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Original Article

Characterization of phenylalanine hydroxylase gene mutations in phenylketonuria in Xinjiang of China

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Abstract: To investigate the spectrum and frequency of phenylalanine hydroxylase (PAH) gene mutations in phenylketonuria (PKU) patients in Xinjiang, China. Polymerase chain reaction (PCR), in combination with single-strand conformation polymorphism (SSCP) and DNA sequencing analyses were performed, to screen potential mutations in the PAH gene in 46 individual PKU patients. Direct DNA sequencing was used to analyze the all of the exons in the PAH gene, including the promoter and flanking intron regions, in another 15 PKU patients. Our results indicated that, 30 different mutation types were identified in all 122 PAH alleles, with the mutation detection rate of 78.7% (96/122). Four novel mutations, i.e., 5'-Flanking -626G>A, 5'-Flanking -480DelACT, S196fsX4, and IVS8+1G>C, were identified for the first time. Similar to other regions in North China, R243Q, EX6-96A>G, IVS4-1A>G, R111X, and Y356X were the most prevalent PAH mutations in PKU patients from Xinjiang. Additionally, common mutations showed different frequencies in Xinjiang, when compared to other areas. Furthermore, sixteen different PAH gene mutation types were identified for the first time in the minorities in Xinjiang. Distinctive mutation spectrum of PAH gene in PKU patients from Xinjiang were characterized, which may promote the construction of PAH gene mutation database and serve as valuable tools for genetic diagnosis and counseling, and prognostic evaluation for PKU cases in the local area.

Keywords: Phenylketonuria, phenylalanine hydroxylase, gene mutation, sequence analysis

Introduction

Defects in the phenylalanine hydroxylase (PAH) gene are the cause of phenylketonuria (PKU), which is one of the most prevalent autosomal recessive inborn errors of hydroxylase deficiency. Blockages in the major pathway of phenylalanine (Phe) metabolism, which involves the irreversible hydroxylation of Phe to tyrosine, result in an increase of Phe in blood and other body fluids, and produces a spectrum of disorders including classical PKU, mild PKU, and mild hyperphenylalaninemia [1]. Increased serum Phe concentration might lead to severe mental retardation, which could be prevented by early diagnosis and implementation of a low-phenylalanine diet [2, 3].

PKU is a heterogeneous metabolic disorder at both the genetic and clinical levels. Mutation analysis of PKU has proved to be clinically advantageous. To date, more than 600 mutations in PAH have been identified and registered in the PAH and HGMD databases (www.pahdb.mcgill.ca and www.hgmd.org). The prevalence of PKU is roughly 1 in 15,000 individuals, which differs among different populations [4, 5]. In China, various PAH mutations have been identified, with significant differences in mutation spectrum and heterogeneity among different ethnic populations from different geographical origins.

In this study, for the first time, we screened potential PAH gene mutations in PKU patients in Xinjiang area of China, with polymerase chain reaction (PCR), in combination with single-strand conformation polymorphism (SSCP) and DNA sequencing analyses. Distinctive mutation spectrums of PAH gene were identified. Our results provide valuable tools for genetic diagnosis and counseling, and prognostic evaluation for PKU cases in southwest China.

Materials and methods

Subjects

A total of 61 patients diagnosed as PKU were included in this study, and their gender, age,

Table 1. Baseline characteristics of PKU patients from Xinjiang, China

	Cohort 1	Cohort 2
Subjects numbers	46	15
Gender		
Male	24	12
Female	22	3
Age	3 d-27 yr	
Ethnicity		
Han	34	8
Uigur	8	3
Hui	3	4
Kazak	1	-
Phe concentration (μM)	480-1800	738-1854

ethnicity, and phenylalanine (Phe) concentrations were summarized in **Table 1**. All the patients were subjected to tetrahydrobiopterin load and Phe tolerance tests, to rule out tetrahydrobiopterin deficiency. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Urumqi General Hospital of Lanzhou Military Region.

