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A preliminary mutation analysis of phenylketonuria in southwest Iran

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ABSTRACT. Phenylketonuria (PKU) is a heterogeneous and autosomal recessive metabolic disorder that is mainly caused by mutations in the hepatic phenylalanine hydroxylase (*PAH*) gene. This study was designed to identify *PAH* mutations within exons 6, 7, and 10-12 in PKU patients from southwest Iran. Forty Iranian patients with clinical and biochemically confirmed PKU were enrolled. The exons were sequenced directly and 13 different mutations were identified including I224T, S231P, R176X, c.592_613del22, R243X, R252W, R261Q, Y356X, V388M, IVS10-11G>A, IVS11+1G>C, IVS11-2A>G, and Q375R, which were associated with 23 genotypes. A novel sequence variant, Q375R (c.1124A>G), was detected in exon 11. In one patient, a typical genotype with more than two mutations (R243X/S231P/S231P) was found. Seven different polymorphisms and three new variants were also detected in intron regions of *PAH*. A high mutation spectrum was predicted in the southwestern region of Iran due to its ethnic heterogeneity, especially the Khuzestan Province. The detection

of 13 different mutations, corresponding to a mutation detection rate of 53.75%, confirmed this phenomenon.

Key words: Phenylketonuria; Phenylalanine hydroxylase; Sequencing; Mutation analysis; Southwest Iran

INTRODUCTION

Deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1) results in phenylketonuria (PKU; OMIM 261600), one of the most prevalent inborn errors of metabolism. In 98% of PKU patients, defects of the PAH enzyme are due to mutations in the *PAH* gene on chromosome 12q23.2. In 1-2% of PKU patients, mutations occur in the genes that encode enzymes for biosynthesis or regeneration of tetrahydrobiopterin (BH4), an obligatory cofactor required for the activity of PAH (Donlon et al., 2008). Blockages in the major pathway of phenylalanine (Phe) metabolism, which involves the irreversible hydroxylation of Phe to tyrosine, result in an increase of Phe in blood and other body fluids, and produces a spectrum of disorders including classical PKU, mild PKU, and mild hyperphenylalaninemia (HPA) (Williams et al., 2008). The worst consequence of increased serum Phe concentration is severe mental retardation that could be prevented by early diagnosis and implementation of a low-phenylalanine diet (Janzen and Nguyen, 2010).

Mutation analysis of PKU has proven to be clinically advantageous. PKU is a heterogeneous metabolic disorder at both the genetic and clinical levels. To date, more than 600 mutations in *PAH* have been identified and have been recorded in the *PAH* and HGMD databases (www.pahdb.mcgill.ca and www.hgmd.org). The prevalence of PKU is roughly 1 in 15,000 individuals, but differs among different populations (Mitchell et al., 2011). In Iran, due to the absence of broad newborn screening programs, there is no precise data of PKU incidence; however, based on the latest report, the incidence of PKU might be approximately 1.6 in 10,000 (Habib et al., 2010).

MATERIAL AND METHODS

Patients

A total of 40 unrelated HPA patients (26 males, 14 females) from southwest Iran were enrolled in the present study. In families with more than one patient, only one member of each sib-pair was enrolled in this study. The age range of patients was 1-28 years.The initial diagnosis of HPA was based on clinical phenotype followed by quantitative analysis of serum amino acids using high-performance liquid chromatography.

The study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences. Based on pre-treatment Phe concentrations, patients were divided into three groups: classical PKU, mild PKU, and mild HPA. The serum Phe level of patients ranged from 179 to 2700 μM (normal range ≈30-85 μM). After obtaining informed written consent from parents, blood samples of 2.5-3.0 mL were collected from each patient at the laboratory unit of Abuzar Children's Hospital, which is associated with the Jundishapur University of Medical Sciences.

Genomic DNA extraction

Sufficient DNA was isolated from total blood using the Diatom DNA Prep 100 Kit. The extraction procedure of this kit is based on the salting-out method.

Primers and polymerase chain reaction (PCR)

Patients were screened for the presence of *PAH* mutations within exons 6, 7, and 10-12, as well as adjacent flanking regions critical to mRNA processing. We selected these exons based on available data about mutation frequencies among regions, which were obtained from the *PAH* database. Primer pairs for amplification of these exons were designed manually (Table 1). PCRs were performed in a 30-µL total volume containing 80-130 ng genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2-0.6 µM of each primer, and 3 U *Super Taq* DNA polymerase (Gen Fanavaran, Tehran, Iran). PCRs were subjected to thermocycling as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 45 s, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

Table 1. Studied exons, primers, annealing temperatures, and amplified fragment length.

