Application of targeted high-throughput sequencing as a diagnostic tool for neonatal genetic metabolic diseases following tandem mass spectrometry screening

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Background: Tandem mass spectrometry (MS/MS) is a crucial technique for detecting inborn errors of metabolism (IEM) in newborns. However, the high false positive rate poses challenges in diagnosing specific types of diseases. Therefore, this study aimed to evaluate the role of targeted next-generation sequencing (NGS) in the accurate diagnosis of positive samples identified through MS/MS screening.

Methods: A cohort study of 260,915 newborns was conducted from January 2018 to June 2023 in Ganzhou City, southern China. Heel blood samples were collected within 72 h of birth and subjected to MS/MS analysis. Infants with positive MS/MS results underwent targeted NGS to confirm the diagnosis and identify genetic variants.

Results: Among 1,265 suspected cases with positive MS/MS results, 73 were confirmed by NGS, and 12 were identified as carriers of recessive diseases. The overall incidence rate was 1 in 3,574, effectively ruling out 94.2% (1,192/1,265) of the MS/MS false-positive. We found 76 variants in 18 genes associated with 15 types of IEM. Among these, 64.47% (49/76) were pathogenic, 10.53% (8/76) were likely pathogenic. Remarkably, 7.89% (6/76) were identified as novel variants. Variants in *SLC22A5* (NM_003060.4) gene was most prevalent, accounting for 41% (77/188), with hotspot variants including c.51C > G, c.1400C > G, and c.338G > A.

Conclusion: Targeted NGS technology can serve as a crucial diagnostic tool for neonatal genetic metabolic diseases following MS/MS screening. Additionally, we identified IEM variant hotspots and some novel variants in our region, which are the underlying causes of disease in patients with IEM.

KEYWORDS

genetic diagnosis, inborn errors of metabolism, newborn screening, targeted sequencing, tandem mass spectrometry

1 Introduction

Newborn screening (NBS) is a successful public health project that employs advanced testing techniques to detect some serious inherited metabolic diseases in newborns. This allows for early diagnosis and treatment before clinical manifestations occur, thereby preventing irreversible damage in children. According to statistics, genetic diseases occur in 3–5% of live births (1). Since Guthrie and Susi (2) first reported the bacterial inhibition test for phenylketonuria (PKU) screening in 1963, NBS has gained global recognition and is now a crucial tool in reducing neonatal morbidity and mortality. The implementation of NBS not only provides immediate health benefits for children diagnosed and treated early but also enables their participation in social activities and alleviates the burden on families. Traditional biochemical screening is currently the mainstream NBS method, including tandem mass spectrometry (MS/MS), electrophoresis technology, enzymology, immunology, and electrophoresis technology-high-pressure liquid chromatography (3). MS/MS technology is characterized by its high efficiency, sensitivity, and convenience, enabling early disease diagnosis (4). However, the spectrum of diseases tested by blood MS/MS is limited, and different diseases can result in elevations of the same metabolites, blood MS/MS testing has limited usefulness in accurate disease diagnosis. Moreover, metabolites can be influenced by various factors such as diet, underlying diseases, and preterm birth, potentially leading to false-positive and false-negative results, requiring further diagnosis (5).

In recent years, with the development of DNA sequencing technology, the focus on inborn errors of metabolism (IEM) screening technology has shifted from the metabolite level to the genetic level. Next-generation sequencing (NGS) technology was employed to discover the genetic factors of thousands of genetic diseases. Therefore, NGS is valuable for genotyping and detecting the genetic factors of IEM. By comprehensively assessing IEM based on the quantification of metabolites and genetic variants, NGS can effectively improve the accuracy of IEM screening, compensating for the limitations of MS technology (6).

In this study, we analyzed data from the NBS program with MS/MS over the past 6 years. Target NGS of genes in a custom panel was employed as a second critical step to diagnose high-risk infants identified by MS/MS, aiming to provide a definitive genetic diagnosis and determine IEM's genetic characterization. This work has enhanced the quality of NBS programs, providing a more accurate diagnosis for children with IEM and consequently enabling more precise targeted therapy.

