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Mutation Analysis of the *CYP21A2* Gene in the Iranian Population

Bahareh Rabbani,[1–3] Nejat Mahdieh,[2,4] Mohammad Tahgi Haghi Ashtiani,[2,5] Bagher Larijani,[6] Mohammad Taghi Akbari,[3] Maria New,[7] Alan Parsa,[7] Jan P. Schouten,[8] and Ali Rabbani[1,2,5]

Background: Defects in the *CYP21A2* gene cause steroid 21-hydroxylase deficiency, which is the most frequent cause of congenital adrenal hyperplasia. Forty four affected families were investigated to identify the mutation spectrum of the *CYP21A2* gene. *Methods:* Families were subjected to clinical, biochemical, and molecular analyses. Allele-specific polymerase chain reaction amplification was used for eight common mutations followed by dosage analysis to exclude *CYP21A2* deletions. *Results:* The most frequent mutations detected were gene deletions and chimera (31.8%). Other mutation frequencies were as follows: Q318X, 15.9%; I2G, 14.8%; I172N, 5.8%; gene duplication, 5.7%; R356W, 8%; and E6 cluster mutations, 2.3%. Direct sequencing of the *CYP21A2* gene revealed R316X, P453S, c.484insT, and a change at the start codon. Different modules carried by patients were classified into five different haplotypes. The genotype phenotype correlation (positive predictive value) for group null, A, B, and C were 92.3%, 85.7%, 100%, and 0, respectively. *Conclusions:* Methods used will be helpful for carrier detection and antenatal diagnosis, especially with inclusion of the multiplex ligation probe dependent amplification technique, which is easier for routine tests in comparison with other methods. Mutation frequencies indicate that Iranians are possible descendants of Asians and Europeans.

Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders caused by inborn errors of steroidogenesis in the adrenal gland. Steroid 21-hydroxylase deficiency (21-OHD) is the most common cause of abnormal sex differentiation in girls and accounts for greater than 90%–95% of all CAH cases (New and Wilson, 1999). Rapid somatic growth, adrenal insufficiency, and precocious pseudopuberty are seen in both sexes in different degrees depending on the type of CAH present. The wide spectrum of clinical features is caused by various combinations of defective genotypes (Speiser *et al.*, 1992; Wilson *et al.*, 1995a). Steroidogenesis within the adrenal gland is regulated by adrenocorticotropic hormone (ACTH) from the pituitary, and its secretion is stimulated by corticotropin releasing hormone (Jameson and De Groot, 2010). Three main groups of 21-OHD exist: salt wasting (SW), simple virilizing (SV), and nonclassic (NC). In SW, the most severe form, patients suffer from SW crises soon after birth and have a defective synthesis of cortisol and aldosterone. Initiating treatment immediately after birth results in strongly reduced mortality and morbidity. The second group, SV, maintains aldosterone synthesis but lacks significant cortisol production. These two groups are known as the classic form of CAH and account for 1 in 10,000–15,000 births (Pang *et al.*, 1988; White and Speiser, 2000; Therrell, 2001). The final group, NC, is mildly affected with a late onset of the disease. Symptoms are mild and include excess androgen and infertility at adolescence (New, 2006).

The *CYP21A2* gene (MIM 201910, Gene ID 1589) has been mapped on chromosome 6p21.3 (Carroll *et al.*, 1985; Higashi *et al.*, 1986). This gene is located in an RCCX module consisting of *RP2* (serine threonin kinase), *C4B* (complementary factor), *CYP21A2* gene, and *TNXB* (cytoskeleton protein). Its pseudogene is located ~30 kb away from the *CYP21A2* gene in another RCCX module in tandem array of *RP1-C4A-CYP21A1P-TNXA* (Levine *et al.*, 1978). *RP2* and *TNXA* are truncated forms of *RP1* and *TNXB*. The *CYP21A2* gene, which consists of ten exons, is 98% similar to its pseudogene, *CYP21A1P*. This similarity leads to recombination events, accounting for 75% of the microconversion derived mutations. About 20%–25% of the recombinations are due to unequal crossing over, causing large gene conversions (GCs), duplications, and deletions (Werkmeister *et al.*, 1986). In fact,

[1]Growth and Development Research Center and [2]Pediatrics Center of Excellence, Tehran University of Medical Sciences, Tehran, Iran.
[3]Medical Genetic Group, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
[4]Medical Genetic Group, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran.
[5]Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran.
[6]Endocrine and Metabolic Research Center, Tehran University of Medical Sciences, Tehran, Iran.
[7]Department of Pediatrics, Mount Sinai School of Medicine, New York, New York.
[8]MRC-Holland, Amsterdam, The Netherlands.

unequal crossing over may generate duplication (Koppens *et al.*, 2002); there is variability of gene duplication frequency depending on the population ethnicity (Haglund-Stengler *et al.*, 1991; Wedell *et al.*, 1994; Lobato *et al.*, 1998; Parajes *et al.*, 2008). Rare mutations account for ~5% of the disorder and for mechanisms other than GC (White and Speiser, 2000).

