Article

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Mutation Characteristics of Phenylalanine Hydroxylase Gene in Children with Phenylketonuria in Yinchuan City

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Background: Phenylalanine hydroxylase deficiency (PAHD) is an autosomal recessive disorder affecting phenylalanine (Phe) metabolism caused by mutations in the phenylalanine hydroxylase (*PAH*) gene. It has a complex phenotype with many variants and genotypes in various populations. This study sets out to analyze the screening results of children with phenylketonuria (PKU) in Yinchuan City and characterize the mutation variants of the *PAH* gene.

Methods: Phenylketonuria screening results were retrospectively analyzed in 398,605 neonates (207,361 males and 191,244 females) born in different maternity hospitals in Yinchuan City between January 2017 and December 2021. Screening for genetic metabolic diseases was performed with parental consent at their own expense. A comprehensive diagnosis was performed by integrating tandem mass spectrometry (MS/MS) findings with clinical presentations. High-throughput sequencing (HTS) was used to detect genetic and metabolic disease-associated genes in children with PKU who were clinically diagnosed and voluntarily tested. The identified loci were validated through Sanger sequencing and parental verification.

Results: Among the screened newborns, 45 (11.3/100,000) PKU cases were diagnosed. In the 38 cases that underwent self-financed *PAH* sequencing, 56 mutations were detected in 76 chromosomes, with an overall detection rate of 73.7%. All patients harbored mutant genes, and the 56 mutations detected identified represented 14 variants, including 8 missense mutations, 2 splicing mutations, 2 nonsense mutations, and 2 silent mutations. The mutations were primarily distributed in *exons 2*, *3*, *6*, *7*, *9*, *11*, and *intron 4*, with the highest frequency observed in *exon 7* (25 [44.7%]), followed by *exon 11* (15 [26.7%]). The most prevalent mutations were *exon 7*-p.R252W (10 [17.9%]) and *exon 7*-p.R261Q (8 [14.3%]).

Conclusions: The *PAH* gene mutations in children with PKU in Yinchuan City are predominantly concentrated in *exons 6*, *7*, and *11*, with the highest detection rates observed for p.R252W and p.R261Q mutations.

Keywords: phenylketonuria; phenylalanine hydroxylase; gene; mutation

Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder characterized by a deficiency in phenylalanine hydroxylase (*PAH*), resulting in impaired phenylalanine (Phe) metabolism [1,2]. Mutations in the *PAH* gene lead to a decrease or loss of *PAH* activity, preventing the conversion of Phe to tyrosine (Tyr). This leads to elevated Phe levels in the blood and its accumulation in the nervous system and blood, potentially causing brain dysfunction [3,4]. The prevalence of PKU varies globally, with an average incidence of approximately 1 in 10,000 newborns [2]. Based on blood Phe concentration, PKU can be classified as either classic or non-classic, with classic PKU representing the majority of cases [5]. The milder form of *PAH* deficiency is known as moderate PKU, mild PKU, mild hyperphenylalaninaemia (HPA), or benign HPA, while the severe form is called classic PKU [2]. The mutation profile of the *PAH* gene varies among different populations, contributing to the genetic diversity of PKU. According to statistics, the incidence of PKU is about 1/11,000 in China, with *PAH* gene mutations causing the majority of PKU [6,7].

Early diagnosis of PKU relies on neonatal screening. Due to its inconspicuous early symptoms, early screening and preventive treatment after birth are crucial to prevent disease progression. Early initiation and continued treatment can help children normal cognitive development, despite certain unavoidable neurocognitive dysfunction [8,9]. Untreated individuals may experience severe intellectual disability, seizures, behavioral and psychiatric issues, motor problems, as well as characteristic physical features such as fair skin, light-colored eyes and hair, eczema, and a moldy odor [2]. Despite being the most common amino acid metabolism disorder, the underlying mechanisms of brain dysfunction in patients with PKU remain poorly understood, warranting further research to facilitate the development of pathophysiology-driven therapies [10,11].

Genetically, PKU exhibits high heterogeneity, with over 1000 *PAH* variants reported in patients with PKU worldwide [12]. Many patients are heterozygous for two different *PAH* mutations, resulting in more than 2600 identified genotypes that cause PKU [13,14]. These *PAH* mutations encompass various types, including missense (58.3%), frameshift (13.9%), splicing (13.1%), nonsense (6.9%), and silent (4.9%) mutations [13]. Approximately 17.9% of pathogenic variants are located in *introns* or untranslated regions of the *PAH* gene. Missense mutations can lead to the production of largely inactive or low *PAH* monomers [2]. While no significant gender differences in PKU incidence have been observed globally, notable geographic and ethnic differences in mutation types and frequencies have been found [15,16]. Therefore, genetic mutation testing in different regions is essential. Given the scarcity of reports on *PAH* gene mutations in Yinchuan City, China, this study aims to investigate the *PAH* gene mutations in this area based on the screening results of neonates with PKU from the past 5 years.

