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Original Article

Detection of IVS4+1G>A mutation in phenylalanine hydroxylase gene in North of Iran using PCR-sequencing

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Abstract

Background and aims: Phenylketonuria (PKU) is an autosomal recessive disorder of phenylalanine (Phe) metabolism. Mutations in the phenylalanine hydroxylase (*PAH*) gene are the main reason for the incidence of PKU. To date, more than 1180 variants have been detected in the *PAH* gene. Given that the distribution pattern of mutations in the *PAH* gene is specific to each population, the present study was conducted to detect exon 4 mutations and adjacent flanking regions of the *PAH* gene in northern Iran.

Methods: This is a descriptive cross-sectional study, in which 24 unrelated PKU patients in Taleghani Hospital in Gorgan were enrolled for a one-year period. After extraction of genomic DNA from leukocytes, identification of exon 4 mutations and adjacent flanking regions was performed using polymerase chain reaction (PCR) and sequencing techniques.

Results: In this study, IVS4+1G>A mutation was detected in one allele (2.08%) among 48 alleles. Moreover, IVS4+47C>T and IVS3-22C>T polymorphisms were observed in 12 alleles (25%) and eight alleles (16.7%), respectively.

Conclusion: In the present study, IVS4+1G>A mutation was only found in 2% of chromosomes. Hence, different mutations are responsible for PKU disease in the north of Iran, and further studies are recommended to identify all mutations in the *PAH* gene in the region.

Keywords: Phenylketonuria, Phenylalanine hydroxylase, Mutation

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Introduction

Phenylketonuria (PKU) is an autosomal recessive genetic disorder caused by a defect in hepatic phenylalanine hydroxylase (PAH). It is one of the most common inborn errors of amino acid metabolism. PAH deficiency prevents phenylalanine (Phe) from being hydroxylated to tyrosine and increases Phe concentration and its derivatives in the blood and other body fluids (1). If left untreated, increased levels of Phe are manifested as mental retardation in patients (2). Hyperphenylalaninemia (HPA) is the result of increased levels of Phe in the blood. This level is less than 2 mg/dL in healthy children, 2-10 in HPA patients, 10-15 in mild PKU, 15-20 in moderate PKU, and more than 20 mg/dL in classic PKU (3). In addition, 98% of all cases of HPA are related to mutations in the *PAH* gene (4). Mutation in the genes that are involved in the synthesis and metabolism of the cofactor tetrahydrobiopterin such as PTS, GCH1, QDPR, and PCBD1 accounts for the other 2% of HPA cases (3). The frequency of PKU is 1 in 10 000 and 1 in 16 500 live births in Caucasian and Oriental populations, respectively. However, the frequency of PKU in Iran is estimated to be more than 1 in 10 000 live births. Early diagnosis and treatment are essential to prevent PKU because the most detrimental outcome of PKU is mental retardation. Therefore, screening programs can be useful tools for the timely treatment and prevention of PKU in patients in different geographical regions of a country (5). *PAH* gene is 90 kb in length with 13 exons which is located on chromosome 12q22-q24.1 and encodes 452 amino acids. The main cause of PKU is mutations in *PAH* gene (6), and more than 1 180 bi-allelic variants have been detected in this gene (7). The frequency of these mutations varies in different geographical regions; as a result, the identification of common mutations in each region is necessary to facilitate genetic screening (5). IVS4+1G>A (c.441+1G>A) is a splicing mutation in the intron 4 of the *PAH* gene. In 2010, Bonyadi et al reported this mutation with a frequency of 3.4% among Iranian Azeri Turkish patients with PKU (8). Moreover, Alibakhshi et al reported *PAH* mutation with a frequency of 0.42% (9). Furthermore, Shirzadeh et al demonstrated this mutation in 3 patients among 635 PKU patients in Iran (10). Since *PAH* gene mutations vary widely among different populations, determining the most common mutations and polymorphisms in each region reduces the time and cost of diagnosing such preventable diseases, thus reducing the burden of disease (11). The present study aimed to conduct a molecular investigation of exon 4 mutations of the *PAH* gene using the sequencing method in patients with PKU in Golestan province and compare the results with studies in other regions of Iran.

