Mutation analysis of Phenylalanine hydroxylase gene in Iranian patients with Phenylketonuria

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Abstract

Background: Phenylketonuria as the most common genetic metabolic disorder is the result of disruption of the phenylalanine hydroxylase gene. This study was carried out to explore the phenylalanine hydroxylase gene mutation status of Iranian phenylketonuria patients.

Methods: Blood samples were collected from 30 patients, and hot spot areas of the phenylalanine hydroxylase gene, including exons 6, 7, 8, 11, and 12 were studied through polymerase chain reaction and sequencing techniques.

Results: Eight different mutations, including 5 missense mutations, 1 splice mutation, 1 nonsense mutation, and 1 Silent/Splice mutation were detected. These mutations were R243X, R261Q, R261X, P281L, R241C, V399V, E280K, and IVS11+1G>C. V399V and R241C were reported for the first time in Iranian population. Three polymorphisms including Q232Q, V245V and L385L and 3 novel intronic variants including IVS10-15A>C, IVS6+44T>G, and IVS6+36 T>G were also detected in this study.

Conclusion: The results of this study prove the heterogeneous status of phenylalanine hydroxylase gene mutations in the Iranian population, which can be useful in carrier testing and genetic counseling.

Keywords: Phenylketonuria, Phenylalanine hydroxylase gene, Sequencing, Mutation, Iran

Introduction

Phenylketonuria (PKU, OMIM 261600), a common metabolic genetic disorder, is identified by the lack of phenylalanine hydroxylase (PAH) enzyme (EC 1.14.16.1) activity (1). Phenylalanine (Phe) conversion to tyrosine by this enzyme is the main pathway for Phe metabolism, and this activity is dependent on tetrahydrobiopterin (BH4) presence as a cofactor (2). If this disease cannot be diagnosed in the first days after birth, it will contribute to the accumulation of phenylalanine in the patient's body fluids with side effects such as failure in growth and brain damages (3). Newborn screening programs for PKU diagnosis are important postnatal requirement tests in many countries. The Guthrie test is the most applicable method for this purpose and has been used for more than 40 years (4). Based on blood Phe concentrations, PKU can be classified to classic PKU, mild PKU and mild hyperphenylalaninemia (HPA) (5).

The human *PAH* gene (GenBank: AF404777) with 13 exons is located on chromosome 12q22-q24.1, spanning about 90 kb, which encodes a polypeptide with 452 amino acids (6). The broad spectrum of mutations in *PAH* gene is the main etiology of this disease, and more than 800 different mutations are registered on the *PAH* mutation database; most of them are missense.

These mutations can make the enzyme structure and activity dysregulated which leads to variable phenotypes

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↑What is "already known" in this topic:
Phenylketonuria is one of the most prevalent metabolic genetic disorders. Mutations in phenylalanine hydroxylase gene cause this disease. More than 800 different mutations are registered on the *PAH* mutation database.

→What this article adds:
Mutation analysis of *PAH* gene study (exons 6, 7, 8, 11 and 12) in Iranian patients with PKU was screened. These mutations showed regional variations useful in genetic counselling and prenatal testing in different ethnic groups. We found three novel intronic variants and two previously unreported mutations. Our data proved the heterogenic status of *PAH* gene mutations.

based on their severity to be observed (7). The presence of different disease-causing mutations in the biallelic *PAH* gene contributes to the compound heterozygosity status in most patients (8). The regional prevalence of PKU varies worldwide, and the highest frequency of this disease is observed among Caucasians with a rate of 1/10,000 per live births (5).

Iran is a country with a high number of consanguine marriages showing a rate of 1.6 in 3,627 (9). Surveying *PAH* gene mutations is applicable in genetic counseling for prenatal diagnosis and presentation of an efficient dietary program for PKU patients (10). This study identifies the genetic mutation status of a group of Iranian PKU patients to expand the mutation database of different ethnic groups within this population proving beneficial for mutation panel designs of laboratories and prevention of the birth of children affected with PKU.

Methods
Subjects

A total of 30 unrelated PKU patients were chosen from the Institute of Endocrinology and Metabolism of Ali Asghar Hospital between August 2015 and July 2016.

