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Research Paper

The Association between Variable Number Tandem Repeat Alleles and Phenylalanine Hydroxylase Gene Mutations in Phenylketonuria Patients in the North of Iran

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Keywords:
IVS10-11G>A, Phenylalanine hydroxylase, Phenylketonuria, VNTR

ABSTRACT

Aims Phenylketonuria (PKU) is one of the essential causes of intellectual disability in infants, and its consequences can only be prevented through timely diagnosis and treatment. Furthermore, mutations in the phenylalanine hydroxylase (PAH) gene are the primary cause of PKU. On the other hand, the variable number tandem repeat (VNTR) is a polymorphic marker within the PAH gene, used to determine carriers and for prenatal diagnosis in PKU families. The present study was conducted to investigate the association between VNTR alleles and *PAH* gene mutations in patients with PKU in Northern Iran (Golestan and Guilan provinces).

Materials & Methods This descriptive cross-sectional study included 51 unrelated PKU patients from various areas of Golestan and Guilan provinces in northern Iran. After extracting genomic DNA from leukocytes, the VNTR-containing fragments of the *PAH* gene were assessed using the polymerase chain reaction (PCR)- sequencing method. Then, the association between the VNTR marker and the *PAH* gene mutations in patients with PKU in northern Iran was investigated.

Findings Our analysis showed the association between VNTR alleles and some *PAH* gene mutations, such as IVS10-11G>A, IVS11+1G>C, p.P416Hfs*36, and p.R408W, in the studied patients. A strong association was mainly observed between the most common mutation of PKU patients in Golestan province, i.e., IVS10-11G>A mutation and VNTR7 allele, which was reported as IVS10-11G>A-VNTR7 (19.2%), IVS11+1G>C-VNTR8 (7.7%), p.P416Hfs*36-VNTR9 (7.7%), and p. R408W-VNTR3 (3.8%).

Conclusion Based on the results of this study, it can be concluded that the most common mutation of PKU in Golestan province (IVS10-11G>A) is exclusively associated with the VNTR7 allele, and the VNTR7-IVS10-11G>A test should be considered for routine carrier screening and prenatal diagnostic settings.

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مقاله پژوهشی

ارتباط بین آلل‌های تعداد تکرارهای پشت سر هم متغیر و جهش‌های ژن فنیل آلانین هیدروکسیلاز در بیماران فنیل کتونوری در شمال ایران

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چکیده

هدف: فنیل کتونوری (PKU) یکی از علل مهم عقب ماندگی ذهنی نوزادان محسوب می‌شود و تنها در صورت تشخیص و درمان به‌موقع، عواقب آن قابل پیشگیری است. بعلاوه جهش‌های ژن فنیل آلانین هیدروکسیلاز (PAH) عامل اصلی فنیل کتونوری (PKU) هستند. از سوی دیگر تعداد تکرارهای پشت سر هم متغیر (VNTR)، یک مارکر چند شکلی درون ژن PAH است که در تعیین ناقلین و تشخیص قبل از تولد در خانواده‌های PKU مورد استفاده قرار می‌گیرد. هدف از این مطالعه بررسی همراهی آلل‌های VNTR و جهش‌های ژن PAH در افراد مبتلا به PKU در شمال ایران (دواستان گلستان و گیلان) بود.

مواد و روش‌ها: در این مطالعه توصیفی-مقطعی تعداد ۵۱ بیمار PKU و غیروابسته از نواحی مختلف دواستان درشمال ایران بررسی شد. پس از استخراج DNA ژنومی از لوکوسیت‌ها، قطعات حامل VNTR ژن PAH با استفاده از تکنیک‌های واکنش زنجیره‌ای پلی‌مراز (PCR) و تعیین توالی بررسی شد.سپس همراهی مارکر VNTR با جهش‌های ژن PAH در افراد مبتلا به PKU در شمال ایران مورد مطالعه قرار گرفت.

یافته‌ها: آنالیز ما همراهی بعضی جهش‌های ژن PAH نظیر IVS۱۰-۱۱G>A، IVS۱۱+۱G>C، p.P۴۱۶Hfs*۳۶ و p.R۴۰۸W رابا آلل‌های VNTR در بیماران مورد مطالعه نشان داد. به ویژه همراهی قوی بین شایع‌ترین جهش بیماران PKU در استان گلستان یعنی جهشIVS۱۰-۱۱G>A و آلل VNTR۷ مشاهده شد.که بصورت (۱۹/۲٪) VNTR۷-IVS۱۰-۱۱G>A، (۷/۷٪) VNTR۸-IVS۱۱+۱G>C، (۷.۷٪) VNTR۹-p.P۴۱۶Hfs*۳۶ و(۳/۸٪) VNTR۳-p.R۴۰۸W گزارش شد.