DNA preparation and PCR amplification

Peripheral blood samples were obtained from each PKU patient, and genomic DNA was isolated from leukocytes by using standard procedure as described previously [6]. The exons and the promoter of PAH gene were amplified by PCR using primers in accordance with literature [6]. PCR reactions were performed in a total volume of 25 μl containing 1× reaction buffer, 0.5 μg of genomic DNA templates, 1.5 U of Taq DNA polymerase, 2 pmol/L primer, and 0.25 mM of each dNTP. PCR conditions were as follows: pre-denaturation at 97°C for 5 min, followed by 45 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s. PCR products were analyzed by electrophoresis on 1.5% agarose gel to validate the generation of the specific promoter and each of the exons.

Single-strand conformation polymorphism (SSCP) analysis

A total of 5 μL of PCR products were mixed with an equal volume of denaturing buffer (containing 0.5 g/L bromophenol blue, 0.5 g/L xylene cyanol, and 95% formamide), and then heated at 97°C for 5 min and cooled on ice immediately. The samples were subjected to electrophoresis analysis by using 8% non-denaturing polyacrylamide gel with following conditions: 30-60 W/550-600 V, 3.5-5.5 h, and 10-28°C, for the 20 cm gel. The gel was stained with silver nitrate to investigate whether abnormal migration patterns existed in PCR products.

Sequence analysis

PCR products with abnormal SSCP migration patterns were subjected to direct sequencing. Briefly, samples were subjected to PCR reaction twice, and then sequenced by using an ABI 377 automatic sequencer (Boya Biotechnology, Shanghai, China) or an ABI 3730 sequencer (Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China).

Results

Spectrum of the PAH gene mutations in PKU patients from Xinjiang, China

To investigate the PAH gene mutation spectrum, PCR, SSCP, and DNA sequencing analyses were performed. As shown in **Table 2**, a total of 30 different mutation types were identified at all 122 PAH alleles, with a mutation detection rate of 78.7% (96/122), which included 18 missense, 4 nonsense, 4 splice site, 2 deletion, and 2 frameshift mutations. The most prevalent PAH gene mutation in PKU patients in Xinjiang was R243Q (21.3%), followed by EX6-96A>G (9.0%), IVS4-1A>G (5.7%), R111X (4.9%), and Y356X (4.1%). Additionally, other commonly detected mutations included V399V (3.3%), F161S (3.3%), L255S (2.5%), P281L (2.5%), R408W (2.5%), and R413P (2.5%). The rest 19 mutations were only detected at 1 or 2 alleles, with mutation rates ranging from 0.8% to 1.6% (**Table 2**). Collectively, 30.3% of these mutation types were found to occur in exon 7 (EX7) and its flanking sequence, followed by EX6 (12.3%), EX11 (7.4%), EX3 (6.6%), EX5 (6.6%), intron 4 (IN4) (5.7%), EX12 (5.7%), 5'-Flanking region (1.6%), IN8 (0.8%), EX8 (0.8%), and EX10 (0.8%) (**Table 2**). These results provide the PAH gene mutation spectrum in PKU patients from Xinjiang, southwest China, forming the basis for the following analysis.

PAH gene mutations in minorities from Xinjiang, China

Based on the PAH mutation spectrum in PKU patients from Xinjiang, China, the gene muta-

Table 2. Distribution of PAH gene mutations in PKU patients from Xinjiang, China

Changes in amino acid	Changes in Bases	Mutation sites	Mutation types	Allele numbers	Relative frequencies (%)
5'-Flanking	-480DelACT	5'-terminal	Deletion	1	0.8
5'-Flanking	-626G>C	5'-terminal	Missense	1	0.8
I65T	c.194T>C	Exon 3	Missense	1	0.8
R111X	c.331C>T	Exon 3	Missense	6	4.9
S70del	208-210delTCT	Exon 3	Deletion	1	0.8
IVS4-1G>A	c.442-1G>A	Intron 4	Splicing	7	5.7
R155H	c.500G>A	Exon 5	Missense	1	0.8
R158Q	c.473G>A	Exon 5	Missense	2	1.6
F161S	c.482T>C	Exon 5	Missense	4	3.3
Y166X	c.498C>G	Exon 5	Nonsense	1	0.8
R176X	c.526C>T	Exon 6	Nonsense	2	1.6
S196fsX4	c.584dupA	Exon 6	Frameshift	1	0.8
Q232X	c.694 C>T c.696 A>G	Exon 6	Nonsense	1	0.8
EX6-96A>G	c.611A>G	Exon 6	Splicing	11	9.0
R241fs	c.722delG	Exon 7	Frameshift	1	0.8
R243Q	c.728G>A	Exon 7	Missense	26	21.3
G247V	c.740G>T	Exon 7	Missense	1	0.8
L255S	c.764T>C	Exon 7	Missense	3	2.5
R261Q	c.782G>A	Exon 7	Missense	1	0.8
E280K	c.838G>A	Exon 7	Missense	1	0.8
E280G	c.839A>G	Exon 7	Missense	1	0.8
P281L	c.842C>T	Exon 7	Missense	3	2.5
IVS8+1G>C	912 + 1G>C	Intron 8	Splicing	1	0.8
A300S	c.898G>T	Exon 8	Missense	1	0.8
L348V	c.1042C>G	Exon 10	Missense	1	0.8
Y356X	c.1068C>A	Exon 11	Nonsense	5	4.1
V399V	c.1197A>T	Exon 11	Splicing	4	3.3
R408W	c.1222C>T	Exon 12	Missense	3	2.5
R413P	c.1238G>C	Exon 12	Missense	3	2.5
A434D	c.1301C>A	Exon 12	Missense	1	0.8

