Original Article

Mutation Analysis of PAH Gene in Phenylketonuria Patients from the North of Iran: Identification of Three Novel Pathogenic Variants

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Abstract

Background: There are more than 1100 different pathogenic variants in the phenylalanine hydroxylase (*PAH*) gene that are responsible for phenylketonuria (PKU) diseases, and the spectrum of these mutations varies in different ethnic groups. The aim of the present study was to identify the frequency of pathogenic variants in all 13 exons of the *PAH* gene among patients with PKU in Mazandaran and Golestan provinces in the north of Iran. **Methods:** Forty unrelated PKU patients from Mazandaran and Golestan provinces were enrolled in the study. Genomic DNA was extracted from leukocytes using a Qiagen DNA extraction kit and polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP), and Sanger sequencing methods were applied to detect the variants. In the case of new variants, the InterVar online tool (PMID: 28132688) was used to classify the variants. **Results:** Twenty-one different pathogenic variants were observed among the 40 investigated patients. The c.106611G>A variant had the highest frequency (27.5%) in the region, and the c.168+5G>C, c.473G>A, and c.782 G>A variants were the other most frequent mutations with allelic frequencies of 7.5, 5, and 5%, respectively. Three novel pathogenic variants including c.773T>G, c.878 T>C, and c. 1245del variants were observed among the investigated patients. **Conclusions:** The introduction of pathogenic variants in the *PAH* gene in each ethnic group provides valuable data regarding the understanding of the pathogenesis of the disease and can be helpful for prenatal diagnosis programs.

Keywords: *Mutation, phenylalanine hydroxylase, phenylketonuria*

Introduction

Phenylketonuria (PKU) or phenylalanine hydroxylase (*PAH*) deficiency is an inborn error of phenylalanine (Phe) metabolism that is caused by pathogenic variants in the *PAH* gene that are responsible for the conversion of Phe to tyrosine (Tyr).[1,2] In untreated PKU patients, blood Phe concentrations are noticeably increased, leading to the formation of phenylketone bodies that are excreted in urine. The increased Phe level will lead to a severe intellectual disability, epilepsy, and behavioral, psychiatric, and movement problems. Light pigmentation of skin, eyes, and hair, eczema, and a musty odor have also been observed in untreated patients.[3-6] Various incidence rates of PKU have been substantially observed among ethnicities and between different geographic regions worldwide. The highest prevalence of PKU is reported among white or East Asian populations (~1:10,000–15,000 live births).[5] The prevalence of PKU in European countries is varied ranging from 1:2,700 live births in Italy to <1:100,000 live births in Finland.[1] The incidence rate of PKU in some regions is comparable to or even higher than what is reported in white or East Asian populations. For example, in Turkey,[6] and the Karachay-Cherkessia Republic of Russia,[7] the incidence rate is 1:4,370 and 1:850 newborns, respectively. PKU in Iran has one of the highest prevalence rates in the world with an estimate of 16.5 per 100,000 newborns.[8] In the Fars province of Iran, the prevalence of PKU is reported to be 1:4,698 live births.[9,10]

PKU disease is usually caused by pathogenic variants in the *PAH* gene (ENSG00000171759), and in rare cases, failures in genes responsible for the synthesis or recycling of tetrahydrobiopterin (BH4), a cofactor for PAH enzyme, may also lead to hyperphenylalaninaemia.[11] The *PAH* gene that is located on chromosome 12 q22–q24.1 consists of 13 exons and occupies a region of about 171 kb in length.[5] Up to

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now, more than 1,100 variants of the *PAH* gene have been identified (the PAHvdb database (http://www.biopku.org/home/pah.asp)), most of which are recognized in exons 7, 6, 3, and 11, respectively. Missense mutations (60%), splice site variants (14%), and deletions (14%) are three common types of reported genetic variants in the PAHvdb database.[12]

Mazandaran and Golestan provinces are located on the southern coastlines of the Caspian Sea with a population of around 5 million. In Golestan, Turkmens are one of the major ethnic groups, and in Mazandaran, the majority of the population has Mazandarani origin. In the first phase of the study, we investigated and published the frequency of five *PAH* gene mutations that were frequently reported from other parts of Iran in Mazandaran and Golestan Provinces. The investigated mutations that were identified using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) methods included c.106611G>A, c.782 G>A, c.754C>T, c.781C>T, and c.1200+1G>C mutations. These mutations were identified in 39.9% of the investigated alleles. The aim of the present study was to determine the pathogenic variants in all 13 exons of the *PAH* gene among those patients.

