</sup> Point mutations in compound state indicates SW form, although a single mutation has mild effect.<sup>[20]</sup>

Across mutations detection in previous studies, p.Q318X and p.R356W had 0% enzyme activity, c. 293-13A/C >G with minimal residual activity and p.I172L with 2–11% enzyme activity were associated with classic form of disease.<sup>[21]</sup>

A large number of methods have been expanded which can detect mutations in *CYP21A2*,<sup>[5]</sup> such as restriction fragment length polymorphism,<sup>[22]</sup> amplification-refractory mutation system (ARMS) polymerase chain reaction (PCR),<sup>[23]</sup> Allele specific oligonucleotide<sup>[24]</sup> and other methods. The biggest problem in *CYP21A2* molecular screening is very highly homology between gene and pseudogene causes to contamination of PCR products with pseudogene.

In this study, we report the results of molecular screening on eight most common mutations in *CYP21A2* gene in 30 NC CAH patients in Isfahan province.

## MATERIALS AND METHODS

### Patients and sample preparation

Thirty children with NC symptom of CAH from Isfahan province participated in our study. The patients were cumulated based on 17-OHP level before and after ACTH stimulation test and premature pubarche from Isfahan endocrine center. Informed consent was obtained from their parents. The patient's age ranged from 3 to 12 years. Basal 17-OHP >10 ng/ml was seen in 60% cases with premature pubarche and hirsutism. Value more than 20 ng/ml was reported only in one case. In other cases, 17-OHP was <10 ng/ml but with NC symptom.

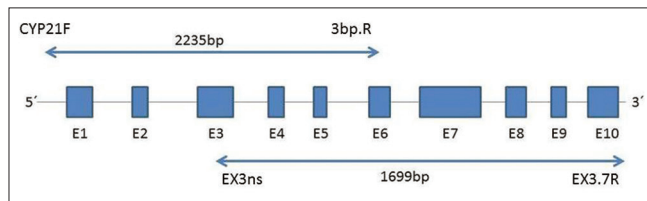
The blood sample was obtained from them. DNA was extracted from peripheral blood samples by GeNet Bio DNA extraction kit (Korea) according to its protocol. Agarose gel electrophoresis and spectrophotometry were used for assessment of DNA quality and quantity.

Positive controls were received from Tehran children's medical center.

### Mutation analysis

Because of high homology between gene and pseudogene, at first a primary couple PCR were performed for specific amplification of the *CYP21A2* gene and discriminate gene from pseudogene [Figure 1]. Primers which used for this step did not amplify *CYP21A1P*. The primers are shown in Table 1. The products of this step were used as a DNA template for ARMS PCR.

ARMS PCR was performed for the detection of eight common mutations in *CYP21A2* gene. Three PCR reaction for splice mutation in intron 2 are required because there are two wild-types and one mutant alleles. Allele-specific PCRs with specific primers were done [Table 2].



**Figure 1:** Typical polymerase chain reaction method for amplification of *CYP21* in two overlapping fragments with 2.2 and 1.7 kb

**Table 1:** The two couples primers for discrimination of gene from pseudogene

Primer	Sequence (5'→3')
CYP21F	F: ATTCCCAATTCTTATTTTTTA
3bp.R	R: CTCAGCTGCATCTCCACGA
Ex3ns	F: CGGACCTGTCCTTGGGAGACTAC
EX3.7_R	R: CCAGCCTCCACCACATTTTGAC

**Table 2:** The primers used for allele specific PCR

Mutation	Forward primer (5'→3')	References	Reverse primer (5'→3')
P30L	5'-CCAGAGCCTCCACCTCCC-3' 5'-TCCAGAGCCTCCACCTCCT-3'		5'-GGAGCCTTTTGCTTGCCC-3'
I2G	5'-TTCCCACCCTCCAGCCCCAA-3' 5'-TTCCCACCCTCCAGCCCCAC-3' 5'-TTCCCACCCTCCAGCCCCAG-3'	[25] [25] [25]	5'-CCCTCCACTGGCCTGCCACG-3'
8bp-deletion	5'-CGGACCTGTCCTTGGGAGACTAC-3' 5'-ACTACCCGGACCTGTCCTTGGTC-3'	[25] [25]	5'-CCAGCCTCCACCACATTTTGAC-3'
I172N	TTCTCTCCTCACCTGCAGCATCAT TTCTCTCCTCACCTGCAGCATCAA	[25] [25]	CCCTCCACTGGCCTGCCACG
EX6 cluster	TCACATCGTGGAGATGCAGCT AGGGACCACAACGAGGAGAA	[25]	GGAGCCTTTTGCTTGTC
V281L	ACAGCTCCTGGAAGGGCAGC ACAGCTCCTGGAAGGGCACT		CCAGCCTCCACCACATTTTGAC
Q318X	TTCGTGGTCTAGCTCCTCTG TTCGTGGTCTAGCTCCTCTA	[25] [25]	GAGGGATCACATCGTGGAGAT
R356W	CTAAGAGCACAACGGGCCG CTAAGAGCACAACGGGCCA	[25] [25]	TAAGAACTACCCGGACCTGTC

PCR: Polymerase chain reaction

### Direct sequencing

To confirm the results of allele-specific PCR, some heterozygote samples have been sequenced. The PCR products were purified and sequenced with SeqF: CCACCTCAGCCTCAAGTGT and Ex3ns: CGGACCTGTCCTTGGGAGACTAC primers.

