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Research Article

Mutations of the phenylalanine hydroxylase gene in patients with phenylketonuria in Shanxi, China

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Abstract

The variation in mutations in exons 3, 6, 7, 11 and 12 of the phenylalanine hydroxylase (PAH) gene was investigated in 59 children with phenylketonuria (PKU) and 100 normal children. Three single nucleotide polymorphisms were detected by sequence analysis. The mutational frequencies of cDNA 696, cDNA 735 and cDNA 1155 in patients were 96.2%, 76.1% and 7.6%, respectively, whereas in healthy children the corresponding frequencies were 97.0%, 77.3% and 8.3%. In addition, 81 mutations accounted for 61.0% of the mutant alleles. R111X, H64 > TfsX9 and S70 del accounted for 5.1%, 0.8% and 0.8% mutation of alleles in exon 3, whereas EX6-96A > G accounted for 10.2% mutation of alleles in exon 6. R243Q had the highest incidence in exon 7 (12.7%), followed by Ivs7 + 2 T > A (5.1%) and T278I (2.5%). G247V, R252Q, L255S, R261Q and E280K accounted for 0.8% while Y356X and V399V accounted for 5.9% and 5.1%, respectively, in exon 11. R413P and A434D accounted for 5.9% and 2.5%, respectively, in exon 12. Seventy-two variant alleles accounted for the 16 mutations observed here. The mutation characteristics and distributions demonstrated that EX6-96A > G and R243Q were the hot regions for mutations in the PAH gene in Shanxi patients with PKU.

Key words: gene mutation, phenylalanine hydroxylase, phenylketonuria.

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Introduction

Phenylketonuria (PKU) is the most common inborn error of amino acid metabolism and shows autosomal recessive inheritance. Mutations in the gene encoding phenylalanine hydroxylase (*PAH*; EC1.14.16.1) cause phenylalanine accumulation in the body fluids and damage to the central nervous system that can result in severe mental retardation and neurobehavioral abnormalities (Xu and Yang, 2005). PKU, which was first described by Folling in 1934, is a worldwide disease with marked regional and ethnic variation in its incidence. The prevalence is high among whites and low amongst blacks and asians (Gu, 2003). A prevalence of 1 in 10,000 was reported in Caucasians (Scriver and Kaufman, 2001) while that in Indians was approximately 1:18,300 (Rama Devi and Naushad, 2004). PKU is more frequent in Tunisia (1:7,631) (Khemir *et al.*, 2011). The incidence of PKU varies considerably across China, with an overall prevalence of 1:11,144 from 1985 to 2001 (Gu and Wang, 2004). The prevalence in the provinces of Shanxi, Jiangxi, Heilongjiang and Shandong was reported to be 1:7,849 (Li *et al.*, 2009), 1:11,188 (Wang and Zhang, 2006), 1:5,346 (Li, 2005) and 1:7,408 (Chen and Tian, 2006), respectively. The incidence among neonates in Shanxi province from 2004 to 2009 was 1:3,425 (Shanxi Provincial Health Bureau , 2009), which suggests that the incidence in northern China is higher than in southern China.

The *PAH* gene was first cloned in 1983 (Woo *et al.*, 1983). To date, 564 mutations of the *PAH* gene (12q22-q24) have been deposited in the PAH Mutation Analysis Consortium Database, of which 60.5% are missense mutations, 13.5% are deletion mutations, 11% are splicing mutations and 5.0% are nonsense mutations, inserts and silent mutations. The *PAH* gene mutations show considerable ethnic and regional variation (Zhang *et al.*, 1995). In the present study, we examined the variation in mutations in exons 3, 6, 7, 11 and 12 of the PAH gene in 59 children with PKU from Shanxi province.

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Subjects and Methods

Subjects

Fifty-nine unrelated children with PKU were recruited for this study after obtaining informed consent from the parents. The patients (30 males, 29 females) were identified during treatment at the Neonatal Screening Center of the Shanxi Province Women and Childrens Hospital in Taiyuan and came from various regions of Shanxi province. The subjects were ≤ 10 years old and were classified as < 2 years old (n = 38), 2-6 years old (n = 18) and 6-10 years old (n = 3). All 59 cases exhibited significant clinical manifestations of PKU and fulfilled the diagnostic criteria for PKU, with blood phenylalanine concentrations > 20 mg/dL (Qian and Wang, 2010). Urinary pterin analysis and blood neopterin dihydropteridine reductase assays were used to exclude tetrahydrobiopterin deficiency. Twenty-five patients were placed on a low Phe diet to decrease Phe levels following the initial diagnosis. The healthy children included 53 males and 47 females, with ages ranging from 1 month to 10 years old. This study was approved by Taiyuan Centre Hospital Ethical Comimittee.