نتیجه‌گیری: از نتایج این مطالعه می‌توان نتیجه‌گیری کرد که شایع‌ترین موتاسیون PKU در استان گلستان، IVS۱۰-۱۱G>A منحصرا با آلل VNTR۷ مرتبط بوده و تست VNTR۷- IVS۱۰-۱۱G>A برای غربالگری معمول ناقلین و تشخیص قبل از تولد باید مورد توجه قرار گیرد.

کلیدواژه‌ها:
IVS10-11G>A
فنیل آلانین هیدروکسیلاز
فنیل کتونوری
VNTR

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Introduction

Hyperphenylalaninemia (HPA) is a group of hereditary diseases characterized by high levels of phenylalanine (Phe) in the blood[1]. Phenylketonuria (PKU) is a rare inherited chronic untreatable disorder associated with intellectual disability and neurological abnormalities [2] caused by the deficiency of the enzyme Phe hydroxylase (*PAH*). This enzyme plays a crucial role in converting the amino acid Phe to tyrosine. Without treatment, the accumulation of Phe can lead to irreversible consequences, such as mental disability [1]. *PAH* gene mutations are the leading cause of PKU [3]. This gene is located on chromosome 12 with a length of 171 kilobases (90 kilobases without lateral regions), has 13 exons, and encodes a polypeptide of 452 amino acids [1,3]. So far, more than 1000 variants of the *PAH* gene have been identified in patients with PKU [4]. The prevalence of this genetic disorder in Caucasians is 1 in 10,000 births; however, it is significantly higher in the eastern Mediterranean region. In fact, the highest prevalence of PKU has been reported in this region of the world: 1 in 4000 in Turkey and 1 in 3627 in Iran. This high prevalence can primarily be associated with the region's high rate of consanguineous marriages [5]. In another study, the highest prevalence was reported in Turkey, while the lowest was in the United Arab Emirates [6]. The frequency of PKU disease and the distribution of *PAH* gene mutations vary among different populations. For example, the IVS10-11G>A mutation is the main mutation among Mediterranean populations, such as those in Iran, southern Italy, Turkey, Spain, Greece, and Egypt [7]. In addition to *PAH* gene mutations, some polymorphic markers in or near this gene have been investigated in various studies, and their association with gene mutation or disease has been confirmed. Some of these markers include multiallelic markers such as short tandem repeats (STRs) located in intron-3, variable number tandem repeat (VNTR) located in the 3′ untranslated region, and biallelic markers, such as r*estriction fragment length polymorphism*s (RFLPs) [8]. These multiallelic markers provide information at higher rates than biallelic markers in identifying affected individuals. VNTR, an AT-rich repeat unit, has 30 base pairs, and several alleles of this polymorphism have been reported worldwide [5]. Polymorphic markers strongly associated with the PAH gene facilitate prenatal diagnosis and the identification of carriers [9].

The association between VNTR alleles and *PAH* mutations has been observed in various studies, including a study in Kermanshah, where an association was found between VNTR and the mutations p.R261* and IVS10-11G>A [3]. In another study in West Azerbaijan Province, Iran, an association was found between VNTR8 and two mutations, IVS10nt546 and p.R261Q [10]. According to studies conducted in Iran, VNTR alleles in the Iranian population were 66% informative [5]. Considering the high prevalence of PKU and consanguineous marriages in the Iranian population, the present study was conducted to investigate the association between VNTR alleles and *PAH* gene mutations in individuals with PKU in northern Iran (Golestan and Guilan Provinces, Iran). By determining the association between specific VNTR alleles and common mutations in these regions, the study of VNTR in the families of patients with mutations can be considered a way to identify carriers of the disease (mutation), because determining the VNTR allele associated with the disease (mutation) is faster, cheaper, and easier than identifying the disease-causing mutation.