### RESULTS

DNA samples of 30 patients in Isfahan were analyzed for the existence of eight common mutations in *CYP21A2* gene. Approximately, 83% of cases were females and consanguinity was seen in 23% of patients. Based on clinical manifestation and biochemical tests of patients, all of them were NC form of CAH.

Results indicated heterozygosity in two patients for I2G mutation with clitoromegaly and premature pubarche manifestation and heterozygosity in some patients for Q318X mutation. Another mutation not found in our patients [Table 3].

This study suggested carriers with heterozygosity for I2G and Q318X mutations may represent as NC form.

### DISCUSSION

This study was directed to detect eight common mutations in 30 patients from Isfahan province, Iran, with NC CAH by ARMS-PCR method.

The molecular diagnosis of the 21-OHD has a large pitfall due to the existence of a pseudogene. Almost all common mutations of *CYP21A2* are present in *CYP21A1P*. Contamination of gene with pseudogene causes false heterozygosity in all normal samples.

**Table 3: The frequency of mutations in *CYP21* in our study**

Mutation	Exon/intron	Nucleotide variation	Frequency (%)
I2G	Intron2	656A/C>G	3.3
Q318X	Exon8	1996C>T	15

Hence, discrimination of gene from pseudogene is a critical point in molecular detection of 21-OHD. To achieve reliable results, *CYP21A2* coding sequence has been divided to two overlapping fragments and amplified by two specific couple primers that amplify an only *CYP21A2* gene, and there was not any contamination with pseudogene. Some mutations have been identified using ARMS PCR on two overlapping amplified fragments to screen eight common mutations.

In according to other studies in Iran, which Q318X is most frequent,<sup>[25,26]</sup> in our study also Q318X mutations is the most common in Isfahan, in contrast to one another study in Iran by Ramazani *et al.*<sup>[23]</sup> and western European cohorts.<sup>[27]</sup>

In the Anglo-Saxons a large deletion is prevalent; an R356W mutation is prevalent in the Croatians; a V281 L mutation is prevalent in Ashkenazi Jews; an IVS2 mutation is prevalent in the Middle-Eastern population and Iranians; and a Q318X mutation is prevalent in East Indians.<sup>[28]</sup>

In 2014, Sharaf *et al.*<sup>[19]</sup> analyzed I2G mutation by allele-specific PCR and reported 76% and 17.2% cases with heterozygous and homozygous mutation respectively. Another study in Macedonia population report 41.5% of patient with I2G mutation by PCR/ACRS method.<sup>[22]</sup> This splice site mutation in intron 2 identify as one of the common mutation in Malaysian patients.<sup>[29]</sup> This splice site mutation by activating another cryptic site in splicing activity change the premature mRNA splicing and, therefore, switch the reading frame.<sup>[30]</sup>

In the Northeast Brazil and Tunisian, Q318X mutation is the most frequent mutation.<sup>[1,31]</sup>

Six other mutations not found in the population of our study. It may require to a large sample size with all form of disease including SW and SV in addition to NC form of the disease. Moreover, it may be due to the prevalence of other common point mutations or deletion in Isfahan.

Various methods for *CYP21A2* point mutation detection was performed in several studies. The biggest problem in this survey is due to pseudogene, and it is important to prevent of pseudogene contamination

in the PCR-based method. In our study, according to previous studies<sup>[7,32]</sup> gene amplified in two parts separating gene from pseudogene and then ARMS PCR for detection of four-point mutations had been used.

ARMS PCR is a rapid method for mutation detection. This method reduce time and cost rather than other methods which used in other study. However, this method is very sensitive and various condition including annealing temperature and primer density can eventuate to false negative or positive result. In our study, two patients with heterozygosity for I2G mutation were detected as homozygote with ARMS PCR, whereas direct sequencing was presented in heterozygote form.

Regarding to high incidence of CAH in Iran and Isfahan province, this highlights the importance of screening of CAH. Q318X and I2G mutations are seen more than other mutations in Iran population, therefore screening for these mutations can be the first step in newborn screening. If any patients are negative for these mutations, molecular diagnosis of other common mutations will assess.

Newborn screening (by molecular method) can predict the phenotypes type and reduce mortality due to SW form of the disease. Moreover, prenatal diagnosis can reduce ambiguous genitalia and genital virilization in the female fetus in SV form and premature puberche in NC form.

Although measurement of 17-OH progesterone with high level can determine form of CAH but cannot recognize SW from SV phenotype<sup>[33]</sup> and also cannot detect carriers in the population. Moreover 17-OHP increases in stresses and can cause to false positive result. Therefore, molecular tests for accurate diagnosis of CAH and type of disease are required. The results of molecular diagnosis of CAH can be used in genetic counseling.

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#### Conflicts of interest

There are no conflicts of interest.

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