They were accepted into this study after their parents filled out the official consent form provided by the Ethical Committee of Iran University of Medical Sciences. The classification of patients into classic and mild PKU was based on their blood phenylalanine concentrations. Most of them were identified during neonatal screening programs by a Guthrie test. These patients, between 8 months to 18 years old, were from different ethnic groups in Iran.

DNA Extraction: A 5 ml blood sample was collected from each patient. The genomic DNA was extracted from leukocytes isolated from EDTA anticoagulated blood, using a DNA purification Genet Bio kit (South Korea). DNA Purity and quality were assessed by NanoDrop Spectrophotometer (Thermo Scientific™, USA) and 1% agarose gel respectively.

PCR: The choice of *PAH* gene exons including 6, 7, 8, 11, and 12 was based on their high mutation rates in previous literature (6, 11). These 5 exons and their adjacent intronic regions were separately amplified by PCR on the T100™ Thermal Cycler system (Bio-Rad Laboratories, Hercules, CA, USA) (Fig. 1).

The primers were chosen from previous studies (Table 1) (12). PCR reactions were carried out in a total volume of 25 µl containing 2.4 µL of 2× reaction buffer, 2 µL of genomic DNA templates, 0.1 µL of Taq DNA polymerase (5 U/µL), 0.7 µL of each primer, 1.5 µL MgCl$_2$ (50 mM) and 0.5 µM of dNTP (10 mM). The cycling program of
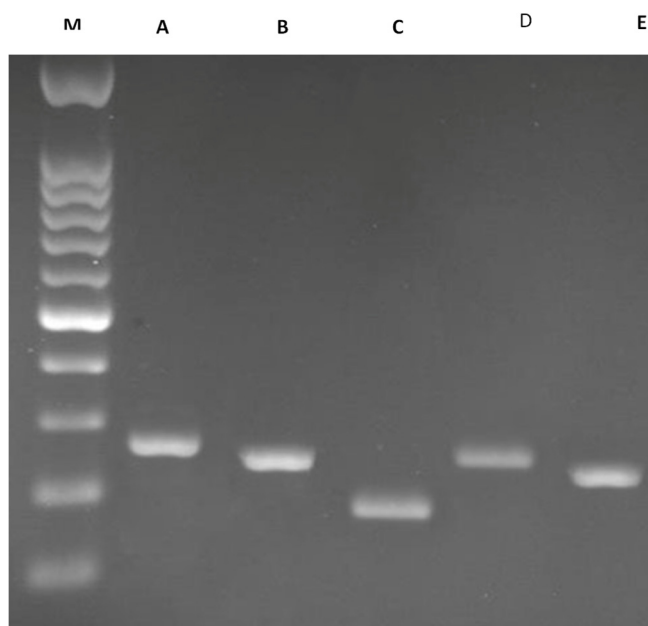
Fig. 1. The analysis of PCR amplification of exons 6, 7, 8, 11 and 12 on 1% agarose gel; M- Marker; A- exon 6 (283 bp); B- exon 7 (256); C- exon 8 (194); D- exon 11 (260); E- exon 12 (232).

Table 1. The list of primer sequences and expected PCR product sizes

Exons	Primers	Annealing temperature (°C)	PCR product length
6	F: TGCCCTGCTTGAGACACCTA	60	283
	R: TCCTCCCCCAACTTTCTGC		
7	F: CCTCTGACTCAGTGGTGA	59	256
	R: CTTGCAGCAGGAAAAG		
8	F: CTGAGTCTGGCTTGGCTTAA	58	194
	R: CTCATTTGAGAAATTCAGGT		
11	F: TGCAGCAGGGAATACTGA	60	260
	R: AGATGAGTGGCACCAGT		
12	F: TCCAAATGGTGCCCT	57	232
	R: GGCGATGGTAGGGAA		

PCR was 94 ˚C denaturation for 3 min, followed by 35 cycles of denaturation at 94 ºC for 45 s, annealing at 59 ˚C for 30 s, extension at 72 ºC for 45 s, and a final extension step at 72 ºC for 3 min. The 1% agarose gel was used for electrophoresis of PCR amplicons.

