Iranian Journal of Basic Medical Sciences

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Mutation analysis of the phenylalanine hydroxylase gene in Azerbaijani population, a report from West Azerbaijan province of Iran

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Nov 9, 2014
Accepted: Apr 8, 2015

Keywords:
Mutation
PAH
PKU

ABSTRACT

Objective(s): Phenylketonuria (PKU) is a genetic inborn error of phenylalanine (Phe) metabolism resulting from insufficiency in the hepatic enzyme, phenylalanine hydroxylase (PAH), which leads to elevated levels of Phe in the blood. The present study was carried out for mutation analysis of the PAH gene in West Azerbaijan province of Iran.

Materials and Methods: A total of 218 alleles from 40 PKU families were studied using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) method.

Results: The frequencies of IVS10-11, S67P, R261Q, R252W, IVS11nt-1 g>c, R408Q, and Q232Q mutations were 28(35), 17(21.25), 15(18.75), 3(3.75), 3(3.75), 2(2.5), and 1(1.25), in cases group, and 51(23.4), 31(14.2), 27(12.4), 6(2.75), 6(2.75), 4(1.83), and 2(0.92) in total group, respectively. The mutations of R243Q, 364delG, L333F, 261X, I65T, and R408W were not detected in our samples.

Conclusion: It can be concluded that the IVS10-11 mutation has the highest frequency in the tested population. To our knowledge, this report is the first in its own kind and provides better understanding of the genetic heterogeneity, the origin and distributions of PAH mutations in West Azerbaijan province of Iran.

► *Please cite this article as:*
Bagheri M, Abdi Rad I, Hosseini Jazani N, Zarrin R, Ghazavi A. Mutation analysis of the phenylalanine hydroxylase gene in Azerbaijani population, a report from West Azerbaijan province of Iran. Iran J Basic Med Sci 2015; 18:649-653.

Introduction

Phenylketonuria (PKU; OMIM 261600) is defined as an autosomal recessive genetic inborn error of phenylalanine (Phe) metabolism (1). Insufficiency in the hepatic specific enzyme, phenylalanine-4-hydroxylase (PAH) (EC 1.14.16.1) leads to hyperphenylalaninemia that is associated with the PKU (1). Deficiency of the PAH enzyme results in the elevation of Phe concentration in blood and biological fluids that is approximately above 2 mg/dl (120 µmol/l) in the pre-treatment condition (1-4). The prevalence of PKU among patients institutionalized for mental retardation varies from 1% to 3% (4-6). The PAH gene is located on q22–q24.1 regions of chromosome 12 (7). More than 530 PAH gene mutations have been identified (PAHdb; http: //www.mcgill.ca/pahdb). The frequency of PKU varies from high incidence in Turkey (about 1 in 2600 births) to low incidence in Japan (about 1 in 125000 births) (7). Overall, the incidence of PKU among Caucasians is about 1 in 10,000, giving a carrier frequency of about 1 in 50 to 1 in 70 (7). The incidence of PKU in Iranian population has been expected at 1 in 3627 live births (8). Genetic structure of Iranian population is highly heterogeneous (8). Genetic overall diversity in Iranian populations is very high and comparable to the other populations from the South Caucasus region, Anatolia and Europe (9). It has been shown that Iranian Azerbaijanis with a population of about 15 to 20 million are more related to the Georgians in comparison to other Iranian groups (9). The finding of Derenko et al (2013) is based on maternal genetic structure on the mitochondrial DNA studies (9). However, this result may change based on paternal genetic structure and Y-chromosome tracing. West Azerbaijan province with a population of about 3 million is in North-West of Iran and closely related to Turks. Regarding to a relatively high incidence of PKU alleles in Iranian population as well as high rate of consanguineous marriages in Iran (10), this study was carried out for mutation analysis of the PAH gene in West Azerbaijan province of Iran.