2 Materials and methods

2.1 Study design and participants

From January 2018 to June 2023, a total of 260,915 newborns underwent screening for IEM at the Ganzhou Maternal and Child Health Hospital in Jiangxi Province, China. Among these, 1,265 infants tested positive and received genetic diagnoses through NGS. Subjects were all newborns who had completed 72 h after birth and had been fed adequately at least eight times. Other inclusion criteria were complete medical history. Additionally, newborns were

excluded if they were undergoing emergency surgery or external blood transfusion. The clinical characteristics of newborns with suspected IEMs were all fully understood by a single physician. The clinical data included sex, major clinical features, and outcomes of IEM. The confirmatory tests vary depending on the disease, including genetic testing or blood biochemical indices testing, enzyme activities testing and urine organic acids analysis, etc. Pretest counseling was performed by physicians. The study was approved by the ethics committee of the Ganzhou Maternal and Child Health Hospital (2020001). The legal guardians of the participating infants gave their written informed consent for their children to be included in the study.

2.2 MS/MS screening method

Heel blood samples were collected from newborns, dripped on filter paper (Schleicher & Schue11 903, Wallac OY Turku, Finland), and dried naturally at room temperature. Dried blood spots were pretreated using a non-derivative MS/MS kit per the manufacturer's instructions (Fenghua, China) and then analyzed using a MS/MS system (Acquity UPLC-TQD, MA). Newborns with abnormal amino acid or carnitine (free carnitine and acylcarnitine) indices were recalled for recollection of heel blood (filter paper dried blood spot specimens) for rescreening. Additionally, mothers of newborns with positive results of free carnitine (CO) and 3-hydroxyisovaleryl-carnitine (C5OH) were recalled for re-examination to rule out maternal origin. If both screens were MS/MS-positive, the newborn was suspected of IEM.

2.3 NGS

Blood samples of patients and any participating family members were collected, and genomic DNA was extracted using the QIAamp DNA Mini Kit (Hilden, Germany) following the manufacturer's protocol. The coding exons of target genes were captured using an Agilent High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA), and libraries generated from enriched DNA were sequenced using the Illumina NovaSeq 6,000 platform (Illumina Inc., San Diego, CA, USA) in the paired-end mode. The average on-target sequencing depth for exome sequencing was 90X. The sequencing reads were aligned to the human reference genome (UCSC GRCh37/hg19) using the Burrows-Wheeler Aligner. Variant filtering was performed with the PhenoPro (7) phenotype-scoring algorithm. Detected variants were confirmed by PCR and subjected to direct automated sequencing using a 3500XL Genetic Analyzer (Applied Biosystems) per the manufacturer's specifications. The variant's pathogenicity was determined using the criteria established by the American College of Medical Genetics and Genomics (8).

3 Results

3.1 General results of NBS

A total of 260,915 newborns underwent MS/MS screening and 1,265 infants (687 male and 578 female) tested positive. The positive results were mainly divided into abnormal amino acid and abnormal

acylcarnitine markers. There were amino acid abnormalities, such as increased phenylalanine (Phe) and citrulline (Cit) (15.3 and 9.4% positivity rates, respectively), and carnitine abnormalities, such as decreased CO and increased isovaleryl-carnitine (C5) and C5OH (21.8, 9.3, and 8.1% positivity rates, respectively). Additionally, 3.7% of positive infants had simultaneously elevated or reduced indicators for certain amino acids or acylcarnitines. Following clinical and genetic diagnoses, 73 cases of IEM in newborns were diagnosed, with an overall incidence rate of 1 in 3,574 (Table 1). There cases were related to 12 IEM diseases, including 3 cases of fatty acid metabolic disease (39/73, 53.4%), 3 cases of amino acid metabolic disease (23/73, 31.5%), and 6 cases of organic acid metabolic disease (11/73, 15.1%). The highest incidence rate was that of primary carnitine deficiency (PCD, 1/7,248), followed by that of phenylketonuria (PKU, 1/15,348) and citrine deficiency (CD, 1/43,486). Additionally, 5 cases of PCD were confirmed in mothers of newborns. The overall detection rate of IEM screening in the 260,915 newborn screening population was 1/3345. Figure 1 shows the workflow of NBS.

