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Original Article

PAH mutation spectrum and correlation with PKU manifestation in north Jiangsu province population

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Abstract Phenylketonuria (PKU) is a common autosomal recessive disorder of phenylalanine metabolism and mainly results a deficiency of phenylalanine hydroxylase gene (*PAH*). The incidence of various *PAH* mutations have race and ethnicity differences. We report a spectrum of *PAH* mutations complied from 35 PKU children who are all Chinese Han population from north Jiangsu in this study. All 13 exons and their flanking intron sequences of *PAH* were determined by Ion Torrent PGM™ sequencing. The relationship of genotype and phenotype was analyzed based on the sum of the arbitrary value (AV) values of the two alleles. We identified 61 mutations, with a frequency of 87.14%, among 70 alleles of 35 patients. The most prevalent mutations were R243Q (26.23%), R241C (9.84%) and V399V (8.20%). Furthermore, the consistency between prediction of the biochemical phenotype and the observed phenotype was 81.25%, with the highest consistency observed in classic PKU (87.50%). A significant correlation was found between pretreatment levels of phenylalanine and AV sum ($r = -0.87$, $P < 0.05$). Finally, our study constructs PAH mutation spectrum by next generation sequencing (NGS), and reveals that the *PAH* genotypes and biochemical phenotypes were significantly correlated. These offers facilitate the provision of appropriate genetic counseling for PKU patients.

Conflicts of interest: All authors declare no conflicts of interest.

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Introduction

Phenylketonuria (PKU, MIM 261600) is a genetic disease widely distributed in human populations [1]. The world wide incidence of PKU is in the range of 1/1000—1/12,000 [1,2], while the average incidence in China is 1/11,572 [2]. PKU is a recessive disorder caused by mutations in the phenylalanine hydroxylase gene (*PAH*) that leads to a loss of enzyme activity and accumulation of phenylalanine [3]. This type of PKU is defined as PAH-deficient PKU. High levels of serum phenylalanine cause injuries in the peripheral and central nervous system, which manifested as neurological complications and intellectual disability [4]. Fortunately, if newborns with PKU are diagnosed and treated properly in a timely manner, mental development problem can be largely prevented. Studies have shown that more than 97% of PKU patients are PAH-deficient subtype [5].

As one of the best studied genetic disorders, PAH-deficient PKU involves mutations in the 13 exons and 12 introns as well as the upstream and downstream regulatory regions of *PAH* gene [6]. More than 900 mutations have been identified in *PAH*, and recorded in the locus-specific database (LSD) known as *PAH*vdb (http://www.biopku.org/pah/) [7]. Constructing *PAH* mutation spectrum can facilitate the diagnosis of PKU, prediction of patients' clinical manifestations, selection of therapeutic options, and design of individualized therapy. Due to the significant regional and ethnic heterogeneity of *PAH* mutations in the world [2,8—10], it is necessary to perform PKU studies in a population-specific manner. To date, there has been little comprehensive PKU research of population genetics focusing on the Chinese Han population which is the major Chinese ethnicity accounting for more than 91% of total population, and there has been no research in the north of Jiangsu Province where is almost all Chinese Han population and a small floating population. This goal of this study is to construct a *PAH* mutation spectrum in patients from north Jiangsu province, a highly populated area in central part of China. Based on the features of mutations, the relationship between genotype and phenotype is analyzed.

Materials and methods

Patients

35 children consisted of 18 boys and 17 girls of PKU (6 months—5 years) and their parents in north Jiangsu province were chosen for this research and no such diseases were found in their parents. All of these children and their parents were Chinese Han population. These children are unrelated and all with PAH-deficient PKU. Serum Phe concentrations of these patients fell in a range from 360 μmol/L to 1800 μmol/L. Depending on serum Phe concentrations of untreated children with PAH deficient PKU, PKU can be categorized into the following four types [11]: classic PKU (Phe, more than 1200 μmol/L), moderate PKU (Phe, 600—1200 μmol/L), mild PKU (Phe, 360—600 μmol/L)and mild hyperphenylalaninemia (HPA) who keeps their Phe levels between 120 μmol/L and 360 μmol/L on a phenylalanine-free diet. The patients were fasted and 2 ml of blood samples were taken and preserved in −20 °C and EDTA-K2 for sequencing. This project was approved by the Hospital Ethics Committee and informed consent was obtained from each participant. All parents of these children provided their written informed consent to participate in the study according to the Declaration of Helsinki.

