Molecular diagnosis of phenylketonuria in 157 Chinese families and the results of prenatal diagnosis in these families

Yang Xiao[1], Qiang Gu[2], Hai-Rong Wu[1], Song-Tao Wang[1], Pei Pei[1], Xue-Fei Zheng[1], Hong Pan[1], Yi-Nan Ma[1]

[1]Department of Central Laboratory, Peking University First Hospital, Beijing 100034, China;
[2]Department of Pediatrics, Peking University First Hospital, Beijing 100034, China.

To the Editor: Phenylketonuria (PKU) is an autosomal recessive genetic disease caused by pathogenic variants in the *phenylalanine hydroxylase* (*PAH*) gene encoding phenylalanine hydroxylase, a key enzyme in the metabolism of phenylalanine. Early low-phenylalanine diet improves most of the neuropsychological disorders, but it is difficult to be maintained for a long period of time.[1] To date, 1184 variants in *PAH* gene, including missense, splicing, nonsense, insertion and deletion variants, have been identified. The distribution of the variants is quite variable in ethnic groups. Genetic testing and prenatal diagnosis are effective to prevent PKU families from transmitting the pathogenic *PAH* alleles to their progeny. However, only a few reports about the prenatal diagnosis of PKU from north China have been found in the literature. Here we summarized the results of variant detection in 157 probands and their parents, and prenatal diagnosis of 103 fetuses from 95 PKU families.

This study was approved by the Research Ethics Committee of Peking University First Hospital. Informed consent was obtained from the probands or their guardians and their family members.

A total of 157 probands with their parents were examined for the variants in *PAH* gene during the period from May 2012 to December 2018. The age of the probands ranged from 1 month to 17 years, the male to female ratio was 1:0.92. Most of them lived in north China. All the probands had higher levels of plasma phenylalanine (>2 mg/dL), and the diagnosis of tetrahydrobiopterin (BH4) deficiency was excluded by a BH4-loading test.

Genomic deoxyribonucleic acid (DNA) was isolated from peripheral lymphocytes of the probands and their parents by a QuickGene DNA Whole Blood Kit (KURABO, Osaka, Japan). The 13 exons and their flanking sequences of the probands were amplified by polymerase chain reaction (PCR) that contained 50 ng DNA, 2.5 mmol/L each deoxy-ribonucleoside triphosphates (dNTPs) 2 μL, 10× reaction buffer 5 μL, 10 μmol/L each primers 1 μL, and 2.5 units of Taq DNA polymerase in a total volume of 50 μL. PCR products were purified and sequenced in an ABI 3130XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing results were compared with the transcript (NM_000277) of *PAH* gene and its genomic sequence (GRCh38/hg38). Detected variants were further searched in the three databases PAHvdb (www.biopku.org/pah/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and HGMD (http://www.hgmd.cf.ac.uk/ac/). A novel variant not found in these databases was evaluated by the online predictive tools of sorting tolerant from intolerant (SIFT) (http://provean.jcvi.org/index.php), PROVEAN (http://provean.jcvi.org/index.php), and PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) to predict pathogenic effect of the mutant protein. Variants found in the probands were then examined in their respective father and mother.

For probands without pathogenic variants or only one pathogenic variant found, the DNA samples were subjected to multiplex ligation-dependent probe amplification (MLPA; MLPA P055 kit, MRC-Holland, Amsterdam, Netherlands) to detect large insertions, deletions, or duplications in *PAH* gene. MLPA products were separated in ABI 3130XL Genetic Analyzer and analyzed by Coffalyser. Net (MRC-Holland).

In the 157 families, prenatal diagnosis was performed for 95 pregnant mothers, in which eight mothers were pregnant twice with prenatal diagnosis twice. DNA samples were extracted from chorionic villi, amniotic fluid, or abortion tissues (for verification of affected fetuses after abortion) using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and subjected to the same PCR-direct sequencing and/or MLPA procedures as described above. In addition, six short tandem repeats (STR) markers nearby *PAH* were amplified by PCR and separated on an ABI 3130XL Genetic Analyzer. Genotypes of the six STR markers were compared between mother

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Correspondence to: Dr. Yi-Nan Ma, Department of Central Laboratory, Peking University First Hospital, No.8 Xishiku Street, Xicheng District, Beijing 100034, China
E-Mail: mayinan1014@163.com

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and the fetus to exclude false results due to maternal blood contamination.