Materials and Methods

Patients

The present study is cross-sectional and descriptive, in which 24 unrelated PKU patients from Taleghani hospital in Gorgan, Golestan province, Iran, were enrolled for one year (2016). After obtaining informed consent, in families with more than one person with PKU, only one person was included. These patients were identified based on their files in Taleghani hospital in Gorgan. The primary diagnosis of these patients was based on clinical criteria and laboratory findings (detection of elevated Phe levels in blood samples using high-performance liquid chromatography). Based on pretreatment serum Phe levels, patients were classified as classic PKU (>20 mg/dL), moderate PKU (15-20 mg/dL), mild PKU (10-15 mg/dL), and HPA (2-10 mg/dL). Blood samples were obtained from the patients after the completion of consent forms and questionnaires by the patients or their families.

DNA analysis

DNA extraction was carried out by a high pure polymerase chain reaction (PCR) template preparation kit (Roche, Germany) according to the manufacturer's instructions. DNA purity and quality were assessed by NanoDrop Spectrophotometer (Thermo Scientific NanoDrop, 2000C, USA) and 1% agarose gel, respectively.

PCR amplification and DNA sequencing

Amplification of exon 4 and adjacent flanking regions was performed by Taq™ 2X PCR Mix kit (Biotechrabbit, Germany), and thermocycler (TC-4000, TECHNE, UK) according to the following plan: initial denaturation at 95°C for 5 minutes, 30 cycles at 95°C for 1 minute, at 55°C for 1 minute, at 72°C for 1 minute, and a final extension step at 72°C for 5 minutes. The primers were chosen from previous studies (12): forward primer: 5′-GACGGGTGGGAGGGAGATGAG-3′ and reverse primer: 5′-AGCACTTGACTTAAACCTCCATAGATG-3′ (12).

Then, PCR products were evaluated by direct sequencing and analyzed by CLC Main Workbench v3.5 for mutation findings.

Results

Phenotypic classification of patients

The examined patients included 12 women and 12 men aged 1-23 years old. They were from different parts of Golestan province, and their ethnicity was also diverse: Fars (20 [83.3%]), Turkmen (3 [12.5%]), and Lor (1 [4.2%]). As mentioned in the methods section, patients were classified into four groups: classic PKU, moderate PKU, mild PKU, and HPA based on pretreatment serum Phe levels (4.5-250 mg/dL).

The results of sequencing

After observing the specific band (678 bp) of PCR products on 1% agarose gel (Figure 1), these products were sequenced.

After examining the nucleotide sequence of exon 4 of the *PAH* gene and the adjacent flanking regions in 24 PKU patients (48 alleles), one splicing mutation IVS4+1G>A (c.441+1G>A) was detected in intron 4, and IVS3-22C>T (rs2037639) and IVS4+47C>T (rs1718301) polymorphisms were detected in introns 3 and 4, respectively. Of the 24 samples, sample No. 21 with moderate PKU phenotype in intron 4 had a splicing mutation (heterozygous). Moreover, IVS4+47C>T (rs1718301) and IVS3-22C>T (rs2037639) polymorphisms were observed in 12 alleles (25%) and 8 alleles (16.7%), respectively. No mutations were found in 27 alleles out of 48 alleles. Furthermore, IVS4+1G>A (c.441+1G>A) mutation was detected in one allele (2.08%). The profile of a patient with this mutation and its electropherogram is presented in Table 1 and Figure 2, respectively.