Materials and Methods

This descriptive cross-sectional study included 51 unrelated patients with HPA from different regions of two northern provinces of Iran (26 patients from Golestan Province and 25 from Guilan Province). It is worth noting that only one patient from each family was included in the study, and patients with BH4 deficiency were excluded from the study. In addition, mutations in the phenylalanine hydroxylase gene have been previously investigated and identified in patients studied [11-12]. Sampling was performed after the patient or parents filled out the consent form. Pre-treatment Phe levels were determined as 4.5-250 mg/dL in 49 patients, and their levels were unavailable in two patients. This study divided patients into four groups: classic PKU, moderate PKU, mild PKU, and HPA based on pre-treatment Phe levels. After obtaining approval from the Research Ethics Committee of Golestan University of Medical Sciences and Health Services (IR.GOUMS.REC.1394.204) and Islamic Azad University, Rasht Branch (IR.IAU.RASHT.REC.1397.138), sampling was conducted in Golestan and Guilan Provinces in northern Iran [9,15]. Then, a 2-5 mL blood sample was prepared from each patient and stored in a tube containing 0.5 M EDTA.

DNA Extraction: A high-purity polymerase chain reaction (PCR) template kit (Roche Co., Germany) was used for DNA extraction according to the manufacturer's instructions. After DNA extraction and before the PCR reaction, the obtained DNA was assessed using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000c, USA) and a 1% agarose gel to evaluate the quantity and quality of the nucleic acid.

Polymerase Chain Reaction (PCR): To amplify VNTR fragments, the PCR reaction mixture was prepared using 2X YourTaq PCR Master Mix (Biotechrabbit, Germany) by adding genomic DNA, primer pair (pM 20), and sterile water. The PCR was performed using a thermocycler (TC-4000, TECHNE, UK) according to the following plan: an initial denaturation step at 95°C for 5 minutes, 33 reaction cycles each including: at 95°C for 15 seconds, at 60°C for 10 seconds, and at 72°C for 40 seconds, and a final extension step at 72 °C for 5 minutes. Primers were selected from previous studies [9]:

Forward primer (5′→3′):
AAACTTAAGAATCCCATCTCTCAGAG
Reverse primer (5′→3′):
GATTTTAATGTTCTCACCCGCC
VNTR primers used in this reaction were synthesized

by Bioneer (South Korea). After completing the PCR reaction, to ensure the amplification of the target fragment and its quality, the PCR products were electrophoresed on a 2% agarose gel.

Sequencing of Polymerase Chain Reaction (PCR) Products: After observing a clear band on the gel, the remaining PCR products were sequenced to confirm the VNTR alleles. Sequencing was performed using an ABI3730 sequencer (Bioneer Co., South Korea). Additionally, CLC Main Workbench version 3.5 was used to read the sequences and compare the results obtained from the sequencing device.

Statistical Method

The statistical method used in the present study was descriptive; accordingly, the frequency distribution (percentage) was used for VNTR alleles**.**

Results

Patient Phenotype

A total of 51 patients with HPA in northern Iran (Golestan and Guilan Provinces) were examined in this study. As mentioned above, patients from Golestan and Guilan Provinces were divided into four groups based on pre-treatment Phe levels: classic PKU (57.7% and 36%), mild PKU (7.7% and 20%), moderate PKU (11.5% and 12%), and HPA (15.4% and 32%, respectively). Notably, the metabolic phenotype of 7.7% of patients in Golestan Province was not available. Furthermore, the patients (51 patients) ranged in age from 1 to 23 years. Their ethnic distribution in Golestan Province was as follows: Fars (76.9%), Turkmen (19.3%), and Lor (3.8%). In Guilan Province, the distribution was as follows: Gilak (76%), Talesh (12%), and Turks (12%). The rate of consanguineous marriages among the parents of patients was 52.9%.

Nano Spectrophotometer Results

The spectrophotometer showed the amount of extracted DNA in the range of 100-500 ng/µL and an absorption ratio of 260/280 in the range of 1.8-2.0, indicating that the extracted DNA, with the mentioned quantity and purity, was suitable for PCR.

Results of Gel Electrophoresis of Polymerase Chain Reaction (PCR) Products

Figure 1 shows the electrophoresis image of PCR products on 2% agarose gel and the bands related to *PAH* VNTR fragments [related to VNTR7 allele (454bp)]. The PCR products of the *PAH* VNTR alleles produced 334,454,484,514, and 604 bp fragments. They corresponded to the presence of alleles with 3, 7, 8, 9, and 12 copies of the repeat units, respectively. In addition, five alleles (4.9%) were "not determined" (ND). Among the 102 investigated alleles, 52 were associated with Golestan Province, and 50 were associated with Guilan Province. Table 1 presents the allelic frequency of the VNTR *PAH* observed in individuals with PKU in northern Iran.