To date, >100 different mutations have been described in the HGMD database (www.hgmd.cf.ac.uk) and consist of point mutations, small deletions or insertions, duplications, GCs, deletions, complex gene rearrangements, and splicing mutations. The molecular genetic basis of CAH has been completely investigated, and different mutation groups were categorized based on *in vitro* enzymatic activities of 21 hydroxylase to predict the related clinical features of the affected individuals. Phenotype predictions of intermediately affected patients, however, remains complicated (Wilson *et al.*, 1995a). Advances in molecular diagnosis of CAH are noteworthy. Previously used techniques for the analysis of the *CYP21A2* gene such as Southern blotting (Werkmeister *et al.*, 1986; Haglund-Stengler *et al.*, 1991) have been replaced by polymerase chain reaction (PCR)-based methods (Wilson *et al.*, 1995b; Lee *et al.*, 2000; Tukel *et al.*, 2003; Lee *et al.*, 2004).

Here, we elucidate the genetic lesions of *CYP21A2* gene in Iranian families. The prevalence of common mutations, deletions, GCs, chimeric genes (CH), duplications, and their genotype-phenotype correlation are discussed with regard to allele-specific PCR, sequencing, and multiplex ligation probe dependent amplification (MLPA). This study came about due to the high consanguineous marriage rate in Iran, and to provide antenatal diagnosis, carrier detection, and genetic counseling for afflicted families.

Materials and Methods

Patients

Forty-four patients affected with 21-OHD from 41 unrelated families and 4 healthy controls from the Children's Center Hospital, Pediatric Center of Excellence in Tehran, were included in the study. These families were counseled and agreed to mutation detection analysis. Informed consent was obtained from parents.

Clinical and biochemical evaluations were performed as indicated. Five microliters of blood was drawn. Mutations were confirmed from parents when available. Samples from normal siblings were obtained in three families. In total, 88 alleles were included.

Girls affected with CAH showed a wide spectrum of virilization from mild to severely virilized external genitalia (0–5 Prader score) (Prader and Gurtner, 1955; Moayeri and Rabbani, 1999; L'Allemand *et al.*, 2000; Razzaghi-Azar *et al.*, 2002). In addition, insufficiency of aldosterone, seen typically in the first 2 weeks of life, would show signs of SW with hyponatremia, hyperkalemia, diarrhea, vomiting, and shock. Affected boys revealed excess androgens and depending on the severity of the enzyme defect, displayed hyponatremia and hyperkalemia, which follow diarrhea, vomiting, gastroesophageal refluxes, and failure to thrive. Hyponatremia and hyperkalemia were observed in those who lacked aldosterone. Patients with severe deficiency of cortisol had increased ACTH with hyperpigmentation and virilization. NC or adult onset form was distinguished on the basis of previous reports (New, 2006).

Mutation analysis

For the purpose of mutation detection, genomic DNA was extracted from peripheral blood cells by using the salting-out method (Miller *et al.*, 1988). Allele-specific PCR amplification was performed for the detection of g.89C>T(p.P30L), g.656A/C>G(I2G), g.708_715delGAGACTAC(p.G110_Y112), g.1001T>A(p.I172N), E6 cluster(p.I236N, p.V237G, p.M239L), g.1685G>T(p.V281L), g.1996C>T(p.Q318X), and g.2110C>T(p.R356W) mutations (Wilson *et al.*, 1995b). Allele-specific primers and conditions used were previously described with some modifications (Wilson *et al.*, 1995b; Tukel *et al.*, 2003;). Modifications for higher specificity were as follows: primer Ex6ns:5′-TCA CATCGTGGAGATGCAGCT-3′, and primer Ex8-1ma:5′-TTC GTGGTCTAGCTCCTCCTA-3′. All the amplification reactions were performed on affected and healthy controls.

MLPA analysis

The concentration of the DNA samples was determined by NanoDrop Spectrophotometer 1000 (Thermo Fisher Scientific), and the DNA was diluted to 55–65 ng/μL before MLPA analysis. The SALSA MLPA Kit P050-B2 CAH (MRC-Holland) was used for *CYP21A2* gene dosage analysis comprising 16 reference probes, 13 specific probes, and 2 probes located elsewhere on 6p21.3. The specific probes detect sequences in, or near, exons 1, 3, 4, 6, and 8 of the *CYP21A2* gene; in, or near, exons 1, 2, and 10 of the *CYP21A1P* pseudogene; in exon 19 of *C4B*; in exons 1, 15, and 32 of the *TNXB* gene; and in the nearby *ATF6B* (*CREBL1*) gene. Included are also four small control fragments indicating the amount of sample DNA, three probes that give a warning for incomplete denaturation of the template, and X and Y-chromosome specific probes that provide differentiate between sexes.

