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Study of Newborn Screening and Genetic Mutation for Phenylketonuria in Yantai City

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ABSTRACT

To provide effective clinical evidence for prediction and genetic diagnosis, comparison of 3 newborn screening techniques for phenylketonuria and analysis of genetic mutations of infants with positive screening results in Yantai city were performed. Guthrie bacterial inhibition assay, fluorescence immunoassay, and tandem mass spectrometry were used for neonatal screening of PKU in 692,703 dried filter-paper blood specimens. MassARRAY was performed to identify mutant sites in 40 cases of phenylketonuria patients. About 30 neonates with positive Guthrie bacterial inhibition assay results were confirmed to be PKU, 57 with fluorometric testing and 1 with tandem mass spectrometry were observed. Total of 31 different mutations was detected in 40 affected children, with the highest mutation frequency of R243Q. The heterogeneity in genetic mutations among infants with phenylketonuria in Yantai was observed, showing that mutation spectrum differed from those reported in other regions.

Keywords: Phenylketonuria, Screening, Guthrie bacterial inhibition assay, Fluorescence immunoassay, Tandem mass spectrometry, MassARRAY, Genetic mutation

INTRODUCTION

Phenylketonuria (PKU) is one of the leading causes of mental retardation among children, where phenylalanine metabolic disorders are characterized by a deficiency in the activity of phenylalanine hydroxylase or coenzymes. As an autosomal recessive genetic disease, its incidence in Chinese population is about 1/11000 [1]. More than 860 different phenylalanine hydroxylase mutations have been reported or registered internationally, and over 70 mutations were identified in China [2]. A diversity of gene mutations and frequency of occurrence were reported among different ethnic groups, races, as well as regions [3]. From August 1998 to December 2013, 692,703 newborns were screened in the newborn screening centers of maternal and child health hospitals in Yantai, of which, 88 were diagnosed with phenylketonuria. This study comparatively explored 3 methods of neonatal phenylketonuria screening in Yantai, and it's the first time for us to use MassARRAY to identify the frequency of mutations in 40 patients in the region of Yantai.

METHODOLOGY

Study Subjects

Total 692,703 cases of live births were screened for PKU in all the hospitals of Yantai city. Among the infants with positive phenylketonuria screening results and conformed to be affected, 40 cases were randomly selected for MassARRAY analysis.

This study has been approved by the ethics committee of the Yantai Hill Hospital in Yantai City and informed consent was signed by the guardian of every subject.

Screening Test

Heel blood samples were collected from newborns 3-7 days after birth on Schleicher and Schuell filter paper, dried naturally in the shade, sealed in plastic bags, and stored at 0-4°C, which was later sent to our laboratory for massive testing. Methods used in the screening test included bacterial inhibition assay, time-resolved fluoroimmunoassay, and

tandem mass spectrometry. Those whose preliminary screening results were above the cut-off value of our laboratory were classified as phenylketonuria-positive suspects, who were recalled for the confirmatory test then.

Guthrie bacterial inhibition assay: The growth of *Bacillus subtilis* can be inhibited by β-2-thiophene alanine, while phenylalanine can relieve this inhibitory effect. When the growth of *Bacillus subtilis* was inhibited in *Bacillus subtilis* culture medium, the addition of neonatal dry blood filter paper into the culture plate and incubation overnight after incubation at 37°C would cause an increase in phenylalanine concentration. Consequently, the obvious growth ring was formed. By comparing the growth ring diameter size with a standard ring with a certain amount of phenylalanine content, phenylalanine concentration of tested samples could be obtained.

Time-resolved fluoroimmunoassay: Fluorescent compounds formed when phenylalanine, Ninhydrin, and copper salt were added into some kind of acidic peptide solution and the fluorescence intensity was measured at the wave length of 485 nm.

Tandem mass spectrometry: With tandem mass spectrometry method, testing substances were ionized into molecules with different charge ratios. According to electromagnetics principle, these charged particles were arranged in a pattern by m/Z size separation molecular weight and a molecular formula of these compounds can be determined by measuring the intensity of the peak particles. Amino acids, organic acids, and acylcarnitine in neonatal dry filter paper can be analysed in this way.

MassArray Genetic Testing

DNA extraction: DNA was extracted of dried blood spots with DNA extraction kit, and quantified with a Nanodrop spectrophotometer. Qualified DNA was adjusted to a concentration 5 ng/µL, and was stored at -20°C for further use.

