

Original Article



Mutation analysis of exon 5 of *PAH* gene in phenylketonuria patients from Golestan Province, Iran

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Abstract

Background and aims: In the phenylalanine hydroxylase (*PAH*) gene, various mutations are mainly responsible for phenylketonuria (PKU). After thalassemia, PKU is considered as the most common autosomal recessive disease among Iranian population. Therefore, identifying the mutations that cause the disease in this population is of great importance. This study aimed to identify exon 5 mutations of *PAH* gene in PKU patients from Golestan, the northern province of Iran, and compare these mutations with those ones found by studies carried out in other parts of the country.

Methods: During a one-year period, 25 unrelated PKU patients aged 1-23 years and from different parts of Golestan province were included in the study. Then, their genomic DNAs were extracted from their blood samples and PCR-sequencing method was used to identify the mutations. The sequencing results were analyzed using Chromas and CLC Main Workbench v3.5 software.

Results: In this study, R158Q mutation was detected with a frequency of 6%. This mutation was homozygous in one PKU patient, but it was heterozygous in another one. These patients had cPKU phenotype.

Conclusion: Evaluation of mutations proved to be a useful technique for molecular diagnosis of PKU and identification of disease carriers in the given population. Taking into account the fact that only one exon of *PAH* gene was explored in this study, however, it is recommended that further studies be conducted to investigate other exons in order for obtaining the complete mutation spectrum of this gene in PKU patients in Golestan province.

Keywords: *PAH*, Exon, Mutation, Phenylketonuria

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Introduction

Phenylketonuria (PKU) is an autosomal recessive genetic disorder (1). This autosomal recessive disorder is caused by a defect in liver enzyme called phenylalanine hydroxylase (*PAH*). Elevated blood phenylalanine due to *PAH* deficiency may lead to severe and irreversible mental retardation in patients (2). Numerous cases of PKU with different prevalence have been reported from all around the world. The lowest and highest prevalence of the disorder have been recorded in the United Arab Emirates and Turkey in Mediterranean region, respectively. The prevalence of PKU also differs in different parts of Iran and its rate varies from 0.0015% to 0.02% in the north and south of the country, respectively (1). Hyperphenylalaninemia (HPA) is divided into four groups based on pre-treatment blood phenylalanine levels as follows: HPA (2-10 mg/dL), Mild PKU (10-15 mg/dL), Moderate PKU (15-20 mg/dL) and Classic PKU (more than 20 mg/dL) (3). Mutations of *PAH* gene mainly cause PKU (4). The *PAH* gene is about 90 kb in length and is located on chromosome 12 in the q22-q24.1 region; it also includes 13 exons and 12 introns (5). *PAH* protein has a tetrameric structure with several subunits, each of which is composed of three domains: N-terminal regulatory domain (residues 1-142), large

catalytic domain (residues 143, 410), and C-terminal domain which is responsible for tetramerization (residues 411, 452) (6). The main cause of PKU is a wide range of mutations in the *PAH* gene (7). About 1180 bi-allelic variants have been identified in the *PAH* gene (8). Numerous studies have shown that the spectrum of PKU mutations varies among different populations (9). PKU is a very heterogeneous disease and more than 20 mutations related to PKU have been already identified among different study populations showing distinct frequency and distribution regarding these mutations (4). The R158Q mutation is a missense mutation that occurs in exon 5 of *PAH* gene. Bonyadi et al reported that the frequency of this mutation in the PKU population of Iran was 2.3% (10). Razipour et al also found a frequency of 1.33% for the above mutation (11). According to a study conducted in Guilan province of Iran, this mutation showed a frequency of 4% for the PKU population (12). Shirzad et al also examined 635 PKU cases from different regions of Iran and identified this mutation in eight cases (13). Taking into consideration the fact that one or more specific mutations may be common in each population, evaluating the common mutations in molecular detection proves more economical in terms of time and cost (4).

Therefore, identification of common mutations in each region is necessary to facilitate prenatal diagnosis and genetic counseling (14). This study aimed to investigate mutations of exon 5 of *PAH* gene by sequencing method in PKU patients in Golestan province, and compare the given mutations with those ones found by studies from other regions of Iran.