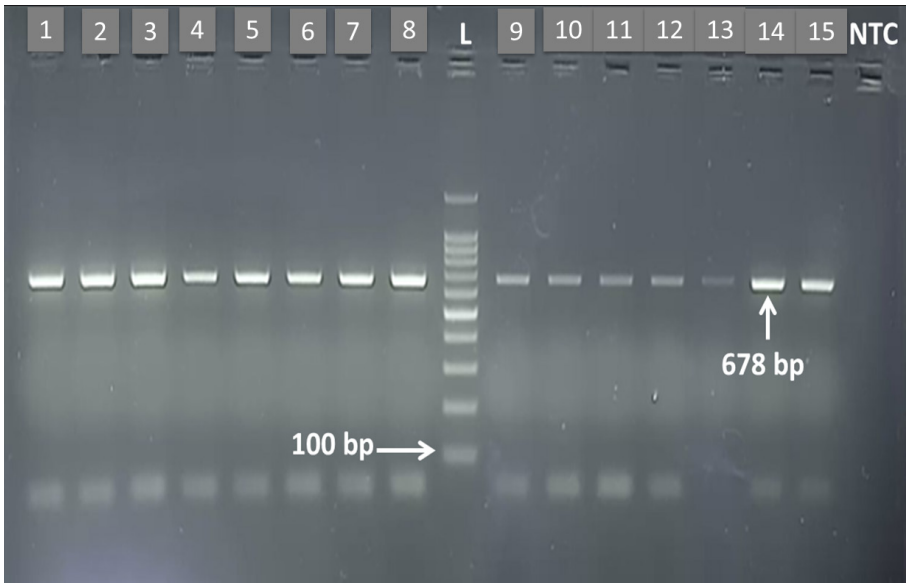

Figure 1. Results of the electrophoresis of PCR products related to exon 4 (678 bp) on 1% agarose gel. *Note.* PCR: Polymerase chain reaction; NTC, negative control sample. L (100 bp DNA Size Marker).

In addition, IVS4+47C>T polymorphism was identified in 12 alleles (in five patients as homozygous and two patients as heterozygous), and IVS3-22C>T polymorphism was identified in eight alleles (in two patients as homozygous and four patients as heterozygous) among the 48 studied alleles. The electropherogram of the forward primer of these two polymorphisms is also illustrated in Figures 3 and 4, respectively.

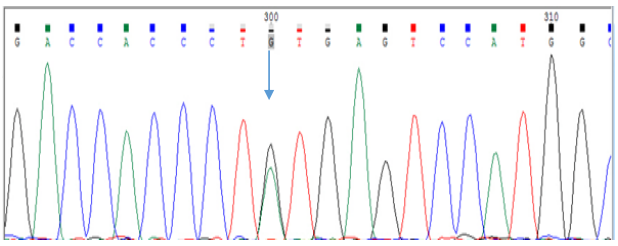

Figure 2: Electropherogram of IVS4+1G>A (c.441+1G>A) mutation (heterozygous).

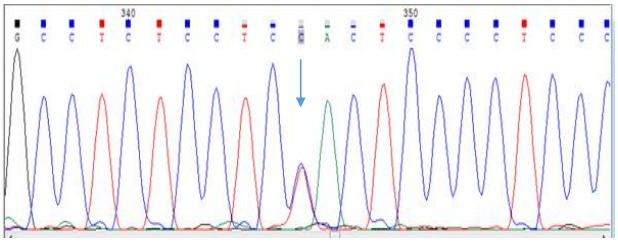

Figure 3. Electropherogram of IVS4+47C>T (rs1718301) polymorphism in heterozygous state (forward sequencing).

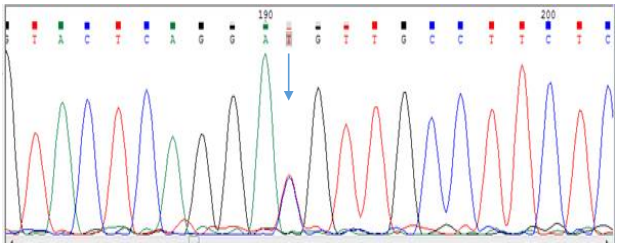

Figure 4. Electropherogram of IVS3-22C>T (rs2037639) polymorphism in heterozygous state (forward sequencing).