DNA extraction

Genomic DNA was extracted from peripheral blood using a DNA extraction kit (Shanghai Ying Jun Biotechnology Co., Ltd., Shanghai, China). Purity was assessed by electrophoresis in 1% agarose gels and the DNA was stored at -20 °C.

PCR amplification

Exons 3, 6, 7, 11 and 12 of the PAH gene were amplified. The primers for exons 3, 6, 11 and 12 were based on a previous report (Gu, 2003) whereas those for exon 7 were designed specifically for this study. All primers were synthesized by Shanghai Jin Kang Biotechnology (Shanghai, China) (Table 1). The PCR mixture consisted of 10X buffer, 2.5 mM Mg^{2+}, 0.2 mM dNTP, 0.5 µM primer, 1 U of *Taq* polymerase and 1 µL of template DNA in a total volume of 20 µL. The reaction conditions were: denaturation at 94 °C for 3 min followed by 35 cycles of amplification at 94 °C for 30 s and annealing (Table 1) for 30 s with a final extension at 72 °C for 5 min. The PCR products were detected by electrophoresis in 2% agarose gels.

Sequencing

The PCR products were sequenced by Shanghai Ying Jun Biotechnology (Shanghai, China) and compared with the human genomic DNA sequence in GenBank to identify the mutations.

Statistical analysis

All statistical analyses, including the χ^2-test, were done using SPSS 13.0 statistical software. A p value ≤ 0.05 indicated significance.

Results

Exons 3, 6, 7, 11 and 12 of the PAH gene from 59 children with PKU and 100 healthy children were aligned with the GenBank cDNA sequence (GI: 209364518) of the PAH gene. Three single nucleotide polymorphisms (SNPs) [Q232 (CAA → CAG), V245V (GTG → GTA), and L385L (CTG → CTC)] were identified in the patients and healthy children. The incidence of the 696 locus SNP in children with PKU (96.2%) was not significantly different from that in healthy children (97%; $\chi^2 = 0.79$, $p > 0.05$). Similarly, there was no significant difference in the incidence of the 735 locus SNP in the patients compared to the healthy controls (76.1% *vs.* 77.3%; ($\chi^2 = 0.327$, $p > 0.05$) or in the incidence of the 1155 locus SNP (7.6% in patients *vs.* 8.3% in healthy controls; $\chi^2 = 0.111$, $p > 0.05$).

In children with PKU, 81 mutations belonging to 16 different types of mutations were observed and accounted for 61% of the PAH gene mutations. The mutations R111X, H64 > TfsX9 and S70 deletion were found in exon 3, with frequencies of 5.1%, 0.8% and 0.8%, respectively. Only the EX6-96A > G mutation was found in exon 6, with a frequency of 10.2%. The R234Q mutation in exon 7 had the highest frequency followed by Ivs7 + 2 T > A (5.1%) and T278I (2.5%). G247V, R252Q, L255S, R261Q and E280K

Table 1 - Primer sequences for PKU exons 3, 6, 7, 11 and 12.

Exon	Sequences	Annealing temperature (°C)	Product length (bp)
3	F: 5'-GTTAGGTTTTCCTGTTCTGG-3' R: 5'-CTTATGTTGCAAAATTCCTC-3'	60	300
6	F: 5'-CACAGGTTCTGGTCCCCGAC-3' R: 5'-CTCTCCTCTCCTCAATCCTC-3'	61	354
7	F: 5'-CTCCTAGTGCCTCTGACTCA-3' R: 5'-CAAACCTCATTCTTGCAGCAGG-3'	60	273
11	F: 5'-AAGGAATCGGGGTGAGATGAGAGAAGGGGC-3' R: 5'-GGTACAAAGTTGCTGTAGACATTGGAGTCC-3'	64	357
12	F: 5'-ATGCCACTGAGAACTCTCTT-3' R: 5'-AGTCTTCGATTACTGAGAAA-3'	60	245

accounted for 0.8% each. Y356X in exon 11 accounted for 5.9%, whereas V399V accounted for 1%. R413P and A434D in exon 12 accounted for 5.9% and 2.5%, respectively (Table 2). Among the 16 mutations, nine were missense, three involved splice sites, two were nonsense and two involved deletions, of which H64 > TfsX9 (Figure 1) was the first to be discovered. All of these mutations have been included in the PAH Mutation Analysis Consortium Database. As shown in Table 3, three alleles were identified in one patient, two alleles were identified in 16 patients and the remaining cases were identified in 33 samples. Six patients with homozygous mutations were identified. The intelligence of the 25 patients in the low Phe diet intervention group is developing well while the remaining children show some degree of mental retardation.

