

Molecular assessment and bioinformatic analysis of two common mutations of phenylalanine hydroxylase (PAH) gene by HRM

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J Babol Univ Med Sci; 19(6); Jun 2017; PP: 42-9

Received: Jan 1st 2017, Revised: feb 22th 2017, Accepted: Apr 30th 2017.

ABSTRACT

BACKGROUND AND OBJECTIVE: Phenylketonuria (PKU) is the most prevalent inborn error of amino acid metabolism in the world and its incidence rate is increasing in Iran. The aim of the present study was to assess the efficiency of HRM technique as a fast and suitable method in identifying common mutations of phenylalanine hydroxylase gene including IVS10-11G>A and P281L in order to improve the early detection of the disease to prevent the occurrence of it.

METHODS: In this case-control study, 20 DNA samples including one sample with IVS10-11G>A mutation, one sample with P281L mutation and 18 control samples were extracted from peripheral blood collected in Medical Genetic Center of Isfahan and were genotyped with HRM technique. To validate the mutations, the mutant samples were genotyped using sequencing. Bioinformatic analyses were used for determining structural and functional effects of P281L mutation on the of PAH protein.

FINDINGS: HRM analysis identified IVS10-11G>A and P281L mutations with a sensitivity and specificity of 100% and the mutant and normal samples differentiated well in normalized and difference plots. Bioinformatic analyses demonstrated instability and pathological effects of PAH protein containing P281L mutation.

CONCLUSION: HRM is a simple and fast technique detecting the two IVS10-11G>A and P281L mutations with 100% sensitivity and specificity.

KEY WORDS: Phenylketonuria, Sensitivity, Specificity, Genotyping.

Please cite this article as follows:

Amir M, Emadi Baygi M, Vallian S, Nikpour P, Akhondi F. Molecular assessment and bioinformatic analysis of two common mutations of phenylalanine hydroxylase (PAH) gene by HRM. J Babol Univ Med Sci. 2017;19(6):42-9.

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Introduction

Phenylketonuria (PKU) is the most common congenital malformation of amino acid metabolism in the world and is caused by the deficiency of the phenylalanine hydroxylase (PAH) in liver. This defect results in the accumulation of phenylalanine and its metabolites in tissues and body fluids such as the brain, blood and urine in PKU patients (1) that one of the most important consequences is mental retardation (2). The PAH enzyme catalyzes the hydroxylation of L-Phe (L enantiomers = L) into L-Tyr (Tyrosine) (3). Its monomer consists of 452 amino acids. The human PAH gene is located on the long arm of chromosome 12 and contains 13 exons and 12 intron. The incidence of PKU disease varies depending on countries or regions. Countries such as Ireland and Turkey (4) have the highest prevalence, and countries such as Japan (5) and Finland (6) have the lowest incidence. Recent studies in Iran indicate a high incidence in Iranian population of about 1 in 4698 births (7).

High incidence of autosomal recessive hereditary diseases is due to familial marriages. More than 900 mutations are known in the PAH gene, introduced in the specific PAHvdb locus database. About 90% of mutations are spotted mutations (8). A study by Vallian et al. in Isfahan indicates a high prevalence of this disease (5%) among mentally retarded individuals, and according to reports, P281L and IVS10-11G> A mutations are common mutations in Isfahan. The mutation IVS10-11G> A (5030855rs) with the systematic name of c.1066-11G> A and the other name IVS10nt546g> a is the most common mutation in Mediterranean regions such as Turkey, Italy, Spain, Egypt (10, 9), and Iran in cities such as Azerbaijan, Khorasan, Semnan and Hamedan (11). The mutation P281L (5030851rs) has the systematic name of C.842C> T (12).