Among the 157 probands, 145 probands have two pathogenic alleles, including one proband with two pathogenic variants in one allele and one pathogenic variant in another allele, ten probands have only one pathogenic allele, and no pathogenic alleles were found in two probands. There were total 80 kinds of pathogenic variants, including 71 point nucleotide substitutions, seven small insertions/deletions, and two large deletions, resulting in 52 missense variants in 159 alleles, 13 splicing variants in 82 alleles, seven premature terminations in 46 alleles, five frame shifts in six alleles, two large deletions in four alleles, and one amino acid deletion in four alleles. The spectrum of the 301 variants listed in Supplementary Table 1, http://links.lww.com/CM9/A525, in which the variant of 163_164insATAT is a novel variant not stored in the above three databases. The most prevalent variants were R243Q, splicing variant due to c.611A>G and splicing variant due to c.1197A>T, accounting for 17.9% (54/301), 9.0% (27/301), and 8.3% (25/301) of the variant alleles, respectively.

The highest frequency of exon and its flanking sequences in which pathogenic variants (excluding large deletions/duplications) locate was exon 7, followed by exon 11, exon 6, exon 12, and exon 3.

MLPA was performed in 13 probands and found one large deletion of exon 1 and its upstream region in two probands and one large deletion of exon 4/exon 5 in the other two probands.

Prenatal diagnosis of PKU was performed in 103 fetuses in 95 of the 157 families. Thirty fetuses (29.1%, 30/103) were identified as PKU (carrying two pathogenic variants); all of the families chose abortion, and the pathogenic variants were confirmed by testing the abortion tissues. Fifty-two (50.5%, 52/103) fetuses were identified as PKU carriers (carrying one pathogenic variant), and 21 (20.4%, 21/103) as normal fetuses (no pathogenic variant found). Most of the carrier fetuses and normal fetuses were born (a few of them were aborted by other reasons), and their genotypes of *PAH* gene were confirmed by testing peripheral blood after birth.

Figure 1 showed the importance of molecular diagnosis and prenatal diagnosis for PKU in a family as an example. After the molecular diagnosis of PKU in the proband (III_1) and her parents (II_1, II_2), the proband's aunt (II_4) and uncle (II_3) were also at the risk to have a PKU baby. Molecular diagnosis revealed that both II_3 and II_4 were the carriers of PKU. Genetic counseling was then provided to prevent the two pairs of couples from delivery of another PKU case.

PAH gene locates in human chromosome 12q23.2, consisting of 13 exons that encode a polypeptide of 452 amino acid residues. Mutant phenylalanine hydroxylase blocks the metabolism of phenylalanine to tyrosine. The accumulation of phenylalanine leads to the alterations of cerebral myelination and protein synthesis and reduced levels of serotonin, dopamine, and noradrenaline in the brain.[2] Eventually, severe mental retardation and neuro-

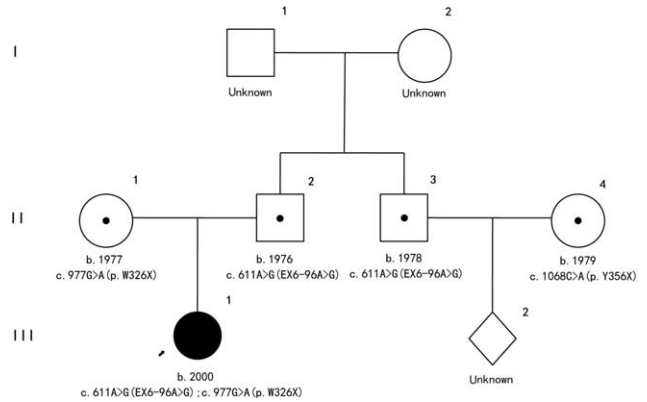

Figure 1: Pedigree of a phenylketonuria family. After the molecular diagnosis of PKU in the proband (III1) and her parents (II1, II2), the proband's aunt (II4) and uncle (II3) were also at the risk to have a PKU baby. Molecular diagnosis revealed that both II3 and II4 were the carriers of PKU.