Discussion

Various PAH gene mutations have been identified in 13 exons, with those of exon 7 accounting for 20% of mutations (Zhang *et al.*, 1995; Song *et al.*, 2001; Fang *et al.*, 1992; Güttler and Guldberg, 1996). Nearly 30 species of PAH gene mutations have been found in the Chinese population and involve mainly exons 7 and 12 (Zhang *et al.*, 1995; Song *et al.*, 2001). In the present study, we identified two relatively high-frequency mutations, EX6-96A > G and R243Q, with frequencies of 10.2% and 12.7%, respectively. Two hot spots for mutations in the PAH gene were identified in Shanxi province patients with PKU and generally agreed with the findings of previous studies (Zhang *et al.*, 2004). Several studies have shown that there is considerable regional and ethnic variation in the distribution of PAH gene mutations in China (Fang *et al.*, 1992; Güttler and Guldberg, 1996; Zhang *et al.*, 1997). In agreement with this, the frequencies of the R243Q mutation in exon 7 of the PAH gene in northern and southern China were found to be 24% and 9.5%, respectively. The frequency of R243Q in exon 7 has been reported to be 18.2% in Chinese and 12% in Koreans; in contrast, R413P is a high-frequency mutation in Japanese (30.5%) (Zhang *et al.*, 1997).

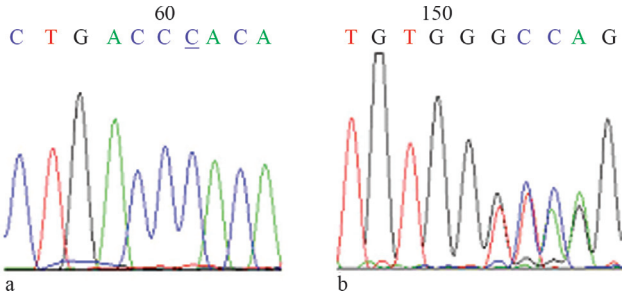

Figure 1 - Sequencing of the H64 > TfsX9 mutation. A: Normal samples for the forward primer sequence. B: Absence of specimens sequenced with the reverse primer.

PAH mutations can be classified into disease-causing and silent mutations, depending on the extent to which enzymatic activity is affected by the mutation. In contrast to disease-causing mutations, silent mutations do not affect PAH activity. Disease-causing mutations often occur in the crosslink areas between exons or between an exon and intron and affect PAH gene transcription and translation; such interference affects protein folding and aggregation and accelerates degradation, thereby influencing the catalytic activity of PAH (Bai and Song, 2003). In the present study, 16 mutations were found, including nine missense mutations, three splice site mutations and two nonsense mutations, as well as two types of deletions. The nonsense,

Table 2 - Shanxi PAH 3, 6, 7, 11 and 12 exon mutations.

Exon site	Amino acid change	cDNA change	Type of mutation	Number of alleles	Mutation frequency (%)
3	R111X	c. 331C > T	nonsense	6	5.1
3	H64 > TfsX9	c. 190delC	loss	1	0.8
3	S70 del	c. 208-210delTCT	loss	1	0.8
6	EX6-96A > G	c. 611A > G	splice site	12	10.2
7	R243Q	c. 728G > A	missense	15	12.7
7	G247V	c. 740G > T	missense	1	0.8
7	R252Q	c. 755G > A	missense	1	0.8
7	L255S	c. 764T > C	missense	1	0.8
7	R261Q	c. 782G > A	missense	1	0.8
7	T278I	c. 833C > T	missense	3	2.5
7	E280K	c. 838G > A	missense	1	0.8
IVS7	IVS7+2T > A	c. 842 +2T > A	splice site	6	5.1
11	V399V	c. 1197A > T	splice site	6	5.1
11	Y356X	c. 1068C > A	nonsense	7	5.9
12	A434D	c. 1301C > A	missense	3	2.5
12	R413P	c. 1238G > C	missense	7	5.9
Number				72	61.0
Total				118	100

Table 3 - Mutations identified in 51 PKU patients.