The procedure was performed according to the manufacturer's protocol including DNA denaturation and hybridization of the probes, ligation reaction, PCR amplification, capillary electrophoresis, and, finally, data analysis. Briefly, 5 μL of the diluted DNA was denatured and hybridized to the MLPA probes overnight at 60°C. Ligase-65 enzyme was used at 54°C for 15 min for sample DNA directed probe ligation. Then, the PCR reaction was performed for 35 cycles. Amplicons were run on ABI 3010XL (Applied BioSystems). The appropriate size standard was used to assign the relative length of fragments. MLPA was performed using three positive controls with known dosage changes, three reference controls (two clinically healthy males and one female), and a negative control in each experiment.

Raw data were analyzed using GeneMarker Software V 1.85 (SoftGenetics). Population normalization of the peaks was done to outline the position of the expected alleles with the use of a defined panel containing all probes. On the basis of peak ratio (peak area of the sample/total peak area of all samples), the copy number was defined as 0 value indicating homozygote or no copy, <0.7 with one copy, 0.7<N<1.3 having two copies, 1.3<N<2 with three copies, and 2< indicating 4 copies. Since a normal individual has two copies of the *CYP21A2* gene, MLPA analysis with one copy or less identified from the peak ratio proves a deletion; thus, three and four copies establish duplication and triplication of the probes, respectively.

Haplotypes were defined by MLPA probes (Fig. 1). These probes lie out of coding regions of *CYP21A1P* and *CYP21A2*

FIG. 1. Position of multiplex ligation probe dependent amplification probes on CYP21A2 and CYP21A1P gene for indicating the haplotypes, gene duplications, gene deletions, and gene conversions. Probe 1 (1) is positioned at 5′CYP21A1P pseudogene at -316 to -264 of regulatory region; this position includes -306G>C, -295T>C, -294A>C, -283A>G, and -281T>G pseudo-derived mutations, which reduce the activity to 50% (Zhang *et al.*, 2009). Probe 2 (2) is located on the complementary sequence of exon 10 of CYP21A1P, which is the opposite strand comprising TNXA gene. Probe 3 (3) shows the C4B gene on exon 19. Probe 4 (4) depicted 18nt upstream of 5′untranslated region of CYP21A2 gene; this site is positioned in -147 to -90 of the regulatory region of CYP21A2, which includes a protein binding site. Variants of -126C>T, -113G>A, -110T>C, and -103A>G are positioned in this probe, which would influence transcriptional activity by fivefold lower than CYP21A2 gene.

genes. 5′*CYP21A2* probe contains a KpnI recognition site that is not included upstream of the *CYP21A1P* gene. The 5′*CYP21A1P* pseudogene probe is positioned at -316 to -264 of the promoter region. Regarding the 3′*CYP21A1P* probe, determination of chimera was confirmed (Fig. 1). Deletion of *CYP21A2* was confirmed by absence of 5′*CYP21A2* probe. Deletion of 30 Kb was confirmed by absence of 5′*CYP21A2*, C4B and 3′*CYP21A1P*. Chimera were determined by absence of 5′*CYP21A2*, 3′*CYP21A1P* probe and normal 5′*CYP21A1P* probe. Partial GC was indicated by the absence of 5′*CYP21A2* and an increase in 5′*CYP21A1P* and normal 3′*CYP21A1P*. Duplication of the *CYP21A2* gene was determined by an increase in the 5′*CYP21A2* gene and normal range of the 5′*CYP21A1P* and 3′*CYP21A1P* probes.

Direct sequencing

For detection of all other mutations, direct sequencing was performed using BigDye terminator cycling conditions (Macrogen) by sequencing analyzer ABI 3730XL (PE Applied BioSystems). The PCR products were purified by ethanol precipitation protocols. Primers used were P1, P2, P5, P6, P10, (Kharrat *et al.*, 2004) 3na, 4 ns, and 7na (Wilson *et al.*, 1995b). Combination of these primers covered the whole coding and intronic sequences. Nucleotide numbering was described as White 2000 (Higashi *et al.*, 1986; White and Speiser, 2000). Indication of mutations in patients was determined by using direct sequencing. All amino acids and proteins were named according to Human Gene Nomenclature guidelines (Wain *et al.*, 2002).