tions in minorities in this area was then detected. For the first time, 16 PAH gene mutation types at 27 alleles were identified in minorities in Xinjiang, including Uigur, Hui, and Kazak nationalities (**Table 3**). The mutation profiles consisted of 11 missense, 3 splice site, 1 nonsense, and 1 deletion mutations, with a total allele mutation rate of 22.1%. At all 27 alleles detected, 15, 10, and 2 mutations were identified for Uigur, Hui, and Kazak nationalities, respectively (**Table 2**). Furthermore, P281L, R111X, EX6-96A>G, R176X, and R243Q were revealed to be common mutations in minorities in Xinjiang, China. These results provide the PAH mutation profiles in PKU minorities in Xinjiang, southwest China.

Novel mutations in the PAH gene in PKU patients in Xinjiang, China

Out of the total 30 mutation types in 61 PKU patients from Xinjiang, the novel mutations were further analyzed. Among them, four mutations, including 5'-Flanking-626G>A, 5'-Flanking-480DelACT, S196fsX4, and IVS8+1G >C, had never been reported previously, and these novel mutations in our study had been registered in PAHdb (www.pahdb.mcgill.ca). In addition, 3 mutations, including R155H, A300S, and L348V, were identified for the first time in China. Moreover, when comparing the PAH gene mutation profiles between PKU patients from Xinjiang and other countries and/

Table 3. Distribution of PAH gene mutations in minorities with PKU in Xinjiang, China

Ethnicities	Change in amino acids	Change in bases	Mutation sites	Mutation types	Allele numbers	Relative frequency (%)
Uigur	5'-Flanking	-480DelACT	5'-terminal	Deletion	1	1.0
	5'-Flanking	-626G>C	5'-terminal	Missense	1	1.0
	R111X	c.331C>T	Exon3	Missense	2	2.1
	R158Q	c.473G>A	Exon5	Missense	1	1.0
	F161S	c.482T>C	Exon5	Missense	1	1.0
	IVS4-1G>A	c.442-1G>A	Exon5	Splicing	1	1.0
	EX6-96A>G	c.611A>G	Exon6	Splicing	2	2.1
	R243Q	c.728G>A	Exon7	Missense	1	1.0
	P281L	c.842C>T	Exon7	Missense	3	3.1
	L348V	c.1042C>G	Exon10	Missense	1	1.0
	R413P	c.1238G>C	Exon12	Missense	1	1.0
Hui	S70del	208-210delTCT	Exon3	Deletion	1	1.0
	R155H	c.500G>A	Exon5	Missense	1	1.0
	IVS4-1G>A	c.442-1G>A	Exon5	Splicing	1	1.0
	R176X	c.526C>T	Exon6	Nonsense	2	2.1
	R243Q	c.728G>A	Exon7	Missense	2	2.1
	R261Q	c.782G>A	Exon7	Missense	1	1.0
	A300S	c.898G>T	Exon8	Missense	1	1.0
	R413P	c.1238G>C	Exon12	Missense	1	1.0
Kazak	F161S	c.482T>C	Exon5	Missense	1	1.0
	EX6-96A>G	c.611A>G	Exon6	Splicing	1	1.0

or regions, very distinguishable and unique mutation pattern was observed for Xinjiang, southwest China (**Table 4**). These findings may contribute to the understanding of the molecular basis for PKU pathogenesis, and the genetic diagnosis and counseling, and prognostic evaluation for PKU, in Xinjiang, southwest China.

