Observational Study

Medicine

OPEN

Exploring a novel model for newborn genetic screening in Ningxia, northern China

A retrospective observational study

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Abstract

The accuracy and precision of quantitative aspects of conventional newborn screening (NBS) are limited due to the complexity of clinical manifestations and the constraints of conventional screening methods. Gene sequencing is commonly employed as an adjunct diagnostic technique to assist in diagnosis. The combined utilization of traditional NBS and newborn genetic screening can effectively reduce false-negative and false-positive rates, thereby enhancing the accuracy and precision of screening, while minimizing the health impact caused by genetic diseases in infants. This study aim to explore the feasibility and effectiveness of newborn genetic screening in Ningxia. For the first time in Ningxia, a genetic sequencing panel based on multiplex PCR technology and next-generation sequencing (NGS) combined with traditional mass spectrometry (MS/MS) was used for initial NBS. This involved the analysis of 134 disease-causing genes covering 74 common inborn disorders. A total of 1837 newborns were screened from January 2020 to December 2021 in the Ningxia region, and 7 positive cases were detected by gene panel among the 1837 newborns including 1 *PAH* disorder, 1 *DUOX2* disorder, 1 *G6PD* disorder and 4 *MT-RNR1* disorders. However, no 1 has yet been detected using traditional NBS. The top ten high-frequency mutant genes detected in the panel test were arranged from high to low as follows: *PAH*, *DUOX2*, *SLC26A4*, *GJB2*, *ATP7B*, *MMACHC*, *SLC22A5*, *ACADS*, *DUOXA2* and *SLC25A13*. Population-specific newborn genetic screening can facilitate the progress of genetic defect prevention and treatment.

Abbreviations: AD = autosomal dominant inheritance, AR = Autosomal recessive inheritance, CH = congenital hypothyroidism, CNVs = copy number variations, G6PDD = glucose-6-phosphate dehydrogenase deficiency, IEM = inborn errors of metabolism, MS = mass spectrometry, NBS = newborn screening, NGS = next-gene sequencing, SNHL = sensorineural hearing loss, WES = whole exon sequencing, WGS = whole genomic sequencing, XLD = X-linked incomplete dominant inheritance.

Keywords: gene mutation spectrum, genetic disorders, newborn screening, panel, sequencing

1. Introduction

Newborn screening (NBS) is a crucial public health initiative that enables prompt identification of genetic disorders in newborns, thereby facilitating timely management and treatment to prevent mortality and disability. The early detection of such conditions is integral to their successful management.[1] The technology and coverage of NBS have been rapidly developing since the first screening was successfully applied in clinical practice in the 1960s for phenylketonuria (PKU) testing.[2] Subsequently, the application of mass spectrometry (MS), especially tandem MS, in multiple inborn metabolic diseases has significantly enhanced the development of NBS.[3] Empirical studies have demonstrated that tandem MS technology possesses the advantages of enhanced efficiency in checking and measuring. In particular, this technology can detect over 40 inborn metabolic disorders in a single test.[4] However, owing to the complex clinical manifestations, where several genetic disorders may exhibit almost identical symptoms, MS and other

MJ, YW, and WZA have no funding to disclose. XMM is supported by a grant from the Key Scientific Research Project of Ningxia Health Commission, grant number 2020-NW-50. TYJ is supported by a grant from the National Key Research and Development Program, grant number 2022YFC2705200.

XMM, MJ, YW, and TYJ have no competing interests to declare. WZA is a contractor with Zhejiang Biosan Biochemical Technologies Co. Ltd and has been financially compensated for performing services.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

The study was approved by the ethics committee of the Peking University First Hospital Ningxia Women and Children's Hospital (Ningxia Hui Autonomous Region Maternal and Child Health Hospital). All parents and/or guardians agreed to participate in the study and provided written informed consent.

Supplemental Digital Content is available for this article.

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How to cite this article: Mao X, Jing M, Wang Y, An W, Ji T. Exploring a novel model for newborn genetic screening in Ningxia, northern China: A retrospective observational study. Medicine 2024;103:52(e41064). XM and TJ contributed equally to this work.

Received: 12 December 2023 / Received in final form: 28 November 2024 / Accepted: 5 December 2024

http://dx.doi.org/10.1097/MD.0000000000041064

biomarker examinations exhibit limited precision and accuracy in quantification. Hence, complementary diagnostic techniques such as genetic sequencing are often utilized to aid in diagnosis.[5] Moreover, several inborn diseases cannot be detected by examining biomarkers, such as hearing loss and cognitive developmental disorders. Such disorders can only be detected at certain stages of development. For an extended period, gene sequencing has served as an essential complementary tool in clinical diagnosis, significantly enhancing our understanding of genetic disorders in humans.[6,7]