Materials and Methods

Study Participants

A retrospective analysis was performed on the PKU screening results of 398,605 newborns (207,361 males and 191,244 females) born in different maternity hospitals in Yinchuan City from January 2017 to December 2021 and who underwent genetic metabolic disease screening at their parents' expense. The screening method involved collecting neonatal heel blood on filter paper using acupuncture within 3 days of birth. After air drying, the Phe content was detected by the ninhydrin-immunofluorescence method. The newborn was suspected of PKU if the Phe concentration was $\geq$120 mol/L, in which case the screening personnel informed the baby's parents by phone or text message for further review. If the Phe concentration remained $\geq$120 mol/L after re-examination, it was considered positive for screening. A comprehensive diagnosis was performed by integrating tandem mass spectrometry (MS/MS) findings with clinical manifestations. Of the 45 confirmed newborns, 38 underwent self-financed genetic testing. All 38 cases originated from 38 non-consanguineous families. The serum Phe concentration of these 38 patients before treatment was >480 µmol/L (mean: 915.52 $\pm$ 513.66). Tetrahydrobiopterin deficiency was ruled out by the Phe loading test as well as urinary neopterin (N) and biopterin (B) analysis combined with clinical symptoms. This retrospective study was approved by the Ethics Committee of the General Hospital of Ningxia Medical University (2020-664).

Methods

Peripheral Blood (PB) DNA Extraction

Peripheral blood samples from children and their parents was collected and anticoagulated for storage at –20 ℃. DNA extraction from the anticoagulated peripheral blood (PB) samples was performed using the DNA separation kit (DP315, Beijing Tiangen Biotech, Beijing, China).

PCR amplification and sequencing: PCR amplification was conducted using an ABI PCR amplifier, following the specified reaction conditions: Initial thermal denaturation at 95 ℃ for 5 min, denaturation at 94 ℃ for 30 s, renaturation at 55–62 ℃ for 30 s, and extension at 72 ℃ for 45 s for a total of 30 cycles, followed by a final extension at 72 ℃ for 10 min.

Sense and anti-sense sequencing of PCR products and identification of mutant loci: The amplified PCR products were electrophoresed and sequenced with a 2% agarose gel. Sample purification and product sequencing and analysis were conducted by Guangzhou KingMed Diagnostics Group Co., Ltd (Guangzhou, China). Primer sequences for the *exon 1–13* region of the *PAH* gene are listed in Table 1.

Table 1. Sequences of primers.

Exon	Sequence
1	Forward: 5′-AATGAGAATCCTGACTGTTTCAGC-3′ Reverse: 5′-GAGGACATTTGTCTGTTGACTTCC-3′
2	Forward: 5′-AGAGTTCATGCTTGCTTTGTCC-3′ Reverse: 5′-TGCCTGTTCCAGATCCTGTG-3′
3	Forward: 5′-TCTGGTTCTGCATCTTTGGC-3′ Reverse: 5′-CTTCCAAGGCATTATTTCCAATAC-3′
4	Forward: 5′-ATCACCATTGGCTGGGATC-3′ Reverse: 5′-AAACCTCCATAGATGTACACAGGC-3′
5	Forward: 5′-GGAGGCTCATGCTAAATCAAAG-3′ Reverse: 5′-CACACACAGAAGGCAGGACTC-3′
6	Forward: 5′-ACTCCCTCTGCTAACCTAACCTG-3′ Reverse: 5′-CTCCTCTGCCTCAATCCTCC-3′
7	Forward: 5′-AGACATCTGAAGCCAAGTCTGC-3′ Reverse: 5′-GAACCCAAACCTCATTCTTGC-3′
8	Forward: 5′-CTGGCTTGGCTTAAACCTCC-3′ Reverse: 5′-GATCTCCGAAATGGGTATTTAGC-3′
9	Forward: 5′-TCTATGTGGGCTGTTCTGAAGG-3′ Reverse: 5′-TAGGAAAGTTTCAAAGACCTGAGG-3′
10	Forward: 5′-GTGTCCTGGTTCCAAGAGAGATAG-3′ Reverse: 5′-AAACGGATACAAATAGGGTTTCAAC-3′
11	Forward: 5′-GGCTGTGATGTAGAAGGAATCG-3′ Reverse: 5′-GATGAGTGGCACCAGTCAGG-3′
12	Forward: 5′-GCTGTTGAAGACCCTGCTCTAG-3′ Reverse: 5′-CATGGCTTACATGGAGGTGC-3′
13	Forward: 5′-CTCATCCAAGAAGCCCACTTATC-3′ Reverse: 5′-AACCAAGCCTTTAGTCAACATCTG-3′