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Table 1. Tested mutations in phenylketonuria families from West Azerbaijan province of Iran

Mutation name	Systematic name	Region	Mutation type	Restriction enzyme
ivs10nt-11	c.1066-11g>a	intron 10	splicing	+DdeI
s67p	c.199 t>c	exon 3	missense	-XbaI
r261q	c.782 g>a	exon 7	missense	-HinfI
r252w	c.754 c>t	exon 7	missense	-AvaI
ivs11nt-1 g>c	c.1199+1 g>c	intron 11	splicing	+DdeI
r408q	c.1223 g>a	exon 12	missense	-Sau96I
q232q	c.696 a>g	exon 6	silent	+DdeI
r243q	c.728 g>a	exon 7	missense	+PflmI
l364del	c.1090-1092delctt	exon 11	deletion	-HindIII
l333f	c.997 c>t	exon 10	missense	-BanII
r261x	c.781 t>c	exon 7	nonsense	+DdeI
i65t	c.194 t>c	exon 3	missense	+DdeI
r408w	c.1222 c>t	exon 12	missense	+StyI

Creation (+) and removal of (-) of a restriction site for related restriction enzyme by a mutation is shown

Materials and Methods

This study was performed regarding the ethical guideline in human genetic research as described by Ethics Committee of Urmia University of Medical Sciences (West-Azerbaijan, Urmia, Iran) and was conducted in accordance with Declaration of Helsinki. We performed this investigation during 2012 to 2014 in Urmia University of Medical Sciences. The procedure of the present study was explained to all families. PKU patients were diagnosed and sequentially selected among patients referred to Motahari Hospital of Urmia University of Medical Sciences. The West Azerbaijan province ethnically has mixed population of Kurdish and Azeri. We studied Azeri cases that were resident in the West Azerbaijan Province of Iran. A total of 109 individuals from 40 PKU families including 40 PKU patients (16 males and 24 females) and their parents entered in the study. All cases were diagnosed by a pediatric neurologist in the department of pediatrics at the University Hospital of Motahary (Urmia, Iran). Medical data recordings, and tests assessments were carried out by the same physician for all cases using criteria for diagnosis of PKU (7,11). Patients with dihydrobiopterin reductase deficiency were excluded from the study. After obtaining an informed written consent from the parents of the kids for research study, 3 to 5 ml whole blood was taken from patients and their parents and collected in ethylenediaminetetraacetic acid (EDTA) tube. Genomic DNA was isolated from blood samples using 'salting out' method (12). A total of 218 alleles from109 individuals from 40 PKU families (40 PKU patients and their parents) were tested for IVS10-11, S67P, R261Q, R252W, IVS11nt-1 g>c, R408Q, Q232Q, R243Q, 364delG, L333F, 261X, I65T, and R408W mutations in the PAH gene using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) as described previously (13,14) (Table 1).

PCR reaction was performed in a 25 µl solution containing 50 ng DNA, 1x reaction buffer, 10 pmol of each primer, 200 µmol of dNTPs, 0.3 unit of Taq DNA polymerase, and 1.5 mmol $MgCl_2$ (Genefanavaran, Tehran, Iran). A 10 to 15 µl of the PCR products were examined for the presence or absence of tested mutations by restriction digestion at 37 °C for 2 hr according to the manufacturer's instructions. 2%-3% agarose gel containing ethidium bromide was used for electrophoresis of PCR amplicons and digested fragments. Presence or absence of a mutation was monitored by UV transilluminator. As seen on PAH database, exon 7 contains the highest number of mutations (87 out of 567) (15.34%) (PAHdb; http://www.mcgill.ca/pahdb). The identified mutations on the exon 7 of the PAH gene were confirmed with the direct sequencing in an ABI 730XL DNA analyzer (Applied Biosystems) using the primer sequences of 5'actaccaaaggtctcctagtgcct3' and 5'ctacacaactagcctgtggaccag3' (15).