Materials and Methods

Patients

In this cross-sectional descriptive study, 25 unrelated patients with HPA (12 females and 12 males) from different parts of Golestan, the northern province of Iran, were examined within a one-year period (i.e., the year 2016). The patients were selected based on the profiles available in Taleghani Hospital in Gorgan, the capital city of the province. Patients and their families were then invited to participate in this study. After explaining the research method and objectives to the participants, they or their parents (if the patient was a child or mentally retarded) were requested to complete the consent form and questionnaire. It is noteworthy that only one patient from each family was included in the study (2). Then patients were divided into four groups based on pretreatment serum phenylalanine(Phe) levels: classic PKU, moderate PKU, mild PKU, and HPA. The sampling was performed after obtaining approval from ethics committee of Golestan University of Medical Sciences and Health Services (IR.GOUMS.REC.1394.204). As for blood sampling, 2-5 mL of blood was obtained from each patient; and in order to prevent blood clotting, 15 mL falcon tubes containing 300 μ L of 0.5 M EDTA were used as anticoagulants (2).

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using High Pure PCR Template Preparation Kit (Roche, Germany).

Polymerase chain reaction (PCR)

PCR was performed on a Thermal Cycler PCR System (ABI, USA). The primers used in this study were designed by employing Oligo 7 software. The sequence of primers used in this reaction is presented in Table 1. Primers were synthesized by Bioneer Co (South Korea).

The 50 μ L of PCR reaction mixture was prepared using Taq[™] PCR Mix, 2x kit (Biotechrrabbit, Germany) with the addition of genomic DNA, primer pair (pmol 20), and sterile water. The length of the final product from PCR reaction was 258 bp. The results of adjusting the PCR conditions for amplification of the target fragment were as follows: initial denaturation temperature of 94°C for 5

minutes; 30 reaction cycles; each cycle with denaturation temperature of 94°C for 30 seconds, annealing temperature of 65°C for 30 seconds, elongation temperature of 72°C for 30 seconds, and final elongation temperature of 72°C for 5 minutes (12).

Electrophoresis of PCR products

In order to evaluate the quality and amplification of the target piece, the reaction products (patient samples) along with 100 bp DNA marker (Ladder, BR0800201, Biotechrrabbit, Germany) were loaded and electrophoresed on 1% agarose gel.

Sequencing

In order to identify variants in amplified fragments, PCR products were sequenced. Sequencing was performed using ABI 3730 DNA Sequencer based on chain termination method.

Software

The sequencing results were analyzed using Chromas and CLC Main Workbench v3.5 software.

Results

Patient phenotype

In this study, 25 HPA patients referring to Taleghani Hospital in Gorgan were examined by a pediatric endocrinologist. These patients were in the 1-23 age range and their ethnic composition were as follows: Fars (20 patients, 80%), Turkmen (4 patients, 16%) and Lor (1 patient, 4%).

Results of electrophoresis of amplified fragments and their sequencing

Once the electrophoresis of PCR products was completed and the formation of a specific band with a length of 258 bp was ensured (Figure 1), the products were sequenced. Evaluating the nucleotide sequencing of exon 5 of the *PAH* gene and surrounding intronic regions in 25 PKU patients (50 alleles) revealed a missense mutation (R158Q) in exon 5. Out of 25 samples, two samples showed this mutation. As for this mutation, a change in codon 158 of this gene caused the conversion of CGG>CAG and change of arginine to glutamine. Out of 50 exon 5 alleles, no mutation was found in 47 alleles. Out of 50 evaluated alleles, R158Q (c.473G>A) mutation was detected in three alleles with a frequency of 6%. The characteristics of the patients with this mutation and the electropherogram of the R158Q mutation are shown in Table 2 and Figures 2 and 3, respectively.

