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Brief Report

# Spectrum of Phenylalanine Hydroxylase Gene Mutations in Hamadan and Lorestan Provinces of Iran and Their Associations with Variable Number of Tandem Repeat Alleles

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## Abstract

Phenylketonuria (PKU) is one of the most common known inherited metabolic diseases. The present study aimed to investigate the status of molecular defects in phenylalanine hydroxylase (*PAH*) gene in western Iranian PKU patients (predominantly from Kermanshah, Hamadan, and Lorestan provinces) during 2014-2016. Additionally, the results were compared with similar studies in Iran. Nucleotide sequence analysis of all 13 exons and their flanking intronic regions of the *PAH* gene was performed in 18 western Iranian PKU patients. Moreover, a variable number of tandem repeat (VNTR) located in the *PAH* gene was studied. The results revealed a mutational spectrum encompassing 11 distinct mutations distributed along the *PAH* gene sequence on 34 of the 36 mutant alleles (diagnostic efficiency of 94.4%). Also, four *PAH* VNTR alleles (with repeats of 3, 7, 8 and 9) were detected. The three most frequent mutations were IVS9+5G>A, IVS7-5T>C, and p.P281L with the frequency of 27.8%, 11%, and 11%, respectively. The results showed that there is not only a consanguineous relation, but also a difference in *PAH* characters of mutations between Kermanshah and the other two parts of western Iran (Hamadan and Lorestan). Also, it seems that the spectrum of mutations in western Iran is relatively distinct from other parts of the country, suggesting that this region might be a special *PAH* gene distribution region. Moreover, our findings can be useful in the identification of genotype to phenotype relationship in patients, and provide future abilities for confirmatory diagnostic testing, prognosis, and predict the severity of PKU patients.

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**Keywords** ● Phenylketonurias ● Phenylalanine hydroxylase ● Variable number of tandem repeats ● Iran

## What's Known

- Phenylketonuria (PKU) is one of the most common of the 300 inherited metabolic diseases that causes microcephaly, motor disorder, seizures, intellectual disability, skin rashes, and other symptoms.
- Phenylketonuria is caused predominantly by mutations in the phenylalanine hydroxylase *(PAH)* gene.

## What's New

- It is the first study on PKU patients in Hamadan and Lorestan provinces. A high degree of heterogeneity of PKU mutations in western Iran is confirmed.
- It is the first report on the association of the novel mutation IVS7-5T>C with VNTR 8.

## Introduction

Phenylketonuria (PKU), with an average incidence of 1:10,000 in Caucasians, is one of the most common inherited metabolic diseases. PKU results from a deficiency of the enzyme phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1). *PAH* catalyzes the irreversible conversion of phenylalanine (Phe) to tyrosine in the presence of $BH_4$, the cofactor of the *PAH* enzyme.[1] This

enzyme assembles into homotetramers with each subunit, consisting of three domains: an N-terminal regulatory domain, a large catalytic domain, and a C-terminal domain.[2]

According to the data on plasma Phe concentration, prior to switching to the Phe restriction diet, PKU patients are classified as having a classical PKU, mild PKU, or mHPA with levels of 1,200 or more, 600-1,200 µmol/l, and less than 600 µmol/l, respectively.[1]

Phenylketonuria is caused predominantly by mutations in the phenylalanine hydroxylase (*PAH*) gene. The *PAH* locus database (http://www.pahdb.mcgill.ca) lists more than 800 *PAH* mutations today, including missense and nonsense mutations, small and large deletions, small insertions, and splicing defects. This gene is located on chromosome 12 in humans. The cDNA sequence contains 13 exons and encodes 452 amino acids.

*PAH* has a rich repertoire of RFLPs and polymorphic markers including a variable number of tandem repeat (VNTR) of 30-bp cassettes harboring at least 10 alleles (differing by the number of repeats), 3 kb downstream from the last exon in *PAH*.[3]

A comprehensive analysis of mutations of the *PAH* gene in 18 western Iranian PKU patients (predominantly from Kermanshah, Hamadan, and Lorestan provinces) was performed and the results were compared with similar studies in Iran.[4-9] Additionally, the association of the identified mutations with *PAH* VNTR alleles was examined.

Kermanshah province is located in the central-western region of Iran. It has borders to the west with Iraq and to the north, east, and south with Kurdistan, Hamadan, Lorestan, and Ilam provinces, respectively. The results of *PAH* gene mutations describing the genotypes of PKU disease in Kermanshah province have already been published.[7] To our knowledge, this is the first study on PKU patients from Hamadan and Lorestan provinces.