Results

Mutation analysis

Based on clinical data, 36 (81.8%), 8 (18.2%), and 0 (0%) of DNA samples were from SW, SV, and NC patients, respectively. In total, 88 alleles were investigated. In fact, 39 of 44 families (88%) were due to first-cousin marriages. Results indicated that gene deletion and CH comprising P30L, I2G, and G110Δ8nt were the most frequent mutations (31.82%). Q318X and I2G were also frequent in the population, 15.9%, and 14.8% of the mutations, respectively. The most frequent mutation of SV form was I172N (5.8%) (Table 1). Multiple mutations included two with the mutations of [P453S;R356W], one for [V281L;Q318X], [I2G;Q318X], [I2G;I172N], three of the duplicated alleles (Q318X/G110Δ;I2G, Q318X/Q318X;E6Cluster, Q318X/Q318X;I2G), and two alleles of E6 cluster (Table 2). Compound heterozygosity was observed in 21 individuals, and homozygosity was seen in 23 patients (52.3%).

Sequencing analysis revealed two rare mutations, P453S, and Q316X, in patients A26 and A55, respectively. An insertion of T, located at codon 484 in exon 10, (c.484insT) and a change in the start codon (ATG>GTG) was found in patient A4 (eletropherograms not shown) (Tables 1 and 2).

Gene dosage and MLPA analysis

Five different haplotypes were found on the basis of previously described haplotypes by Koppen *et al*. (1992) (Table 2, Fig. 2).

Deletions and chimera accounted for 13 and 15 alleles, respectively, including 27.3% SW and 4.5% of SV alleles. Altogether, gene deletions, CH, and duplications comprised 36.4% of all mutations. Four individuals carried duplication of either the *CYP21A2* or *CYP21A1P* genes. Three of them had Q318X mutation in one of the copies (Table 2).

Haplotype A included 58/88 (65.9%) of the alleles (Fig. 2a). 15/88 alleles included the haplotype D having two *CYP21A1P*. Most of the D haplotypes were chimera (Fig. 2d) except four alleles in patient A12, A22, and A25 (Fig. 2c). 11 of 88 alleles were haplotype E indicating gene deletion. Patient A45 had haplotype E carrying CH (Fig. 2f). Higher incidence of gene deletion/conversions was observed than gene duplication (haplotype F) (Fig. 2b) (Table 1). A high incidence of deletion might be due to the tendency for shorter fragments during meiosis, because longer fragments would be lost by looping out (Koppens *et al.*, 1992).

Genotype-phenotype correlation

Patients were grouped into mutation groups (Speiser *et al.*, 1992) to correlate their genotypes to the clinical diagnosis. Genotypes were categorized into 5 mutation groups (which could be used for analysis of the expected phenotype): null (no enzyme activity), A (SW, severely affected), B (SV, moderately affected with 2% enzymatic activity), C (NC, mildly affected with 20%–30% enzyme activity), and D (others) (Table 3). 21-OHD severity is related to enzymatic activity, and the less severely impaired allele will express the phenotype (White and Speiser, 2000). Therefore, patients with SW have severe/severe (gene deletion/conversion, Δ8bp, E6 cluster, Q318X, R356W) genotypes, patients with SV have moderate/moderate or moderate/severe (I2G homozygotes or heterozygotes with group A), and patients with NC have mild/mild, mild/severe, or mild/moderate (including P30L, V281L, and P453S homozygous or compound heterozygous with group A, and B mutations) genotypes.

In Table 3, the genotypes are arranged in groups from A–D. In the null group, all cases except two were confirmed salt wasters with positive predictive value (PPV) of 92.3%. In group A, the PPV was 85.7%. In group B, PPV was 100%, and in group C, no mild phenotype was observed.

Discussion

The frequency of *CYP21A2* mutations has been determined in the Iranian population but not without some disparities

Table 1. CYP21A2 Mutations Found in the Current Study on 21-Patients with Hydroxylase Deficiency Compared with Previous Studies in Iranian Population and the Clinical Features