Table 2. Neonatal PKU screening in Yinchuan City from 2017 to 2021.

Year	Number of people screened	Number of suspect positives in the initial screening	Number of suspect positives recalled for re-examination	Recall rate (%)	Number of diagnosed cases	Incidence rate
2017	63,287	125	124	99.2	7	11.1/100,000
2018	74,566	146	114	78.1	11	14.8/100,000
2019	86,534	102	85	83.3	15	17.3/100,000
2020	91,245	97	36	37.1	6	6.6/100,000
2021	82,973	108	25	23.1	6	7.2/100,000
Total	398,605	578	384	66.4	45	11.3/100,000

PKU, phenylketonuria.

Statistical Analysis

All data were analyzed using Microsoft Excel (2019, Microsoft, Redmond, WA, USA). Categorical and continuous variables were denoted by the number of cases and percentages (%) and the mean ± standard deviation, respectively.

Results

Analysis of Neonatal PKU Screening in Yinchuan

Of the 398,605 newborns screened for PKU, 578 were suspected positive in the initial screening, 384 cases (66.4%) were recalled for reexamination, and finally, 45 cases (11.3/100,000) were confirmed (Table 2).

The Detection Rate of PAH Gene Mutations

Genetic testing was performed on 38 out of the 45 patients with PKU. Upon analyzing *PAH exons* and *introns* in these 38 patients with PKU, 56 mutations were detected in 76 chromosomes, resulting in an overall detection rate of 73.7%. All patients had at least one mutated gene, with 18 cases having 2 mutant loci and 20 cases having 1 mutant locus.

Types and Distribution Characteristics of PAH Gene Mutations

The 56 detected mutations were classified into 14 types, including 8 missense mutations, 2 splicing mutations, 2 nonsense mutations, and 2 silent mutations. These mutations were primarily distributed in *exons 2*, *3*, *6*, *7*, *9*, *11*, and *intron 4*. *Exon 7* exhibited the highest frequency of mutation, with a total of 25 mutations, followed by *exon 11*, with 15 mutations. *Exons 2*, *3*, *6*, and *9* had 2, 3, 6, and 2 mutations, respectively, while *intron 4* had 3 (Table 3).

Typical Case Analysis

Child A presented a heterozygous mutation in the *exon* region of the *PAH* gene, specifically the c.754C>T mutation, which resulted in a missense mutation leading to an amino acid change (p.R252W). The mother of the child carried a c.754C>T mutation, while the father had no mutations (Fig. 1A). In addition, Child B, diagnosed with *PAH*, exhibited a heterozygous mutation in the *exon* region of the *PAH* gene, specifically the c.782G>A missense mutation. No mutations were identified in the mother, while the father displayed a heterozygous mutation (Fig. 1B).

Discussion

PKU occurs worldwide, with varying incidence rates based on region and race. It is one of the few severe monogenic disorders that can be treated, and genetic diagnosis is the only effective prenatal diagnosis measure. Early identification of PKU newborns through genetic screening allows for timely intervention and management, alleviating the impact of the condition [17]. However, such children need long-term treatment, which brings heavy economic and psychological burdens on families and society. Therefore, genetic diagnosis plays a vital role in preventing the birth of children with PKU.

The *PAH* gene is located on chromosome 12q22-q24.1 and consists of 13 *exons* and 12 *introns*, spanning a length of 90 kb. It encodes a monomeric enzyme containing 452 amino acid residues, which polymerizes into functional *PAH* and participates in Phe metabolism [18,19]. Homozygous or compound heterozygous mutations in any loci of the *PAH* gene have been reported to affect *PAH* activity in humans, leading to alterations in Phe metabolism and eventually causing PKU [20]. The prevalence of *PAH* gene mutations can vary significantly due to different testing modalities, sample sizes and genetic variations among regions and ethnic groups. In this study, we analyzed the neonatal PKU screening results of 315,632 cases in Yinchuan from 2017–2021. The data revealed a significant decrease in the number of recalls in 2021 compared to 2017. We speculated that the probability of parents carrying undesirable gene variants might be influenced by race, geography, and population mobility. The increased likelihood of carriers marrying within economically underdeveloped areas or regions with low population mobility and low intermarriage rates in 2017 could explain the observed increase in recalls. Additionally, the COVID-19 pandemic and subsequent quarantine measures since 2020 may have restricted the ability of *PAH* children's parents, who work in other locations, to return or maintain contact, resulting in a decrease in the actual number of recalls. By analyzing the

Table 3. Mutation types and distribution characteristics of *PAH* gene in children with PKU.