Discussion

Phenylketonuria (PKU) is an autosomal recessive disorder caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH). To date, more than 500 PAH gene mutations have already been described. Using PCR-SSCP combined with DNA sequencing, 30 different mutation types in PAH gene of PKU patients from Xinjiang were identified in this study. A higher mutation frequency (94.8%) were observed in PKU patients from Xinjiang, southwest China, compared with what was reported in North China (84.6%) [7]. The detected mutations were distributed in the promoter and introns, as well as exons 3, 5-8, and 10-12, among which exons 3, 5-7, 11-12, and intron 4 were the popular regions. In addition, our results revealed that the PAH gene mutation spectrum in Xinjiang was significantly different from that in Europe, where PAH mutations mainly occur in exons 3, 10, and 12 [8]. Therefore, the popular exons and introns we detected merit first consideration when developing antepartum genetic diagnosis and mutation screening for PKU in Xinjiang, southwest China.

It is well known that allelic heterogeneity at the PAH locus account for highly heterogeneous PAH deficiency in PKU patients [9, 10]. In the present study, four novel mutations in PAH gene are identified for the first time worldwide. Among them, S196fsX4 frameshift mutation can result in the premature termination of PAH gene translation and the subsequent truncated PAH enzyme. IVS8+1G>C mutation is located at the 8th exon-intron boundary, which will cause splicing error during PAH mRNA processing. In addition, both mutations of -626 G>C and -480DelACT might cause the failure in the translation and expression of PAH. Therefore, all these mutations can be recognized as pathogenic mutations of PKU.

Five common mutations among the total 30 mutations, including R243Q, EX6-96A>G, IVS4-

Table 4. Comparisons of the PAH gene mutation frequencies between PKU patients from Xinjiang and other countries and/or regions

Mutations	Xinjiang	North China	South China	Taiwan	Japan	Korea	Iceland	Holland	Czech	Ireland	Belgium	Germany	Croatia	Lithuania	Greece	Brazil	Texas
R243Q	21.3	21.7	9.5	6.0	7.0	12.0	-	-	3.0	3.0	2.0	1.9	-	-	-	1.3	-
EX6-96A→G	9.0	10.2	10.7	4.0	6.0	10.0	-	-	-	-	-	-	-	-	-	-	-
R111X	4.9	8.3	5.2	4.0	3.7	0.7	3.0	-	-	-	-	-	1.3	0.5	-	-	-
Y356X	4.1	6.1	7.7	-	5.0	6.0	-	-	-	-	-	-	-	-	-	-	-
R413P	2.5	6.5	7.1	4.0	31.0	3.0	-	-	-	-	-	-	-	-	-	-	-
IVS4-1G→A	5.7	3.5	-	2.0	7.0	10.0	-	-	-	-	-	-	-	-	-	-	-
P281L	2.5	0.4	-	-	-	2.0	19.0	1.0	1.0	1.0	6.0	3.6	11.0	1.0	10.0	2.1	-
R158Q	1.6	0.9	-	-	-	-	-	13.0	5.0	1.0	9.0	4.5	1.3	7.1	3.0	3.5	1.0
R408W	2.5	0.4	-	-	-	-	3.0	1.0	55.0	41.0	5.1	24.9	37.0	73.4	-	3.5	19.0
R261Q	0.8	0.7	-	-	-	1.0	-	18.0	2.0	1.0	4.0	6.0	9.0	0.5	-	12.2	-
I65T	0.8	0.4	-	-	-	-	-	-	1.0	20.0	2.0	2.0	-	-	-	3.5	1.0
E280K	0.8	0.7	-	-	-	-	-	4.0	-	1.0	1.0	-	-	1.0	5.0	-	3.0
R176X	1.6	1.1	-	-	-	-	-	-	-	-	-	1.1	-	0.5	-	1.7	-
V399V	3.3	4.1	7.7	2.0	-	-	-	-	-	-	-	-	-	-	-	-	-
Y166X	0.8	1.7	7.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G247V	0.8	1.1	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F161S	3.3	1.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L255S	2.5	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E280G	0.8	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A434D	0.8	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Q232X	0.8	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R241fsX5	0.8	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S70del	0.8	1.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A300S	0.8	-	-	-	-	-	-	4.0	-	-	1.0	0.8	2.5	-	-	0.4	-
L348V	0.8	-	-	-	-	-	-	4.0	-	8.0	1.0	1.1	-	-	-	3.2	1.0
R155H	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S196fsX4	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IVS8+1G→A	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5'-480DelACT	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5'-626G→A	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: North China is comprised of provincial regions, such as Peking, Hebei, Inner Mongolia, Jilin, and Liaoning provinces. South China is comprised of Shanghai, Jiangsu, Zhejiang, Anhui, and Yunnan provinces. References: North China [4]; South China [9, 10]; Taiwan [11]; Japan [12]; Korea [13]; Iceland, Holland, Czech, Ireland, Belgium, Germany, Croatia, Lithuania, and Greece [5]; Brazil [14]; Texas [15]. The R155H mutant genotype was first found in Boston and Southern California in the United States of America [16].