Mutations (protein level)	*Exon/intron*	*Nucleotide variation*	*SW*	*SV*	*Nonclassic*	*Number of alleles*	*Frequency (%)*	*Enzyme activity (%)*	*Reported frequency (%) (White and Speiser 2000; Goncalves et al., 2007; Parajes et al., 2008)*	*Frequency of the previous studies in Iran (%)*	
										(Vakili et al., 2005)	*(Ramazani and others 2008)*
Deletions	All		11	2	0	13	14.77	0	20–45	25	ND
Gene conversions[a]	Exon 1, 2, 3	89C>T, 656A/C>G, Δ708–715	13	2	0	15	17.05	0		ND	ND
Duplications	CYP21A2/CYP21A1P gene	Vary	3	1	0	4	4.55	ND	1–7	ND	ND
P30L	Exon 1	89C>T	0	0	0	0	0	20–30	0–3	0	0
I2G	Intron 2	656A/C>G	11	2	0	13	14.77	0–1	12–56	15	28
G110Δ8nt	Exon 3	Δ708–715	0	0	0	0	0	0	0–5	10	13
I172N	Exon 4	1001T>A	1	4	0	5	5.68	1–5	6–29	11.7	9
V281L	Exon 7	1685G>T	0	1	0	1	1.14	30–50	0–17	5	3
Q318X	Exon 8	1996C>T	13	1	0	14	15.91	0	0–14	6.7	9
R356W	Exon 8	2110C>T	7	0	0	7	7.95	0	3–13	0	5
E6 Cluster	Exon 6		2	0	0	2	2.27	0	1–5	1.7	4
I236N		1382T>A									
V237E		1385T>A									
M239K		1391T>A									
Multiple mutations		Vary	4	1	0	5	5.68			ND	ND
R316X	Exon 8	946C>T	2	0	0	2	2.27	0		0	ND
c.484insT	Exon 10	GGtGG	1	0	0	1	1.14	0		0	ND
Start codon		ATG>GTG	1	0	0	1	1.14			0	ND
5′gene conversion of CYP21A2 gene		-306G>C, -295T>C, -294A>C, -283A>G, -281T>G (Zhang *et al.*, 2009)	5	0	0	5	5.68	20	ND	ND	ND
Total						88	100			100	100

[a]Large gene conversions would result in chimeric genes (CH-1) (Lee, 2004).
ND, not determined; SW, salt wasting; SV, simple virilizing.

Table 2. CYP21A2 Genotypes of Each Studied Individual with the Predicted Haplotypes

Number	*Paternal-Maternal allele*	*Sex*	*CYP21A1P copy number*	*CYP21A2 copy number*	*Clinical feature*	*Genotype*	*Predicated haplotype*[a]
A1	ND-Q318X[b]	M	3	1	SW	CH/Q318X	A/E
A2	CH-Del[b]	F	3	0	SW	CH/Del	D/E
A3	CH-Del[b]	F	3	0	SW	CH/Del	D/E
A4	ND-ND[b]	M	2	2	SW	ATG>GTG, 484insT)	A/A
A6	Q318X-Q318X	M	2	2	SW	Q318X/Q318X	A/A
A8	R356W-R356W[b]	F	2	2	SW	R356W/R356W	A/A
A9	Q318X-G110Δ;I2G[b,c]	F	3	2	SW	Q318X/G110Δ;I2G	A/C
A10	I2G-I2G[b]	F	2	2	SW	I2G/I2G	A/A
A12	Del-Del[b]	M	4	0	SW	Del/Del	D/D
A14a	Del-CH[b]	M	2	1	SW	Del/CH	A/E
A14b	Del-CH[b]	M	2	1	SW	Del/CH	A/E
A15	Q318X;E6 cluster-Q318X[b]	F	2	3	SW	Q318X;E6 Cluster/Q318X	F/A
A16	R356W-R356W[b]	F	2	2	SW	R356W/R356W	A/A
A18	CH-CH[b]	F	4	0	SW	CH/CH	D/D
A19	ND-ND[b]	F	2	2	SW	Regulatory mutation	A/A
A20	R356W-I2G;I172N	F	2	2	SW	R356W/I2G;I172N	A/A
A21	I2G-I2G;Q318X[b]	F	2	2	SW	Q318X;I2G/I2G	A/A
A22	Del-Del[b]	F	3	0	SV	Del/Del	D/E
A23	CH-Del[b]	F	3	0	SW	CH/Del	D/E
A24	N-N[b]	M	2	2	SW	R356W/R356W	A/A
A25	Del-Del[b]	M	3	0	SW	Del/Del	D/E
A26	R356W;P453S-R356W; P453S[b]	M	2	2	SW	R356W;P453S/R356W;P453S	A/A
A27	Del-Del	F	2	0	SW	Del/Del	E/E
A28	I2G-Q318X	M	2	2	SW	I2G/Q318X	A/A
A29	ND-ND[b]	F	3	2	SW	Regulatory mutation	A/A
A31	CH-CH[b]	M	4	0	SW	CH/CH	D/D
A32	I2G-I2G[b]	F	2	2	SW	I2G/I2G	A/A
A35	Q318X-Q318X[b]	F	2	2	SW	Q318X/Q318X	A/A
A36	V281L-Dup[b]	M	3	2	SV	V281L/DupCYP21P	A/C
A37	V281;Q318X-I172N	F	2	2	SV	V281L;Q318X/I172N	A/A
A39	Q318X-I172N[b]	M	2	2	SV	Q318X/I172N	A/A
A40	I2G-I2G[b]	F	2	2	SV	I2G/I2G	A/A
A42	ND-ND[b]	M	2	2	SW	I2G/I2G	A/A
A43a	I172N-I172N[b]	F	2	2	SV	I172N/I172N	A/A
A43b	I172N-I172N[b]	F	2	2	SV	I172N/I172N	A/A
A44	I2G-I2G[b]	M	2	2	SW	I2G/I2G	A/A
A45	CH-CH[b]	F	3	0	SV	CH/CH	D/E
A48	E6Cluster-E6Cluster[b]	F	2	2	SW	E6Cluster/E6Cluster	A/A
A49	Q318X-Q318X;I2G[b]	F	2	3	SW	Q318X/Q318X;I2G	A/F
A53	Q318X-Q318X[b]	F	2	2	SW	Q318X/Q318X	A/A
A54	CH-Q318X[b]	F	3	1	SW	Q318X/CH	A/D
A55	R316X-R316X[b]	M	2	2	SW	R316X/R316X	A/A
A56	I2G-Q318X[b]	F	2	2	SW	Q318X/I2G	A/A
A57	CH-CH[b]	F	4	0	SW	CH/CH	D/D

