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Association Between PAH Mutations and VNTR Alleles in the West Azerbaijani PKU Patients

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ABSTRACT

Introduction: *We report the frequency of IVS10nt546, R261Q, S67P, R252W, and R408W mutations linked to PAH VNTR alleles in the west Azerbaijani PKU patients.*

Material and methods: *VNTR alleles and IVS10nt546, R261Q, S67P, R252W, R408W mutations were studied in a total of 20 PKU patients by PCR and RFLP-PCR.*

Outcomes: *Our analysis showed that 95% of cases were homozygote for an allele containing eight-repeat VNTR (VNTR8); while 5% were homozygote for an allele containing three-repeat VNTR (VNTR3). The IVS10nt546, R252W, and R261Q mutations were associated with VNTR8 allele, and also, R252W and S67P mutations were associated with VNTR3 allele. VNTR8 was common among mutant alleles as were IVS10nt546–VNTR8 (50%), R252W–VNTR8 (2.5%), and R261Q–VNTR8 (22.5%). The association of VNTR3 was found as R252W–VNTR3 (2.5%) and S67P–VNTR3 (2.5%) among studied cases. The frequency of IVS10nt546–VNTR8/IVS10nt546–VNTR8, IVS10nt546–VNTR8/ND–VNTR8, IVS10nt546–VNTR8/R252W–VNTR8, R261Q–VNTR8/R261Q–VNTR8, R261Q–VNTR8/ND–VNTR8, and S67P–VNTR3/ R252W–VNTR3 were 30%, 35%, 5%, 20%, 5%, and 5%, respectively. R408W mutation was not found in this study. Conclusions: The present report is the first in its own kind in the west Azerbaijani population (Iran) and implies that the most common PKU mutation in this population, IVS10nt546, is exclusively associated with VNTR8 allele, and IVS10nt546–VNTR8 alleles testing should be considered for routine carrier screening and prenatal diagnostic setting.*

Keywords: PAH gene, VNTR alleles, west Azerbaijan, PKU

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INTRODUCTION

Phenylketonuria (PKU) and hyperphenylalaninemia are resulted from hepatic phenylalanine hydroxylase (PAH) deficiency (1). The frequency of PKU among Iranian is approximately 1 in 3627 live births (2). The PAH deficiency leads to abnormally higher levels of serum phenylalanine (Phe), that is, higher than 120 μmol/L, which resulting in irreversible mental retardation in untreated patients (3,4). Maternal HPA/PKU is a risk factor for abnormalities such as intrauterine and postnatal growth retardation, microcephaly, decreased skin and hair pigmentation, congenital heart disease, eczema, intellectual disability, and epilepsy, as well as other brain problems in a fetus (5-9). The PAH gene contains 13 exons and is located on the long arm of chromosome 12 (12q24.1.) (3,4). Over 530 PAH mutations and polymorphisms have been identified in PKU patients in different ethnic groups (PAHdb; http://www.mcgill.ca/pahdb). The high rate of heterozygosity in Variable-Number Tandem Repeats (VNTR) provides a Polymorphism Information Content (PIC) of 66% for Iranian population (10). Regarding the high rate of PKU and consanguineous marriages among Iranian population (11), this investigation was performed for analysis of association between IVS10nt546, R261Q, S67P, R252W, R408W mutations and PAH VNTR alleles in the west Azerbaijani PKU patients. ❑

MATERIALS AND METHODS

This study was approved by ethics committee of the Institutional Review Board (Urmia University of Medical Sciences). In accordance with the criteria mentioned by Scriver and Kaufman (3), a total of 20 PKU patients were studied. This number of cases was collected during 2 years. The average ages of patients were 4.4±4.8 years (range 1-19). A written consent was obtained from the PKU families. From each patient, 3-4 ml of whole blood was collected in EDTA-contained tube. The genomic DNA was extracted using the standard salting-out method (Miller et al. 1988) with some modifications (12). After detection of patients who were homozygote for the PAH VNTR alleles, analysis of IVS10nt546, R261Q, S67P, R252W, and R408W mutations were carried out via RFLP-PCR. ❑