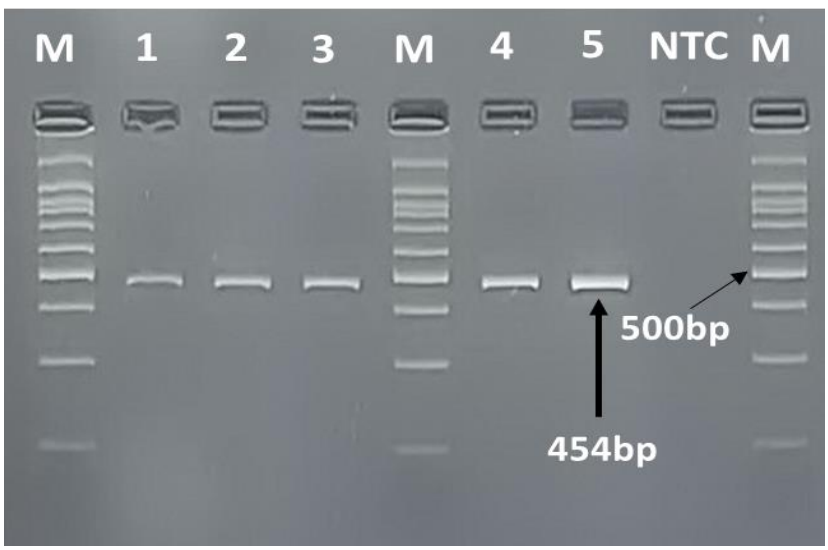

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Figure 1. Image related to variable number tandem repeat (VNTR)7 allele (454bp) on 2% agarose gel from five patients with IVS10-11g>a mutation
Abbreviations: NTC, no template control; M, marker size: 100 bp.

Table 1. The allelic frequency of the VNTR in the studied patients (n = 51)

VNTR	No. (%)
VNTR3	21(20.6 %)
VNTR7	21 (20.6 %)
VNTR8	42 (41.2 %)
VNTR9	10 (9.8 %)
VNTR12	3 (2.9 %)
ND	5 (4.9 %)

Abbreviations: VNTR, variable number tandem repeat; ND, not determined.

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Sequencing Results

After PCR, the samples were sequenced to confirm the number of repeats in the VNTR alleles. The base sequence of the *PAH* VNTR alleles was determined using CLC Main Workbench v3.5. For example, Figure 2 illustrates an electropherogram associated with VNTR7.

Table 2 presents the frequency of VNTR Genotypes observed in individuals with PKU in northern Iran. Analysis of VNTR alleles showed that the VNTR8 allele and the VNTR8/VNTR8 genotype have the highest frequency in the studied population (Tables 1 and 2).

Tables 3 and 4 present the association between different mutations and PAH VNTR alleles in the present study; the association of IVS10-11G>A mutation, the most common mutation in the studied patients from Golestan Province, was observed exclusively with VNTR7_(IVS10-11G>A-VNTR7 [19.2%]). However, IVS10-11G>A mutation in Guilan Province was associated with VNTR8 in one patient (IVS10-11G>A-VNTR8 [2%]) and VNTR7 in two patients (IVS10-11G>A –VNTR7 [8%]).