3.2 Results of IME genetic diagnosis

Among the 1,265 infants suspected of having IEM, we performed genetic diagnosis utilizing targeted NGS technology. Following genotyping and interpretation, 73 cases were confirmed as IEM (Supplementary Table S1), comprising 46 cases of compound heterozygosity and 27 cases of homozygosity. Additionally, 12 cases were identified as carriers of recessive disorders (Supplementary Table S2). Specifically, among 73 infants with IEM, MS/MS testing suggested some forms of IEM in 49 cases (49/73, 67.1%). The result in P59 via NGS was inconsistent with that of MS/MS. The results of NGS revealed the homozygosity of *SLC25A13* c.851_854del (p.M285Pfs), classified as CD. In the remaining 48 infants (48/73, 65.8%), the genetic result was consistent with that of

MS/MS. Additionally, MS/MS revealed that 24 infants (24/73, 32.9%) suffered from certain kinds of IEM, and then disease types were identified by NGS. Among 24 infants, 17 (P40–P56) showed an increase in Phe and Phe/Tyr by MS/MS detection and 13 were cases of phenylalanine hydroxylase deficiency, while four were cases of tetrahydrobiopterin deficiency by NGS. Three infants (P70–P72) showed an increase in C3 and C3/ C2 by MS/MS detection and two were cases of methylmalonic acidemia and one was a case of propionic acidemia by NGS. Four infants (P63-P66) were detected by MS/MS with C$_5$OH increasing; one was a case of biotinidase deficiency and three were cases of 3-Methylcrotonyl-CoA carboxylase deficiency by (3MCC) NGS. Results from confirmatory biochemical tests were employed to verify the genetic findings (Supplementary Table S3). The consistency was observed in the outcomes of 73 cases of genetically confirmed IEM abnormalities. Overall, 72 true positive cases and one false negative case were identified through NGS, and 94.2% (1,192/1,265) of the false positivity results were excluded (Supplementary Table S4).

3.3 Analysis of genetic variation

Among 1,265 infants with suspected IEM, 76 variants involving 18 IEM-related genes were detected by NGS. Approximately 64.47% (49/76) of the variants were classified as pathogenic, 10.53% (8/76) were likely pathogenic, and 18.42% (14/76) were categorized as being of uncertain significance, based on the ACMG guidelines and criteria (Supplementary Table S5). The annotation results indicated that 71.1% (54/76) were missense variants, 7.9% (6/76) were frameshift variants, 10.5% (8/76) were splice variants, 6.6% (5/76) were nonsense variants, and 3.9% (3/76) were inframe variants. Additionally, 7.89% (6/76) were novel variants which has not yet been included in the Human Gene Mutation Database, the 1,000 Genomes Project and the Exome Aggregation in the Consortium. These six novel variants were located

TABLE 1 The incidence and spectrum of 260,915 newborns in the screening program.

Types of diseases	Cases (*n*)	Accounting for patients (%)	Incidence
Fatty acid metabolic disease	39	53.4	
Primary carnitine deficiency	36	49.3	1/7,248
Short-chain acyl-CoA dehydrogenase deficiency	2	2.7	1/130,458
Medium chain acyl CoA dehydrogenase deficiency	1	1.4	1/260,915
Amino acid metabolic disease	23	31.5	
phenylalanine hydroxylase deficiency	13	17.8	1/20,070
Citrin deficiency	6	8.2	1/43,486
Tetrahydrobiopterin deficiency	4	5.5	1/65,229
Organic acid metabolic disease	11	15.1	
3-Methylcrotonyl-CoA carboxylase deficiency	3	4.1	1/86,972
Glutaric acidemia type I	3	4.1	1/86,972
Methylmalonic acidemia	2	2.7	1/130,458
Biotinidase deficiency	1	1.4	1/260,915
Propionic acidemia	1	1.4	1/260,915
Isovaleric acidemia	1	1.4	1/260,915
Total	73	100%	1/3,574