Recently, there has been a surge in the application of gene sequencing-based technologies in NBS, owing to the advancements in next-generation sequencing (NGS). Examples of such technologies include massive parallel sequencing of target gene panels, whole exome sequencing (WES), and whole genome sequencing (WGS).[8–10] A comprehensive NGS based assay that targets a single gene has been developed to detect deletions, duplications, and single-nucleotide variants in a single test. Such approach allows for the identification of pathogenic variants in deep intronic regions and facilitates precise detection of breakpoints for copy number variations (CNVs) that involve similar exons within the gene of interest.[11] WES and WGS can detect more pathogenic, potential pathogenic variations and variants of uncertain significance associated with disease, having significant potential for disease screening including NBS.[12–15] To demonstrate the importance of investigating the applications of DNA sequencing in newborn healthcare, the National Institutes of Health provided funding for the Newborn Sequencing in Genomic Medicine and Public Health network in the early 2010s.[16] The North Carolina Newborn Exome Sequencing for Universal Screening project identified a sensitivity of 88% for detecting cases with abnormal NBS results for inborn errors of metabolism (IEMs) and a sensitivity of 18% for detecting cases with hearing loss in the screened cohort.[17] The NBSeq project, which conducted a retrospective analysis of cases diagnosed with known IEMs through conventional NBS in California using WES, reported insufficient sensitivity (88%) and specificity (94%) of WES as a substitute for current NBS methods, including MS, biomarker, and other conventional pathways. As such, WES could not be recommended as a replacement for conventional NBS methods for IEMs.[18] Compared with WES, WGS can detect the wider scope of detectable variants beyond coding regions. According to clinical research, in some IEMs, WGS detected large variations of deletions in IEM genes that were not observed by WES alone.[19] Despite such findings, WES and WGS testing could be beneficial in reducing false-positive results and in identifying disease diagnosis. However, the challenges arising from WES and WGS cannot be disregarded. Such challenges include difficulties in interpreting the detection results, high expenses, prolonged cycle times, and more intricate genetic counseling. Given the aforementioned challenges, it is essential to consider the marked genetic heterogeneity among different populations worldwide. As a result, a targeted gene panel that covers nearly all specific pathogenic and potentially pathogenic variations in genes that cause common diseases is receiving increased attention owing to its advantages, including being more feasible, efficient, and cost-effective. In fact, the targeted gene panel has been attempted for use in NBS for both single[10] and multiple monogenic genetic disorders.[9,20,21] Genetic screening is a significant factor in preventing monogenic disorders. Therefore, there is an urgent need for an economical and efficient genetic screening method. Given the complexity of the causes of genetic disorders and their wide variation across regions and populations, it is imperative to perform newborn genetic screening using a targeted gene panel that reflects the genetic characteristics of the specific population and region. As such, comprehending the spectrum of gene mutations in a given area holds considerable significance for optimizing the effectiveness of newborn genetic screening.

In this study, a gene panel covering 134 genes directly associated with 74 common inborn disorders in Chinese newborns was designed. The panel was designed based on multiplex PCR to conduct newborn genetic screening and comprehensive analysis combined with conventional NBS for 74 diseases in 1837 neonates from the Ningxia region for the first time. The aim of the present study was to analyze the validity and feasibility of genetic screening in the Ningxia region.

2. Materials and methods

2.1. Participants and samples collection

From January 2020 to December 2021, 1837 newborns (975 males and 862 females) in the Ningxia Hui Autonomous Region of China were recruited for newborn genetic screening. Newborns who met specific criteria were enrolled in a genetic screening program to assess the mutation spectrum present in the general population with Ningxia. The newborns were of Chinese ethnicity, born as single births, and had healthy parents with no known genetic diseases or a history of serious acute or chronic illnesses. Additionally, newborns underwent comprehensive screening, which included screening for inherited metabolic diseases using MS/MS methods, as well as biochemical screening for congenital hypothyroidism (CH) and glucose-6-phosphate dehydrogenase deficiency (G6PDD). At least 2 dry blood spots with a diameter of more than 8 mm were obtained from each newborn's heels on a 903-filter paper (Wallace Oy, Turku, Finland) 72 hours after birth. In the present study, at least 2 mL of peripheral blood samples collected from recalled parents were treated with EDTA anticoagulant and used for pedigree analysis. The sample size was determined based on the statistical requirements.

The present study was reviewed and approved by the Ethics Committee of Peking University First Hospital Ningxia Women and Children's Hospital (Ningxia Hui Autonomous Region Maternal and Child Health Hospital). Written informed consent was obtained from all participants' parents and/or guardians.

