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Mutation analysis of the *CYP21A2* gene in congenital adrenal hyperplasia

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Abstract

Congenital adrenal hyperplasia (CAH) is an inherited autosomal recessive enzymatic disorder involving the synthesis of adrenal corticosteroids. 21-Hydroxylase deficiency (21-OHD) is the most common form of the disease which is observed in more than 90% of patients with CAH. Early identification of mutations in the genes involved in this disease is critical. A marker of the disease, errors in the *CYP21A2* gene, is thought to be part of the pathophysiology of CAH. Therefore, the identification of gene mutations would be very beneficial in the early detection of CAH. This research was a descriptive epidemiological study conducted on individuals elected by the inclusion criteria whom were referred to the Genetic Diagnosis Center of Tabriz during 2012 to 2013. After sampling and DNA extraction, PCR for the detection of mutations in the CYP21A2 gene was performed followed by sequencing. For data analysis, the results of sequencing were compared with the reference gene by blast, Gene Runner and MEGA-5 software. Obtained changes were compared with NCBI databases. The analysis of the sequencing determined the mutations located in Exons 6, 7, 8 and 10. The most frequent findings were Q318X (53%) and R356W (28%). Exon 6 cluster (7%), E431k (4%), V237E (2%), V281L (2%), E351K (2%), R426C (2%) were also frequent in our patients. The most frequent genotype was compound heterozygote, Q318X/R356W. Three rare mutations in our study were E431K, E351K and R426C. Observed mutation frequencies in this study were much higher than those reported in previous studies in Iranian populations. Thus, it seems that it is necessary to follow-up screening programs and use sequencing methods to better identify mutations in the development of the disease.

Key words: Congenital adrenal hyperplasia, *CYP21A2* gene, mutation.

Introduction

Congenital adrenal hyperplasia (CAH; OMIM 201910), is one of the most common form of inborn metabolic disorders with autosomal recessive inheritance. It is the most common cause of genital ambiguity (1, 2). In about 90-95% of cases, 21-hydroxylase deficiency (21-OHD) is the most common cause of congenital adrenal hyperplasia playing the main role in 17-hydroxyprogesterone (17-OHP) and progesterone conversion into 11-deoxycortisol (3, 4). 21-OHD results in a reduced ability to synthesize cortisol and aldosterone in adrenal glands leading to produce excessive androgen and adrenal hyperplasia (5, 6). CAH is characterized by reduced or complete absence enzymatic activity of steroid biosynthesis pathway, cortisol and aldosterone, which are the final products of the pathway. Thus, various degrees of impaired synthesis of them shunt the loop into other pathway (particularly androgen pathway), leading to produce excessive androgen and adrenal hyperplasia (5, 6). Phenotypes vary from classic form with severe enzyme deficiency and prenatal onset of virilisation including simple virilising form (25%) and salt-wasting form (75%) to a non-classic form which is the mildest form with late onset symptoms (4, 7). The affected enzyme, 21-OH, is encoded by *CYP21A2* (Gene ID: 1589) gene located adjacent to a highly homologous pseudogene, *CYP21P*. It consists of 10 exons and is mapped to chromosome 6p21.3 being transcribed into a 1488bpcDNA, which encodes a 495-residue glycopeptide (CCDS4735.1) (8, 9). *CYP21A2* and *CYP21P* have a high similarity with a nucleotide identity of 98% in their exons and 96% in their intron sequences (8). To date, almost 250 different types mutations underlying CAH have been identified (Human Gene Mutation Database [HGMD, 2015]). Gene conversion events involving the functional gene and the pseudogene are thought to account for many cases of steroid 21-hydroxylase deficiency (7). Approximately 25% of 21-OHD caused by large deletion and about 75% of it seems to carry point mutations. Some rare mutations are most commonly found in a single family or a specific population (10).