Exon	Primer	Annealing temperature (°C)	PCR product length (bp)
6	F: 5'-GTGATGGCAGCTCACAGGTTCTGG-3' R: 5'-CAGGTACACGGCAAAATCCACAGC-3'	64	550
7	F: 5'-TGCCTAGCGTCAAAGCCTATGTCC-3' R: 5'-CTGTGGACCAGCCAGCAATGAACC-3'	66	358
10-11	F: 5'-AGGTATCCCTTCATCCAGTCAAGG-3' R: 5'-GATGAGTGGCACCAGTCAGGAGG-3'	63	927
12	F: 5'-CTGCTCTAGGGAGGTGTCCGTG-3' R: 5'-GAGGTGGAGTGGAATCTAGGAAGG-3'	64	459

DNA sequencing

The PCR products were purified, and automated DNA sequencing was carried out in the Applied Biosystems 3730 DNA Analyzer (Macrogen, Seoul, Korea). Sequence analyses were performed using Chromas and mutation surveyor softwares. The National Center for Biotechnology Information (NCBI) BLAST tool was also used for sequence alignment.

RESULTS

Phenotypic classification of patients

Among the 40 patients, 18 were classified as classical PKU, 14 were classified as mild PKU, and five were classified as mild HPA. The pre-treatment Phe levels of three patients were not available.

PAH mutations and polymorphisms

Direct sequencing of five exons and the related exon-intron boundaries of *PAH* in 40 patients enabled the characterization of 53.75% of the studied alleles. A total of 13 different mutations were identified, including six missense mutations, three nonsense mutations, three splice site mutations, and one deletion (Table 2). In one patient, we detected a typical genotype

with more than two mutations (R243X/S231P/S231P). The splice site mutation IVS10-11G>A had the highest relative frequency at 10%. Ten mutations, I224T, R176X, c.592_613del22, R243X, R252W, R261Q, IVS11+1G>C, IVS11-2A>G, V388M, and Q375R, accounted for 48.86% of mutant alleles, and three mutations, IVS10-11G>A, Y356X, and S231P, accounted for 51.16% of mutant alleles. These results demonstrate the high heterogeneity of PKU in this population. Three silent mutations, Q232Q, V245V, and L385L, were detected with high frequency in exons 6, 7, and 11, respectively. We also detected seven polymorphisms, of which three were exonic silent mutations with relatively high frequency (Table 3).

Table 2. Detected mutations from the studied sample.

Mutation		Location	Mutation type	Number of alleles	Relative frequency (%)
DNA level	Protein level				
c.1066-11G>A	IVS10-11G>A	Intron 10	Splice	8	10.0
c.1068C>A	Y356X	Exon 11	Nonsense	7	8.75
c.691T>C	S231P	Exon 6	Missense	7	8.75
c.727C>T	R243X	Exon 7	Nonsense	5	6.25
c.526C>T	R176X	Exon 6	Nonsense	2	2.50
c.672T>C	I224T	Exon 6	Missense	2	2.50
c.592_613del22	p.Y198_E205>Sfs	Exon 6	Deletion	2	2.50
c.754C>T	R252W	Exon 7	Missense	2	2.50
c.782G>A	R261Q	Exon 7	Missense	2	2.50
c.1199+1G>C	IVS11+1G>C	Intron 11	Splice	2	2.50
c.1200-2A>G	IVS11-2A>G	Intron 11	Splice	2	2.50
c.1124A>G	Q375R*	Exon 11	Missense	1	1.25
c.1162G>A	V388M	Exon 11	Missense	1	1.25
Total				43	53.75

*Novel mutation.

Table 3. Polymorphisms found in the *PAH* gene of 40 phenylketonuria patients from southwest Iran.

Polymorphism	Location	Frequency (%)
L385L	Exon 11	56.25
Q232Q	Exon 6	36.25
IVS5-54G>A	Intron 5	32.50
IVS10-236C>T	Intron 10	23.75
V245V	Exon 11	22.50
IVS10+97G>A	Intron 10	16.25
IVS10-193G>C	Intron 10	15.00
IVS10+205A>T	Intron 10	15.00
IVS10+155T>G	Intron 10	13.75
IVS10 +156T>G	Intron 10	13.75

Novel sequence variants

In exon 11, we identified the alteration c.1124A>G (Q375R) as a new variant (Table 2). The new variants IVS10+32(+A), IVS12+163(-C), and IVS13+30C>T were detected in introns 10, 12, and 13, respectively (Table 4).

Table 4. New intronic variants identified in this study.

Variant	Location	Frequency (%)
IVS10+32(+A)	Intron 10	2.50
IVS12+163(-C)	Intron 12	1.25
IVS13+30C>T	Intron 13	1.25

Genotyping

Genotyping was carried out in 23 of the 40 HPA patients: 16 were homozygous, 4 were heterozygous, and 3 were compound heterozygotes (Table 5).