2.2. The gene panel design

The gene panel (Hangzhou Biosan Clinical Laboratory Co. LTD) for NBS contained 134 pathogenic genes (*PAH*, *DUOX2*, *PTS*, *SLC22A5*, *MMACHC*, *MMUT*, *MMAA*, and *GAA* etc.) covering 74 diseases. Such diseases primarily include inborn metabolic disorders that can be screened using conventional NBS, including hearing loss and mitochondrial genetic diseases. In the present study, 41 diseases could be screened using biochemical screening, including those covered by MS/MS, G6PDD, and CH. Conversely, 33 diseases were found to be unsuitable for biochemical screening. The 74 diseases and 134 genes, as well as their high-frequency variations, are listed in Tables S1 and S2, Supplemental Digital Content, http://links.lww.com/MD/O235. The selection of the 74 target diseases was based on several criteria. First, the diseases were identified through regulations issued by the Secretary's Advisory Committee on Heritable Disorders in Newborns, as well as by applying the Wilson and Jungner criteria. Second, diseases can be detected through various methods, including MS screening, biochemical testing, or hearing tests. Lastly, the selected diseases had high incidence rates yet could not be screened using conventional NBS methods. The frequency of the variation sites in the normal population was obtained by searching the following databases: dbSNP (http://www.ncbi.nlm.nih.gov/snp/) and genomAD/1000 Genomes Project database (https://gnomad.broadinstitute.org/). Pathogenic genes and pathogenic sites were annotated, and correlations between variations and diseases were established by

Table 1

The biochemical and genetic information of 5 cases with abnormal MS/MS value in retest.

Case number	TSH (μIU/mL)	G6PD (U/gHb)	17-OHP (nmol/L)	Initial screening value of MS/MS	MS/MS value range	follow-up results	Mutant gene	Gene mutation
1	3.68	7.6	3.34	C3 = 6.77 μmol/L > 5 μmol/L↑ C2 = 28.81 μmol/L C3/C2 = 0.235 > 0.22↑	C3: 0.3 to 5 μmol/L C2: 4 to 50 μmol/L C3/C2: 0 to 0.22	Normal	Not detected	Not detected
2	2.65	5.5	3.51	C0 = 7.98 μmol/L < 8 μmol/L↓	C0: 8.0 to 60 mol/L	Normal	Not detected	Not detected
3	6.15	6.4	1.41	C3 = 3.02 μmol/L C2 = 8.00 μmol/L C3/C2 = 0.378 > 0.22↑	C3: 0.3 to 5 μmol/L C2: 4 to 50 μmol/L C3/C2: 0 to 0.22	Normal	Not detected	Not detected
4	1.77	6.3	1.86	C3 = 7.43 μmol/L > 5 μmol/L↑ C0 = 27.05 μmol/L C2 = 25.02 μmol/L C3/C0 = 0.275 > 0.25↑ C3/C2 = 0.297 > 0.22↑	C3: 0.3 to 5 μmol/L C0: 8.0 to 60 mol/L C2: 4 to 50 μmol/L C3/C0: 0 to 0.25 C3/C2: 0 to 0.22	Normal	*GBA*	Exon11, C.1448T > C (p.L483P)
5	1.77	6.1	10.3	PHE = 136.08 μmol/L > 100↑ TYR = 45.79 μmol/L PHE/TYR = 2.972 > 1.2↑	PHE: 20 to 100 mol/L TYR: 30 to 350 mol/L PHE/TYR: 0.15 to 1.2	Normal	*PAH*	Exon3, c.331C > T (p.R111*)

Abbreviations: TSH = thyroid-stimulating hormone, G6PDD = glucose-6-phosphate dehydrogenase deficiency, 17-OHP = 17-hydroxyprogesterone, MS = mass spectrometry, PHE = phenylalanine, TYR = tyrosine, ↑ = higher than reference, ↓ = lower than reference.

referencing several databases, including OMIM (http://www.omim.org), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar) and the Human Gene Variation Database (http://www.hgmd.org).

2.3. Library construction and sequence analysis

Genomic DNA was extracted from the dried blood spots of the newborns using an automatic nucleic acid extraction system (Bioer, China). DNA libraries were prepared based on multiplex PCR technology using the StemLoop inhibition mediated amplification (SLMamp) method. High-throughput sequencing was performed using Illumina NextSeq 500 according to the manufacturer's instructions. Raw sequencing-data were processed using conventional procedures. Low-quality sequencing reads were removed, and the acceptable reads were mapped to the NCBI human reference genome (hg19/GRCh37). Pathogenic and likely pathogenic variants were identified, reported, and subsequently used for further analyses.

2.4. Statistical analysis

We analyzed the data using GraphPad Prism software (version 8.0) and Adobe Photoshop software (version CS6). We respectively counted the genes with a mutation frequency of greater than or equal to 3 and listed the specific gene names and mutation frequencies. The cases of 1837 participants were divided into 3 types: positive, carrier, and negative. Statistical analysis was conducted on the data of carriers and positive cases, and the data distribution was presented in the forms of histograms, pie charts, etc. Descriptive statistics were used to represent categorical data in numbers and percentages.

