


## ORIGINAL ARTICLE

# Targeted exome sequencing strategy (NeoEXOME) for Chinese newborns using a pilot study with 3423 neonates

Ziyang Cao<sup>1,2</sup> | Xiaoyan He<sup>3</sup> | Dongjuan Wang<sup>3</sup> | Maosheng Gu<sup>4</sup> | Feng Suo<sup>4</sup> | Rong Qiang<sup>5</sup> | Ruixue Zhang<sup>5</sup> | Chengrong Song<sup>5</sup> | Xiaohua Wang<sup>6</sup> | Bo Zhu<sup>6</sup> | Donghua Cao<sup>7,8</sup> | Haihua Yu<sup>7</sup> | Yiping Qu<sup>9</sup> | Guosong Shen<sup>10</sup> | Jian Wu<sup>11</sup> | Pengpeng Wang<sup>11</sup> | Jinxia Wang<sup>1,2</sup> | Hongyang Zhang<sup>1,2</sup> | Zijun Yan<sup>1,2</sup> | Guangjun Yu<sup>1,2</sup> | Lin Zou<sup>1,2,3</sup> 

<sup>1</sup>Clinical Research Unit, Shanghai Children's Hospital, Shanghai Jiao Tong University Medical School, Shanghai, China

<sup>2</sup>Institute of Pediatric Infection, Immunity, and Critical Care Medicine, Shanghai Jiao Tong University School of Medicine, Shanghai, China

<sup>3</sup>Center for Clinical Molecular Laboratory Medicine of Children's Hospital of Chongqing Medical University, Chongqing, China

<sup>4</sup>Center of Medical Genetics, Xuzhou Maternal and Child Health Care Hospital, Xuzhou, China

<sup>5</sup>Center of Medical Genetics, Northwest Women and Children's Hospital, Xi'an, China

<sup>6</sup>Center of Medical Genetics, Inner Mongolia Maternal and Child Health Care Hospital, Hohhot, China

<sup>7</sup>Medical Genetic Laboratory, Dalian Municipal Women and Children's Medical Center (Group), Dalian, China

<sup>8</sup>Genetic Laboratory, Shenyang Jinghua Hospital Co., Ltd, Shenyang, China

<sup>9</sup>Newborn Screening Center of Children's Hospital of Zhejiang University School of Medicine, Hangzhou, China

<sup>10</sup>Medical Laboratory Center of Huzhou Maternal and Child Health Care Hospital, Huzhou, China

<sup>11</sup>Research and Development Department, MyGenostics Inc., Beijing, China

## Correspondence

Guangjun Yu

Email: [gju@shchildren.com.cn](mailto:gju@shchildren.com.cn)

Lin Zou

Email: [zoulin@shchildren.com.cn](mailto:zoulin@shchildren.com.cn) and [zoulin74@126.com](mailto:zoulin74@126.com)

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## Abstract

**Background:** Newborn screening (NBS) aims to detect congenital anomalies, and next-generation sequencing (NGS) has shown promise in this aspect. However, the NBS strategy for monogenic inherited diseases in China remains insufficient.

**Methods:** We developed a NeoEXOME panel comprising 601 genes that are relevant to the Chinese population found through extensive research on available databases. An interpretation system to grade the results into positive (high-risk, moderate-risk, and low-risk genotypes), negative, and carrier according to the American College of Medical Genetics (ACMG) guidelines was also developed. We validated the panel to evaluate its efficacy by using data from the "1000 Genomes Project" and conducted a pilot multicenter study involving 3423 neonates.

**Results:** The NGS positive rate in the 1000 Genomes Project was 7.6% (23/301), whereas the rate was 12.0% in the multicenter study, including 3249 recruited neonates. Notably, in 200 neonates, positive per conventional NBS, 58.5% (69/118)

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showed results consistent with NGS. In the remaining 3049 neonates showing negative results in conventional NBS, 271 (8.9%) were positive per NGS, and nine of them were clinically diagnosed with diseases in the follow-up.