[a]Alternative haplotype would be possible for some cases.
[b]Consanguineous marriages (first cousins).
[c]Reference (Lee *et al.*, 2003).
F, female; M, male; ND, not determined; Del, deletion; dup, duplication.

from other reports that may be due to restricted sampling (Vakili *et al.*, 2005) or the gene dosage analysis was not performed for the individuals (Ramazani *et al.*, 2008). In this study, the frequency of mutations in 44 new patients was investigated in the Iranian population. Our results allowed for a reliable mutation analysis within the country as compared with other reports (White and Speiser, 2000). In this regard, the frequency of mutations detected in this study fall into the midrange of previously published studies (Table 1). HLA typing of Iranian 21-OHD patients has been previously performed, which shows association with HLA-B18 and HLA-B21 (Haghi Ashtiani *et al.*, 2008). Gene deletions and chimeric genes encompass 31.8% of all gene mutations. The I2G has been mentioned as the most frequent mutation in western European cohorts (White and Speiser, 2000); but with an exception, the Q318X mutation was frequent in our study, which corresponds to Tunisia (Kharrat *et al.*, 2004) and confirms the previous study in Iran (Vakili *et al.*, 2005). By far, the I172N mutation is associated with the SV form. V281L frequency was low, which might be due to an inadequate inclusion of the NC form in the study, that is, NCs may present later in life (our patients were pediatrics) (New, 2006) and are,

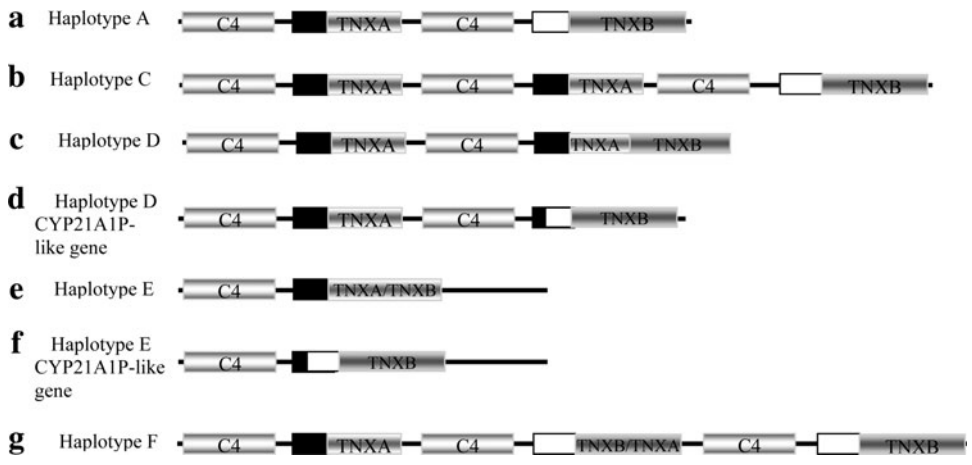

FIG. 2. A scheme representing different haplotypes found in the present study. The black box indicates *CYP21A1P* pseudogene, and the white box shows *CYP21A2* gene. **(a)** haplotype A: A bimodule form of RCCX comprising a *CYP21A1P* and a *CYP21A2* gene, **(b)** haplotype C: a trimodule with two *CYP21A1P* pseudogene formed from unequal crossing over events of C4 gene **(c)** haplotype D: complete *CYP21A2* gene conversion to *CYP21A1P*, **(d)** haplotype D *CYP21A1P*-like gene: partial gene conversion of *CYP21A2* to *CYP21A1P*, **(e)** haplotype E: a *CYP21A1P* gene due to deletion of *TNXA-RP2-C4B-CYP21A2-TNXB*, **(f)** haplotype E indicated as *CYP21A1P*-like gene: *CYP21A1P-CYP21A2* chimers resulting from gene deletions of *CYP21A1P-TNXA-RP2-C4B-CYP21A2*, **(g)** haplotype F: a trimodule with two *CYP21A2* genes resulting from *TNXA-TNXB* chimer.