Method for phenylanaline level measurement

The concentration of blood phenylanaline was determined by Ninhydrin fluorescence method [12]. The detection instrument was DEFIA-1420 Semi-auto time-resolved fluoroimmunoassay analyser (Wallac, Finland). Reagent kits were provided by PerkinElmer company (USA) and the test process was strictly accordance with the Operation instruction.

Next generation sequencing

The whole blood genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) following protocols provided by the manufacturer. The DNA samples were frozen on dry ice and sent to a commercial lab (Biosan, China) specialized in NGS. The NGS procedures, including amplification of exons and exon—intron junction regions, library construction, sequencing, and data analysis, were carried out according to previous publication [13].

Sanger sequencing

The extracted samples were further verified using Sanger sequencing method. This procedure was completed by the commercial lab (Biosan, China). The modified gene was named in accordance with the standard of the Human Genome Variation Society (HGVS, www.Hgvs.Org/mutnomen/) [14].

Phenotypic prediction system

In accordance with the residual activity of PAH *in vitro*, mutations were assigned to four phenotype categories including: classic, moderate, mild, and HPA by the method of Guldberg [11]. In brief, an arbitrary value (AV) was assigned to each mutation: AV = 1 for classical PKU mutation, including nonsense mutations, splicing mutations, deletion and insertion mutations, which lead to almost undetectable levels of residual activities by *in vitro* expression and biochemical assays. The missense mutations in this class lead to residual activities less than 10% of the wild type PAH activity. AV = 2 for moderate PKU mutation, including mutations leading to residual activities only 10%—30% of the wild type PAH. AV = 4 for mild PKU mutations, including mutations leading to residual activities 30%—70% of the wild type PAH activity. AV = 8 for mild HPA mutation, including mutations leading to residual activity more than 70% of the wild type PAH activity. Phenotypes resulting from a combination of the two mutant alleles were expressed as the sum of AVs of the two mutations. The individuals whose mutations without AV estimates in the literature were excluded from this specific analysis.

Statistical analysis

The data statistical analysis was performed with the use of SPSS 17.0. Qualitative data was expressed as the percentage. The Pearson correlation analysis was used to evaluate the relationship between AV score and patients' blood Phe levels. $P \leq 0.05$ was used as a level of significance in all analyses.

Results

Type and distribution of phenylalanine hydroxylase mutations

The study identified 26 children with mutations in two alleles of *PAH*, and 9 children with mutations in one allele. Thus, totally 61 mutations were detected. The mutation detection rate among the totally 70 *PAH* alleles were 87.14% (61/70). Three high-frequency mutations were R243Q (11/50, 22%), R241C (4/50, 8%) and V399V (4/50, 8%), respectively (Table 1). Mutations from these patients belong to 24 different types, with 22 types of mutations found in exons and 2 found in introns. In these different mutation types, 15 were missense mutations, 4 were nonsense mutations, 1 were deletion mutation and 4 were splice mutations. Among the 61 individual mutations detected, 43 (70.5%) are missense mutations, with 40.9% in Exon 7, followed by Exon 11 (16.4%) and Exon 12 (13.1%). All the mutations in this study were confirmed by Sanger Sequencing technology. In addition, the mutations of children with PKU in this study were all derived from their parents, rather than spontaneous mutation. Information on the mutations and their distribution in different countries are summarized in Table 2.

Genotype—phenotype relationship

19 patients meet the requirements for analysis on genotype—phenotype relationship. Comparison of the predicted phenotype based on the sum of AVs of two alleles and the actual biochemical metabolic phenotype observed from children showed that 75% or higher consistency can be achieved in all the four groups (Table 3). Besides, there was a significant negative correlation between the AV scores before treatment and the blood Phe levels in children ($r = -0.87$, $P < 0.05$).

Discussion

In this study, 61 *PAH* mutations were detected using NGS method from 35 children with *PAH* deficient PKU. The detection rate was about 87.14% and the results were comparable to those of Zhu [9], Murad [18], and Polak [19]. *PAH* was consisted of 13 exons and 12 introns. The study found that exons 7 and 11—12 harbor most mutations, with exons 3, 5—8, 10—12 and intron 4 and 5 showing lower

Table 1 Distribution of *PAH* mutations in PKU patients from north Jiangsu, China.