**Conclusion:** We successfully designed a NeoEXOME panel for targeted sequencing of monogenic inherited diseases in NBS. The panel demonstrated high performance in the Chinese population, particularly for the early detection of diseases with no biochemical markers.

#### KEYWORDS

monogenic inherited diseases, NeoEXOME panel performance, newborn screening, pilot study, targeted sequencing

## 1 | INTRODUCTION

Congenital anomalies are significantly responsible for neonatal mortality globally. The Ministry of Public Health of China in 2012 reported that approximately 5.6% of births in China have congenital anomalies, resulting in approximately 900,000 new cases each year (Ministry of Health of the People's Republic of China, 2012). To address this, the World Health Organization (WHO) has developed a three-level prevention strategy based on the causes and epidemiology of congenital anomalies. The strategy includes preconception screening to increase the likelihood of a healthy birth, peri-conception screening to help predict the risk of abnormalities and neonatal screening to reduce mortality and morbidity due to congenital disorders (World Health Organization, 2010). Newborn screening (NBS), the third step in the prevention of congenital anomalies, aims to identify serious diseases during the neonatal period to facilitate earlier referral and the initiation of medical or surgical treatment, thus potentially reducing child mortality and morbidity (Fabie et al., 2019; Zhou & Zhao, 2021).

NBS was initiated in the 1960s when Professor Guthrie first applied the method of bacterial inhibition to screen for phenylketonuria (PKU) in dried blood spots (DBS; Guthrie & Susi, 1963). Since then, NBS has advanced under the several screening guidelines (Rajabi, 2018; Wilson & Jungner, 1968). In 2006, the American College of Medical Genetics (ACMG) published a Recommended Uniform Screening Panel for NBS, which has undergone continuous updates (Watson et al., 2006). In China, NBS began in 1981 and focused mainly on screening for PKU and congenital hypothyroidism (CH; Zhan et al., 2009). Tandem mass spectrometry (MS) is the main conventional NBS method that has been widely used in clinical practice for the last 20 years (Ombrone et al., 2016). Although MS accelerated the development of NBS, it

is associated with a false-positive/negative ratio (Fabie et al., 2019; Mendell et al., 2012; Slaughter et al., 2010). In addition, MS and other conventional NBS methods have a limited coverage of diseases that can be detected. New methods are thus urgently needed to address the limitations of NBS.

Recently, the high-throughput sequencing (NGS) technology has been widely used in tumor-targeted gene testing (Rizvi et al., 2018), pathogen detection (Han et al., 2019), as well as in three-level screening for congenital anomalies (Swedish Council on Health Technology A, 2016). NGS has been successfully applied for NBS and has been shown to be useful for second-tier confirmation of some diseases (Lin et al., 2021; Parad et al., 2020). In addition, the ability of NGS to detect disease-genes in patients in the neonatal intensive care units (NICU) has also been uncovered (Meng et al., 2017; Zhu et al., 2020). However, there are challenges associated with the use of NGS in NBS, including a large number of variants with uncertain significance (VUS) and the lack of recognized and recommended list of gene–disease association conditions.

To address these challenges, researchers have been working to establish an appropriate criterion for the gene–disease association list and report strategies in NGS for NBS (Ceyhan-Birsoy et al., 2017; Milko et al., 2019). However, the high incidence of monogenic disorders in the Chinese population is quite different from that of other nations due to diverse ethnicities and geographical areas. Therefore, the gene–disease association list and reporting strategy cannot be fully implemented. To address the unique genetic landscape of the Chinese population, we designed a severe and actionable inherited gene–disease list called NeoEXOME panel for NBS. We then conducted a multicenter study including 3423 neonates to evaluate the feasibility of the NeoEXOME panel and compare it with other NGS panels for NBS.

## 2 | METHODS

### 2.1 | Establishment of the NGS panel

#### 2.1.1 | Generation of gene–disease association list

We researched monogenic inherited diseases that are severe and actionable (outline in the result section) and screened for heritable genes corresponding to the diseases using the Online Mendelian Inheritance in Man (OMIM) database (<https://www.omim.org/>). Genes with a “phenotype mapping key” of “3,” which indicates that the molecular basis of the disease is known, were included.