Table 1. Primers Used in the Present Study

Primers name	Primers sequence	Tm	Length of primer	Product size
Exon5-F	5'-GTATAACCAAGGGAAGGAGACAT-3'	63	23	258 bp
Exon5-R	5'-GGGCAAGGGAGAAGCAGGCTA-3'	68	21	

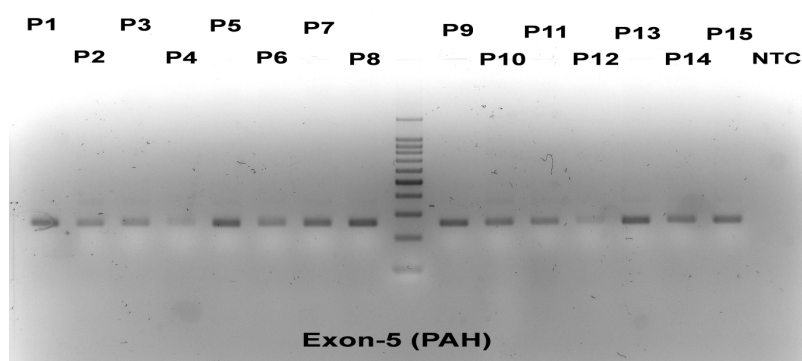


Figure 1. Results of electrophoresis of amplified fragments of 15 patient samples (258 bp) on 1% agarose gel, 100 bp marker size, non-template control (NTC).

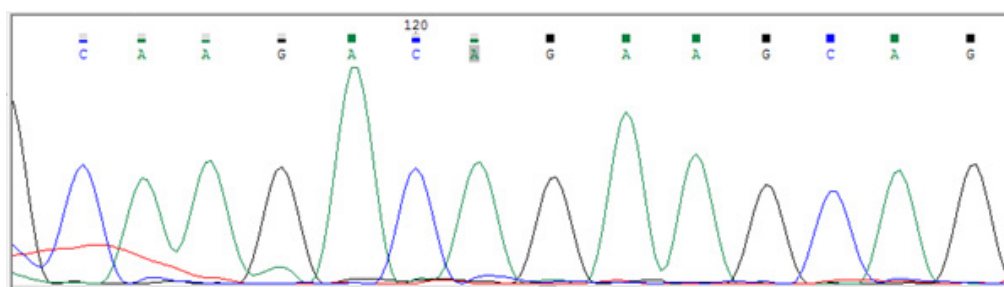


Figure 2. Electropherogram of R158Q mutation (CGG>CAG) in sample No. 8 (forward sequencing).

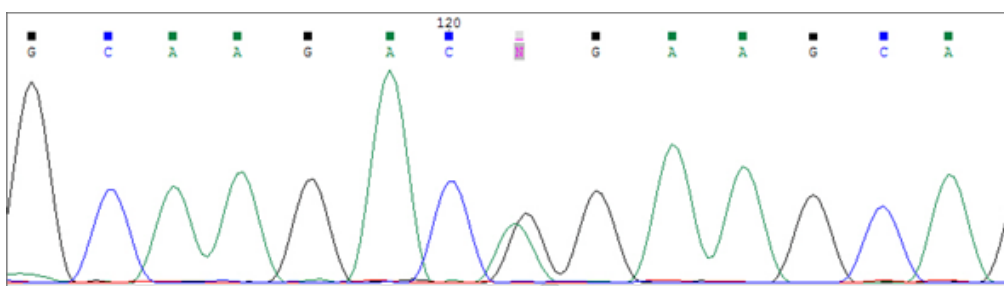


Figure 3. Electropherogram of R158Q mutation (CGG>CAG) in sample No. 17 in the heterozygous state (forward sequencing).

Discussion

Genetic diseases are undoubtedly responsible for major emotional and financial problems in human societies. The prevalence of PKU has led to neonatal screening and treatment with restricted diet as a common practice in many developed countries (2). Treatment limitations increase the need for a neonatal screening program so that at-risk couples can be identified with confidence. Therefore, it is necessary to explore the prevalence and spectrum of *PAH* mutations causing this disease in infected people from different geographical regions so that those at higher risk of the disease can be identified by genetic screening and timely medical interventions be applied (2).