Primer design: Primers were designed with Sequenom's Typer 4.0 software, all primers for Polymerase Chain Reaction amplification (PCR) of test mutation sites and for single base extension were synthesized by Integrated DNA Technologies, Inc. USA.

Screening of mutation site: Total 161 different mutations of phenylalanine hydroxylase (PAH) and 30 different mutations of 6-pyruvoyl tetrahydropterin synthase (PTS) in Chinese population was screened according to the disclosed inclusion criteria for gene mutations in NCBI PubMed, OMIM and HGMD literature.

Experimental procedure: Reagents were prepared according to SequnomiPLEX genotyping manual. Total PCR reaction volume was 5 µl, including 1 pmol of PCR primers and 5 ng of genomic DNA and reactant mixture. PCR reaction conditions were 94°C for 15 min followed by 40 cycles of 20 sec at 94°C, 30 sec at 56°C, 60 sec at 72°C and a final step at 72°C for 3 min. Then, the remaining PCR reaction products (dNTP) were removed by SAP enzyme; total reaction volume of SAP was 7 µl (operated on 384 well plates) (37°C for 40 min, 85°C for 5 min). Next, the single genome amplification reaction was performed (total reaction volume of 9 µl; 94°C for 5 min followed by 40 cycles of 5 sec at 94°C, 5 sec at 52°C and 5 sec at 72°C. Afterwards, the reaction products were dispensed onto the chip with a Sequenom Nanodispensor RS1000. Laser excitation and data collection were done with a Sequenom MassARRAY mass spectrometer, and finally, mutations were interpreted according to the preset genotypes using Sequenom Typer 4.0 software.

Instruments and reagents: DNA extraction kit was purchased from Qiagen Company, PCR and iPLEX Gold Reagent Kits from Sequenom Company were prepared for genotyping mutation analysis, and Sequenom's Nanodispensor RS1000, MassARRAY Analyzer 4 system and Typer 4.0 software were prepared for mass spectrometry analysis.

RESULTS

Testing results of Guthrie bacterial inhibition assay, fluorescence immunoassay, and tandem mass spectrometry,

692,703 neonates received phenylketonuria screening within the 3 methods and 88 cases were confirmed to be positive, 30 of which with Guthrie bacterial inhibition assay, 57 with fluorescence immunoassay and 1 with tandem mass spectrometry, and of which 38 were boys and 50 were girls. The false-negative rate was 0. PKU affected children were provided with a stringent diet of low-phenylalanine and/or with pharmacologic treatment of BH4 (Table 1).

Table 1 Comparison of detection results of three methods

Screening Methods	Number of Screening	Suspicious Positive	Confirmed Positive
Bacterial inhibition	267950	119	30
Fluorescence immunoassay	424753	145	57
Tandem Mass Spectrometry	2475	1	1

Comparison of the 3 Screening Methods

The variation coefficients of quality controls of the 3 screening methods, which were the bacterial inhibition method, fluorescence method, and tandem mass spectrometry, decreased successively, inversely, the accuracy, stability, and repeatability increased successively (Table 2).

Table 2 Comparison of batch quality controls of the three methods

Screening Methods	Average	Median	Standard	The coefficient of variation
Bacterial inhibition	5.30	5.20	0.46	8.68%
Fluorescence immunoassay	4.80	4.80	0.35	7.32%
Tandem Mass Spectrometry	233.80	233.50	15.00	6.42%

Mutations Detection Result of MassArray Test

The total mutations detection rate was 93.75%, of which 3 mutations were identified in 3 cases, accounting for 7.5%, 2 mutations were detected in 29 cases, occupying 72.5% and 1 mutation was found in 8 cases, accounting for 20%. 71 were heterozygous mutations and 4 homozygous mutations. 75 mutation sites were found on the 80 chromosomes with 70 mutation sites in the exons and 5 in the introns. Our findings also included 53 missense mutations, 9 nonsense mutations, 1 deletion mutations, and 12 splice mutation. The highest frequency of R243Q mutation was 23%, followed by V399V and Y356X (8%), with R241C ranking the third (7%), A434D, R413P and R53H took up 4% each, followed by R400T and Y204C (3% each). Two mutations p.P87S and p.D96N were of 6-pyruvoyl-tetrahydropterin synthase gene, which encodes BH4 coenzyme of phenylalanine hydroxylase. Table 3 showed 31 different gene mutations identified in all PKU affected children.