#### 2.1.2 | Age of onset

We manually sorted diseases records in the OMIM database to identify the known minimum onset age of diseases and categorized them into six groups based on the age of onset: <1 year of age (infants), 1–3 years of age (toddlers), 3–6 years of age (preschoolers), 6–12 years of age (middle childhood), 12–18 years of age (young teens and teenagers), >18 years of age (adulthood).

### 2.2 | Ethical compliance

This study was approved by the Ethics Committee of Children's Hospital of Chongqing Medical University (2019-R-171-1). Guardians of neonates provided written and signed informed consent.

### 2.3 | Participants

The study has been registered in [ClinicalTrials.gov](https://www.clinicaltrials.gov) (Number: NCT03984266). The *inclusion criteria* were meeting both of the following: all newborns (including hospitalized infants and infants with abnormal results of conventional NBS) and the guardian's agreement to sign the informed consent and participate. The *exclusion criteria* were meeting one or more of the following criteria: other similar clinical studies are underway and receiving transfusion of allogeneic blood products in the past 2 weeks. *Rejection criteria*: Specimen cannot be tested due to improper collection or storage; samples that failed quality control; participants with no follow-up data; guardian's request to withdraw.

From October 2019 to September 2021, a total of 3423 neonatal subjects were enrolled from five hospitals:

Children's Hospital of Chongqing Medical University, Inner Mongolia Maternal and Child Health Hospital, Northwest Women and Children's Medical Center, Dalian Maternal and Child Health Hospital, and Xuzhou Maternal and Child Health Care Hospital.

### 2.4 | Sample preparation

The heel blood of the participants was collected into a special filter paper and allowed to dry naturally at room temperature forming DBS. After conventional NBS, the DBS was stored at 4°C. Genomic DNA was extracted from DBS using QIAamp DNA Mini Kit (Qiagen, German) according to the operation manual. In brief, a punched-out circle from a DBS was placed into a 1.5 mL microtube with Buffer ATL to dissolve DNA and incubated at 85°C for 10 min. Protein K was added to digest proteins. After incubation at 70°C and the addition of ethanol, the buffer was transferred into a Mini spin column. Finally, the DNA solution was eluted using the elution buffer after a series of centrifugation. The quality and quantity of the DNA were assessed by Qubit® 3.0 Fluorometer (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions.

### 2.5 | Library construction and sequencing

A library was constructed according to the standard procedures of Illumina (Illumina, Inc., USA), including terminal repair, adaptor connection, and polymerase chain reaction enrichment. The Neonatal Gene Capture Kit NeoExome™ (MyGenostics GenCap® Enrichment technologies, China) was designed based on the targeted genes in our study and used for hybridization capture of relevant target regions. The final library was determined using the Qubit® 3.0 Fluorometer and Agilent 2100 Bioanalyzer system (Agilent Technologies, USA) and sequenced using the HiSeq X Ten System (Illumina, Inc., USA) for 150-bp double-terminal sequencing (Zhu et al., 2020). After sequencing, we compared the reads to the UCSC HG19 reference genome and identified variants using a bioinformatics process set by MyGenostics (based on GATK, The Genome Analysis Toolkit). Variations were annotated using the ANNOVAR databases. Variants with high population frequency (>1/1000) were filtered out using the dbSNP 1381000 Genome Project, ESP6500SI, and ExAC (the Exome Aggregation Consortium) browser.

## 2.6 | Probe design description

The design of the liquid-phase hybridization capture probe was based on the GenCap technology of MyGenostics (Yu et al., 2020). In addition to designing probes that cover the exon region of related genes (covering the exon region, exon flanks ~50bp, and non-coding disease-causing regions reported by the Human Gene Mutation Database, HGMD), encryption designing probes were designed for 25 CNV high-risk genes (increasing the coverage of 300bp intron region by designing probes on the exon flanks of related genes). We also designed probes that included full-length coverage of *HBA1/HBA2/HBB/SMN1/SMN2* genes (coding region and intron region of coverage gene). The probes covered the hot spot variations of the mitochondrial genes, *MT-RNR1*, *MT-TL1*, *MT-ND3*, and *MT-ATP6*. We covered some regional genes of Prader-William/Angleman, DiGeorge, and Williams in the microdeletion syndrome and encrypted the probe for the analysis of copy number variation in related regions. The length, density, and position of the probe were adjusted based on the Probe design software BaitDesigner of MyGenostics to improve the capture efficiency of the probe according to the GC content, region size,  $T_m$  value, and other parameters of the target region.