3. Results

3.1. Results of biochemical screening

In the conventional screening process, a total of 40 cases were identified as biochemical positives, resulting in a primary screening positivity rate of 2.18%. Upon further confirmatory testing, 35 cases were found to be false positives. The remaining 5 cases, while still testing abnormal, were not diagnosed with any disease. During the follow-up, all 5 cases performed normally and showed no severe clinical symptoms. Of the 5 cases that were identified as abnormal but not diagnosed, 2 cases were found to be carriers through gene panel testing, while no genetic variations were detected in the remaining 3 cases. A summary of the information regarding the 5 cases is presented in Table 1.

3.2. Analysis of popular variant genes and variation sites

A total of 1837 newborns, consisting of 975 males and 862 females underwent gene panel screening. In the screening process, 393 pathogenic variations, 117 likely pathogenic variations, and 1 variant of unknown significance were detected across 60 genes. The 60 mutant genes and their mutation frequencies are shown in Figure 1A and a full summary of the mutation information is provided in Table S3, Supplemental Digital Content, http://links.lww.com/MD/O235. As summarized in Table 2, the top ten high-frequency mutant genes, as well as their corresponding high-frequency variation sites could be arranged from high to low as follows: *PAH* (Exon7, c.728G > A), *DUOX2* (Exon14, c.1588A > T), *SLC26A4* (Intron7, c.919–2A > G), *GJB2* (Exon2, c.235del), *ATP7B* (Exon8, c.2333G > T), *MMACHC* (Exon4, c.567dup), *SLC22A5* (Exon8, c.1400C > G), *ACADS* (Exon9, c.1031A > G), *DUOXA2* (Exon5, c.738C > G), and *SLC25A13* (Exon9, c.852_855del).

3.3. The detection of positives, carriers and negatives

According to the results of the genetic screening, 7 positive cases were detected, including 4 *MT-RNR1* mutations, 1 *DUOX2* mutation, 1 *G6PD* mutation and 1 *PAH* mutation (Fig. 1C and Table 3). The PAH-positive case also carried a *BCKDHA* (Exon9, c.1270C > T) variation. In total 429 carriers and 1401 negatives were detected (Fig. 1B). Positive, carrier, and negative cases accounted for 0.38%, 23.35%, and 76.27% of the total newborns, respectively (Fig. 1B). Among the 429 carriers (192 females and 237 males), 373 individuals (169 females and 204 males) carried 1 variation site, 51 individuals (23 females and 28 males) that carried 2 variation sites and 5 individuals (two females and 3 males) carried 3 or more variation sites (Fig. 1D). The 3 aforementioned group accounted for 86.95%, 11.89% and 1.16% of the carriers, respectively (Fig. 1E). Notably, in all the variations observed, the 2 variation sites (c.752C > T and c.761C > T) of the *GAA* gene were found to be in cis with a considerably close distance between them.

3.4. Analysis of mutation sites in Ningxia region

The mutant genes and variations were analyzed according to the screening results of newborn genetic screening (NGS) from the Ningxia region, of the 74 targeted genes 60 were found to contain mutated sites. Only one type of target mutation was detected in 18 genes, 2 types of mutations were detected in 11 genes, and 3 or more kinds of mutations were detected in 31 genes. Mutant genes with a proportion >2.00% ranged from high to low as follows: *PAH* (18.16%), *DUOX2* (8.78%), *SLC26A4* (8.18%), *GJB2* (7.58%), *ATP7B* (5.79%), *MMACHC* (4.39%), *SLC22A5* (3.79%), *ACADS* (3.59%), *DUOXA2* (2.79%), *SLC25A13* (2.59%), *GAA* (2.59%), *PTS* (2.40%) *ACADSB* (2.20%) and *DPYD* (2.00%). Mutation frequencies proportions are listed in Table S4, Supplemental Digital Content, http://links.lww.com/MD/O235 As shown in Figure 2A, the carrier rates of the top ten most frequently observed partial mutant genes in the Ningxia region were *PAH* (4.90%), *DUOX2* (2.34%), *SLC26A4* (2.23%), *GJB2* (2.07%), *ATP7B* (1.58%), *MMACHC* (1.20%), *SLC22A5* (1.03%), *ACADS* (1.03%), *DUOXA2* (0.98%), and *SLC25A13* (0.76%). The mutation locations and corresponding mutation frequencies were summarized in Figure 2B.

4. Discussion

Clarifying the spectrum of gene mutations is essential to gain a deeper understanding and to more precisely prevent birth defects. The differentiation of gene profiles can be mainly attributed to ethnic characteristics. In the present study, a gene panel was designed and attempts were made to analyze the gene mutation spectrum of the Chinese population and explore a