Mutant loci	Base change	Amino acid change	Mutation property	Number of mutations	Frequency (%)
Exon 7	c.754C>T	p.R252W	Missense mutation	10	17.9
Exon 11	c.1159T>G	p.Y387D	Missense mutation	6	10.7
Exon 7	c.728G>A	p.Arg243Gln	Missense mutation	7	12.5
Exon 11	c.1197A>T	p.V399V	Silent mutation	5	8.9
Exon 3	c.320A>G	p.H107R	Missense mutation	3	5.4
Exon 6	C.611A>G	p.Y204C	Missense mutation	2	3.6
Intron 4	G442-1G>A	IVS4-1G>A	Splicing mutation	3	5.4
Exon 6	c.694C>T	p.Q232X	Nonsense mutation	2	3.6
Exon 6	c.650G>A	p.C217Y	Missense mutation	1	1.8
Exon 7	c.782G>A	p.R261Q	Missense mutation	8	14.3
Exon 11	c.1068C>A	p.Y356X	Nonsense mutation	4	7.1
Exon 2	c.168G>A	p.E56E	Silent mutation	2	3.6
Exon 6	c.611A>G	EX6-96A>G	Splicing mutation	1	1.8
Exon 9	c.964G>A	A322T	Missense mutation	2	3.6

PAH, phenylalanine hydroxylase; PKU, phenylketonuria.