Results

Totally, 218 chromosomes from 40 PKU families were studied. Results of the present investigation are summarized in Tables 2 and 3. The rate of consanguineous marriages was 47.5% (19/40) among all studied families. The frequencies of IVS10-11, S67P, R261Q, R252W, IVS11nt-1 g>c, R408Q, and Q232Q mutations were 28(35), 17(21.25), 15(18.75), 3(3.75), 3(3.75), 2(2.5), and 1(1.25), in case group and 51(23.4), 31(14.2), 27(12.4), 6(2.75), 6(2.75), 4(1.83), and 2(0.92) in total group, respectively. Observed mutations in the patients were inherited from their parents. The mutations of R243Q, 364delG, L333F, 261X, I65T, and R408W were not found in this study. Of the alleles studied, the most frequent mutation was IVS10nt546 (35%). Seven mutations represent approximately 86.25% and 83% of PKU chromosomes analyzed in cases and total groups, respectively. Our analysis showed that 37.5% (15/40) and 62.5% (25/40) of the cases have homozygote and compound heterozygote genotypes regarding the studied mutations (Table 3). The most common mutations of IVS10-11, S67P, and R261Q can be as a result of the high rate of consanguineous marriages. The frequencies of missense, splice, and silent mutations were 37 (46.25), 31 (38.75), and 1 (1.25), in cases and 68 (31.18), 57 (26.15), and 2 (0.92) in total groups,

Table 2. Mutation analysis in the phenylalanine hydroxylase gene of 40 cases and total (patients and parents) groups in West Azerbaijan province of Iran

Mutation	Total [a], n=109, f (%)	Cases [b], n=40, f (%)
ivs10nt-11	51 (23.4)	28 (35)
s67p	31 (14.2)	17 (21.25)
r261q	27 (12.4)	15 (18.75)
r252w	6 (2.75)	3 (3.75)
ivs11nt-1 g>c	6 (2.75)	3 (3.75)
r408q	4 (1.83)	2 (2.5)
q232q	2 (0.92)	1 (1.25)
other	26 (11.9)	11 (13.75)

a: The allele frequency was based on 218 alleles including mutant (153/218) and normal (65/218) alleles in total (patients and parents) group; b: The allele frequency was based on 80 mutant alleles in case group; The mutation detection rate was 86.25% (69 out of 80 PKU chromosomes) and 83% (127 out of 153 PKU chromosomes) in case and total (patients and parents) groups in the West Azerbaijani population. F: Frequency

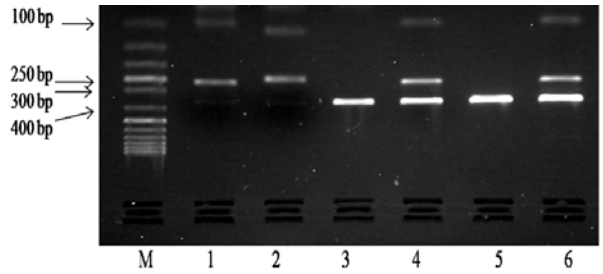

Figure 1. Detection of IVS10nt-11 (c.1066-11g>a) and IVS11nt1 g>c (c.1199+1g>c) mutations in six samples
PCR products (357 bp) were digested with DdeI. The presence of IVS11nt1 g>c mutation naturally produces restriction site for DdeI enzyme. Individuals homozygous for normal sequence regarding IVS11nt1 g>c mutation show a single un-cut band of 357 bp. Individuals homozygous for IVS11nt1 g>c mutation show two bands of 244- and 113- bp. Individuals heterozygous for IVS11nt1 g>c mutation show three bands of 357-, 244- and 113- bp. As well as, individuals homozygous for normal sequence regarding IVS10nt-11 mutation show a single un-cut band of 357 bp. Individuals homozygous for IVS10nt-11 mutation show two bands of 261- and 96- bp. Individuals heterozygous for IVS10nt-11 (c.1066-11G>A) mutation show three bands of 357-, 261- and 96- bp
Lane M: 50 bp marker (Fermentas). Lanes 1: Homozygous for IVS10nt-11 g>a mutation. Lanes 2: Homozygous for IVS11nt1 g>c mutation. Lanes 3 and 5: Without mutation. Lanes 4 and 6: Heterozygous for IVS10nt-11 g>a mutation