Mutation sites	Changes in Bases	Changes in amino acid	Mutation types	Allele numbers and percentage (%)	AV
Exon 3	c.208_210del TCT	p.S70del	Deletion	2 (3.28)	1
Exon 3	c.331C>T	p.R111X	Nonsense	1 (1.64)	1
Intron 4	c.442-1G>A	IVS4-1G>A	Splice	1 (1.64)	1
Exon 5	c.472C>T	p.R158W	Missense	1 (1.64)	1
Exon 5	c.482T>C	p.F161S	Missense	2 (3.28)	1
Exon 5	c.505C>A	p.R169S	Missense	1 (1.64)	?
Intron5	c.509+1G>A	IVS5+1G>A	Splice	1 (1.64)	1
Exon 6	c.611A>G	p.EX6-96 A>G	Splice	3 (4.92)	1
Exon 6	c.699C>A	p.F233L	Missense	1 (1.64)	?
Exon 7	c.721C>T	p.R241C	Missense	6 (9.84)	4
Exon 7	c.727C>T	p.R243X	Nonsense	1 (1.64)	1
Exon 7	c.728G>A[a]	p.R243Q	Missense	16 (26.23)	1
Exon 7	c.833C>A	p.T278N	Missense	2 (3.28)	?
Exon 8	c.875C>T	p.P292L	Missense	1 (1.64)	?
Exon 10	c.1006C>T	p.Q336X	Nonsense	1 (1.64)	1
Exon 10	c.1045T>G	p.S349A	Missense	3 (4.92)	?
Exon 11	c.1068C>A	p.Y356X	Nonsense	3 (4.92)	1
Exon 11	c.1197A>T	p.V399V	Splice	5 (8.20)	1
Exon 11	c.1139C>T	p.T380M	Missense	1 (1.64)	8
Exon 11	c.1199G>A	p.R400T	Missense	1 (1.64)	1
Exon 12	c.1223G>A	p.R408Q	Missense	1 (1.64)	4
Exon 12	c.1222C>T	p.R408W	Missense	1 (1.64)	1
Exon 12	c.1238G>C	p.R413P	Missense	2 (3.28)	1
Exon 12	c.1301C>A	p.A434D	Missense	4 (6.56)	2

Note: a. The highest frequency of mutations in this study.

Table 2 Comparisons of the *PAH* mutation frequencies between PKU patients from north Jiangsu and other countries and/or regions.

Mutations	North Jiangsu (%)	North China (%) [6]	South China (%) [15]	Taiwan (%) [8]	Korea (%) [15]	Czech (%) [16]	Lithuania (%) [16]	Brazil (%) [17]
R243Q	26.23	21.70	9.50	4.20	12.00	3.00	—	1.30
R241C	9.84	—	—	23.20	—	—	—	—
V399V	8.20	7.70	—	3.50	—	—	—	—
A434D	6.56	—	—	2.80	—	—	—	—
EX6-96A>G	4.92	10.02	10.07	9.20	10.00	—	—	—
S349A	4.92	—	—	—	—	—	—	—
Y356X	4.92	7.70	7.70	—	6.00	—	—	—
R413P	3.28	7.10	7.10	—	3.00	—	—	—
F161S	3.28	—	—	—	—	—	—	—
T278N	3.28	—	—	—	—	—	—	—
S70del	3.28	—	—	—		—	—	—
R111X	1.64	5.20	5.20	3.50	0.70	—	0.50	—
R158W	1.64	—	—	—	—	—	—	—
R169S	1.64	—	—	—	—	—	—	—
F233L	1.64	—	—	—	—	—	—	—
R243X	1.64	—	—	—	—	—	—	—
P292L	1.64	—	—	—	—	—	—	—
Q336X	1.64	—	—	—	—	—	—	—
T380M	1.64	—	—	—	—	—	—	—
R400T	1.64	—	—	—	—	—	—	—
R408Q	1.64	—	—	12.00	—	—	—	—
R408W	1.64	—	—	—	—	55.00	73.40	—
IVS4-1G.>A	1.64	—	—	—	10.00	—	—	—
Splicing Error	1.64	—	—	—	—	—	—	—
R261Q	—	—			1.00	2.00	0.50	12.20