Incidence of severe form of 21-OHD is ranging from one per 10000 live births to one per 20000, all over the world (7). The prevalence of non-classic form is one in 1700 in general population (7). Frequent consanguineous, first cousin marriages and underestimation contribute to a very high prevelance of the disease in our

population (7, 11). As our country has a heterogeneous population, a comprehensive study on different ethnic groups in the country is necessary to determine the mutation frequency based on each ethnic cohort. In current study, we report the mutation spectra of *CYP21A2* gene on a group of patients from northwest Iranian suffering from classical form of CAH. The aims of this research are to identify common frequency of mutations in *CYP21A2* and then correlate genotype with phenotype.

Materials and methods

Patients

Twenty one unrelated patients with CAH (11 Male, 10 Female) were clinically diagnosed by pediatric endocrinologist. Informed consent was obtained from all parents for DNA analysis and the study was approved by the Ethics Committee and Research Council of Tabriz University of Medical Sciences.

Clinical evaluation

The criteria used to diagnose a SW form was either a SW crisis in the newborn period or elevated plasma renin activity (PRA) levels, hyponatremia and hyperkalemia. The SW patients usually presented ambiguous external genitalia in girls or precocious puberty in boys, with no salt loss crisis. Consanguinity had been observed in 12 families (57.1%), not seen in 3 families (14%) and unknown in 6 families (28%). All patients had increased serum level of 17-OH-progesterone.

Molecular analysis

Peripheral blood was obtained from patients and their parents and genomic DNA was extracted according to the standard salting out protocol (12). The entire sequence of the *CYP21A2* gene including exons and adjacent intron were amplified by the polymerase chain reaction (PCR) followed by sequencing. Primer sequence sets and PCR cycling conditions were previously described (11). The products of amplification were sequenced using the ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA).

Results

We analyzed 21 patients with classic form of CAH. Patients were classified with the SW, SV, or NC forms of CAH via clinical presentations and hormonal investigations. Fifteen patients (71.40%, Mean ±SEM 6±1.2 years) had SW form consisting of 7 females and 8 males and 3 of them (14.30%, Mean ±SEM 301±8 days) were in SV form consisting of 1 female and 2 males. Among 42 chromosomes both chromosomes were affected by mutations in 18 patients (85%). Two patients (9.50%, Mean ±SEM 18±0.5 years) carried only 1 copy of mutation (NC form) and in one patient (4.80%, 23 days) no mutation was detected. We found five homozygote (23.80%) and eleven compound heterozygote (52.40%) for these mutations and one patient who is affected by 3 mutations (Q318X, R356W and R426C). We also had one patient whose both alleles were homozygous for 2 mutations (Q318X and R356W). These complex mutation alleles are likely the result of large gene conversions or multiple mutation events.

The most frequent finding were Q318X (53%) and R356W (28%). Exon 6 cluster (7%), E431k (4%), V237E (2%), V281L (2%), E351K (2%), R426C (2%) were also frequent in these patients. The most frequent genotype was compound heterozygote, Q318X/R356W. Three rare mutations in our study were E431K, E351K and R426C. The frequency of Q318X mutation in male was higher than females (47% and 28% respectively), but, R356W distributed in both sex equally. The frequency of SW CAH is similar in both sexes because equally severe enzyme disruption in males and females lead to a salt-wasting crisis shortly after birth.

Exon 6 cluster includes I236N, V237E and M239K. The mutation V237E abolished enzyme function and is a null mutation whereas enzyme activity in I236N mutation is very low, but, measurable. M239K had no effect on enzyme activity, thus, V237E has the main role in CAH. It could confirm the SW phenotype in a patient with Q318X/V237E genotype. Conversely patients who were heterozygous for Q318X/V281L, E431k/E351K and Q318X/E431K had SV form. As we know, mild mutation has the main role of phenotype in heterozygote genotypes due to codominance, E431K and V281L could be responsible for SV type. All molecular detected mutations were distributed in Exon 6, 7, 8 and 10,as demonstrated in table 1. The most frequent mutations were Q318X (stop codon) and R356W.