In studies by Zare-Karizi et al., the mutation IVS10-11G> A is the most common mutation in the Iranian population and the mutation P281L has a relatively high prevalence in Iranian population (11). High Resolution Melting (HRM) is a new method after PCR to detect known and unknown mutations, including PAH mutations. In this method, the target amplification is performed in the presence of a color attached to a two-string DNA in a closed tube during a PCR reaction, followed by starting the HRM phase and increasing the temperature from about 65 ° C to about 95 ° C. DNA is created. During the melting, only the color attached to the double strand DNA beams and the fluorescence

light is constantly detected by the optical system and appears as a graph. With the melting of DNA, molecules of color are separated from it, resulting in a decrease in the fluorescence intensity. The turning point of the corresponding graph is considered as the melting temperature (T_m) of the DNA. The DNA fragments of the same length, even if different in a nucleotide, will have different T_m (13). In the studies of Dobrowolski et al., HRM method was used to determine the genotype of PKU patients and with specificity and sensitivity 99% mutations were identified (14). Also, HRM analysis has been used to determine the genotype and scanning of other autosomal recessive diseases such as cystic fibrosis (15) and B thalassemia (16) as well as cancer (17). The aim of this study was to determine the specificity and sensitivity of the HRM method in identifying and determining the genotype of IVS10-11G> A and P281L mutations in the PAH gene.

Methods

DNA extraction from total blood: In this case-control study, 20 blood samples including two mutated samples containing IVS10-11G> A mutation and the other containing P281L mutation and 18 non-disease control samples from Isfahan Medical Center were collected and DNA samples were extracted by method of Miller et al. (18). Laboratory procedures were approved by the Ethics Council of Shahrekord University with the code of 272. A written informed consent was obtained from all subjects at Isfahan Medical Center.

Primer Design: To design the primers for the two mutations IVS10-11G> A and P281L, the first sequence of the PAH gene with its access number 008690NG was taken from the National Center for Biotechnology Information (NCBI) and, with the help of the Gene runner software (<http://www.generunner.com>) Version 4.7 primers were designed (Table 1) and the 123 bp sequence containing the mutations P281L and the 125 bp sequence of amplicon containing IVS10-11G> A mutations were amplified using primers.

PCR reaction: PCR reaction at a volume of 25 µL in the presence of a combination of: 2.5 µL of the buffer x10, 0.5 µL of dNTP (Deoxynucleotide triphosphate) (40 mM), 75 µL/. MgCl₂ (100 mM) from each primer (10 pmol/µlit), 0.1 µL, 0.25 µL of Taq DNA polymerase (5 unit/µlit), 3 µL of DNA (200 ng/µL) and 17 µL of deionized water. PCR proliferation by TC-XP-G and during initial denaturation at 95°C for 5 minutes and then 35 cycles including denaturation at 95°C for 30

seconds, coupling step at 59 °C for P281L mutation and a temperature of 57 °C for IVS10-11G> A mutation for 30 seconds, the expansion step was carried out at a temperature of 72 °C for 30 seconds and the end extension at 72 °C for 5 minutes were done. In order to

obtain the appropriate temperature, the binding for the specific proliferation of the desired component for the P281L mutation was applied from a slope of 57° C to 59 °C, and for the IVS10-11G> A mutation, a slope of 54 °C to 57 °C was used in PCR.

Table 1. Sequence of primers for mutations P281L and IVS10-11G> A

The name of oligo	Sequence	Piece size
PAHP281L-Forward	GGATCCAAGCCCATGTATAC-3'-5'	123bp
PAHP281L-Reverse	GACCAGCCAGCAATGAAC -3'-5'	
PAHIVS10-11-Forward	GGATGCAGCAGGGAATAC -3'-5'	125bp
PAHIVS10-11-Reverse	TTGGATGGCTGTCTTCTC -3'-5'	

Detection of DNA molecules on agarose gel: 1% agarose gel was used to ensure the correct reproduction of the desired DNA samples.

HRM Response: For HRM, the Type It Kit (Germany Qiagen) was used and performed on the 6000 Gene Router (Qiagen, Hilden, Germany). The HRM kit contains PCR master, which includes: HotStarTaqPlus DNA Polymer, PCR Buffer with EvaGreen color, Q solution and dNTP (dATP, dCTP, dGTP, dTTP).HRM reaction in a volume of 10 µL and with a combination of materials: 5 µL DNA master, 5 µL. HRM template (200 ng / µL), 3 µL. of each primer (10 pmol / µlit) and 9.3 µL RNase-free water were performed.Prior to the HRM process, the PCR reaction was performed in the presence of a color attached to a double-strand DNA, in which the initial denturization step was performed at 95 ° C for 5 minutes, then 40 cycles including 95 C for 10 seconds, 59 C for P281L mutation and 57 C for mutation of IVS10-11G> A for 30 seconds and 72 C for 10 seconds followed by HRM stage and temperature rise from low to high temperature for both mutations from temperature78 C to 95 C.