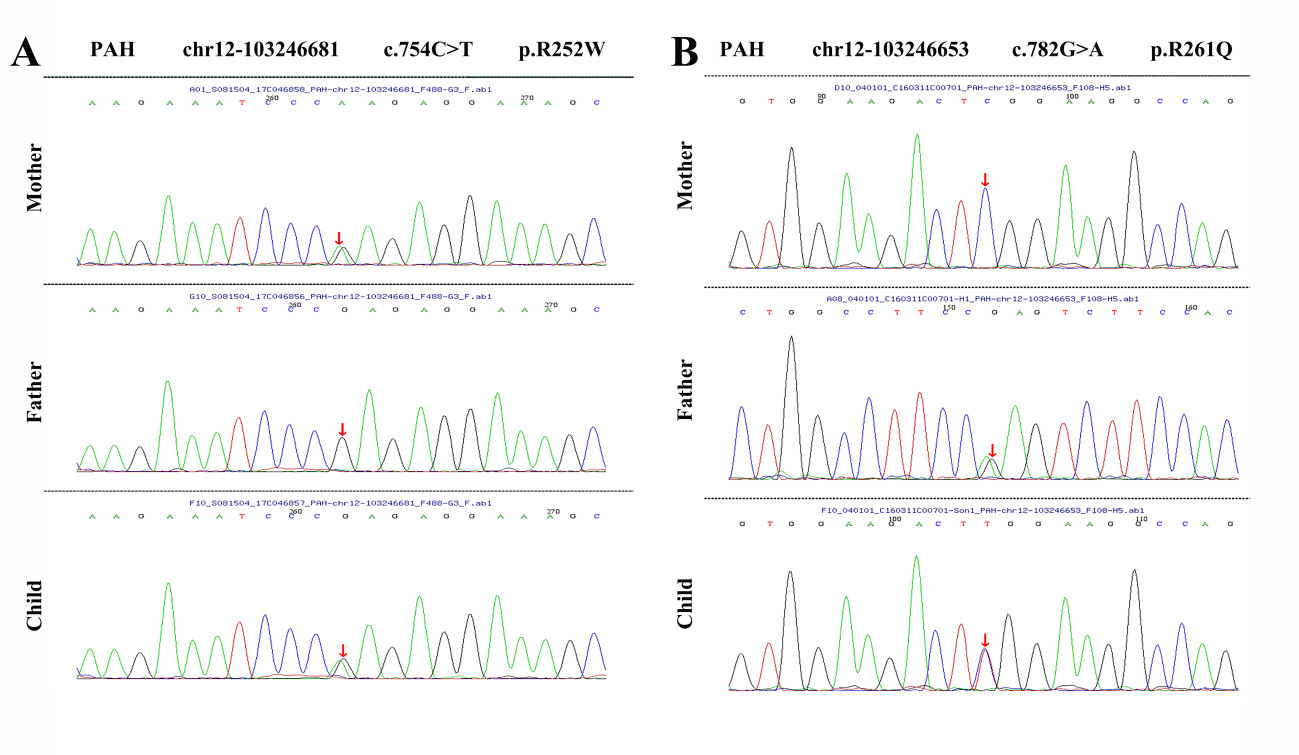

Fig. 1. Sanger sequencing diagram of gene mutation loci in children with phenylketonuria (PKU) and their parents. (A) Child A has a heterozygous c.754C>T mutation. (B) Child B has a heterozygous c.782G>A mutation. The red arrow indicates the location of the mutation in the gene. *PAH*, phenylalanine hydroxylase.

PAH gene in 38 children with PKU from Yinchuan, we detected 56 mutations in 76 chromosomes, resulting in an overall detection rate of 73.7%. These 56 mutations were classified into 14 types, including 8 missense mutations, 2 splicing mutations, 2 nonsense mutations, and 2 silent mutations. Currently, 564 *PAH* gene mutations have been reported worldwide, involving 13 *exons* of the *PAH* gene, with missense mutations being the most common. Researchers worldwide are trying to identify high-frequency mutation loci of the *PAH* gene for prenatal screening of patients with PKU. Our study observed a concentration of *PAH* gene mutations, with the highest detection rates found for p.R252W and p.R261Q. *Exon 7* exhibited the highest mutation frequency (25 [44.7%]). In a study conducted in Hainan, China, 29 PKU patients exhibited 58 mutant alleles representing 15 different types, with the top four genotypes

being c.611A>G (20.7%), c.728G>A (17.2%), c.158G>A (15.2%), and c.721C>T (13.8%) [21]. These findings differ from our study results. In the European population, the common mutations include R408W, IVS12+1G>A, and IVS10-11G>A [22]. In Asia, R413, R243Q, R241C, and IVS4-1G>A were identified by Okano *et al.* [23] to be the common mutations in Japan. In Korea, R243Q, IVS4-1G>A, and E696A>G were the main mutations that accounted for about 32% of all mutant genes [24]. These variations indicate the presence of differences in the high-frequency mutation loci of the *PAH* gene among different regions and ethnic groups. Interestingly, our study revealed many mutation loci in *exon 6*, consistent with some previous studies suggesting that the deletion of human *exon 6* is associated with classical PKU [25,26].

The distribution and types of PKU mutations have been widely recognized to exhibit significant regional and ethnic variations, making the study of PKU distribution and mutation types crucial for prenatal diagnosis and genetic counseling [27]. Our experimental data suggest that when conducting mutation analysis on patients with PKU, the 1st, 3rd, 6th, 7th, 10th, and 11th loci and *exons* can be preferentially selected for direct sequencing. Subsequent sequencing of other *exons* can be performed later, thus avoiding unnecessary sequencing of the full-length coding region and improving the efficiency of prenatal diagnosis. However, it is important to note that our study solely utilized Sanger sequencing, which lacks the ability to detect large fragment deletions in the *PAH* gene. Meanwhile, the sample size was also limited. Future investigations should incorporate novel methods to expand the detection range and the sample size for more comprehensive analyses.

Conclusions

In conclusion, our study has identified the types, distribution, and high-frequency mutation loci of *PAH* gene mutations in children with PKU in Yinchuan City. Our findings contribute to the existing knowledge of *PAH* gene mutations and the *PAH* gene mutation spectrum among the Chinese population, thereby holding significant implications for future prenatal diagnosis of PKU and the development of eugenics in China.

Availability of Data and Materials

The original data in this study is included in the article. Further inquiries can be directed to the corresponding author.

Author Contributions

XY and FL contributed equally to this work; XY, FL and LP contributed to concept and design of the study, and wrote the first draft of the manuscript; XY, FL, BW, RL and ML contributed to data analysis and manuscript revision; XY, FL and RL contributed to manuscript revision and project management. All authors approved the submitted version. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

This study was reviewed and approved by the Ethics Committee of the General Hospital of Ningxia Medical University (approval number: 2020-664) and written informed consent was given by the patient. This report was prepared in accordance with the Helsinki Declaration.

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Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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