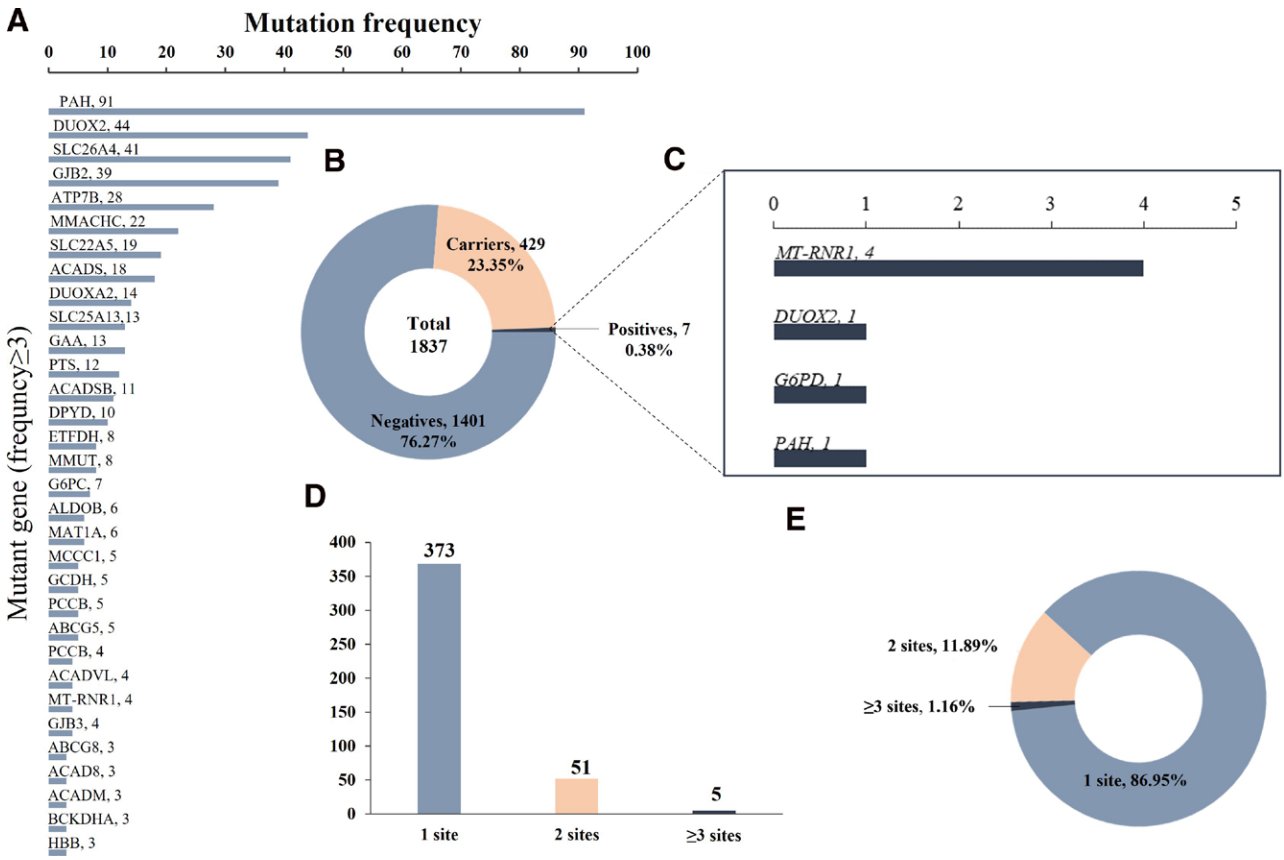

Figure 1. The mutant genes (frequency ≥ 3) and their corresponding mutation frequency among 1837 newborns were summarized in (A) there were 7 positives, 429 carriers and 1401 negatives that have been detected; (B) 7 positives, consisting of 4 *MT-RNR1* mutations, 1 *G6PD*, 1 *PAHD* and 1 *DUOX2*; (C) the carriers with 1 mutation site, 2 mutation sites and ≥ 3 mutation sites were respectively counted 373, 51, and 5, and (D) their corresponding proportion were 86.95%, 11.89%, and 1.16% (E).

Table 2

The top10 mutant genes and hotspot mutation site were detected in Ningxia region.

No.	Gene	Frequency of detection	Number of carriers	Carrying rate	The most popular mutation sites	Frequency of most popular mutation site
1	*PAH*	91	89	4.90%	E7, c.728G > A, p.R243Q	9
2	*DUOX2*	44	43	2.34%	E14, c.1588A > T, p.K530*	25
3	*SLC26A4*	42	42	2.28%	I7, c.919-2A > G	17
4	*GJB2*	40	40	2.18%	E2, c.235del, p.L79Cfs*3	21
5	*ATP7B*	28	28	1.52%	E8, c.2333G > T, p.R778L	9
6	*MMACHC*	22	22	1.20%	E4, c.567dup, p.I190Yfs*13	6
7	*SLC22A5*	19	19	1.03%	E8, c.1400C > G, p.S467C	13
8	*ACADS*	18	18	0.98%	E9, c.1031A > G, p.E344G	9
9	*DUOXA2*	14	14	0.76%	E5, c.738C > G	9
10	*SLC25A13*	13	13	0.71%	E9, c.852_855del	5

Table 3

The information of positive cases by genes panel in newborn screening.