DNA Sequencing: The PCR products were purified and sequenced using PCR forward and reverse primers by the Applied Biosystems 3730 DNA Analyzer (Macrogen, Seoul, Korea) using the classic Sanger's method.

Data Analysis

Mutations and polymorphisms were detected by software and programs including ChromasPro (Technelysium, South Brisbane Australia), Sequence Scanner (Applied Biosystems, US), Mutation Surveyor (SoftGenetics, Pennsylvania, USA), and nucleotide blast program of NCBI. These ones were then identified by comparison with the reference sequence in GenBank (www.ncbi.nlm.nih.gov/genbank), available genetic data in PAH database (http://www.pahdb.mcgill.ca), and SNP database of NCBI (www.ncbi.nlm.nih.gov/snp).

The clinical significance of mutations was characterized by using ClinVar (www.ncbi.nlm.nih.gov/clinvar/) database. The analysis of new variants was carried out by Mutation Taster (http://www.mutationtaster.org/) and CADD (http://cadd.gs.washington.edu/) online bioinformatics tools.

Results

The patients (15 females and 15 males), aged between 8 months to 18 years old, were examined. They were from different parts of Iran, and their ethnicity was also diverse such as Kurdish, Azeri, and Persian. The rate of consanguine marriage was 53%, and most of the parents were third cousins. A total of 8 different mutations consisting of 5 missense mutations, 1 splice mutation, 1 nonsense mutation, and 1 Silent/Splice mutation were characterized in 14 alleles. The rate of mutation detection was about 23.3%. Three polymorphisms and four intronic novel variations were characterized as well.

The mutations, including c.782G>A, c.838 G>A, c.842C>T, c.721C>T, c.727C>T, c.1197A>T, c.781C>T and IVS11+1G>C, the polymorphisms including c.696A>G, c.735G>A and c.1155G>C and the novel intronic variants including IVS6+44T>G, IVS6+36T>G, and IVS10-15A>C were identified (Table 2). The survey of pathogenic variations in ClinVar database also proved their pathogenic effect (Table 3). The genotype and phenotype information of patients were demonstrated in Table 4. Some associations were also observed in this study and

Table 2. Relative frequency of variations and prediction of their effect

Location	Systematic name (DNA level)	Trivial name (protein effect)	No. of alleles	Relative frequency (%)	phyloP	phastCons	Prediction
Exon7	c.782G>A	p .R261Q	4	6.66	6.198	1	Disease causing
Exon7	c.781C>T	p .R261X	2	3.33	2.582	1	Disease causing
Exon7	c.838G>A	p .E280K	1	1.66	6.203	1	Disease causing
Exon7	c.842C>T	p .P281L	2	3.33	6.203	1	Disease causing
Exon7	c.721C>T	p .R241C	1	1.66	4.439	1	Disease causing
Exon7	c.727C>T	p .R243X	2	3.33	2.751	1	Disease causing
Exon 11	c.1197A>T	p .V399V	1	1.66	0.423	1	Disease causing
Intron11	c.1199+1G>C	IVS11+1G>C	1	1.66	6.012	1	Disease causing
Exon 6	c.696A>G	p .Q232Q	7	11.6	-3.252	0.005	polymorphism
Exon7	c.735G>A	p .V245V	7	11.6	0.873	1	Polymorphism
Exon11	c.1155G>C	p .L385L	2	3.3	-0.213	0.749	Polymorphism
Intron6	c.706+36T>G	IVS6+36 T>G	1	1.6	-0.172	0	Polymorphism
Intron 6	c.706+44T>G	IVS6+44T>G	1	1.6	-0.986	0.004	Polymorphism
Intron 10	c.1066-15A>C	IVS10-15A>C	3	5	0.479	0	Polymorphism