therefore, likely missed in childhood. The chimeric form of the *CYP21A1P-CYP21A2* gene included only exon 1 through exon 3. No other form of chimera was found in this study compared with other reports (Lee, 2004). The R356W mutation was also somewhat high in our population (~8%) in comparison with other populations (2%–4%). Gene duplication was responsible for about 5% (4/88 allele) of the mutations, which could also be considered common in comparison with other mutations. This kind of gene lesion was a higher frequency than in the Netherlands (Koppens *et al.*, 2002) and was similar to values published in studies from Spain (Parajes *et al.*, 2008). A higher duplicated version of the pseudogene (only upstream of the *CYP21A1P* was available) as compared with the *CYP21A2* gene was observed in our study, similar to a study by Lobato *et al.* (1998). Some reports have not shown any duplication in the Chinese population (Lee *et al.*, 2004). This demonstrates that our population might somehow be a bridge between Europeans and Asians. This highlights the importance of Iran's position on the Silk Road for migration between Europeans and Asians as it has been described for other genes and mutations such as 35delG mutation in the *GJB2* gene, (Mahdieh and Rabbani, 2009), though it should be noted that the R316X found in the Chinese population was detected in our population (Lee *et al.*, 1998). R316X produces a nonsense mutation that leads to the SW form and loss of heme binding domain. Therefore, gene dosage analysis should also be performed to determine the increase of the *CYP21A2* or *CYP21A1P* gene for the purpose of antenatal diagnosis and genetic counseling.

Two new mutations were found for which no expression study was performed, but they were evaluated *in silico* (Desmet *et al.*, 2009). One was a change in the start codon. The other mutation was an insertion in exon 10 that could result in a longer protein at the C terminus. An ATG>GTG in start codon was discerned with another cryptic ATG as the start codon producing a shorter protein with no exon 1. Also, in the same patient, a T insertion at codon 484 caused a frameshift in the C terminus of the 21 hydroxylase protein.

Based on molecular methods used in this study and with the use of MLPA analysis, a rapid and reliable strategy for dose-dependent identification of *CYP21A1P* and *CYP21A2* genes was developed with a diagnostic sensitivity of 100%. Gene duplication was more easily evaluated using this procedure compared with real-time PCR and long-range PCR or Southern blot analysis. However, some modifications in MLPA probes would improve the haplotype analysis of the results. Dosage analysis can help quantify the pseudogene/gene. In addition, quantification distinguishes between chimers and *CYP21A2* deletions (Fig. 2c in comparison with d). Overall, the MLPA assay is a powerful tool for analyzing gene copy number and deletion based on a comparative quantification of hybridized probes. It is a quantitative not a qualitative detection analysis. Therefore, a defective *CYP21A2* in a duplicated *CYP21A2* appearing in the trimodule may not exactly distinguish whether the defect was downstream of *TNXA* gene or downstream of *TNXB* gene. Further, the exact location of the mutation in the related gene could not be identified.

A chimeric *CYP21A1P/CYP21A2* gene has a fivefold lower enzymatic activity in the *CYP21A2* gene due to changes in the promoter region in binding to Sp1, which makes a difference from gene deletions (Lee, 2004). In addition, MLPA revealed a 5'*CYP21A1P* regulatory region before a normal *CYP21A2* gene, which expresses the normal allele insufficiently as in A53 (haplotype A/A); also, a single mutation (Q318X) in the allele with the normal regulatory region may produce an impaired protein, thus leading to impaired enzyme activity. The R356W/R356W genotype in patient A24 may be due to *de novo* mutations; parental testing of the alleles could be validated by STR analysis (Rabbani *et al.*, 2008).

Complete sequencing of the regulatory regions of the *CYP21A2* gene were not carried out in this study. Therefore, six individuals were excluded from the study, even though complete exonic and intronic sequencing had been performed for these individuals. Further regulatory changes far upstream (-2574 to −2489) of the regulatory regions located in intron 35 *C4B* could be responsible for the misinterpretation (Wijesuriya *et al.*, 1999) that should be considered for additional analyses; despite the fact that MLPA probes detected regulatory changes in patient A19 and A29.