PAH VNTR ALLELES

Analysis of PAH VNTR alleles was performed according to the previously described method of polymerase chain reaction (PCR) using the 5′-ttttaatgttctcacccgcc-3′ and 5′-aagaatcccatctctcagag-3′ primers with an annealing temperature of 55°C (13). PCR reaction was performed in a 25-μl solution containing 100 ng DNA, 1x reaction buffer, 10 pmol of each primer, 200 μmol of each dNTPs, 0.2 unit of Taq DNA polymerase, and 1.5 mmol MgCl2 (Genefanavaran, Tehran, Iran). PCR products of the PAH VNTR alleles produced fragments with 325, 445, 475, 505, 565, 595 and 625 bp. They are corresponding to the presence of alleles with 3, 7, 8, 9, 11, 12, and 13 copies of the repeated units, respectively. Electrophoresis of PCR products was performed on 1.5% - 2.5% agarose gel. Presence or absence of PCR products were visualized via UV transilluminator.

Exon/ Intron	Mutation	Primers (5′→3′)	Annealing Temperature	Length (bp)	Restriction Enzymes
Intron 10	IVS10nt546(IVS10nt-11g->a)	gtatcccttcatccagtcaagg cccaggttgcatatcaaaacgg	57	290	+DdeI
Exon 7	R252W	actaccaaaggtctcctagtgc caaacctcattcttgcagcagg	57	285	-AvaI
Exon 7	R261Q	actaccaaaggtctcctagtgc caaacctcattcttgcagcagg	57	285	-Hinfl
Exon 3	S67P	ggttttcctgttctggttctgc gtccactcatttaatccccc	55	463	-XbaI
Exon 12	R408W	ccaaatggtgcccttcactcaagcc tatttttcctatggcgatgg	53	241	+StyI

TABLE 1. Mutations, set of primers, annealing temperature, length of PCR products, and appropriate restriction enzymes for analysis of exons 3,7,10, and 12 at the PAH gene.

Formation (+) and abolition (-) of a restriction site for related restriction enzyme by a mutation is indicated.

Mutation Analysis

Patients with homozygote VNTR alleles studied by a set of primers and appropriate restriction enzymes regarding IVS10nt546, S67P, R261Q, R252W, and R408W mutations as shown in table 1 (13,14). Each PCR was performed in a 25-μl solution containing 100 ng DNA, 1xreaction buffer, 10 pmol of each primer, 200 μmol of each dNTPs, 0.2 unit of Taq DNA polymerase, and 1.5 mmol MgCl2 (Genefanavaran, Tehran, Iran). PCR program was as follows: denaturation at 95°C for 5 min, 35 cycles of 95°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min, and final extension at 72°C for 10 min. 10 μl of the PCR products were digested with appropriate restriction enzymes at 37oC for 2 hours according to the manufacturer's instructions. ❑

OUTCOMES

We studied 20 PKU patients. Our results are summarized in table 2. Analysis of VNTR alleles showed that 19 out of 20 patients (95%) were homozygote for VNTR8 allele; while only one out of 20 patients (5%) was homozygote for VNTR3 allele. PAH VNTR allele analysis in PKU patients demonstrates that the IVS10nt546, R252W, and R261Q mutations were associated with VNTR8 allele, and also, R252W and S67P mutations were associated with VNTR3 allele. VNTR8 was common among mutant alleles as were IVS10nt546–VNTR8 (50%), R252W–VNTR8 (2.5%), and R261Q–VNTR8 (22.5%). VNTR3 was found as R252W–VNTR3 (2.5%) and S67P–VNTR3 (2.5%) among studied cases. The frequency of IVS10nt546–VNTR8/IVS10nt546–VNTR8, IVS10nt546–VNTR8/ND–VNTR8, IVS10nt546–VNTR8/ R252W–VNTR8, R261Q–VNTR8/R261Q–VNTR8, R261Q–VNTR8/ND–VNTR8, and S67P–VNTR3/R252W–VNTR3 were 30%, 35%, 5%, 20%, 5%, and 5%, respectively. Relative frequency of R408W mutation was zero among studied patients. Figure 1 indicates the frequency (%) of IVS10nt546–VNTR8, R252W–VNTR8, R261Q–VNTR8, R252W–VNTR3, and S67P–VNTR3 in the west Azerbaijani PKU patients. ❑