Materials and Methods

Sampling

The research project was approved by the ethics committee of Mazandaran University of Medical Sciences (ethics code: IR.MAZUMS.REC.1398.308). The parents of all patients signed an informed consent and agreed that their children could participate in the study. Forty unrelated PKU patients (22 from Mazandaran and 18 from Golestan provinces) were enrolled in the study.

DNA extraction

Genomic DNA was extracted from 200 µl of peripheral blood using a Qiagen DNA extraction kit. In the first phase of the study, as published before.[13]

PCR-RFLP

PCR-RFLP method was used to identify five mutations including c.106611G>A, c.782G>A, c.754C>T, c.781C, and c.1200+1G>C. For the detection of the mentioned mutations, the amplified fragments were treated with specific restriction enzymes, as published before.[14]

Sanger sequencing

For the detection of other pathogenic variants among patients with unidentified mutations after the first phase of the study, 13 exons of the *PAH* gene were amplified with specific primers, and the subsequent Sanger sequencing method was used via 3130 × 1 Genetic Analyzers (Applied Biosystems, USA). In the case of new variants, the InterVar online tool (PMID: 28132688) was used to classify the variants with adjusted criteria according to American College of Medical Genetics and Genomics (ACMG) recommendations (PMID: 25741868). Finally, the allelic frequencies of each variant were calculated.

Results

Twenty-one different pathogenic variants were observed among 40 investigated patients (16 male and 24 female cases) [Table 1]. The pathogenic variants were identified in 68 alleles (85%). The c.106611G>A variant had the frequency among the patients, and 27.5% of the alleles carried this mutation. The c.168+5G>C, c.473G>A, and c.782G>A variants were other frequent mutations among the investigated patients with frequencies of 7.5, 5, and 5%, respectively. The c.727C>T, c.782G>A, and c. 113_115delTCT variants were detected in three, and the c.441+5G>T and c.603T>G variants were identified in two alleles. Nine different pathogenic variants were just identified in one allele. Twenty different genotypes were observed in patients with two identified mutations [Table 1]. The c.106611G>A/c.106611G>A was the most frequent genotype among the patients (22.5%), especially in Golestan Province (33.3%). Eight cases carried two different mutations, and in two cases, the mutation was detected just in one allele.

In the present study, three novel pathogenic variants were observed among the investigated patients including c.773T>G (NM_001354304.2), c.878T>C (NM_001354304.2), and c.1245del (NM_001354304.2) variants that were located on exons 7, 8, and 12, respectively. The identified variants have not been reported before. The c.773T>G variant was identified in combination with the c.969+5G>A variant, while c.878 T>C, and c.1245del variants were detected in a homozygous state [Figure 1]. All of the new variants were categorized as pathogenic based on InterVar online tool analysis.

Discussion

The *PAH* gene that was mapped to chromosome 12 (12q22–q24.2) is 90 kb in length and contains 13 exons.[3] PKU is genetically heterogeneous, and more than 1,100 PAH variants have been reported in patients with PKU worldwide.[15] These PAH variants are presented in the locus-specific databases PAHvdb and BioPKU (http://www.biopku.org). In the present study, we have reported the pathogenic variants of the *PAH* gene among patients with classic PKU in the north of Iran in which 21 different mutations including three novel pathogenic variants were identified.