behavioral abnormalities are present in these children. Neonatal screening for PKU is only useful for the early treatment of PKU. During the period from 2014 to 2017 in the Haidian District of Beijing city, screened for PKU among 176,340 newborns, in which 33 newborns were confirmed to have PKU with the incidence of 1/5344.[3]

In this cohort of PKU probands, the most prevalent variants of R243Q, splicing variants of c.611A>G and c.1197A>T accounted for 35.2% (106/301) of the variants, similar to the reports from other regions in China and Korea.[4,5] In contrast in Japan, the most prevalent variant was R413P.[6] The R243Q variant causes a mutant phenylalanine hydroxylase which has only <10% normal activity in the eukaryotic cell expression system.[7] The novel variant of 163_164insATAT we found causes frameshift and premature termination of the polypeptide, which is a definite pathogenic variant.

Two large deletions, exon 1 and its upstream region in two cases and exon 4/exon 5 in other two cases, were identified by MLPA in the 13 probands in which two variant alleles were not found by PCR-Sanger sequencing. Chen *et al*[8] reported that three large deletion alleles (exon 1 and its upstream region, exon 4/exon 5, and exon 5) were disclosed in 17 PKU families without two pathogenic variants. Yan *et al*[9] examined 43 PKU patients with none or only one variant allele by MLPA and identified that 22 PKU patients had 24 (51.1%) large deletion/duplication alleles, of which Ex1del3758 was detected in ten cases and Ex4_5del in four cases, similar to our findings. Therefore, the large deletions of exon 1 and exon 4/exon 5 may be relatively common in Chinese PKU patients.

No variant hotspot in *PAH* gene exists in this cohort of PKU patients. The variants were distributed in all 13 exons. The highest frequency of exon and its flanking sequences in which variants locate was exon 7, followed by exon 11, exon 6, exon 12, and exon 3. Zhang *et al*[10] examined the variants in exons 3, 5, 6, 7, 10, 11, and 12 of *PAH* gene in 40 PKU families and demonstrated that most variants concentrated in exon 7, followed by exons 6, 11,

and 3, which was similar to our results. Therefore, these exons can be chosen first for variant screening.

The next-generation sequencing technology has become a powerful tool for the diagnosis of genetic diseases.[4,11] Regular PCR-Sanger sequencing and MLPA could detect 95.6% (301/314) pathogenic alleles in *PAH* gene in this cohort of PKU patients, suggesting that the classic methods are still effective for the genetic diagnosis of PKU.

In families that both the husband and wife who carrying a pathogenic variant in *PAH*, the possibility of giving birth of a PKU baby is 25%, theoretically. Prenatal diagnosis is the unique way for PKU families to prevent the birth of infant with PKU case. Technologically, genotyping of several STR markers must be included to prevent misdiagnosis due to maternal blood contamination in fetal samples.[12] The six highly polymorphic STR markers that we used for linkage analysis were located around the *PAH* gene, two upstream, three downstream, and one in intron 3 of *PAH* gene. In case the fetal samples was contaminated by maternal blood, DNA extracted from cultured amniotic fluid cells or chorionic villi cells must be used to obtain accurate results.

Prenatal diagnosis using chorionic villi is usually performed at 11 to 13th week of gestation, and the earlier molecular diagnosis of the fetus is obtained, the less physical and psychological damages to the pregnant woman when the fetus is affected and the pregnancy is terminated. However, abortion due to the manipulation of chorionic villi sampling is relatively high, and the presence of placental chimerism may affect the accuracy of the results. In contrast, amniocentesis is usually performed at 16 to 23rd week of gestation and is relatively safe. However, the later the molecular diagnosis of the fetus, the higher the risk of abortion when the fetus is affected.

Here we present the spectrum of variants in *PAH* gene in PKU patients in north China. No variant hotspot in *PAH* gene was found. The variants were frequently detected in exon 7. Prenatal diagnosis is the unique way to prevent the progeny of heterozygous couples from PKU.

Conflicts of interest

None.

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