Discussion

In current study, molecular testing of 21 patients with classic form of CAH identified eight mutations, which were previously reported. Of the changes in *CYP21A2* identified in this study, two known mutations, Q318X (53%) and R356W (28%), were the most frequent mutations in the affected patients from northwest of Iran (Table 1). CAH is one of the common disorder in Iran due to high rate of consanguineous marriages (61%) in our population (13). To date, several reports from Iran investigated clinical and molecular bases of CAH patients (13, 14). Due to existence of different ethnic groups and criteria of CAH for newborn screening in our country, epidemiologic study should be considered for prevalence of CAH. Molecular genetic testing would significantly decrease the costs, hospitalization

Table 1. Molecular features *CYP21A2* in patients with CAH.

Alteration in nucleotide sequence[a]	Location	Frequency	Predicted effect on protein[b]
710T>A	Exon 6	2%	V237E
841G>T	Exon 7	2%	V281L
952C>T	Exon 8	53%	Q318X
1066C>T	Exon 8	28%	R356W
1051 G>A	Exon 8	2%	E351K
1291 G>A	Exon 10	4%	E431K
1276 C>T	Exon 10	2%	R426C
	Exon 6	7%	E6 cluster

[a] Numbering of the nucleotides refers to the cDNA sequence (GenBank accession no. NM_000500.7).

[b] Numbering of the amino acids refers to the deduced peptide sequence, NP_000491.4, with the ATG initiation codon as 1.

Table 2. *CYP21A2*common mutations found in the current study on 21-OHD compared with previous studies in Iranian population.

Amino acid change	Exon/ intron	Nucleotide change	SW	SV	Allele No	Mutation Frequency (%)			
						Vakili et al., 2005	Ramazaniet al., 2008	Rabbani et al., 2012	Current study
I2G	Intron2	656A/C>G			0	15	28	14.77	0
V281L	Exon7	1585G>T	0	1	1	5	3	1.14	2
Q318X	Exon8	1996C>T	18	1	19	6.7	9	15.91	53
R356W	Exon8	2110C>T	10	0	10	0	5	7.95	28
E6 cluster	Exon6		3	0	3	1.7	4	2.27	7
Total					42	100	100	88	

SW, salt wasting; SV, simple virilising.

Table 3. Allele frequencies in different populations (%).

Nationality	Allele No	Q318X	R356W	Ex6 cluster	V281L	Ref.
USA	394	4	4.0	4.0	9	[21]
Sweden	400	2	4.0	5.0	6	[22]
England	284	0	10.0	0.0	0	[23]
French	258	4		6.0	17	[24]
Italy	146	8		12.0	11	[25]
China	40	8	10.0	5.0		[26]
Turkey	31	8	9.6	3.2	0	[27]
Spain	58	4	4.0	5.0	17	[28]
Chilly	126	9	11.0	10.0		[29]
Mexico	94	4	7.0	1.0	9	[30]
Brazil	74	11	8.0	5.0	4	[31]
Argentina	72	14	6.0			[32]
Fenland	102	2		10.0	3	[33]
Iran	330	76.6	40.95	14.97	11.14	[13-15]

Iran , Our study and other reports of Iran.

and time to correct sex assignment. Newborn screening is necessary to reduce morbidity, mortality and treatment costs of CAH patients. For special interest of physicians in performing prenatal diagnosis of CAH, fetal DNA could be obtained by amniocentesis or chorionic villus samples could be considered for predicting phenotype.