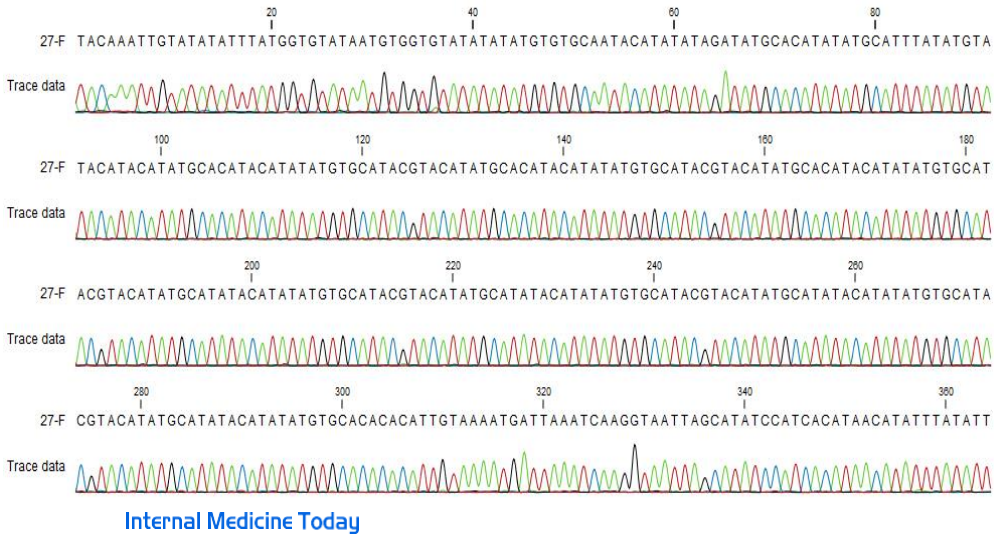

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Figure 2. Electropherogram related to the variable number tandem repeat (VNTR7) allele

Table 2. Frequency of VNTR Genotypes in the Studied Patients (n = 51)

VNTR Genotypes	No. (%)
VNTR3/VNTR3	7 (13.7%)
VNTR7/VNTR7	9 (17.6%)
VNTR8/VNTR8	19 (37.2%)
VNTR9/VNTR9	5(9.8%)
VNTR12/VNTR12	1 (2%)
VNTR7/VNTR3	3 (5.9%)
VNTR8/VNTR3	3 (5.9%)
VNTR12/VNTR3	1 (2%)
VNTR8/ND	1 (2%)
ND/ND	2 (3.9%)

Abbreviations: VNTR, variable number tandem repeat; ND, not determined.

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Table 3. Association between VNTR and *PAH* gene mutations in people with phenylketonuria (PKU) in Golestan Province (n = 26)

Mutation (11-13)	**VNTR**	**Number of Alleles**	**Relative Frequency**
IVS10-11G>A	VNTR7	10	19.23%
IVS11+1G>C	VNTR8	4	7.7%
p.R261Q	VNTR8	4	7.7%
p.R408W	VNTR3	2	3.8%
p.P416Hfs*36	VNTR9	4	7.7%
p.E178G	VNTR7	1	1.9%
p.R158 Q	VNTR3	3	5.8%
p.V399V	VNTR3	1	1.9%
p.V399V	VNTR7	1	1.9%
p.R241H	VNTR3	2	3.8%
IVS9-1G>T	VNTR12	2	3.8%
p.R261*	VNTR3	2	3.8%
IVS4+1G>A	VNTR8	1	1.9%

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Table 4. Association between VNTR and *PAH* gene mutations in people with phenylketonuria (PKU) in Guilan Province (n = 25)

Mutation (14)	VNTR	Number of Alleles	Relative Frequency
IVS10-11G>A	VNTR7	4	8%
IVS10-11G>A	VNTR8	1	2%
p.R400K	VNTR8	4	8%
p.R400K	VNTR7	2	4%
p.R261*	VNTR8	10	20%
p. R261Q	VNTR8	3	6%
p.R241H	VNTR8	2	4%
IVS4+5G>T	VNTR8	5	10%
p.R158Q	VNTR8	2	4%
p.R176X	VNTR8	2	4%
p.N376Ifs*24	VNTR7	2	4%
p.Q304*	VNTR7	2	4%
p. E305*	VNTR3	2	4%
p.A300S	VNTR8	1	2%

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Discussion

In the present study, an association was observed between VNTR3, VNTR7, VNTR8, VNTR9, and VNTR12 alleles and *PAH* mutations in individuals with PKU in Golestan Province (Table 3). However, in Guilan Province, an association was observed between three VNTR alleles (VNTR3, VNTR7, and VNTR8) and PAH mutation in individuals with PKU (Table 4). This difference in allelic diversity of VNTRs in different regions of the country can be attributed to variations in ethnic groups and differences in the mutation spectrum across various areas of Iran. Notably, in the present study, a strong correlation was observed between the most common mutation [11], IVS10-11G>A, and the VNTR7 allele in individuals with PKU in Golestan Province. This mutation was associated with VNTR8 in one patient and VNTR7 in two patients in Guilan Province.