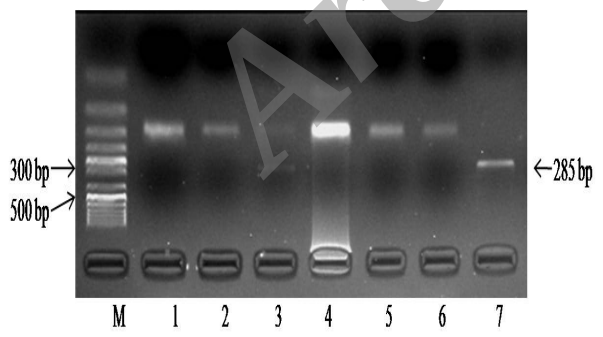

Figure 2a. Detection of R261Q mutation in 7 samples. PCR products (285 bp) were digested with HinfI. The presence of R261Q mutation removes restriction site for HinfI enzyme. Individuals homozygous for R261Q mutation show a single un-cut band of 285 bp. Individuals heterozygous for R261Q mutation show two bands of 285 and 123 bp
Lane M: 50 bp marker (Fermentas). Lanes 1,2,4,5 &6: Without R261Q mutation. Lane 3: Heterozygous for R261Q mutation. Lane 7: Homozygous for R261Q mutation

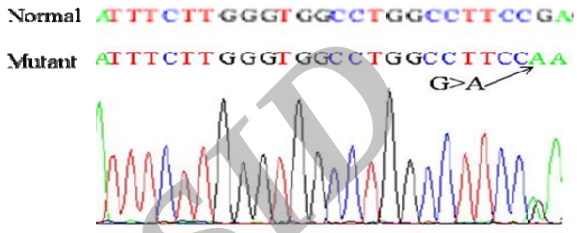

Figure 2b. PKU Nucleotide sequences of PKU mutation of c.782G>A (p.R261Q) of exon 7 of the PAH gene in a sample

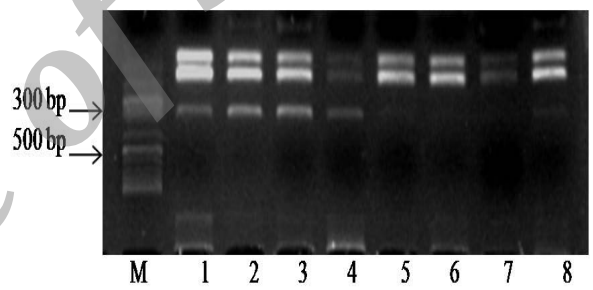

Figure 3. Detection of R252W mutation in 8 samples. PCR products (285 bp) were digested with AvaI. The presence of R252W mutation removes restriction site for AvaI enzyme. Individuals homozygous for R252W mutation show a single un-cut band of 285 bp. Individuals heterozygous for R252W mutation show three bands of 285, 162 and 123 bp
Lane M: 50 bp marker (Fermentas). Lanes 1-4: Heterozygous for R252W mutation. Lanes 5-8: Without R252W mutation

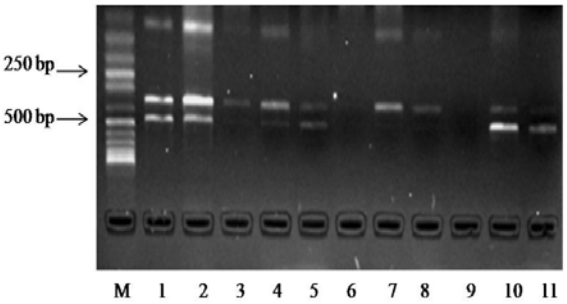

Figure 4. Detection of S67P mutation in 11 samples. PCR products (463 bp) were digested with XbaI.The presence of S67P mutation removes restriction site for XbaI enzyme
Individuals homozygous for S67P mutation show a single un-cut band of 463 bp. Individuals heterozygous for S67P mutation show three bands of 463, 348 and 115 bp
Lane M: 50 bp marker (Fermentas). Lanes 1,2,4,5,10: Heterozygous for S67P mutation. Lanes 3,6,7,8: Normal sequence

Table 3. Distribution of homozygote and compound heterozygote genotypes in the West Azerbaijani among Phenylketonuria patients