Sequencing: Sequence is the final method and the gold standard for the detection of mutations. Therefore, sequencing was used to confirm the presence of mutations in mutated specimens. For sequencing, the ABI3500 (Life Technologies, Foster City, CA, USA) device and the chaining method were used. Bioinformatics studies: Bioinformatics tools provide computational predictions about the structural and functional effects of protein sequence changes.

In this study, we used bioinformatics methods to investigate the effects of P281L mutation on the structure and function of PAH protein, and various databases and tools including I-Mutant, PROVEAN, SNP & GO, PhD-SNP, PANTHER, NetSurfP, HOPE and I- TASSER were used as described in the study of Akhoundi et al. (19).

Results

The results of PCR in Agarose Gel: In this study, after extraction of DNA samples and amplification of them, a non-smear bond was observed in the gel and the confidence of the reproduction was obtained. After

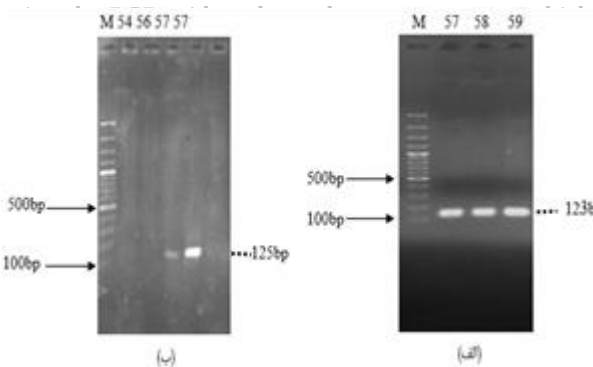


Figure 1. The results of electrophoresis of a 123-bp multiplication component related to the P281L mutation (A) and 125 bp related to the IVS10-11G> A mutation (B) in the PAH gene with the desired temperature slope. No. 1 well (M) The molecular weight marker (GeneRuler 100 bp DNA Ladder) and no. 2, 3 and 4 wells show the result of fragment proliferation at the desired temperature. In fig. A, a better bond was observed at 59 ° C and in band B, only bands were observed at 57 ° C.

HRM reaction and sequencing: HRM reaction was performed to determine the genotype of 20 DNA samples and DNA replication was performed in 40 cycles. Using the 6000 Gene Router software version 2/0/2 normalized graphs, difference graph and melt curve analysis were depicted. Homozygote samples are distinct from normal samples based on the difference in the melting point, and the heterozygote samples are differentiated according to the shape of the curve. In the analysis of the melting curve, the difference in the single strand DNA pattern, which is completely specific for different DNA fragments was shown (Fig. 2).

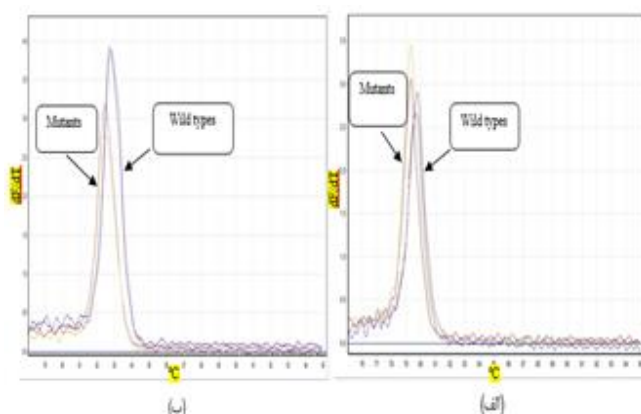


Figure 2. The melting curve of the data relates to the mutations IVS10-11G> A (a) and P281L (b). As shown, the melting temperature of the normal and mutated sample is different. Red and mustard colors graphs of mutated samples and blue and violet colors show normal sample diagrams.