Number	Gene	Correlated disease	Mutation information	Case number	Genetic model	Male/Female	Results of biochemical screening
1	*MT-RNR1*	Non-syndromic sensorineural hearing loss, NSSNHL	m.1555A > G	4	Mitochondrial inheritance	2 males and 2 females	Negative
2	*DUOX2*	Type 6 thyroid hormone endocrine disorders	c.1588A > T, c.602dup	1	AR	Male	Negative
3	*G6PD*	Glucose-6-phosphate dehydrogenase deficiency, G6PDD	c.844G > C	1	XLD	Male	Negative
4	*PAH*	Phenylalanine hydroxylase deficiency	c.688G > A, c.158G > A	1	AR	Male	Negative

Abbreviations: AD = autosomal dominant inheritance, AR = Autosomal recessive inheritance, G6PDD = glucose-6-phosphate dehydrogenase deficiency, XLD = X-linked incomplete dominant inheritance.

new method of NBS based on NGS. The present results show that the high-frequency mutated genes observed in Ningxia are different from those observed in Zhejiang and Jiangsu provinces, where this gene panel has also been used for NBS.[9,20,21] The Ningxia Hui Autonomous Region lies in Northwest China, while Zhejiang Province and Jiangsu Province lie in the south. The significant differences between the 2 regions can be attributed to the presence of different local minority populations and their distinct social and cultural features. These subtle differences may be reflected in the specific variants detected in each region. In the present study, the prevalence of certain genetic disorders, including G6PDD, PKU/HPA, and DUOX2D, as well as the deafness-related gene *MT-RNR1*, was investigated in the Ningxia region. The prevalence of each of these disorders was 0.054% (1/1837). The prevalence of G6PDD and PKU varied significantly among the different regions. Specifically, the prevalence of G6PDD was higher in South China (2.15%) and Southwest China (0.96%) than in North China (0.04%) and Northwest China (0.06%).[22,23] The present results are consistent with those of previous research, indicating a G6PDD prevalence of approaching 0.06%.[22] However, the detection rates for PKU and DUOX2D in the present study (0.054%) were slightly different from those previously reported (PKU, 0.10%, and DUOX2D, 0.028%).[23] Moreover, during conventional screening, 5 cases were identified with an abnormal MS/MS index, but all of them remained undiagnosed and exhibited no clinical symptoms during the follow-up period. Consequently, it could be assumed that the biochemical true-positive rate was zero in the present biochemical screening. In general, the combined use of genetic and biochemical screening can minimize false-negative rates. However, due to the low prevalence of the genes and the limited sample size, further verification of the present findings is required. Overall, the present findings contribute to enriching the repertoire of gene mutations associated with characteristics of the Chinese population. The results of the present study further suggest that the criteria for genetic diseases and newborn genetic screening should be further refined according to regional and ethnic characteristics.

PKU/HPA is a rare inherited disorder caused by changes in *PAH* gene expression. The present screening results show that *PAH* is a gene with a high-frequency of mutations in Ningxia, which is in agreement with the results of previous studies based on biochemical screening.[24,25] Among the Ningxia population, hot mutations of the *PAH* gene are slightly different from those in Japan, Korea, and other East Asian countries, or significantly different from those in West Asian, European, and American countries.[26] The *PAH* gene profiles in Northwest China were independent and conservative. Based on previous studies,[27] c.721C > T and c.694C > T mutations are predominantly found in individuals of the Hui ethnicity. Therefore, it can be assumed that the 2 variations likely originated from a Hui individual. Consistent with a previous study in central China, exons 7 and 6 of the *PAH* gene were found to be the most common mutation locations in the Chinese population.[28] Newborns diagnosed with *PKU* require lifelong adherence to a phenylalanine-restricted diet because failure to do so can result in severe health complications. While there is currently no cure for *PKU*, early detection and immediate treatment can greatly improve prognosis and prevent nervous system damage, which can manifest as impairments in cognition, comprehension, and communication. Providing carriers with early genetic counseling and facilitating a trouble-free pregnancy with healthy offspring through preimplantation genetic testing/diagnosis is crucial not only for confirmed patients but also for carriers to conduct effective prognoses.[29,30] Similarly, it is important to emphasize that all carriers identified should undergo a thorough pedigree analysis, and that their parents should seek genetic counseling if they plan to have another pregnancy. Additionally, carriers should receive genetic counseling when preparing for pregnancy to ensure the best possible outcome, as stated by current scientific understanding. Table 2 shows the identification of an infant with a biallelic mutation in *PAH* (c.688G > A and c.158G > A). The c.158G > A variant of the *PAH* gene was first reported in a Korean patient with classic PKU etiology,[31] and later categorized as a "likely benign" variant due to its high population frequency and low incident rate.[32] However, in patients with the c.158G > A variant, the phenylalanine hydroxylase concentration slightly increased within the normal range in the absence of low-phenylalanine diet management,[9] as was observed in the follow-up outcomes in the present study. The c.158G > A variant is also considered to be related to mild hyperphenylalaninemia. High variability of the *PAH* gene is a typical genetic characteristic in Northwestern China, possibly associated with the features of local population.