Table 3. Genotype Grouping of Mutations for Phenotype Predication of the Patients Compared with the Clinical Observations

Mutation group	Genotype	Number of affected	Clinical observations		
			SW	SV	NC
Null	CH/Del	5	5		
	CH/CH	4	3	1	
	CH/Q318X	2	2		
	Del/Del	4	3	1	
	Del/G110Δ;I2G	1	1		
	R356W/R356W	3	3		
	R356W/I2G;I172N	1	1		
	Q318X/Q318X	2	2		
	Duplication:				
	Q318X/G110Δ;I2G	1	1		
	Q318X/Q318X; E6Cluster	1	1		
	Q318X/Q318X;I2G	1	1		
	E6Cluster/E6Cluster	1	1		
A					
	I2G/Q318X	1	1		
	I2G/I2G	5	4	1	
	I2G/I2G;Q318X	1	1		
B					
	I172N/Q318X	1		1	
	I172N/V281L;Q318X	1		1	
	I172N/I172N	2		2	
C					
	V281L/DupCYP21A1P	1		1	
D (others)					
	Regulatory mutation	3	3		
	R316X/R316X	1	1		
	ATG codon/484insT	1	1		
	R356W;P453S/R356W; P453S	1	1		

NC, nonclassic.

In this study, haplotype analysis of each individual would provide information about *CYP21A2* configurations. Although *C4* genes could not be distinguished for each subdivision, they could reveal different rearrangements of alleles. We mentioned five different haplotypes that have been previously described, but there are difficulties in predicting the exact haplotype, as other patterns could also be predicated, for example, A/C and D/F. Family studies reduced this problem, though there maybe some bias. Similar to other reports, the frequency of bimodule (>50%) was higher in our study, similar to other reports. Trimodule and monomodule also existed in our study population.

Genotype phenotype correlation was performed in five groups (Table 3). PPVs set forth high degrees of concordance in null, A, and B groups. Patients with several mutations presented the clinical phenotype of the least severe mutated allele (White and Speiser, 2000), which is not the case for multiple mutations on one allele. Two patients with severe mutation in the null group displayed SV form of CAH. Del/del and del/CH mutation led to unusual phenotypes as previously reported by L'Allemand *et al.* (2000). This may be due to microconversions of the *CYP21A1P* regulatory region, which may cause lower transcriptional activity to *CYP21A2*. In group A, one patient (A40) had SV form instead of SW form. I2G/I2G is predicated to present SW form, but a girl with SV showed a no-salt losing crisis. The MLPA results indicated one copy of the regulatory 5'*CYP21A1P* that may cause a leaky transcription of the mutated alleles. In Group C, a boy heterozygous for V281L mutation was expected to explain the NC form (mild form), but this individual (A36) displayed SV form of the disease; this might be due to the regulatory region of the *CYP21A1P*, which may influence the phenotypic expression; his parents were also carriers of the V281L mutation. In group D, regulatory mutations were determined by MLPA probes and were not confirmed by other methods in patient A19 and A29 who disclosed the SW form of the disease. Although it is mentioned that these mutations would cause the SV or NC form, it is not known whether they could be related to the classic form of the disease (Zhang *et al.*, 2009). Microconversion in the regulatory region occurs in steroidogenic cells, which could change the protein binding sites (Sp1, SF1, nuclear factor NF-GM, adrenal-specific protein, Nur77) (Chang and Chung, 1995; Kyllo *et al.*, 1995). In addition, the severe phenotype (SW form) may be due to mutations in highly methylated CpG dinucleotides in the *CYP21A2* gene, possibly leading to a reduction of enzyme activity (Jiddou *et al.*, 1999; Zhang *et al.*, 2009).

Phenotypic variability may be due to alternative splicing mutations, presence of additional mutations, the influence of other loci in steroidogenesis or their mutations in phenotypic variations, receptors, and their polymorphisms and modifier genes (Wilson *et al.*, 1995a). Therefore, *in vitro* expression analysis may not always predict the *in vivo* effects (Krone *et al.*, 2000).

The prevalence of CAH has not been epidemiologically determined throughout the country, but studies from different parts of Iran have shown that there is a higher rate in children born from consanguineous marriages (Mokhtari and Bagga, 2003; Saadat *et al.*, 2004; Fathzadeh *et al.*, 2008; Akrami *et al.*, 2009) and our results fit in this range. However, the prevalence of the classic form of the disease is higher than other countries, estimated to be 1 in 6000–7000 individuals (Moayeri and Rabbani, 1999; Razzaghi-Azar *et al.*, 2002). The prevalence of heterozygotes and NC form should also be evaluated.

In conclusion, due to the need for newborn screening and carrier detection, genotype analysis should be done throughout the country. Prenatal diagnosis is also needed to reduce the psycho-economical problems that these patients and their families are dealing with.

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Address correspondence to:
Ali Rabbani, M.D.
Growth and Development Research Center
Tehran University of Medical Sciences
Gharib St.
Tehran 14155-6386
Iran

E-mail: rabania@tums.ac.ir;
baharehrabbani@yahoo.com