Different molecular methods have been used to identify changes in *CYP21A2* gene including ARMS-PCR, PCR-sequencing and Multiplex ligation-dependent probe amplification (MLPA) analysis for detection of known, novel and copy number alteration, respectively. In this study, sequence analysis was used to ascertain molecular basis of affected CAH patients from northwest of Iran. The results of our study were different from previous studies in mutation frequency due to differences in the ethnicity. In this report, frequency of Q318X and R356W were much more than other reports of Iran, in which no R356W mutation was reported by Vakili et al. (14). Another bold point in our result was no report of 12G which has been mentioned as the most frequent mutation in Iranian population (Tale 2) (13-15). Both Q318X and R356W mutations underlie no activity of enzyme leading to SW form, which is a common phenotype among Iranian patients with CAH. As Tabriz in northwest of Iran has less heterogeneous and more consanguineous marriages, we observed high rate of homozygous condition in patients (28%) for the affected patients in this study. High frequency of Q318X in Iranian CAH patients suggests screening for this mutation in the first step of molecular diagnosis (Table 1 and 2). By considering other reports from Iran, CAH patients of our study had common mutations that are the most frequent mutations of *CYP21A2* in the world (Table 3). Taken together, it could be concluded that Q318X mutation has likely ancestral relationship among these patients from northwest of Iran or it was originated from this region and then disseminate gradually to other parts of Iran. Also, the R356W mutation had also high frequency in our population (28%) in comparison with other populations (2-4%) (13-15). Increasing the number of patients may reveal precise frequency for *CYP21A* alterations.

Our findings show a close correlation between genotype and phenotype (Table 4). Phenotype of our patients was assigned by hormonal profiles and physical examination and we know that phenotype-genotype predicting in CAH is ambiguous due to diversity of this disorder. In agreement with other reports, the most variability of clinical phenotypes associated with SW form of CAH (16). Phenotype-genotype correlation could be drawn by considering the phenotypes in patients with homozygous genotypes. The patients who were homozygous for Q318X, R356W and E6 cluster had SW form of CAH, indicating these mutations would be responsible for this type of classic CAH (Table 4). As Q318X has high frequency among our patients, a screening for this mutation in Iranian population for prenatal screening between families with CAH background or consanguinity should be helpful. Results of this study showed no significant sex difference among SW affected individuals for common mutations in *CY*P21A2 gene, as

Table 4. Genotype–phenotype correlation in the patients.

Genotype	Clinical features	Total
Q318X / V23vE	SW	1
Q318X / Q318X	SW	3
Q318X / Q318X + R356w / R356W	SW	1
Q318X / R356W, R426C	SW	1
Q318X / R356W	SW	5
Q318X , R356W / R356W	SW	1
Q318X / E431K	SV	1
R356W / R356W	SW	1
E6 / E6	SW	1
Q318X / E6	SW	1
E431 / E351K	SV	1
Q318X/V281L	SV	1

severe enzymatic disruption in both sex leading to SW crisis shortly after birth. Females with classic form of CAH are prenatally exposed to excess androgen and born with external genitalia virilisation (17). Most of patients with SW form of CAH are unable to produce sufficient aldosterone to maintain a sodium balance and may cause fatal crisis if not treated (18). In males, signs of adrenal hyperandrogenemia are difficult to ascertain due to high secretion of testosterone by testis (16). It was described that males with SW CAH are more likely suffering from a delayed or incorrect diagnosis because there is no genital ambiguity to alert clinician. Therefore, the death rate due to an adrenal crisis in male fetal with SW form is higher than similarly affected females (19, 20). A newborn with positive test screening using 17-OHP progesterone measurements does not differentiate between SW and SV forms. Thus, a catalog of genotype-phenotype correlation as showed here would be useful for newborn screening programs. For special interest of physicians in performing prenatal diagnosis of CAH, fetal DNA could be obtained by amniocentesis or chorionic villus samples would be used for predicting phenotype. Due to high rate of consanguinity in our country, *CYP21A2* mutation analysis is suggested in different ethnic group. Results of this study will be useful for genetic counseling, newborn screening and carrier detection particularly in cases that both parents are heterozygote and want to know the risk of having a child with CAH.

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