Variations in *GJB3*, *MT-RNR1*, *SLC26A4* and *GJB2* are known to be common causes of sensorineural hearing loss (SNHL).[33,34] In the present study, a gene panel analysis detected 4 positive cases of *MT-RNR1* variations, 4 cases of *GJB3* variations, 36 cases carrying variations in the *SLC26A4* gene, and 39 cases carrying variations in the *GJB2* gene among 1837 newborns. The most common sites of variation were c.919-2A > G in *SLC26A4*, c.235del in *GJB2*, m.1555A > G in *MT-RNR1* and c.538C > T in *GJB3*. Variations in the mitochondrial gene *MT-RNR1* are associated with aminoglycoside ototoxicity. Carriers of these variations are unable to take aminoglycosides throughout their lifetime owing to the risk of drug-induced hearing loss. Screening results can provide clinical guidance for the use of drugs.[33] The mutation c.538C > T in the *GJB3* gene was originally known as an autosomal dominant form of non-syndromic deafness DFNA2 in Chinese patients, however, recent studies have shown that the *GJB3* c.538C > T variant has a considerably low incidence in the Chinese population, but there is no clear evidence to support the role of the *GJB3* c.538C > T variant in the autosomal dominant form of non-syndromic deafness.[35] Because of the controversial genotype-phenotype correlation, the pathogenic variants of *GJB3* c.538C > T were not reported as positive cases in reference to a recent report.[23] Nevertheless, it is still recommended that individuals with