Since one or more specific mutations may be common in each population, the identification of such mutations at the country level may be an effective factor in the design of national PKU screening programs. Moreover, the

Table 1: The frequency of detected pathogenic variants and identified genotypes in the *PAH* gene among the PKU patients in the north of Iran

Variant name	Number of alleles (frequency, %)	Mazandaran	Golestan	Genotype	Number (%)	Mazandaran	Golestan
c. 1066-11G>A	22 (27.5)	8 (18.2)	14 (38.9)	c. 1066-11G>A/c. 1066-11G>A	9 (22.5)	3 (13.6)	6 (33.3)
c. 168+5G>C	6 (7.5)	4 (9.1)	2 (5.55)	c. 168+5G>C/c. 168+5G>C	3 (7.5)	2 (9.1)	1 (5.55)
c. 782 G>A	4 (5)	4 (9.1)	0	c. 1066-11G>A/c. 473G>A	2 (5)	2 (9.1)	0
c. 473G>A	4 (5)	2 (4.5)	2 (5.55)	c. 1066-11G>A/c. 1197 A>T	2 (5)	0	2 (11.1)
c. 113_115TCT	3 (3.75)	3 (6.8)	0	c. 782 G>A/c. 782 G>A	2 (5)	1 (4.5)	1 (5.55)
c. 727C>T	3 (3.75)	3 (6.8)	0	c. 113_115delTCT/c. 113_115delTCT	1 (2.5)	1 (4.5)	0
c. 1208 C>T	3 (3.75)	3 (6.8)	0	c. 728G>A/c. 728G>A	1 (2.5)	1 (4.5)	0
c. 441+5G>T	2 (2.5)	2 (4.5)	0	c. 727C>T/c. 727C>T	1 (2.5)	1 (4.5)	0
c. 728G>A	2 (2.5)	2 (4.5)	0	c. 727C>T/c. 603T>G	1 (2.5)	1 (4.5)	0
c. 878 T>C (Novel)	2 (2.5)	2 (4.5)	0	c. 441+5G>T/c. 441+5G>T	1 (2.5)	1 (4.5)	0
c. 688G>A	2 (2.5)	2 (4.5)	0	c. 773T>G/C.969+5G>A	1 (2.5)	1 (4.5)	0
c. 603 T>G	2 (2.5)	2 (4.5)	0	c. 781C>T/C.688G>A	1 (2.5)	1 (4.5)	0
c. 1197A>T	2 (2.5)	0	2 (5.55)	c. 143T>C/C.1208 C>T	1 (2.5)	1 (4.5)	0
c. 1245 del (novel)	2 (2.5)	0	2 (5.55)	c. 754 C>T/c. 782 G>A	1 (2.5)	1 (4.5)	0
c. 773T>G (novel)	1 (1.25)	1 (2.3)	0	c. 473 G>A/c. 473 G>A	1 (2.5)	0	1 (5.55)
c. 969+5G>A	1 (1.25)	1 (2.3)	0	c. 838G>A/c. 441+1G>A	1 (2.5)	0	1 (5.55)
c. 781C>T	1 (1.25)	1 (2.3)	0	C.878 T>C/C.878 T>C	1 (2.5)	1 (4.5)	0
c. 143T>C	1 (1.25)	1 (2.3)	0	c. 1245 del/c. 1245 del	1 (2.5)	0	1 (5.55)
c. 838 G>A	1 (1.25)	0	1 (2.8)	c. 603 T>G/c. 1208 C>T	1 (2.5)	1 (4.5)	0
c. 441+1G>A	1 (1.25)	0	1 (2.8)	c. 688G>A/c. 1208 C>T	1 (2.5)	1 (4.5)	0
c. 754 C>T	1 (1.25)	1 (2.3)	0	c. 782 G>A/?	1 (2.5)	1 (4.5)	0
				c. 113_115delTCT/?	1 (2.5)	1 (4.5)	0