Table 5. Genotypes of 23 phenylketonuria (PKU) patients.

Patient	Genotype		Polymorphism	Pre-treatment Phe levels (μM)
	Allele 1	Allele 2		
2	IVS11+1G>C	IVS11+1G>C	Q232Q	1560
3	R252W	R252W		1380
5	Y356X	Y356X	Q232Q	1200
8	I224T	I224T	Q232Q	1440
9	IVS10-11G>A	IVS10-11G>A	IVS10+155T>C IVS10+156T>G IVS10-193G>C L385L	1700
11	IVS10-11G>A	-	Q232Q/- V245V/- IVS10+97G>A/- IVS10+155T>C/- IVS10 +156T>G/- IVS10-193G>C/- L385L	2700
12	R176X	R176X	IVS5-54G>A	1440
13	Y356X	Y356X	Q232Q	1320
14	IVS11-2A>G	IVS11-2A>G	Q232Q	1620
16	IVS10-11G>A	IVS10-11G>A	IVS10+15T>C IVS10 +156T>G IVS10-193G>C L385	2034
19	R243X	R243X	V245V	1440
20	R243X /S231P	S231P	IVS5-54G>A V245V/-	2040
21[a]	S231P	S231P	IVS5-54G>A	930
27	R243X	-	V245V IVS10+32(+A) IVS10+97G>A IVS10+205A>T L385L	1600
28	S231P	S231P	IVS5-54G>A Q232Q	Unknown
33[a]	R261Q	R261Q		1186
34[a]	S231P	Y356X	IVS5-54G>A/- Q232Q/- IVS10-236C>T/- L385L/-	984
35	p.Y198_E205>Sfs	p.Y198_E205>Sfs		1990
36[a]	Q375R	-	IVS10+155T>C/- IVS10 +156T>G/- IVS10-193G>C/- L385L/-	608
37	R243X	-	V245V IVS10+97G>A IVS10+205A>T L385L	1560
38	IVS10-11G>A	IVS10-11G>A	IVS10+155T>C IVS10 +156T>G IVS10-193G>C L385L	1850
39[a]	Y356X	Y356X		1063
40[a]	IVS10-11G>A	V388M	IVS10+155T>C/- IVS10 +156T>G/- IVS10-193G>C L385L/- IVS13+30C>T/-	1087

[a]Mild PKU patients, other patients are classical PKU; (-) = another allele unknown; Phe = phenylalanine.

DISCUSSION

Early diagnosis via neonatal screening and initiation of treatment has prevented nearly all complications of PKU disease. Therefore, PKU represents the epitome of human biochemical genetics for the paradigm of a treatable genetic disorder (Scriver and Waters, 1999; Dobrowolski et al., 2009; Antshel, 2010; Harding and Blau, 2010). *PAH* mutation analysis has utility in evaluating the clinical phenotype of PKU, for genetic consultation of patients' families, prenatal diagnosis, carrier detection, and also in refining diagnoses and anticipating dietary requirements (Guldberg et al., 1998; Güttler and Guldberg, 2000; Dobrowolski et al., 2007, 2009). Many BH4-responsive mutations have been identified in various studies, and it has been estimated that more than 30% of all HPA patients respond to BH4, thus revealing that identification of *PAH* mutations could be useful for BH4-based therapies (Zurflüh et al., 2008; Staudigl et al., 2011).

In the present study, we described the molecular basis of PKU in a population from the southwest of Iran by analyzing mutations in *PAH*, and evaluating correlations between genotype and phenotype. The lack of standardized methods for the classification of HPA phenotypes can complicate the interpretation of genotype-phenotype correlations (Daniele et al., 2009). In this study, we used pre-treatment plasma Phe levels for phenotypic classification.

Because patients enrolled in this study were of different ages at diagnosis and had different feeding habits and diets, a wide range of Phe levels was observed. Nonetheless, the vast majority of patients showed high pre-treatment Phe levels, as well as severe clinical manifestations. This finding could be due to the lack of systematic and comprehensive neonatal screening of PKU in Iran, and consequently the late diagnosis of PKU. Furthermore, insufficient knowledge of parents about the importance of dietary management programs likely contributed to these effects.

Among the 40 patients analyzed, 13 distinct mutations were found in combinations of 23 genotypes. In this study, the mutations IVS10-11G>A, Y356X, and S231P were found in both classical and mild PKU cases. The patient with the homozygous R261Q mutation suffered from mild PKU. In spite of diagnosis at 5 years old, this patient did not present general consequences of late diagnosis, such as severe mental retardation, and the IQ was estimated to be 85. The sibling of this patient showed the same condition, although he was diagnosed earlier, at 3 years old. This finding shows that PKU is a heterogeneous disorder at both the genotype and phenotype levels. In this study, the genotypes S231P/S231P, R261Q/R261Q, Y356X/Y356X, S231P/Y356X, R243X/-, Q375R/-, and V388M/IVS10-11G>A were each identified in patients with mild PKU. The remaining 15 genotypes appeared in classical PKU cases.