1A>G, R111X, and Y356X, display similar mutation frequencies to that found in North China. However, the frequencies of V399V, F161S, L255S, P281L, R408W, and R413P mutations in Xinjiang, southwest China, are significantly different from other domestic areas. Additionally, A300S and L348V mutations, although very common in PKU patients in Europe and South America, were identified in China for the first time. However, mutations of Q232X, R241fsX5, S70del, E280G, A434D, L255S, P281L, R261Q, and I65T have been previously reported in China [11-13]. Comparing the data from domestic and abroad, we can find that mutations of R243Q, EX6-96A>G, R111X, Y356X, V399V, R413P, and IVS4-1A>G are common in oriental population, while P281L, R158Q, R408W, R261Q, I65T, E280K, A300S, and L348V are popular mutants in Europe and South America. Mutations specific for Chinese, such as L255S, F161S, Y166X, E280G, G247V, and A434D, were identified in PKU patients in Xinjiang. Most importantly, 5'-Flanking -626G>A, 5'-Flanking -480DelACT, S196fsX4, and IVS8+1G>C in Xinjiang PKU patients turn out to be navel mutations, which have never been reported previously (**Table 4**), indicating a distinctive mutation pattern of PAH gene in Xinjiang.

In addition, 16 different mutations were detected for the first time in minorities of Uigur, Hui, and Kazak in Xinjiang, which may promote the study on PKU in minorities in this area. Although the frequency of mutant alleles in these nationalities was only 22.1% (27/122), diverse mutations that ever identified in other areas, both domestic and aboard, have also been detected in these populations. Interestingly, we find that P281L, a common PAH mutation in Europe and South America but a rare mutation in Asia, is however the most prevalent mutation in these minority populations, reflecting the complicated genetic characteristics of these minorities in Xinjiang.

Xinjiang is geologically located at the center of Asia, and therefore has been serving as the platform for cultural communication and international trade between the East and the West for a long time. Since the Han dynasty, the famous Silk Road through Xinjiang has contributed greatly to the cultural and commercial communications between China and Asia-Europe countries. Therefore, there were good chances for miscegenation between people with different ethnicities in this area (including Sacae, Qiangs, Dinglings, Rouzhi, Huns, Turks, Mongols, Greeks, Arabs, Aryans, and Sogdians), which might lead to frequent genetic alterations and combinations between these peoples. Given the high degree of miscegenation in this multiethnic area, diversities and variances in the type and frequency of PKU alleles would be expected, both in comparison with other countries as well as other regions in China. This genetic profile may serve as valuable genetic markers for researches on genetic diversity and heterogeneity, as well as origin and migration of peoples, in Xinjiang.

In summary, this study characterizes the mutation spectrum for PAH gene in PKU patients from Xinjiang, China, and it is found that there are distinctive PAH mutations in these patients. Our results provide valuable tools for genetic diagnosis and counseling, and prognostic evaluation for PKU, and contribute to the further understanding of the correlation between the genotype and phenotype of PKU cases in Xinjiang.

Disclosure of conflict of interest

None.

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