	Mutation linked to VNTR	Frequency(%)
Alleles N=40 alleles	IVS10nt546 - VNTR8	20(50)
	R252W - VNTR8	1(2.5)
	R261Q - VNTR8	9(22.5)
	ND - VNTR8	8(20)
	R252W - VNTR3	1(2.5)
	S67P - VNTR3	1(2.5)
Genotypes N=20 patients	IVS10nt546 - VNTR8/ IVS10nt546 - VNTR8	6(30)
	IVS10nt546 - VNTR8/ ND-VNTR8	7(35)
	IVS10nt546 - VNTR8/ R252W-VNTR8	1(5)
	R261Q - VNTR8/ R261Q-VNTR8	4(20)
	R261Q - VNTR8/ ND- VNTR8	1(5)
	S67P - VNTR 3/ R252W-VNTR3	1(5)

TABLE 2. Association between PAH mutations and VNTR alleles and genotypes in the west Azerbaijani PKU patients.
ND: Not Determined.

DISCUSSION

The PAH gene has been associated with several polymorphic markers including restriction enzyme based RFLPs, intragenic short tandem repeats (STR) and intragenic variable number of tandem repeats (15). The study of intragenic polymorphic markers linked to the mutations provides better understanding regarding of the origin and mutations flow (16). One of the famous multiallele markers that has been recognized within the PAH gene, is minisatellite of variable number of tandem repeats (15,16). This polymorphic marker has more than ten alleles in different ethnic groups regarding 30-bp tandem repeats at 3´ end of the PAH gene (17). The polymorphic marker of PAH VNTRs could be understood as a suitable informative tool for carrier screening and prenatal diagnosis of diseases in PKU families (18). Results of several investigations have been demonstrated that these alleles are individual and population specific (19). In this regard, the 13-repeat VNTR alleles are found in Iranian population (19), while, this allele was not found in United State of America, Russia, Brazil, Poland, Italy, Sweden, Switzerland, England, Turkey, Denmark, Spain, and Ireland (19). Relative frequencies of R408W mutation were 0.323, 0.111, 0.141, 0.257, 0.182, 0.20, 0.11, 0.219, 0.486, 0.622, 0.549, 0.35, 0.734, 0.57, and 0.565 in Scotland, England, France, Germany, Denmark, NW Norway, SE Norway, Sweden, Hungary, Poland, Czech Republic, Bulgaria, Lithuania, Ukraine, and Russia, respectively (20); and this mutation accounts for 0.4% of PKU alleles in Iranian population (21). The

Mutation	VNTR	Haplotype	STR	Location	Ethnicity	Reference
IVS10nt-11g->a	9	ND	242	N Ireland	NW European	13
	ND	36	ND	SE Europe	ND	25
	7	34	240	UK	English	26
	8	6	240	UK	English	26
	8	6	250	UK	English	26
	7	6	246/2	UK	English	26
	7	34	240	Spain	Spanish	27
	7	34	230	Germany	German	28
	7	5	ND	Turkey	Turkish	29
	9	6	ND	Italy	ND	30
	7	6	250	Australia	Australian	31
	7	6	230	Turkey	Turkish	32
	7	34	240	Spain	Gypsy	33
	7	6	ND	Brazil	Brazilian	34
	8	ND	ND	west Azerbaijan (Iran)	Iranian azeri Turkish	This study
R261Q	8	1	238	Spain	Spanish	7
	3	4	246	Germany	German	28
	8	1	ND	Turkey	Turkish	29
	3	28	246/2	Australia	Australian	31
	8	1	238	Australia	Australian	31
	8	1	ND	Canada	Anglo-Saxon	35
	3	ND	ND	Fmr Soviet Union	ND	36
	8	ND	ND	Fmr Soviet Union	ND	36
	8	1	ND	Spain	Spanish	37
	8	ND	ND	west Azerbaijan (Iran)	Iranian azeri Turkish	This study
R252W				Germany	German	
	8	7	238	Germany	German	28
	8	novel	242	Australia	Australian	28
	8	1	242	Norway	Norwegian	31
	3	2	ND	west Azerbaijan	Iranian azeri	38
	8	ND	ND	(Iran)	Turkish	This study
	3	ND	ND	west Azerbaijan (Iran)	Iranian azeri Turkish	This study
S67P	3	240	ND	N Ireland	Irish	13
	3	4	ND	UK	English	26
	ND	4	ND	Italy	Italian	39
	3	ND	ND	west Azerbaijan (Iran)	Iranian azeri Turkish	This study