Table 3. Clinical significance of mutations

| Mutation	rs Number	Clinical significance
R261Q	rs62508698	Pathogenic
R261X	rs5030850	Pathogenic
E280K	rs62508698	Pathogenic
P281L	rs5030851	Pathogenic
R241C	rs76687508	Pathogenic
R243X	rs5030846	Pathogenic
V399V	rs199475584	Pathogenic
IVS11+1G>C	rs62509015	Likely pathogenic

Table 4. Genotypes and characteristics of 12 patients

Patients ID	Location	Genotypes	Classification	HPA Type	Ethnicity	Pre-treatment Phe levels (mg/dl)
5	Exon 11	L385L/U	Heterozygote	Classic	Azeri	21.5
6	Exon 7	V245V/U	Heterozygote	Mild HPA	Persian	18.4
		E280K/U				
7	Exon 7	R261Q/ U	Heterozygote	Mild HPA		
7	Exon 6	Q232Q/ U	Heterozygote		Persian	16.7
8	Exon 7	V245V/U	Heterozygote	Mild HPA	Kurdish	14
9	Exon 6	Q232Q/ Q232Q	Homozygote	Mild HPA	Persian	15
11	Exon 7	P281L/ P281L	Homozygote	Classic	Persian	25
12	Exon 7	R241C/U	Heterozygote	Classic	Azeri	21.1
		V245V/U				

Table 4. Cntd

| 14	Exon 7	R261Q/U	Heterozygote	Classic	Persian	24
15	Exon 6	Q232Q/ Q232Q	Homozygote	Classic	Persian	26
		R261X/R261X	Homozygote			
16	Exon 6	Q232Q/ Q232Q	Homozygote	Classic	Azeri	25
18	Exon 11	V399V/U	Heterozygote	Classic	Persian	28.8
	Exon 7	V245V	Homozygote			
19	Exon 7	R243X/ R243X	Homozygote	Classic	Azeri	22.9
		V245V/ V245V				
22	Exon 11	L385L/U	Heterozygote	Classic	Persian	23.4
	Intron 11	IVS11+1G>C/U				
25	Exon 7	R261Q	Homozygote	Classic	Azeri	25.5

A
Normal
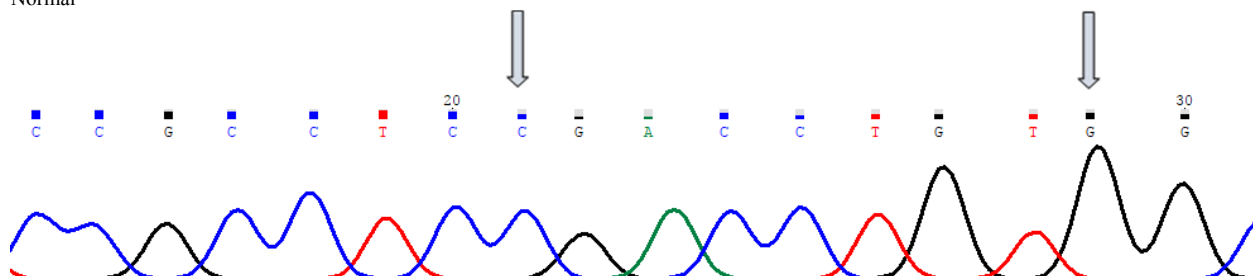
B
Mutant
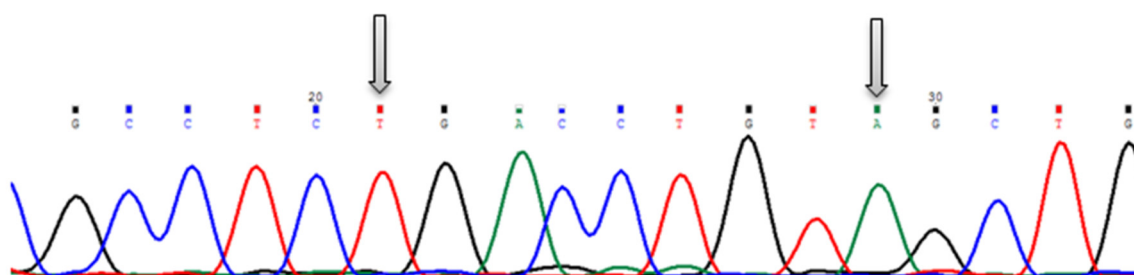
Fig. 2. Mutation analysis of R243X (c.727C>T) nonsense mutation and V245V (c.735G>A) polymorphism in exon 7 of PAH gene

one of them, including R243X mutation and V245V polymorphism was shown in Fig. 2. The mean quality value of surrounding nucleotides of these variants was higher than 50 which proved their accuracy. The PHRED score of these novel variants showed their non-pathogenic effect (Table 5).