FIGURE 1
Screening and diagnosis of IEM.
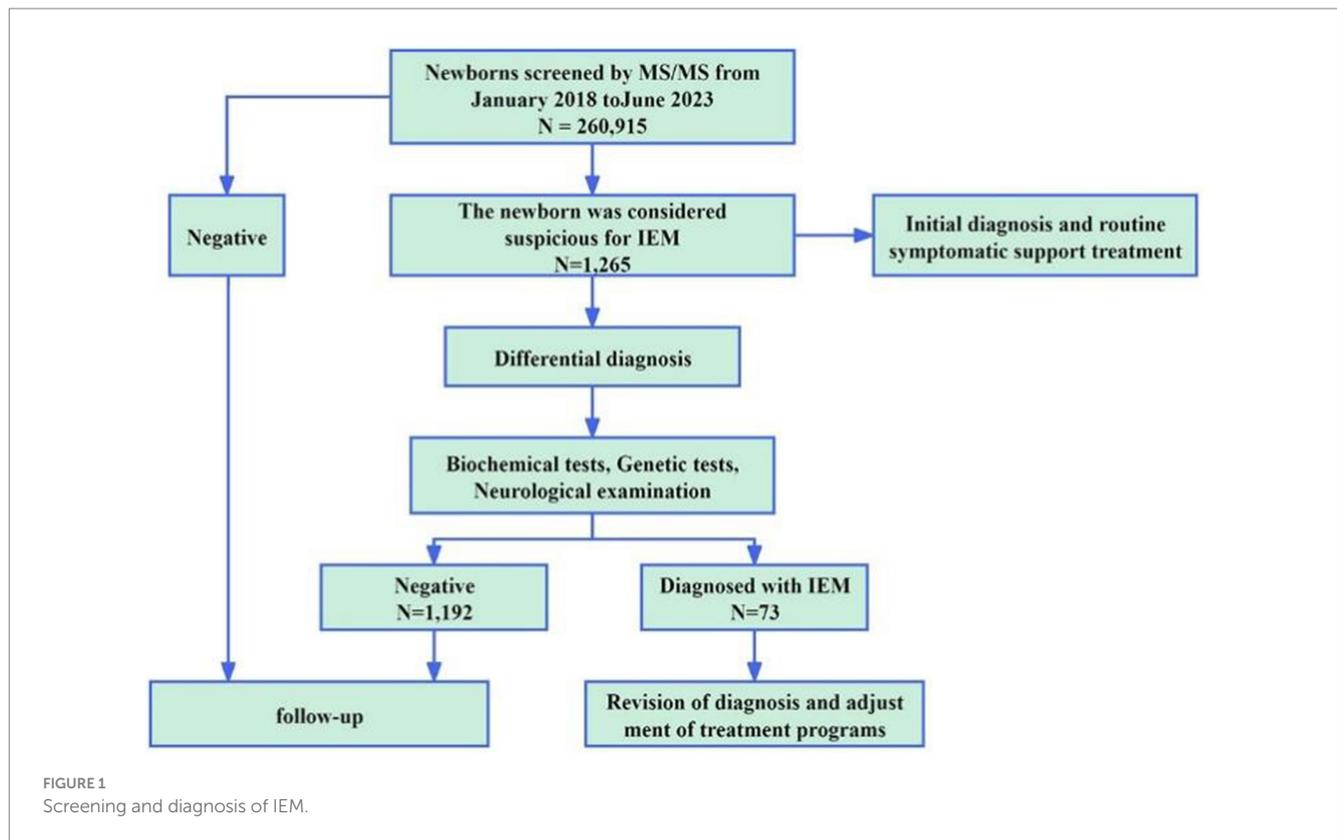
in four genes, including c.547G > T (p.E183Ter) and c.948delA (p.E316Ter) in *PAH* (NM_000277.1) associated with PKU, c.628G > A (p.E228K) and c.79A > C (p.T27P) in *ACADS* (NM_000017.4) causing short-chain acyl-CoA dehydrogenase deficiency, c.1364G > C (p.R455P) in *SLC25A13* (NM_014251.3) causing CD, and c.493A > C (p.T165P) in *MCCC1* (NM_020166.5) causing 3MCC. These results broadened our understanding of the IEM diseases. Additionally, our findings revealed that variants of the *SLC22A5* gene were the most prevalent, accounting for 41% (77/188) of all identified variants. In 40 cases of primary carnitine, c.51C > G in NM_003060.4 is one of the most common variant, accounting for 36.4% of all variants (28/77) and affecting 55% (22/40) of patients, followed by c.1400C > G (17/77, 22.1% and 17/40, 42.5%). Additionally, variants of the *PAH* gene were also common, accounting for 18.1% (34/188). The most common variant was c.728G > A in NM_000277.3, accounting for 26.5% (9/34) of all variants and affecting 41.2% (7/17) of patients, followed by c.611A > G (5/34, 14.7% and 4/17, 23.5%). Additionally, variants of *SLC25A13* were also common, accounting for 8.0% (15/188). The most common variant was c.852_855del in NM_014251.3, which accounted for 66.7% (10/15) of all variants and affecting 66.7% (6/9) of patients. These variants are pathogenic. These results reflected the variant characteristics of IEM diseases in Ganzhou and provided important information for the clinical diagnosis of other samples in the future.