TABLE 3. PAH VNTR, Haplotype, and STR markers in association with IVS10nt-11g->a (c.1066-11g->a), R261Q (c.782G->A), R252W (c.754C->T), and S67P (c.199T->C) mutations.

ND: Not Determined.

polymorphic markers of PAH VNTRs are used in the linkage study of the affected alleles and their transmission in a family (15). Linkage study is performed by at least two informative markers (15). Each marker has a PIC value that is population specific (15). The majority of studies have been implied that the three-repeat VNTR alleles are the most common marker in ethnic groups and could be considered as the first natural allele among different populations (15). Expansion of this repeats could be explained by dynamic bases of repeated sequences and replication slippage (22,23). DNA replication slippage occurs in the parents' genome and results in insertion of extra repeated units to the recently synthesized sequences (22, 23). It has been indicated that a single haplotype has been associated with several VNTR alleles (13). Most of common mutations are associated with several VNTRs and haplotypes (13). But, rare mutations are associated with specific VNTRs and haplotypes (13). The VNTRs and haplotypes are inherited by Mendelian pattern and are associated with specific mutations (13). Haplotype 12 contained 12 repeated unites and the most of haplotype 1 alleles has been linked with eight-repeated VNTR alleles (24). Analysis of PAH VNTR multialleles

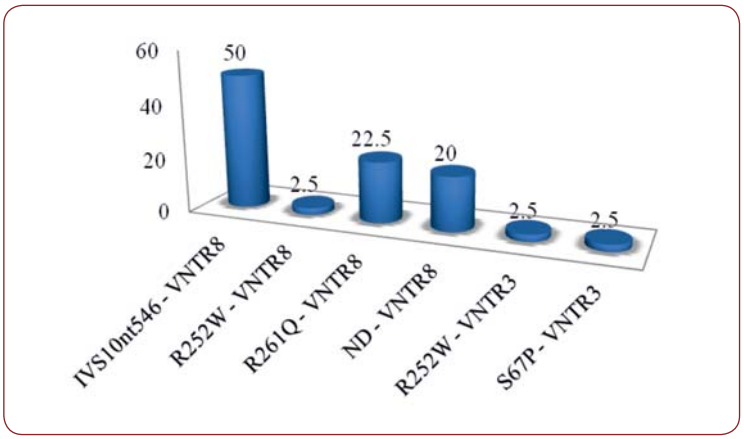

FIGURE 1. Frequency (%) of IVS10nt546 - VNTR8, R252W - VNTR8, R261Q - VNTR8, ND - VNTR8, R252W - VNTR3, and S67P - VNTR3 in the west Azerbaijani PKU patients (N=40 alleles).
ND: Not Determined.

linked to the IVS10nt546 mutation was carried out in several investigations. Table 3 lists the PAH VNTRs, haplotypes, and STR markers in association with IVS10nt-11g->a (IVS10nt546), R261Q, R252W, and S67P mutations that were identified in this study and compared with others. The sample size in our investigation was 20 and this amount is bigger than any previous study in Iran, therefore, its foremost power relates to the number of cases. A further study is needed to be carried out with considering more details regarding possible heterogeneity in the number of repeated unites on chromosomes with different haplotypes, ethnicity, Mendelian pattern of inheritance, VNTR alleles, restriction enzyme based RFLPs, intragenic short tandem repeats and PAH mutations. It can be concluded that the most common PKU mutation in the west Azerbaijani population (Iran), IVS10nt546, is exclusively associated with VNTR8 allele, and IVS10nt546–VNTR8 alleles testing should be considered for routine carrier screening and prenatal diagnostic setting. This analysis provides molecular based device for population-genetic studies regarding the origin and expansion of the PAH gene mutation in different ethnic groups.

Conflict of interests: none declared.

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