frequency of mutation. This distribution pattern was generally consistent with previous findings [8,9,18]. In addition, the percentage of missense mutations was at the highest frequency, also consistent with previous findings. Recently, the spectrum of *PAH* mutations in Chinese Han population have been constructed by scholars [9]. It was reported that the most common *PAH* mutation in Chinese Han population were R243Q which was consistent with our study, and other frequent mutations include: EX6-96A>G, R241C and R111X. So, there were differences between above studies [9,20] and our study (R241C, V399V and A343D) in other frequent mutations. Furthermore, frequent mutations in Jiangsu province from Chen [21] were R241C, Y356X and EX6-96 A>G, and this appeared to be not consistent with our study. Moreover, some studies showed that in south China and Taiwan region the R243Q was not the highest, and the most common mutations in Taiwan [8] were R241C and R408Q, while the R241C in the northern of China was relatively low [6]. The EX6-96A>G was found to be the highest in south China in previous studies [15]. Other studies found that the most common *PAH* mutations in Eastern Europe (Czech and Lithuania), south America (Brazil) and Eastern Asia (China and Korea) were different [8,9,15], which were R408W, R261Q and R243Q, respectively. It was mainly due to the significant regional and ethnic heterogeneity of *PAH* mutations in the world. In this study, R243Q (22%) was found to be the most frequent *PAH* mutation in north Jiangsu area. R241C and V399V, both reaching 8%, were the second most frequent mutations. The frequency of R241C ranks higher than in other studies

Table 3 The analysis of genotype—phenotypic on the basis of AV.

AV	The predicted phenotype	Number	The actual phenotype				The concordance rate
			Classic PKU	Moderate PKU	Mild PKU	Mild HPA	
2	Classic PKU	8	7	1	0	0	87.50%
3—4	Moderate PKU	4	0	3	1	0	75.00%
5—8	Mild PKU	6	0	1	5	0	83.33%
9—16	Mild HPA	1	0	0	0	1	100.00%
Total	—	19	—	—	—	—	84.21%

[9,20]. Although the exact reason behind the specific *PAH* mutation pattern in this area and in Northern and Western regions of China remains unclear, investigation on *PAH* mutation spectrum of north Jiangsu can facilitate the diagnosis and prognosis of PKU patients in this region.

NGS is an advanced method to build the PAH mutation spectrum [7]. The NGS procedures were optimized constantly to ensure the data quality. To confirm the results, all the mutations in this study were verified by Sanger sequencing and the technology. In this study, only one mutation was identified by NGS and Sanger sequencing in some patients. Probably this was because the pathogenic mutation was out of the detection range, such as the 5′- or 3′-noncoding region (UTR) and noncoding RNA binding sites. Of course, we should not completely rule out other pathogenesis which has not yet been found. One drawback of this study is the limited sample size. Future studies with larger cohort of patients should be performed to verify the observations.

Some studies have shown a close quantitative correlation between genotype and phenotype [22,23]. The residual PAH activity was recorded from *in vitro* experiments using recombinant proteins expressed in eukaryotic cells [11]. Since the corresponding enzyme activity of some specific mutants had not been tested and only one mutation type had been tested in some individuals, some patients were excluded from the analysis. Nevertheless, the data (Table 3) revealed that the genotypic score based on the biochemical phenotype could well predict the actual phenotype, in 84.21% cases actually. One previous study in Beijing area reported a 81.50% overall consistency rate, with a 87.50% consistency for classic PKU. These numbers were close to the result of current study. The consistency rate in another study in Shanghai area was 54.41%, in which classic PKU was 61.83%, which were lower than the result of this study [9]. The consistency rate in Jiangsu province was 38.10%, that was lower than the result of this study, and the 45.45% consistency for classic PKU was relatively low [21]. Taken together, the current results strongly support a close correlation between *PAH* genotypes and biochemical phenotypes.

In conclusion, the main mutation regions and sites in north Jiangsu where is almost all Chinese Han population were determined by constructing *PAH* mutation spectrum in this study. Based on reported *in vitro* enzyme activity of mutants, the relationship between genotype and phenotype was analyzed. An appropriate genotype—biochemistry phenotype prediction system could be formed through characterization of a large number of mutants in the future [24]. Such an approach could be a productive strategy in the development of tools useful for genetic counseling and prenatal diagnosis.

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