## Patients and Methods

The present study was conducted over a period of 3 years (2014-2016) at the Medical Genetics Laboratory, Kermanshah University of Medical Sciences, Kermanshah, Iran. After ruling out patients who were suffering from $BH_4$ deficiency, 18 unrelated PKU patients from Kermanshah, Hamadan, and Lorestan provinces were tested. Consanguinity among parents was proven in 67% of patients. The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (project code: 89184) and a written informed consent was obtained from all patients.

Genomic DNA was collected from peripheral whole blood with a QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions. PCR amplification was performed using a GeneAmp PCR system 9700 (Applied Biosystems, USA). PCR conditions were as follows: an initial denaturation at 95 °C for 5 min (each cycle), denaturation at 94 °C for 30 sec, annealing at 56-60 °C (depending on primers' Tm) for 30 sec, elongation at 72 °C for 45 sec, and 30 cycles of the final elongation at 72 °C for 7 min. The agarose gel was stained with a green dye for visualizing the fragment migration. The primer sequences can be provided upon request. For the amplification of *PAH* VNTR marker, a set of primers including 5'-GCTTGAAACTTGAAAGTTGC-3' (as forward primer) and 5'-GGAAACTTAAGAATCCCATC-3' (as reverse primer) were used.

PCR products were purified using QIAquick PCR purification kit. Then, the samples were precipitated with ethanol-sodium acetate precipitation and used for cycle sequencing. For sequencing analysis, samples were analyzed by direct sequencing of all 13 exons of the *PAH* gene and their flanking introns in an ABI-3130 DNA analyzer (Applied Biosystems, USA). PCR products of the *PAH* VNTR alleles were loaded on a 2% agarose gel and visualized by staining with the green dye. Since the amplified products of VNTR may differ by their lengths, a 100-bp DNA-marker was used.

## Results

The results revealed a mutational spectrum, encompassing 11 distinct mutations distributed along the *PAH* gene sequence on 34 of the 36 mutant alleles (diagnostic efficiency of 94.4%) (table 1). In addition, 11 polymorphisms and four *PAH* VNTR alleles (with repeats of 3, 7, 8, and 9) were detected (table 2).

Of the 18 unrelated families studied, 13 (72%) were homozygous (12 of them belonging to consanguineous families), 3 (17%) were compound heterozygous, and 2 (11%) were heterozygous (only one mutant allele identified). The greatest prevalence of *PAH* disease-causing mutations, in decreasing frequency, were found in exons 9, 7, 8, 2, 11, 6, or their exon-flanking intronic sequences. No mutation was detected in exons 1, 3, 4, 5, 10, 12, and 13 or their exon-flanking intronic sequences. The majority of the mutations were located in the catalytic domain and intronic regions.

PCR products of the *PAH* VNTR alleles produced fragments with 364, 584, 514, and 544. They were corresponding to the presence of

**Table 1:** The identified *PAH* gene mutations and comparison with other studies in Iran

| Mutation name | Systematic name | Allele frequencies (current study) | | | | West Azerbaijan (80 alleles)[9] | Khorasan Razavi (62 alleles)[4] | Qazvin/Zanjan (78 alleles)[6] | EastAzerbaijan (88 alleles)[5] | Kermanshah (54 alleles)[7] | Khuzestan (80 alleles)[8] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Hamadan | Lorestan | Kermanshah | Total | | | | | | |
| IVS9+5G>A | c. 969+5G>A | 4/12 | 2/14 | 4/10 | 10/36 (27.8%) | | | 2.56% | | 17% | |
| IVS7-5T>C | c. 843-5T>C | | 4/14 | | 4/36 (11%) | | | | | 7.4% | |
| p.P281L | c. 842C>T | 2/12 | 2/14 | | 4/36 (11%) | | 12.9% | 10.25% | 19.3% | | |
| IVS8-7A>G | c. 913-7A>G | | 1/14 | 2/10 | 3/36 (8.3%) | | | | | 1.8% | |
| IVS2+5G>C | c. 168+5G>C | 2/12 | 1/14 | | 3/36 (8.3%) | | | 2.56% | 3.4% | 26% | |
| IVS10-11G>A | c. 1066-11G>A | 2/12 | | | 2/36 (5.6%) | 35% | 19.35% | | 19.3% | 7.4% | 10% |
| p.K363>Nfs | c. 1089delG | | | 2/10 | 2/36 (5.6%) | | | | | 7.4% | |
| p.R261Q | c. 781G>A | | | 2/10 | 2/36 (5.6%) | 18.75% | | 2.56% | 5.7% | 1.8% | 2.5% |
| p.R176X | c. 526C>T | | 2/14 | | 2/36 (5.6%) | | 9.7% | 10.25% | | 3.7% | 2.5% |
| p.F39delTTC | c. 115_117delTTC | 1/12 | | | 1/36 (2.8%) | | | | | | |
| p.A300S | c. 898G>T | 1/12 | | | 1/36 (2.8%) | | | | | | |

*PAH*: Phenylalanine hydroxylase

alleles with 3, 7, 8, and 9 copies of the repeated units, respectively (table 2).