In the naturalization diagram (Fig. 3), which shows only the temperature range in the melting stage, the initial fluorescence was 100% and the residual fluorescence after DNA separation was zero. In the differentiation diagram (Figure 4), which provides a better representation of the small differences between the melting curves of each individual, and each genotype can be selected as a reference that usually is a normal sample. A normal sample was used as control, and the separation of sample with PAH mutation from a normal sample was easily accomplished. To confirm the genotype of the mutated specimens, sequencing was performed to confirm that the mutations were listed in the samples (Fig. 5)

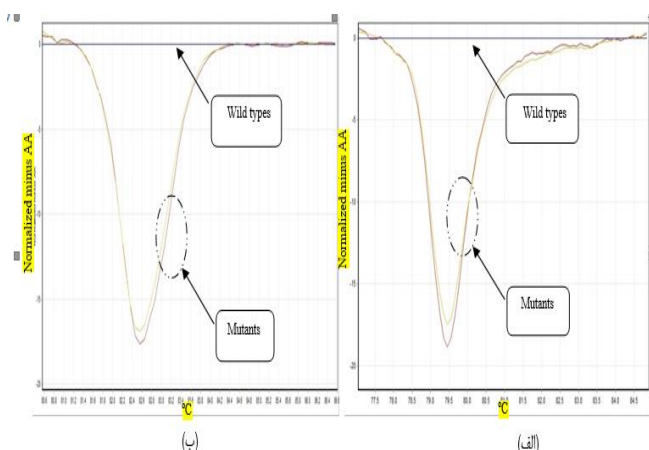


Figure 3. Naturalization diagram of normal and mutant samples related to mutations IVS10-11G> A (a) and P281L (b). In this type of graph, normal and mutated samples were shown to be separated. Red and mustard colors indicate mutated samples and blue and violet colors indicate normal samples.

The results of bioinformatics studies: By using different bioinformatics databases, instability and pathogenicity of PAH protein containing P281L mutation were shown and the results of bioinformatics (In silico) and experimental (in vivo) were obtained. Overall, the HRM method, with 100% specificity and sensitivity, was able to distinguish PKU subjects from the two mutants of P281L and IVS10-11G> A from other individuals.

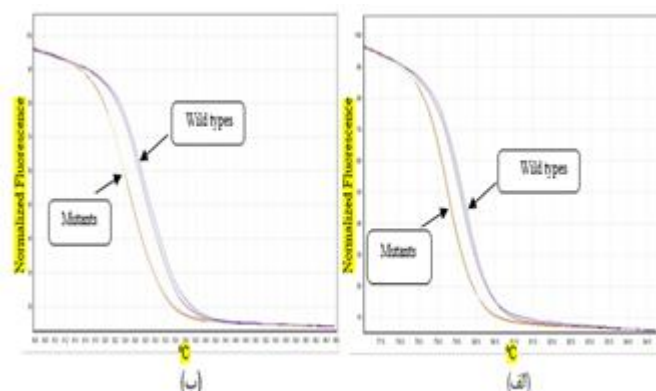


Figure 4. Differentiation of mutated specimens from normal using differentiation diagram of the mutations IVS10-11G> A (a) and P281L (b). As shown in the figure, this type of graph makes it possible to distinguish mutated homozygous samples from the normal sample. Graphs with red and mustard colors indicate mutated samples and blue and violet colors show normal sample diagrams.

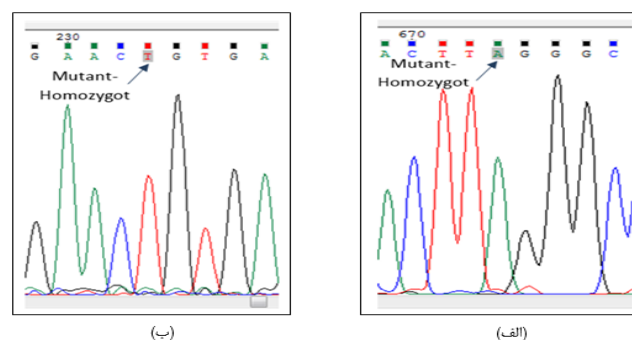


Figure 5. Results from sequencing for IVS10-11G> A (a) and P281L (b) mutations. In Fig. A, nucleotide G has been transformed into A and in Fig. B, nucleotide C has been transformed into T.