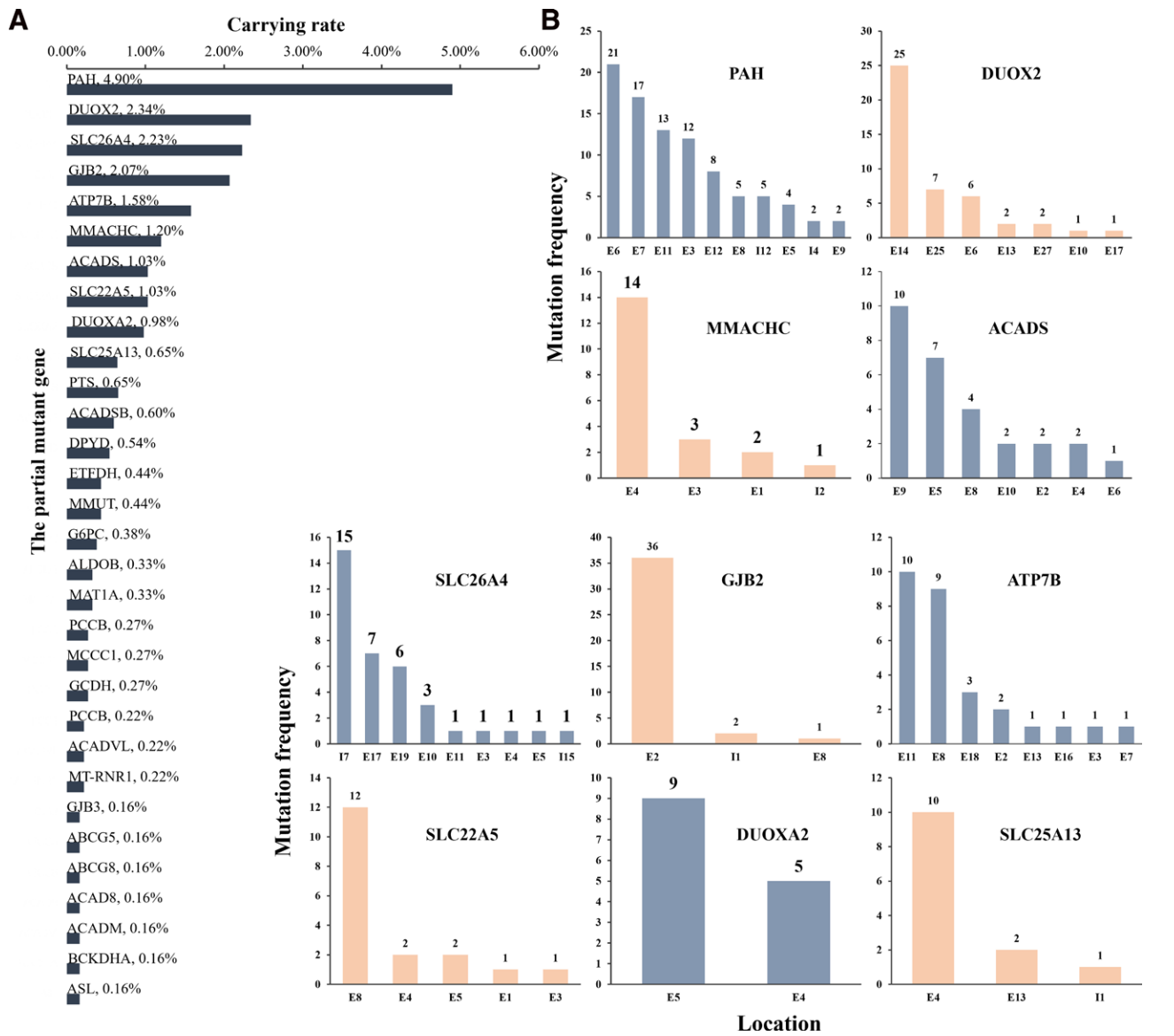

Figure 2. The top 10 mutant genes and their corresponding carrier rates (A) as well as location-distribution of mutations (B).

such variations undergo regular hearing checkups. Previous research has shown that patients with a homozygous mutation in *SLC26A4* c.919-2A > G were at a higher risk of progressive hearing loss, which was found to be strongly correlated with enlargement of the vestibular aqueduct.[34] However, the frequency of high-frequency variations and genotype-phenotype correlations in patients with SNHL has not been clearly established. Long-term follow-up for such cases is needed, and the data will provide a basis for newborn hearing screening. The present results further reveal the importance of enrolling genes involved in hereditary hearing loss in NBS based on NGS.

Variants of *G6PD* are the most common cause of hereditary glucose-6-phosphate dehydrogenase (G6PD) deficiency, commonly known as broad bean disease. Approximately 200 million people suffer from this disease worldwide. China is 1 of the most prevalent areas of the disease, and distribution characteristics of high incidence in the south and low incidence in the north have been shown.[22] One *G6PD* mutation positive case was detected in 1837 newborns, and the patient was diagnosed during follow-up. These findings suggest that genetic screening is beneficial for medical management of patients.

DUOX2 variants are a common cause of congenital primary hypothyroidism, with c.1588A > T and c.3329G > A being the most common variants in the Ningxia region based on the present screening data. Recent studies have already shown that *DUOX2* missense variants (c.1588A > T and c.3329G > A) might cause abnormal cell proliferation, suggesting that high-frequency variations in *DUOX2* may correlate with adenomatous polyposis. Moreover, *ATP7B*, *MMACHC*, *ACADS*, *DUOXA2* and *SLC22A5* were detected as high-frequency variation genes in the Ningxia region, and the corresponding high-frequency variation sites were identified as follows: *ATP7B* (c.2333G > T and c.2605G > A), *MMACHC* (c.567dup and c.658_660del), *ACADS* (c.1031A > G and c.655G > A), *DUOXA2* (c.413dup and c.738C > G) and *SLC22A5* (c.1400C > G). Based on a survey of the Chinese population, the high-frequency mutation site of c.2333G > T in *ATP7B* was significantly associated with lower levels of serum ceruloplasmin.[36] The c.1031A > G mutation in *ACADS* is a high-frequency variation related to SCADD. Cases of SCADD have been reported to have no clinical symptoms.[37] Therefore, early genetic screening and diagnosis are important for the prevention and treatment of SCADD. Consistent with research in other areas of China, the mutation site c.1400C > T in *SLC22A5* was found to be a high-frequency mutation in Chinese patients with primary carnitine deficiency.[38]

While the gene panel can partially represent the regional gene mutation spectrum, there are various limitations that must be considered when employing it for neonatal screening assays. Because the gene panel based on multiplex PCR only covers preselected target genes and high-frequency variants, several pathogenic variants will inevitably be missed, including some de novo mutations, which may lead to false-negative results and low overall detection rates. Moreover, although capture-based genetic screening methods can provide almost complete coverage of the coding regions of targeted genes, variants in noncoding regions such as introns and untranslated regions, as well as large CNVs and complex structural variations, are usually not easily detected. In addition, because the genetic screening test only reports pathogenic and likely pathogenic variants, the number of rare variants will be interpreted as VUS due to a lack of clinical or research evidence.[18] Therefore, conventional NBS and newborn genetic screening were concurrently applied in the present study, with both tests being reciprocally optimizable when joint analyzing biochemical and sequence data.

According to the analysis of gene mutations based on populations from the Ningxia region, the gene mutation spectrum was significantly different between Hui and Han populations, and there was a high detection rate of novel mutations in the Ningxia region.[27] However, there was no significant difference in the high-frequency mutation genes and mutation sites between the Han and Hui populations in this screening. The reason for this inconsistency is the selection of target genes and pathogenic mutation sites in the gene panel design. Therefore, large-scale genetic screening needs to be conducted in Ningxia to further clarify the gene mutation spectrum. WES and WGS may be more suitable screening methods than gene panels because of their ethnic and geographic characteristics, which can improve the accuracy of screening.

Acknowledgments

We thank all newborn family members for their cooperation and continued support for this study.

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