Discussion

One of the most common inborn errors of amino acid metabolism, PKU, can be diagnosed by neonatal screening (1). Mutations in the *PAH* gene and genes involved in the synthesis and metabolism of the cofactor BH4 have been reported to be associated with the pathogenesis of the PKU disease. Since approximately 97% of disease-causing mutations are located in the *PAH* gene, analysis of the above mutations is necessary for populations at risk (1). One of the provinces located in the north of Iran is Golestan province. A part of the population of Golestan includes Turkmen, which is an ethnic group in Iran (13). This is the first report dedicated to the analysis of exon 4 mutations in the *PAH* gene among PKU patients in Golestan province. Based on direct sequencing in the present study, one splicing mutation IVS4+1G>A (c.441+1G>A) with 2.08% frequency and two polymorphisms of IVS4+47C>T (rs1718301) and IVS3-22C>T (rs2037639) were detected with 25% and 16.7% frequencies, respectively.

In 2011, Zare-Karizi et al reported IVS4+47C>T polymorphism in the PKU population of Iran (14). Likewise, Alibakhshi et al identified two polymorphisms of IVS4+47C>T and IVS3-22C>T in PKU patients in the Kermanshah province in western Iran (15). IVS3-22C>T polymorphism was reported by Biglari et al in Qazvin and Zanjan provinces with a frequency of 2.56% (16) and by Alavinejad et al with a frequency of 70% (17).

PAH gene mutations have been studied in many populations. IVS4+1G>A is a splicing mutation that occurs in the intron 4 of the *PAH* gene. In the present study, this mutation was heterozygous in one patient with a frequency of 2.08%, and base-pair changes in intron 4 of this gene created a splicing site in the *PAH* gene. There are reports about the PKU population in Iran regarding IVS4+1G>A mutation, including the studies by Bonyadi et al (8), Alibakhshi et al (9), and Shirzadeh et al (10). Bonyadi et al examined 13 exons and exon/intron boundaries of the *PAH* gene in Iranian Azeri Turk patients with PKU, detecting 13 different mutations in the study. Among the reported mutations, IVS4+1G>A with a frequency of 3.4% was related to intron 4 (8). In addition, Alibakhshi et al reported the above mutation with a frequency of 0.42% (9). Moreover, Shirzadeh et al evaluated 635 PKU patients in Iran and reported this mutation in three cases (10). Other studies in Iran did not find IVS4+1G>A mutation among different PKU populations in terms of *PAH* mutation spectrum, including the studies by Biglari et al in two provinces of Zanjan and Qazvin (16), Razipour et al (18), Shaykholeslam Esfahani and Vallian (1), Alibakhshi et al in Hamadan and Lorestan provinces (19), Jafarzadeh-Esfehani et al (11), and Nemati et al in Guilan province (20). On the other hand, Song et al reported that 0.5% of the northern Chinese population have IVS4+1G>A mutation (21). Moreover, the frequency of IVS4+1G>A mutation in PKU patients in Georgia was reported to be 0.7% by Gundorova et al (22). The limitations of the present study were the number of exons, the length of the study, and the lack of sufficient funds to investigate the mutations of the entire gene. Hence, exon 4 and adjacent flanking regions were evaluated in this study to identify mutations.

Table 1. Profile of a patient with IVS4+1G>A mutation in the present study

Patient	Age at diagnosis	Pretreatment Phe level (mg/dl)	Phenotype	Ethnicity	IVS4+1G>A
21	One year of age	16.8	Moderate PKU	Fars	+/-

-/+: Heterozygous

Conclusion

Identification of *PAH* gene mutations, especially local mutations in each region of the country with regard to the prevalence of consanguineous marriage in it, is particularly necessary to design a screening program. Since only one exon of the *PAH* gene has been evaluated in this study, other exons must be evaluated to obtain the full mutation spectrum of this gene in PKU patients in Golestan province.

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Writing – review & editing: Zeinab Khazaei Koohpar.

Conflict of Interests

The authors have no conflicts of interests.

Ethical Approval

This research project was approved by the Ethics Committee of Golestan University of Medical Sciences (IR.GOUMS.REC.1394.204).

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