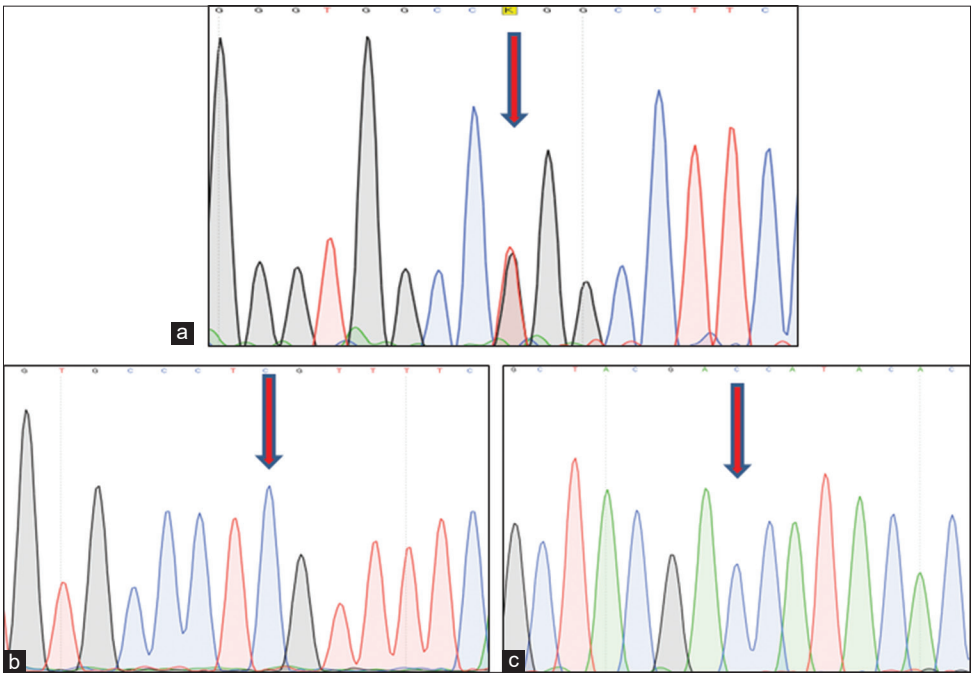

Figure 1: The Sanger sequencing results indicate three novel variants identified in the *PAH* gene of the patients with PKU. a) The c.773 T>G (NM_001354304.2) variant in heterozygous state at exon 7; b) the c.878 T>C (NM_001354304.2) variant at exon 8 in homozygous state; c) the c. 1245del (NM_001354304.2) variant in homozygous state at exon 12

introduction of new pathogenic variants in the *PAH* gene provides valuable data regarding the understanding of the pathogenesis of the disease and can be helpful for prenatal diagnosis programs. In the present study, the spectrum and frequency of *PAH* gene mutations were investigated in the north of Iran, and three novel pathogenic variants in the *PAH* gene were introduced.

Alibakhshi *et al*.,[16] in a systematic review study, analyzed the results of 21 articles with a sample size of 1,547 Iranian

PKU patients, published between 2003 and 2020. Based on the results of that study, a total of 129 different PAH gene mutations were identified in Iranian PKU patients. They have reported that c.1066-11G>A (19.23%), c.782G>A (7.63%), c.842C>T (6.24%), c.168+5G>C(5.75%), c.727C>T (3.59%), c.969+5G>A (2.84%), c.526C>T (2.42%), c.1089delG (2.13%), c.1199+1G>C (2.07%), and c.143T>C (2.04%) are the most common mutations in Iran. The spectrum and frequency of mutations reported in that study were closer to those identified in Mediterranean countries. The results of the present study were also similar to the previous studies in Iran.

Based on the BIOPKU database, the c.1066-11G>A variant with a frequency of 6.8% is the second most frequent mutation among all reported mutations.[11] This variant is a major cause of PKU in the Mediterranean region and the Middle East. It is also the most frequent variant (21.11%) among PKU patients in Iran, except in western provinces, where c.969+5 G>A is the most frequent variant and c.1066-11G>A is the fourth common mutation (6.67%).[16] In the present study like in other parts of the country the c. 1066-11G>A variant is the most common mutation (27.5%) and in Golestan province with high Turkmen settlements its frequency is 38.9% which is the highest frequency of this variant in Iran and the Middle East that may be related to genetic drift, founder effect, and consanguinity.

Iran has a very heterogeneous population, and a variety of different ethnic groups live in different parts of the country. Hence, PAH gene mutations in Iranian PKU patients are very heterogeneous. In the present study, 21 different mutations were identified; three of them (c. 1245 del, c. 878 T>C, and c.773T>G) have not been reported so far. Nemati *et al*.[17] have also reported 15 different variants containing the c.913G>T and c.1330C>A novel variants in Gilan province, which is also located in the north of Iran.[17] This variety of mutations and identification of new variants, especially in the north of Iran is indicative of the heterogeneity of the population in the region.

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Conflicts of interest

There are no conflicts of interest.

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