Patient	Sex	Age (years)	*PHA* allele 1		*PHA* allele 2	
			Codon	Het/Hom	Codon	Het/Hom
17	Male	1.5	Y356X	Het		
18	Male	3	Y356X	Het		
21	Male	5	R111X	Hom		
22	Female	2	R252Q	Het		
25	Male	2.5	H64 > TfsX9	Het		
26	Female	7	T278I	Het	G247V	Het
27	Female	7	IVS7+2T > A	Het		
28	Male	6	R413P	Het		
33	Male	1.5	EX6-96A > G	Het		
36	Male	5	EX6-96A > G	Het		
37	Male	5	R111X	Het	R243Q	Het
38	Female	4	EX6-96A > G	Het	Y356X	Het
40	Female	10	R413P	Het		
41	Male	2.5	A434D	Het		
47	Female	4	EX6-96A > G	Het	R243Q	Het
50	Male	35 days	T278I	Het	R243Q	Het
54	Female	35 days	T278I	Het		
60	Male	6	R261Q	Het		
63	Male	2	EX6-96A > G	Het	R413P	Het
69	Female	1	R243Q	Het		
72	Female	18 days	IVS7+2T > A	Het		
78	Male	7 months	R111X	Het		
81	Female	23 days	R243Q	Het		
84	Female	1	IVS7+2T > A	Het	R243Q	Het
87	Female	4	Y356X	Hom		
90	Male	3	EX6-96A > G	Hom		
99	Male	15 months	V399V	Het		
104	Female	1	IVS7+2T > A	Het	R413P	Het
107	Female	1	R111X	Hom		
112	Female	18 days	EX6-96A > G	Het		
115	Male	15 days	V399V	Het		
118	Male	34 days	R243Q	Het	Y356X	Het
121	Female	1 month	R413P	Het		
124	Female	8 months	EX6-96A > G	Het	R243Q	Het
130	Female	12 months	A434D	Het		
132	Female	32 days	EX6-96A > G	Het	R243Q	Het
134	Male	34 days	IVS7+2T > A	Het	R243Q	Het
141	Female	4	V399V	Het	R413P	Het
144	Male	13 months	R243Q	Het		
147	Male	1	IVS7+2T > A	Het		
153	Male	2	R243Q	Het		
166	Male	6 months	Y356X	Het		
172	Male	1	V399V	Het		
175	Female	23 days	R243Q	Het	E280K V399V	Het
180	Male	8 months	EX6-96A > G	Het	R413P	Het
186	Female	2.5	R413P	Het	L255S	Het
192	Male	36 days	EX6-96A > G	Het		
195	Male	43 days	S70 del		R243Q	Het
198	Female	11 months	V399V	Hom		
201	Female	4.5	A434D	Het		
204	Male	3.5	R243Q	Hom		

splice site and deletion mutations interfere with protein transcription or translation and are generally considered to be pathogenic mutations. The pathogenicity of missense mutations requires further investigation involving gene expression and protein structure analysis (Song *et al.*, 2008). To our knowledge, this is the first report to identify the mutant site H64 > TfsX9, a single base deletion that resulted in a frameshift mutation in exon 3 of the PAH gene. The mutation at codon 64 (c. 190), which replaces histidine by threonine, may change the PAH structure and affect the corresponding enzymatic activity.

PKU should be detected and treated early since the earlier the treatment, the better the prognosis (Lu and Jiang, 2003). Early treatment is the key factor to changing the outcome of this disease (Zhang and Qi, 2001). The prenatal diagnosis of PAH gene mutations using the approach described here could be useful in reducing the incidence of PKU in Shanxi province. Screening for mutations could also be useful in the detection, genetic counseling and treatment of potential PKU carriers, thereby improving the quality of life of affected individuals.

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Internet Resources

The PAH Mutation Analysis Consortium Database, http://www.mcgill.ca/pahdb (Aug 31, 2009).

Shanxi Provincial Health Bureau http://www.sxws.cn/bureau/ShowLeaderActionBeta.asp?strNewsId = 5252 (May 27, 2009).

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