In this study, exon 5 mutations and surrounding intronic regions were evaluated in 25 PKU patients in Golestan

province, and R158Q mutation was observed with a frequency of 6%. The R158Q mutation locating in the exon 5 of the *PAH* gene is a missense mutation. Bonyadi et al examined 44 Azeri Turkish patients and reported a frequency of 2.3% for R158Q mutation (10).

Razipour et al investigated the *PAH* locus and its mutation among Iranian population and identified various mutations. They found a frequency of 1.23% for the Arg158Gln (R158Q) mutation in exon 5 (11). Furthermore, Shirzad et al examined 635 PKU cases from different regions of Iran and detected several mutations including R158Q mutations in eight cases out of the total 635 cases (13). This mutation showed a frequency of 4% when it was explored by another study conducted on the PKU population of Guilan province (12). A study on 140 Iranian PKU patients by Shaykholeslam Esfahani

Table 2. the characteristics of patients with R158Q mutation in the present study

Patient	Parents	Age at diagnosis	Pretreatment Phenylalanine levels (mg/dL)	Phenotype	Ethnicity	R158Q mutation
8	Unrelated	One year of age	21.6	*cPKU	Fars	+/+
17	Related	Two weeks of age	46.9	cPKU	Fars	-/+

*Classic PKU, Homozygous: +/+, Heterozygous: +/-

and Vallian found 34 different mutations, including the R158Q mutation with frequency of 0.71% (15). Other studies investigating *PAH* mutation spectrum in PKU populations from different parts of Iran failed to report the R158Q mutation. These investigations included the studies by: Zare-Karizi et al (9), Biglari et al in Zanzan and Qazvin provinces (16), Alibakhshi et al in Hamadan and Lorestan provinces (17), as well as Jafarzadeh-Esfahani et al (2020) (1). Reports on the R158Q mutation in other parts of the world included the study by Song et al, which examined all *PAH* gene exons in 185 patients with PKU in north of China and reported 70 different mutations. Among the reported mutations, the frequency of R158Q mutation was determined to be 0.8% (18). Daniele et al (2009) also identified the sequence of the entire *PAH* gene in 51 HPA patients from south of Italy and found 32 different mutations, including the R158Q mutation (19). Baturina et al (2014) investigated mutations associated with HPA in *PAH* locus among 76 unrelated patients and their family members in Novosibirsk region of Russia. In the given study, 21 types of mutations were identified, including the R158Q mutation (4.67%) (20). Moreover, Yu et al examined 61 patients with PKU from Xinjiang, China and identified 30 different mutations in the *PAH* gene; R158Q (1.6%) was one of the mutations reported in their study (21).

Then Baturina and Morozov performed a comparative analysis on *PAH* mutation spectrum in Novosibirsk and Kemerovo regions in western Siberia, Russia and detected the R158Q mutation with a frequency of 4.32% and 2.7%, respectively (22). In our study, the R158Q mutation in PKU patients from Golestan province showed a relatively high frequency compared to that reported by other studies performed in Iran; however, the above mutation was only observed in 6% of chromosomes in our study. Obtaining the full spectrum of above gene mutations in patients with PKU in Golestan required examination of the other 12 exons. This study faced few limitations including the high length of *PAH* gene and the lack of sufficient funding for analyzing the mutation of the gene's total length (total exons). Therefore, only exon 5 and its surrounding intronic regions were examined in this study in order to identify the mutations.

Conclusion

Since there is a noticeable difference in the spectrum of *PAH* gene mutations in different regions of Iran, it is recommended that comprehensive studies be conducted in different regions of the country in order to identify specific mutations in each region and use them for developing future preventive and therapeutic measures.

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Authors' Contributions

MZ: collection of data, data analysis, explanation.

ZKK:investigational plan, data analysis, explanation, manuscript writing and final approval of the manuscript.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Approval

The patients or their parents signed an informed consent and formally approved their children's participation in the study. The study was approved by the Ethics Committee of Golestan University of Medical Sciences (IR.GOUMS.REC.1394.204).

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