Table 5. The PHRED score of new variants

Variant	PHRED Score
IVS6+36 T>G	2.980
IVS6+44T>G	2.380
IVS10-15A>C	2.560

Discussion

Phenylketonuria, a metabolic autosomal recessive genetic disorder, is the consequence of *PAH* gene deficiency. The hallmark of this disease is high blood serum Phe levels with a low-Phe diet as the cornerstone of its treatment (13). Mutations in the BH4 gene also resulted in hyperphenylalaninemia, and identification of related mutations to PAH enzyme can be applicable in the prescription of BH4 based therapies (14). Patients were diagnosed based on their high blood Phe and in order to confirm mild HPA cases for having *PAH* mutations, analysis of DHPR and biopterin-neopterin was carried out.

In this study, molecular characterization of exons 6, 7, 8, 11, and 12 in 30 Iranian patients showed a 23.3% mutation detection rate. Ten percent of patients were homozygote for mutations. Three polymorphisms including Q232Q (11.6%) in exon 6, V245V (11.6%) in exon 7 (11.6%) and L385L (3.33%) in exon 11 were also identified. The R261Q mutation (6.66%) in exon 7 was observed in two patients in heterozygote form and one patient in homozygote form which affects a CpG mutation hot spot in nucleotide 782 of *PAH* gene. The explanation of this mutation mechanism is by the 5-methylcytosine deamination at a CpG dinucleotide site which contributes to the change of amino acid Arg to Glutamine. This one is the second most common mutation in Turkey, which is also common in European nations like France (17%), and Italy (16%) (15, 16). The presence of Mediterranean mutations in our patients may prove the historical connection between Iranian and Mediterranean populations.

The R261X mutation which is the result of C to T conversion at codon 781 of *PAH* gene is placed in its catalytic domain (residues 118-427) (17). P281L (3.33%), a missense mutation in homozygote form, is the result of C to T substitution at codon 842 of *PAH* gene. This nucleotide position is in the region of cofactor binding domain.

P281L has been identified in classic PKU patients frequently. Previous studies in Iran have also reported this mutation (11).

R243X (3.3%) as a nonsense mutation is located in exon 7 of *PAH* gene, which was observed along with V245V as a polymorphism in one classic patient. These ones were in homozygote form and had located in 21th and 29th base of exon 7 respectively. Two associations in heterozygote patients, including R241C (1.66%) with V245V and E280K (1.66%) with V245V were also reported, which have not been reported previously in PAHdb. R241C is classified as the BH4-responsiveness mutation reported for the first time in the Iranian population.

The splice mutation IVS11+1G>C/U (1.66%) and L385L polymorphism were observed in intron 11 and exon 11 of one classic PKU patient. The V399V (1.66%) mutation had been identified in the Chinese population for the first time (17). The c.1197 nucleotide is located at the 3'-end of exon 11 at position -3' of the exon-intron boundary, and transition of A to T in this position cause exon skipping as a post-transcriptional event which can result in deficient PAH mRNA (18). This heterozygote one was observed in a classic patient and was the first report of this mutation in Iran.

Conclusion

The results of our study proved the existence of heterogeneous mutations in different ethnic groups in Iran. The highest rate of mutations was observed in exon 7, and no mutation was observed in exon 8. It should be considered that our results showed two mutations which had not been reported previously in Iran.

So by monitoring diverse ethnic groups in Iran, other novel mutations will be easier to find and identify. This data can be useful for genetic counseling and carrier detection tests and as a base to start further research from.

Acknowledgment

Conflict of Interests

The authors declare that they have no competing interests.

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