Discussion

In the present study, HRM method was able to identify two mutations IVS10-11G> A and P281L with 100% specificity and sensitivity. Investigations that are clearly mutated by using the HRM method to determine

the genotype of these two mutations are very limited. In this regard, Dobrowolski et al. identified 95.5% of the mutated alleles of the PAH gene in 67 patients with PKU using HRM. The main reason for failure to identify 100% of PAH mutations in this study was the inability of this method to identify deletion mutations (9).

However, for the molecular diagnosis of these two mutations, other methods have also been used. For example, sequencing was used to identify mutations IVS10-11G> A and P281L as common mutations in Khorasan Razavi (20). Also, to identify these mutations in Turkey, DHPLC method was first used and sequencing was used (21).

SSCP method and sequencing in northern China (22) and DGGE, RFLP and sequencing methods were used in Germany (23). The rate of diagnosis of the SSCP process is about 70%, but the main problem is the lack of recognition of many mutations due to the lack of effect of many on the mutated field. As a result, there is no difference in the pattern of migration of the pieces. The ability to detect the DGGE method is above 95%, but its method is complex and requires relatively sophisticated equipment.

The ability to diagnose DHPLC is higher than 96%. The disadvantages of this are that the optimization of the method, although short, requires the direct entry of the operator, which impedes automation. In addition, some of the components have several second melts that are easily ignored by the empirical melting temperature selection method. In RFLP, the limiting parts are separated by electrophoresis.

The disadvantages of this technique are the need to create or eliminate a site where the restriction enzyme is diagnosed. In addition, some limiting enzymes are expensive. The sequencing method is the final method and the gold standard for the diagnosis of mutations that determines genotype and screening simultaneously. Its disadvantages are that it costs a lot, and PCR products need to be purified (25, 24).

Other mentioned methods except HRM method are expensive and time consuming and have the risk of contamination, they also need to isolate the sample on a gel or matrix, additional processing, and enzymatic or chemical reactions. Each process increases the risk of contamination in future reactions as the PCR products are exposed to the environment. But the HRM method is quicker and more cost effective than other methods, and it has a lower risk of contamination, because it is done in a closed tube; it does not need a matrix and a spatula; therefore, it can be compared to the other

method preferably (24). The HRM method is used to find a single nucleotide variant of sequence such as single nucleotide polymorphism (26), to determine the genotype of the probe without the label (27) and the conformance of HLA (Human Leukocyte Antigen) (28).

The main limitation of the HRM method is that the exact nature of the mutation is not easily identifiable, so if necessary, it should be used in conjunction with the sequencing method. Other disadvantages are limitation during amplicon, but in general, HRM analysis has several advantages including reducing human resources, saving time and reducing the risk of contamination than the above mentioned methods (24). In studies by Okano et al. and Dworniczak et al., the analysis of the expression of mutant alleles in mammalian cultured cells indicated the absence of PAH in transfected cells with this mutation and the absence of PAH activity in transfected mutated alleles (30, 29). However, according to these studies, the protein that has this mutation has a functional expression and little activity, but in the present study, the purpose of *In silico* analysis was to show that this nucleotide change at the DNA level affects protein stability, its free energy change, the availability of wild-type amino acids and mutated to solvent (protein biophysical properties). In addition, the effect of these biophysical effects on the pathogenicity of modified protein has been estimated, which is well in agreement with the experimental findings.

Regarding the functional effect of IVS10-11G> A on the PAH protein, it has also been shown that although the content of liver PAH in homozygous patients is normal, it does not have any catalytic activity.

This lack of enzyme activity is most likely due to changes in the configuration caused by the presence of three additional amino acids (Gly, Leu, Gln) between the normal sequences encoded by exons 10 and exons 11 (31).

With the exception of sequencing method as the gold standard, based on studies on the advantages and disadvantages of mutation identification methods focusing on PAH gene mutations in PKU, it can be concluded that the HRM method identifies mutations with at least 96% specificity and sensitivity and with less time and risk of contamination; however, in this study due to the nature of mutations, this method was able to identify IVS10-11G> A and P281L mutations in the PAH gene with 100% specificity and sensitivity.

Acknowledgment

We would like to thank Vice-Chancellor for Research and Technology of Shahrekord University for

the financial support of this research, as well as the cooperation and assistance of Mr. Farhad Bani Mehdi.

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