## Discussion

The *PAH* mutation profile was obtained for 34 of 36 (94.4%) western Iranian PKU chromosomes (table 1). The three most frequent mutations were IVS9+5G>A, IVS7-5T>C, and p.P281L with the frequency of 27.8%, 11%, and 11%, respectively. Eight other mutations, including IVS8-7A>G, IVS2+5G>C, IVS10-11G>A, p.K363>Nfs; p.R261Q, p.R176X, p.F39delTTC, and p.A300S represented 44.6 % of the total PKU chromosomes. The genotypes of 100% of chromosomes from Kermanshah and Hamadan provinces (10/10 and 12/12, respectively) and 85.7% from Lorestan province (12/14) were identified. The most frequent PKU allele was IVS9+5G>A in Kermanshah and Hamadan (4/10 and 4/12, respectively) and IVS7-5T>C in Lorestan (4/14). Genotypes and phenotypes of the patients participated in the study are shown in table 2.

The most frequent mutation among our patients, IVS9+5G>A (28.7 %), was first identified in a mild HPA Turkish patient combined with the mutation p.A300S (a mutation causing mHPA).[7] It was recently found in an Iranian PKU patient in compound heterozygous form (IVS10-11G>A/IVS9+5G>A) with the unreported phenotype.[10] Also, Biglari et al. reported this mutation in patients from Qazvin and Zanjan provinces with the frequency of 2.56% (2/78).[6] According to our previous study,[7] IVS9+5G>A is the second most common mutation among PKU patients from Kermanshah province (table 1). Therefore, it seems that this mutation is one of the most prevalent mutations in western Iran. Our results showed that the IVS9+5G>A mutation is strongly linked to VNTR8 (table 2). Following a search in academic literature and the *PAH* locus database (http://www.pahdb.mcgill.ca), we did not find any reports on the association between IVS9+5G>A mutation and *PAH* VNTR.

IVS7-5T>C accompanied by p.P281L constitutes 22% of causing mutations (11% for each of them). IVS7-5T>C in intron 7 changes the fifth nucleotide in the acceptor splice site and is a novel mutation that was reported in our previous article.[7] The association of this mutation with VNTR8 is proven in the present study. Either of the severe or moderate phenotypes are associated with the mutation P281L in homozygous state. The PAH enzymatic activity with the p.P281L missense mutation is known to be <1%.[11] This mutation has previously been reported as the third most common mutation

**Table 2:** Distributional genotypes in 18 PKU patients

| Patient # | Genotype | Polymorphism (s) | Class | VNTR |
|---|---|---|---|---|
| 2 | IVS9+5G>A/IVS9+5G>A | –71A>C/–71A>C and IVS1+62C>T/IVS1+62C>T | NA* | 8 |
| 3 | | | NA* | |
| 10 | | | mPKU | |
| 14 | | | cPKU | |
| 17 | | | NA* | |
| 1 | IVS10-11G>A/IVS10-11G>A | IVS1+62C>T/IVS1+62C>T and IVS2+19T>C/IVS2+19T>C | cPKU | 7 |
| 4 | IVS2+5G>C/IVS2+5G>C | | NA* | 9 |
| 5 | P.A300S/p.F39delTTC | IVS5-54G>A/--- and IVS1+62C>T/----- | cPKU | NI[K] |
| 6 | P.P281L/p.P281L | IVS5-54G>A/IVS5-54G>A | cPKU | NI[K] |
| 7 | P.P281L/p.P281L | IVS5-54G>A/IVS5-54G>A, V245V/V245V, and IVS9+43G>T/IVS9+43G>T | NA* | NI[K] |
| 8 | IVS7-5T>C/p.R176X | IVS9+43G>T/----, IVS4+47C>T/----, and IVS5-54G>A/--- | cPKU | 8 |
| 9 | IVS7-5T>C/IVS7-5T>C | –81C>T/-81C>T and IVS1+62C>T/IVS1+62C>T | cPKU | 8 |
| 11 | P.R176X/---- | IVS1+62C>T/----, p.Q232Q/----, and V245V/---- | mHPA | 3, 8 |
| 12 | IVS2+5G>C/IVS7-5T>C | –81C>T/---, IVS1+62C>T/IVS1+62C>T, IVS2+19T>C/----, and IVS12-35C>T/---- | mPKU | 8, 9 |
| 13 | IVS8-7A>G/--- | –71A>C/-71A>C, IVS1+62C>T/IVS1+62C>T, Q232Q/----, IVS9+43G>T/IVS9+43G>T, and p.L385L/---- | cPKU | 7, 8 |
| 15 | P.R261Q/p.R261Q | IVS9+43G>T/IVS9+43G>T | mPKU | NI[K] |
| 16 | IVS8-7A>G/IVS8-7A>G | | NA* | 7 |
| 18 | P.K363>Nfs/p.K363>Nfs | IVS1+62C>T/IVS1+62C>T | cPKU | 8 |