Classification	Genotypes	No. of patients n=40	Frequency (%)
Homozygote	ivs10nt-11/ivs10nt-11	7	17.5
	r261q/r261q	5	12.5
	s67p/s67p	2	5
	ivs11nt-1/ivs11nt-1	1	2.5
Compound heterozygote	s67p/ ivs10nt-11	7	17.5
	s67p/r261q	3	7.5
	ivs10nt-11/r261q	2	5
	ivs10nt-11/r408q	1	2.5
	r252w/q232q	1	2.5
	r408q/s67p	1	2.5
	ivs10nt-11/r252w	1	2.5
	s67p/r252w	1	2.5
	ivs10nt-11/nd	3	7.5
	ivs11nt-1/nd	1	2.5
	s67p/nd	1	2.5
	other	3	7.5

nd: not determined

respectively. Deletion and nonsense mutations were not identified in this investigation. Figures 1 to 4 show PKU mutation analysis in this local population.

Discussion

The tested mutations were chosen based on similarity between population in the West Azerbaijan province and the Mediterranean groups. The IVS10nt-11g>a mutation with systematic name of c.1066-11g>a also known by the trivial name IVS10nt546 g>a, is the most common Mediterranean PKU mutation (1). Dworniczak et al (1991) reported transition of G to A at location 546 in intron 10 of the PAH gene (1). This mutation activates a splicing site and leads to insertion of nine nucleotides between exons 10 and 11 during splicing. IVS10nt546g>a is the major cause of PKU in parts of southern and southeastern Europe, mainly in Turkey. Several investigations have been conducted to study the spectrum of PKU causing mutations in various groups (8, 11, 13, 16-23). Interestingly, the frequency of IVS10nt-11 mutation in our cases is higher compared to other reports (8, 11, 13, 16-23). The high rate of consanguineous marriages (47.5%) is a contributing reason to this high prevalence in tested families. It has been suggested that the world-wide distribution of IVS10nt546 mutation has Turkish origin with expansion in different geographic regions (7). In this study, it was established that the IVS10-11 mutation has the highest frequency in the PKU patients among the Iranian Azerbaijanis, and it can be considered for molecular diagnosis in this population. The findings of the present study may be a sign of close familial link between West Azerbaijanis and Turks, which is consistent with the historical as well as geographical relations between West Azerbaijan and Turkey. The second most common mutation identified in our investigation, S67P (c.199T>C), is a missense mutation with low frequency in other populations (4). Unexpectedly, the mutation of S67P with 25% frequency seemed to be explained by the high rate of consanguineous marriages in the tested group (47.5%). The third most common mutation identified in our investigation, R261Q (c.782G>A), is a Mediterranean missense mutation and occurs on a CpG dinucleotide on exon 7 in the PAH gene. This mutation results in conversion of Arg→Gln at codon 261 and is the second most frequent mutation in Turks (19). The remaining mutations of R252W (c.754C>T), IVS11nt-1 G>C (c.1199+1G>C), R408Q (c.1223G>A), and Q232Q (c.696A/G) account for 12.47% of the identifiable mutations. This study indicated high level of heterogeneity of the PAH gene in PKU families in the West Azerbaijan, and thus further investigation should be carried out. Mutation analysis in the PAH gene can be used in carrier detection, prenatal diagnosis and prevent the incidence of PKU phenotypes in the West Azerbaijani population. The results of this study would be useful for biomedicine and molecular anthropology studies by tracing the anthropological characters of the population regarding autosomal chromosomes. This study had some limitations including the small sample size and poor quality of medical records.

Conclusion

Exon 11 and its intronic regions carry the most prevalent mutant alleles in PKU families in the West Azerbaijan. Screening of IVS10nt-11 g>a Mediterranean mutation should be tested for detection of possible mutations of PAH in the West Azerbaijan (Iran). This report reveals the genetic heterogeneity in the West Azerbaijani PKU population with a high frequency of IVS10nt-11 g>a mutation (35%) that shows similarity to Turks.

Acknowledgment

The results described in this paper were part of a PhD thesis. This study was financially supported by Urmia Medical Science University (Grant No: 1276). We are grateful to the patients and their families, for providing the blood samples, and to medical staff of Motahari Hospital for collecting samples.

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