Various studies have observed an association between VNTR alleles and *PAH* gene mutations. Hosseini Mazinani et al. observed an association between VNTR3, VNTR7, VNTR8, and VNTR9 alleles and *PAH* gene mutations in individuals with PKU in Iran [5]. Parivar et al. in Yazd Province, Iran, reported an association between VNTR3, VNTR7, VNTR8, and VNTR12 alleles and *PAH* gene mutations in patients with PKU [16]. Alibakshi et al. observed an association between VNTR3, VNTR7, VNTR8, and VNTR9 alleles and *PAH* gene mutations in individuals with PKU in Kermanshah Province, Iran [3]. Among them, the association between three mutations IVS10-11G>A, p.R261*, and IVS4+1G>C and the VNTR7 allele and the association between the P.R243Q mutation and the VNTR9 were reported [3]. In another study by Alibakhshi et al., *PAH* gene mutations were investigated in Hamedan and Lorestan Provinces, Iran, and their relationship with VNTR alleles was assessed. In this study, the IVS10-11G>A mutation, a common mutation in southern Europe, Mediterranean countries, and Iran, was also associated with VNTR7 [17].

Furthermore, the analysis of VNTR polymorphism in association with IVS10-11G>A mutation in Turkey, Israel, Italy, Spain, Germany, Switzerland, and Denmark showed that IVS10-11G>A mutation is associated with VNTR7 in these populations. Only three cases from Italy were associated with VNTR8 [18]. In our study, the VNTR7 allele exhibited a strong association with the IVS10-11G>A mutation in patients with PKU in Golestan Province. In the study of Bagheri et al. in West Azerbaijan Province, the relationship between VNTR8 and IVS10-11G>A mutation in the *PAH* gene was reported in 50% of patients. In their study, the association between VNTR8 and p.R261Q mutation was also observed [10]. Saadat et al. reported an association between the VNTR8 and IVS10-11G>A mutations in the *PAH* gene in Fars Province, Iran [18]. Razipour et al. reported an association between IVS10-11G>A and VNTR7, as well as p.R158Q and VNTR3, in different regions of Iran. This study also observed an association between p.R261Q, p.R261*, and IVS4+5G>T mutations and VNTR8 [8]. The present study demonstrated an association between VNTR alleles and mutation in PKU patients in two northern provinces of Iran (Tables 3 and 4). A strong association was found between the VNTR7 allele and the most common mutation in Golestan Province [11], IVS10-11G>A. When a strong association is observed between VNTR and a specific mutation, the VNTR should be evaluated in the family of a patient with that mutation, which is a proper way to identify carriers, because the identification of VNTR alleles related to the disease (mutation) is cheaper and easier than the identification of the disease-causing mutation. It is a fast and simple method. However, this was not the case in Guilan Province; no strong association was observed between a crucial mutation and a specific VNTR allele. Various factors, such as ethnic groups and the resulting genetic diversity, may be involved in this. Because the efficiency of VNTR alleles in identifying carriers in the Iranian population is

approximately 66% [5], and considering that checking VNTR alleles is easily feasible, it is recommended to investigate the efficiency of these alleles in identifying carriers in PKU families in this region. If the efficiency of this marker in identifying the carriers in each population is confirmed. In this case, there is no need to detect the PKU mutation to identify the carriers directly. In genetic counseling of families, it is possible to recommend VNTR examination of the PAH gene to identify carriers, as checking the VNTR allele is easily feasible. As a result, this method is recommended as a simple, cost-effective, informative, and quick method for identifying PKU carriers in that population. The limitations of the present study include the small sample size and insufficient funds to investigate another polymorphic marker, STR related to the *PAH* gene, in patients with PKU.

Conclusion

According to the results of this study, the most common PKU mutation in Golestan Province, IVS10-11G>A, is exclusively associated with the VNTR7 allele, and the VNTR7-IVS10-11G>A test should be considered for routine carrier screening and prenatal diagnosis. However, due to the population diversity in Iran, the type of VNTR alleles in the *PAH* gene may differ in different regions. Therefore, it is necessary to investigate the frequency and distribution of VNTR marker alleles in the various areas of the country.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Golestan University of Medical Sciences and Health Services (IR.GOUMS.REC.1394.204) and Islamic Azad University, Rasht Branch (IR.IAU.RASHT.REC.1397.138), Iran.

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Authors' contributions

All authors contributed to this research project.

Conflicts of interest

The authors declared no conflict of interest.

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