4 Discussion

IEM is a group of diseases that affect the growth and development of newborns and children and even lead to death. Their occurrence is associated with genetic defects in the biosynthesis process of the skin, protease, receptor, carrier, and membrane pump, which the body needs to maintain normal metabolism (9). IEM often leads to progressive and irreversible nerve damage and physical and mental disability, posing a major threat to families and society. In this study, 260,915 neonates were screened for IEM using MS/MS technology, and 12 diseases were detected, with an overall incidence rate of 1 in 3,574. Compared with other regions of China such as Taiwan (10) (1/7,030) and Liuzhou (11) (1/3,733), the overall incidence rate is higher. Hence, performing early IEM screening and accurate diagnosis in this area is of particularly importance.

MS/MS has proven to be a reliable method suitable for clinical use, offering many advantages such as high efficiency, sensitivity, and convenience (4). However, biochemical screening has limitations and *in vivo* metabolism is influenced by many factors, leading to the existence of false positives and lowering the positive predictive value. In this study, 1,265 infants were positive in the MS/MS screening, and 73 cases were ultimately diagnosed by NGS, indicating a 94.2% false-positive rate. The simultaneous increase or decrease of several indicators of amino acids or acylcarnitines in the positive results of MS/MS screening can be influenced by various factors such as gestational age at birth, certain diseases, medications, diet and maternal factors, of which can lead to transient or secondary metabolic disorders (5). Per our findings, C5 was common in acylcarnitine-positive indicators in MS/MS; however, only one case of isovaleric acidemia was diagnosed via NGS. False-positive cases should be followed up on. It was reported that there was the presence of isomers in metabolites, including isovalerylcarnitine, tervalerylcarnitine, and 2-methylbutyrylcarnitine, that are difficult to distinguish by MS/MS (12). It is critical to further clarify the nature of

the disease, implement targeted therapy, and exclude false alarms to reduce the unnecessary economic, physical, and mental burden on children and their families (13).

In recent years, an increasing number of genetic detection techniques have been employed in the field of NBS (14, 15). Most studies have indicated that the application of targeted NGS technology advanced NBS diagnosis and treatment and reduced the diagnostic delay (16). In this study, we designed an NGS-based genetic diagnostic panel for IEM. All children underwent identification using the NGS panel and received a definitive diagnosis. Among the identified IEM cases, PCD was the most frequently diagnosed, accounting for 49.3% (36/73) of the total. PCD, also known as the carnitine transport disorder or carnitine uptake disorder, is a fatty acid beta-oxidation disorder resulting from a variant of *SLC22A5* that encodes the carnitine transporter OCTN2 located in the cell membrane (17, 18). PCD is an autosomal recessive inherited disease with an incidence of approximately 1/300–142,000, varying across different countries and ethnic groups (19). The incidence of PCD in NBS in this area is 1/7,248, making it one of the highest incidence areas in China, comparable to Liuzhou (11), Quanzhou (20), and other areas. The most reliable and rapid method for early PCD diagnosis is the MS/MS detection of the CO level (21). However, the CO results of MS/MS screening can be affected by various factors, including the maternal CO level, prematurity, and inadequate intake, and other fatty acid oxidation defects (22). In this study, we employed targeted NGS to advance the differential diagnosis of children with suspected PCD, thereby eliminating false positives resulting from these factors. Several pathogenic variants of *SLC22A5* (NM_003060.4) were found, including c.51C > G, c.1400C > G, c.428C > T, c.338G > A, and c.760C > T, with c.51C > G and c.1400C > G having the highest frequency. It has been reported that c.760C > T (p.R254X) and c.1400C > G (p.S467C) are the two most common variants in patients with PCD (20). However, in this study, the main variants observed were c.51C > G (p.F17L) and c.1400C > G (p.S467C). Previous studies have reported a low variant frequency of c.760C > T (p.R254X) in asymptomatic neonates (23). This finding is consistent with the results of the present study.