Table 3 Spectrum of PAH gene and PTS gene identified in 40 PKU affected newborns

System name (DNA level)	Trivial name (protein effect)	Location	Characters of mutation	No. of alleles	Relative frequency
c.158G>A	p.R53H	exon2	Missense	3	0.041
c.168G>T	p.E56D	exon 2	Missense	1	0.014
c. 194T>C	p.I65T	exon 3	Missense	1	0.014
c.208-210 delT	p.S70 del	exon 3	Missense	1	0.014
PTS c.259C>A	p.P87S	exon 3	Missense	1	0.014
259C>T	p.P87S	exon 3	Missense	1	0.014
PTS c.286G>A	p.D96N	exon 3	Missense	1	0.014
c.331C>T	p.R111X	exon 3	Nonsense	1	0.014
c.442-1G>A	Splicing Error	Intron 4	Splicing	3	0.041
c.473G>A	p.R158Q	exon 5	Missense	1	0.014
c. 482T>C	p.F161S	exon 5	Missense	1	0.014
c. 498C>G	p.Y166X	exon 5	Nonsense	1	0.014
c. 526C>T	p.R176X	exon 6	Nonsense	1	0.014
c.611A>G	p.Y204C/EX6-96A>G/splicing	exon 6	Splicing	2	0.027
c. 721G>A	p.R241C	exon 7	Missense	5	0.068
c.728G>A	p.R243Q	exon 7	Missense	17	0.233
c.739G>C	p.G247R	exon 7	Missense	1	0.014
c.755G>A	p.R252Q	exon 7	Missense	1	0.014
c. 782G>A	p.R261Q	exon 7	Missense	1	0.014
c. 800A>T	p.Q267L	exon 7	Missense	1	0.014
c.824+2T>A	Splicing Error	Intron7	Splicing	1	0.014
c.842+2T>A	Splicing Error	Intron7	Splicing	1	0.014

<table>
<tr><td>c.975C>G</td><td>p.Y325X</td><td>exon 10</td><td>Nonsense</td><td>1</td><td>0.014</td></tr>
<tr><td>c.1024G>A</td><td>p.A342T</td><td>exon 10</td><td>Missense</td><td>3</td><td>0.041</td></tr>
<tr><td>c. 1068C>A</td><td>p.Y356X</td><td>exon 11</td><td>Nonsense</td><td>6</td><td>0.082</td></tr>
<tr><td rowspan="2">c.1101G>A</td><td>p.L367L/</td><td rowspan="2">exon 11</td><td rowspan="2">Silence/splicing</td><td rowspan="2">1</td><td rowspan="2">0.014</td></tr>
<tr><td>Splicing Error</td></tr>
<tr><td rowspan="2">c.1197A>T</td><td>p.V399V/</td><td rowspan="2">exon 11</td><td rowspan="2">Silence/splicing</td><td rowspan="2">6</td><td rowspan="2">0.082</td></tr>
<tr><td>Splicing Error</td></tr>
<tr><td>c. 1197G>C</td><td>p.R400T</td><td>exon 11</td><td>Missense</td><td>2</td><td>0.027</td></tr>
<tr><td>c. 1199G>C</td><td>p.R400T</td><td>exon 11</td><td>Missense</td><td>1</td><td>1.014</td></tr>
<tr><td>c.1238G>C</td><td>p.R413P</td><td>exon 12</td><td>Missense</td><td>3</td><td>0.041</td></tr>
<tr><td>c.1301C>A</td><td>p.A434D</td><td>exon 12</td><td>Missense</td><td>3</td><td>0.041</td></tr>
</table>