The splice mutation IVS10-11G>A was detected with the highest frequency (10%) in three homozygous patients and in one compound heterozygous patient along with V388M. A G to A transition at position 546 in intron 10 of the *PAH* gene, located 11 bp upstream from the intron 10/exon 11 boundary, caused the IVS10-11G>A mutation. This mutation activates an alternative splice site and results in an in-frame insertion of nine nucleotides between exon 10 and exon 11 of the processed mRNA. Although the liver PAH protein content is normal in homozygous patients, no catalytic activity can be detected. This loss of enzymatic function is most likely caused by conformational changes due to the presence of three additional amino acids (Gly-Leu-Gln) between the normal sequences encoded by exon 10 and exon 11 (Dworniczak et al., 1991). This mutation has been identified as the most common mutation in Mediterranean populations such as Turkey (Dobrowolski et al., 2011), Italy (Daniele et al.,

2007), Spain (Desviat et al., 1999), Egypt (Effat et al., 1999), and Israel (Bercovich et al., 2008). In previous studies that have been performed in Iran, IVS10-11G>A was also identified with the highest frequency (Bonyadi et al., 2010; Zare-Karizi et al., 2011).

The Y356X and S231P mutations showed similar frequencies of 8.75%. Y356X was first reported in China (Wang et al., 1992), and is relatively common in East Asian countries including China, Japan, and Korea (Zhu, 2010; Okano et al., 2011). Based on phylogenetic studies, S231P is most similar to c.592_613del22, R261Q, and IVS10-11G>A, which are all common Mediterranean mutations (Effat et al., 1999). The mutations I224T, R176X, c.592_613del22, R243X, R252W, R261Q, IVS11+1G>C, and IVS11-2A>G were equally frequent (2.5%) in the present study. IVS11-2A>G was first identified in a Turkish population (Dobrowolski et al., 2011), and the present study represents the second report of this mutation. The two missense mutations, V388M and Q375R, were each detected in one allele. Kinetic studies of the mutant protein with the V388M mutation showed reduced affinity of the enzyme for L-Phe and tetrahydrobiopterin (Leandro et al., 2000). This mutation is relatively common in Brazil, Portugal, and Spain (Santos et al., 2006). The Q375R mutation is a new sequence variant that is reported in the present study for the first time, and was entered into the *PAH* and HGMD databases. However, phylogenetic comparison revealed the conserved position of the 375-PAH residue (Protein knowledgebase: http://www.uniprot.org/; Table 6). Therefore, population and *in vitro* expression analyses are necessary to reveal the pathological effect of this mutation. These procedures should also be performed for the three new variants detected in intronic regions, because these variants might also have pathogenic effects.

Table 6. Conserved position of the 375-PAH residue.

Organism/amino acid residue	372	373	374	375	376	377	378
Human	T	A	I	Q	N	Y	T
Rat	I	A	C	Q	E	Y	S
Mouse	T	A	C	Q	E	Y	T
Caenorhabditis elegans	C	C	V	T	K	Y	P
Drosophyla melanogaster	T	A	I	Q	N	Y	T

PAH = phenylalanine hydroxylase. Obtained from Protein knowledgebase [http://www.uniprot.org/].

To date, more than 30 different mutations have been detected in patients with PKU living in Iran (Bonyadi et al., 2010; Zare-Karizi et al., 2011). This study is the first to report five other mutations, Y356X, S231P, I224T, IVS11-2A>G, and V388M, in an Iranian population. Southwestern Iran, especially the Khuzestan Province, is comprised of heterogeneous ethnic groups; therefore, diversity in the mutation spectrum was expected. In summary, we sequenced five exons of the *PAH* gene in 40 PKU-affected families in southwestern Iran, and identified 13 different mutations, including one novel variant. Although the Iran mutation profile of *PAH* is similar to those of Mediterranean populations, there are several different characteristic features. The genotype-phenotype relationship, based on pre-treatment Phe levels, was also described. One notable feature of this population is its high rate of consanguineous marriage (77.5%). With a mutation detection rate of 53.75%, approximately 69.6% of patients were homozygous, 17.40% were heterozygous, and 13% were compound heterozygotes. The high homozygosity rate indicates a high rate of consanguinity in the studied cohort. Finally, the results of the present study could be advantageous for the diagnosis, genetic counseling, and planning of dietary-based treatment and other therapeutic strategies for PKU patients.

Conflicts of interest

The authors declare no conflict of interest.

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