PKU is the most common disease of abnormal amino acid metabolism and has the second-highest incidence in this study (1/15,348). This incidence is close to the prevalence rate of live births (1/15,924) in China (24). The incidence of PKU varies considerably between geographical regions, with China having the highest incidence among Asian countries (25). Its pathogenesis is associated with the variants in *PAH,* which encodes the phenylalanine lightening enzyme (26). If patients are not treated promptly, severe and irreversible mental impairment, growth retardation, psychological behavior, acquired microcephaly, systemic skin hypopigmentation, and musty sweat odor may occur (27). The use of MS/MS to detect the Phe concentration and the Phe/Tyr ratio in newborns enable the early detection of PKU in children. However, this method cannot distinguish between different phenotypes; therefore, it may not be suitable for timely and appropriate treatment (28). Thus, the key to treating PKU lies in further clarifying the exact type of PKU. This study demonstrates the effectiveness of targeted NGS technology in eliminating false positives in MS/MS screening and identifying the PKU type and genotype. This enables accurate targeted therapy for infants with specific types of PKU. Consistent with previous reports, early diagnosis and treatment contributed to favorable outcomes for patients with PKU (29). There is a high degree of variability in *PAH* (NM_000277.3), as two of the

first variants found were c.1315 + 1G > A and c.1222C > T (p.Arg408Trp) (30). Within a few years, many new variants were discovered, and two of these new variants c.547G > T (p.E183Ter) and c.948delA (p.E316Ter) were also found in this study. According to ACMG and the available evidence, these new variants were classified as pathogenic. To date, over 800 variants in *PAH* have been identified, encompassing more than 100 different types of variants in children with PKU in China (31). It is noteworthy that there were variations in hotspot variants in *PAH* among different regions and populations. According to the results of large-sample research conducted in mainland China (26), the most common variant sites included c.728G > A, c.611A > G, c.331C > T, c.1238G > C, and c.442-1G > A, with c.728G > A having the highest variant frequency. The variant characteristics of *PAH* in this study were consistent with the results of large-cohort studies conducted in mainland China, including eastern China (18) and Nanjing (32). However, the hotspot variant c.158G > A detected in this study is uncertain and requires further validation.

CD, which is inherited in an autosomal recessive manner, is the most common disorder of the urea cycle. It is caused by pathogenic variants of *SLC25A13* (33) and results in a broad spectrum of phenotypes ranging from life-threatening hyperammonemia in neonates to adult-onset hyperammonemia with mild symptoms or no manifestations at all. The detection of neonatal blood amino acids (Cit, Cit/Arg) by MS/MS has a high sensitivity for the early diagnosis of CD children. However, an increasing number of case reports have found that the clinical manifestations and laboratory abnormalities of CD patients are varied and transient (34). This study identified six cases of CD through targeted NGS. One case was detected due to abnormal levels of CO, while the initial results for Cit or Cit/Arg were within the normal range. Therefore, while highly biochemical indicators are not strictly necessary, a combination of clinical manifestations and genetic analyses is essential for making an accurate diagnosis (35). With an incidence rate of 1/43,486, it ranked third in our study. A previous study indicated that the incidence rate in southern China is significantly higher than that in northern China, with provinces at lower latitudes having significantly higher incidence rates than those at higher latitudes (36). In *SLC25A13* (NM_014251.3), c.852_855del, c.1638_1660dup, c.615 + 5G > A, and c.1751-5_1751-4ins were the most common variants in China, accounting for 82.9% of all variants (37). In our study, we observed that c.852_855del was the most prevalent, accounting for 66.7% of cases. These findings are in line with results of previous studies (38).

Although targeted NGS technology has demonstrated many advantages in clinical applications, its high cost compared with MS/MS technology may limit its widespread use in resource-limited areas (39). Furthermore, targeted NGS primarily focuses on detecting known potential targets and exhibits limitations when addressing complex genomic variants, such as structural and copy number variations (40). This limitation could lead to the omission of certain disease-associated variants, potentially impacting clinical decision-making.

5 Conclusion

In summary, targeted NGS technology can serve as a crucial diagnostic tool for neonatal genetic metabolic diseases. Its combination with MS/MS technology proves effective and suitable for clinical screening and diagnosis. Additionally, we identified IEM variant hotspots and some novel variants in our region. These variants

are the cause of IEM in certain patients, helping to elucidate the etiology of the disease at the genetic level.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ganzhou Maternal and Child Health Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

GL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. QG: Data curation, Software, Writing – original draft. ZL: Investigation, Methodology, Software, Writing – original draft. HC: Data curation, Formal analysis, Methodology, Project administration, Supervision, Writing – original draft. XT: Data curation, Project administration, Supervision, Writing – review & editing. JC: Conceptualization, Data curation, Methodology, Writing – review & editing. JH: Methodology, Project administration, Software, Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2024.1461141/full#supplementary-material

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