*NA: Not available; [K]NI: Not identified; PKU: Phenylketonuria

among Iranian PKU patients[10] and as the second major PKU-causing mutation in patients from East Azerbaijan[5] and Khorasan Razavi[4] provinces (table 1). The two homozygote patients, carrying p.P281L mutation in our study, were from Hamadan and Lorestan provinces. One of the patients (#6) had a classic phenotype (c.PKU), but the associated phenotype for the other patient (#7) could not be defined (table 2).

IVS2+5G>C, the most frequent mutation in Kermanshah,[7] was detected in a PKU patient from Hamadan province (in homozygous form) and in a PKU patient from Lorestan province (in heterozygous form) (table 2). There are some reports of this mutation in Iranian studies, including Biglari et al.[6] and Bonyadi et al.[5] In the present study, the IVS2+5G>C mutation was strongly associated with VNTR9, which is in line with the results of a study by Zschocke and Hoffman.[12] The IVS10-11G>A mutation, a mutation common in parts of Southern Europe, the Mediterranean countries, and Iran,[13] is probably an ancient mutation that originated long before the end of the last ice age and separated into different alleles early in prehistory.[12] According to Zare-Karizi et al.,[10] the frequency of this mutation is higher throughout the northern part of Iran (including Khorasan, Semnan, Tehran, Markazi, Hamadan, and Azarbaijan provinces). In the present study, we found IVS10-11G>A in one PKU patient from Hamadan province, which is in line with the results of Zare-Karizi et al.

In addition to mutations discussed above, we also found six other mutations (IVS8-7A>G, p.K363>Nfs, p.R261Q, p.R176X, p.F39delTTC, and p.A300S). p.K363>Nfs, a mutation with a relatively high frequency among Romanian patients, has also been found among American, German, Italian, Kuwaiti, Turkish, and Iranian patients.[7]

As shown in table 2, some associations between mutations and polymorphisms in cis were observed including IVS9+5G>A with -71A>C and IVS1+62C>T; IVS10-11G>A with IVS1+62C>T and IVS2+19T>C; p.P281L with IVS5-54G>A, V245V, and IVS9+43G>T; IVS7-5 T>C with -81C>T and IVS1+62C>T; p.R261Q with IVS9+43G>T; and p.K363>Nfs with IVS1+62C>T.

It is well known that different ethnic groups have their own distinctive and diverse *PAH* mutant allele series that include one or a few prevalent founder alleles.[12] According to previous studies in Iran, the prevalent mutations in different parts of Iran were IVS10-11G>A, P281L, p.S67P, and p.R261Q in the northwest;[5,6,9] IVS10-11G>A in the southwest;[8] and IVS10-11G>A, p.P281L, and p.R176X in the northeast.[4] Therefore, as stated by Zare-Karizi et al., the IVS10-11G>A is the most prevalent mutation among the Iranian PKU patients. On the other hand, according to our current and past[7] studies, IVS2+5G>C and IVS9+5G>A are much more frequent in the western region of Iran. The unexpected

high-frequency of these two mutations might be explained by consanguinity and other factors (e.g. genetic drift) operating on this population. According to these results along with the spectrum and frequencies of mutations detected in western Iran in comparison to the other parts of the country (table 1), it seems that this region might be a special *PAH* gene distribution region.

The association of *PAH* gene mutations with a VNTR located at the 3´ end of the gene has been investigated frequently by researchers (http://www.pahdb.mcgill.ca). As a suitable informative tool, an investigation of this association could help us in carrier screening and prenatal diagnosis in PKU families. More than ten alleles of this 30-bp tandem repeats have been reported in the literature.[14] Since there was only one comprehensive study regarding the above-mentioned association in Iran,[15] an adequate comparison with our results could not be made.

## Conclusion

The results of the present study confirm a high degree of heterogeneity of PKU mutations in western Iran. It is also shown that there is not only a consanguineous relation, but also a difference in *PAH* characters of mutations between Kermanshah and the two other provinces of western Iran (Hamadan and Lorestan). Moreover, our findings can be useful in the identification of genotype to phenotype relationship in patients and provide future abilities for confirmatory diagnostic testing, prognosis, and predict the severity of PKU patients. Finally, because of a small sample size for each of the above-mentioned provinces, it is recommended to conduct future studies with a larger sample size.

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**Conflict of Interest:** None declared.

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