DISCUSSION AND CONCLUSION

The incidence of phenylketonuria varies among different regions in China. Neonatal morbidity is around 1/8000 in Yantai, higher than the average national incidence. Studies on genetic and biochemical metabolism and pathogenesis have confirmed that phenylketonuria is associated with mutations in the phenylalanine hydroxylase (PAH) gene, which is located on chromosome 12q23 and is an autosomal recessive disease, with equal incidence in both sexes. Clinically, the disease is divided into classic type, temporary and tetrahydrobiopterin deficiency type. The simplest differential diagnosis method is urinary pterin analysis. There are 5 methods used for phenylketonuria screening, including bacterial inhibition assay, enzyme assay, fluorescence assay, series mass spectrometry, and ferric chloride test. Ferric chloride test can only be applied to neonates 4 weeks after birth, so we compared the rest 4 methods in this study. As a semi-quantitative screening method, bacterial inhibition assay is easy to use, however, its disadvantages are obvious, including a large coefficient of variation and relatively poor accuracy. According to statistics data from the Chinese health department, some cases of phenylketonuria patients were missed by bacterial inhibition method. Advantages of fluorimetric determination of phenylalanine include high sensitivity and specificity, trace sample, wide linear range, high precision, and good reproducibility, while it is time-consuming. Tandem mass spectrometry is reported to be efficient, fast, highly sensitive and specific, and has wide linear range, high precision, and good repeatability. Accuracy is improved by using the ratio of phenylalanine to tyrosine as a screening marker to assess urine of neonates. Currently, the widespread application of new technologies such as tandem mass spectrometry in clinical practice has made early detection of the neonatal congenital metabolic disease possible [4].

Among gene testing methods for phenylketonuria, Desviat LR et al., identified 2 large deletion mutations (6.6 kb and 1.8 kb respectively) on PAH gene of 22 PKU affected patients with multiple ligation probe amplification methods innovatively [5]. Kim, et al., performed a tetrahydrobioprerin sensitivity study *in vitro* with transient overexpression system and identified some affected mutations, including 6 novel mutations (P69S, G103S, L293M, G332V, S391I, A447P) [6]. Ding, et al., adopted molecular biology method on the treatment of phenylketonuria [7]. In our study, MassARRAY was used to analyse genes of phenylketonuria patients tentatively. Only 40 cases were included in our study due to limited funding. Primers for 161 different phenylalanine hydroxylase gene mutations were designed, as well as 30 different 6-pyruvoyl-tetrahydropterin synthase gene mutations. MassARRAY turned out to be a novel clinical technique for the detection and diagnosis of PKU, with advantages of flexible experimental design and fast detection. MassARRAY is supposed to identify 98% of clinically validated single point mutations, deletions, and insertions of genes. While those unreported mutations, large deletions, and inversions are likely to be missed by this method. Other techniques such as next sequencing can be added to confirm detected mutations and identify potential mutations undetectable with MassARRAY. For example, 2 pedigree analysis was performed in our study with MassARRAY and next sequencing, and one more mutation was identified in one patient with the next sequencing, which was undetectable with MassARRAY. The pedigree analysis also demonstrated that all mutations of the two PKU affected children turned out to be inherited from their parents, not novo mutations. So further genetic analysis of pedigree can help the patient's family to establish a reliable basis for the prenatal diagnosis of subsequent pregnancies.

Racial and regional differences of mutation spectrums of the PAH gene and PTS gene have been reported. The detection rate of gene mutations associated with PKU was 93.45% in our study, 93.94% in Tianjin and surrounding areas, 84.6% in north China, 89.10% in Shanxi and 98.2% in Hebei with the highest incidence reported so far [8-11].

It would be better to carry out a pedigree linkage analysis to reach a detection rate of 100%. Xuefan, et al., showed that the majority of PAH gene mutations in Chinese Han population were located on exons 3, 5, 6, 7, 11, 12 and intron 4 [1]. While Song, et al., reported that mutations in Xinjiang region of China were located in the regions of exons 3, 5, 6, 7, 11 and 12 mainly, while those in north China reported by Song, et al., were exons 2, 3, 5, 6, 7, 8, 10, 11, 12 and intron 4 [2,9]. Our study showed mutations in Yantai region distributed on exons 2, 3, 5, 6, 7, 10, 11, 12 and introns 4, 7. The highest mutation frequency in Israeli was IVS10-11G>A (13.3%) [12], while that of the Croation area was R408W (36%) [13]. Hotspot mutations in the Taiwan region of China were R241Q (63%) and R408Q (28%) [14]. The highest mutation frequency of our study was up to 23% in R243Q, which is consistent with one literature reported, in which mutation p.R243Q ranked the first with 15.62% [15].

Yantai Newborn Disease Screening Center has achieved over 98% of screening coverage for PKU and no missed cases were identified since it was established, thus providing a reliable basis for clinical screening and diagnosis of PKU in neonates.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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