## 2.7 | Interpretation of gene variants

Based on the ACMG guidelines, the pathogenicity of the variation was classified into pathogenic, likely pathogenic, unknown, likely benign, and benign. The gene results were graded to positive (high-, moderate-, and low-risk genotypes), negative, and carrier according to the pathogenicity of the variation and the genetic inheritance pattern (Figure S1).

### 2.7.1 | High risk

High-risk genotypes were defined as one pathogenic or likely pathogenic (P/LP) variant in the autosomal dominant or Y-linked genes; two P/LP variants in the autosomal recessive genes; one P/LP variant in females in X-linked dominant genes, or two P/LP variants in X-linked recessive genes; and one P/LP variant in males in X-linked genes.

### 2.7.2 | Moderate risk

Moderate risk genotypes were defined as one P/LP variant, and one VUS variant with Bayesian point  $>3$  in autosomal recessive genes; one P/LP variant, and one VUS variant with Bayesian point  $>3$  in females in X-linked recessive genes.

### 2.7.3 | Low risk

Low-risk genotypes were defined as one P/LP/VUS variant, and one VUS variant with Bayesian point  $<3$  in the autosomal recessive genes; one VUS variant with Bayesian point  $<3$  in the autosomal dominant or Y-linked genes; one P/LP/VUS variant, and one VUS variant with Bayesian point  $<3$  in the X-linked recessive genes in females.

### 2.7.4 | Carrier

Carrier genotypes were defined as one P/LP variant in autosomal recessive genes; one P/LP variant in females in X-linked recessive genes.

## 2.8 | Venny analysis

The Venny 2.1 software was used to compare different NBS NGS panels with our panel through an interactive tool (Oliveros, 2007; <https://bioinfo.gp.cnb.csic.es/tools/venny/>). Multivariate Venn's Diagrams were drawn using the R version 4.0.5 software (<https://www.r-project.org/>).

## 3 | RESULTS

### 3.1 | Generation and features of the NeoExome panel

To design an NGS panel for NBS that covers severe and actionable monogenic diseases for the Chinese population, we combined the categorization of diseases based on the China National Catalog of Rare Diseases (The People's Republic of China, 2018), Preventable and Treatable Rare Diseases in China (Shanghai Municipal Health Commission, 2016), conventional MS/MS NBS project in China (Interlaboratory Quality Evaluation Committee of Neonatal Genetic and Metabolic Disease Screening CTC, Ministry of Health, 2019), conventional NBS project in China (National Health Commission of the People's Republic of China, 2010), ACMG recommendations (Fabie et al., 2019), Chinese NBS Laboratory Committee recommendations, and the MyGenostics database of monogenic diseases in 40,000 ill newborns. We collected the diseases list from each database, and then the disease panel was created. The OMIM database was used to standardize and screen out the diseases-related genes and the disease-genes panel (named NeoEXOME) was created (Figure 1a). Our NeoEXOME panel comprised of 601 genes and 542 diseases (Table S1).



The panel covered diseases with several systems, including skeletal, respiratory, urinary, immune system, nervous system, cardiovascular system, blood, endocrine system, metabolism, and mitochondria-related diseases. Among them, metabolic system diseases accounted for the highest proportion (Figure 1b). In addition, most of the 601 gene-diseases have an onset age in childhood (96%), with 418 having onset before 1 year and 577 having onset before 18 years (Figure 1c).

## 3.2 | Performance of NeoEXOME

### 3.2.1 | Validation results of “1000 Genomes project”

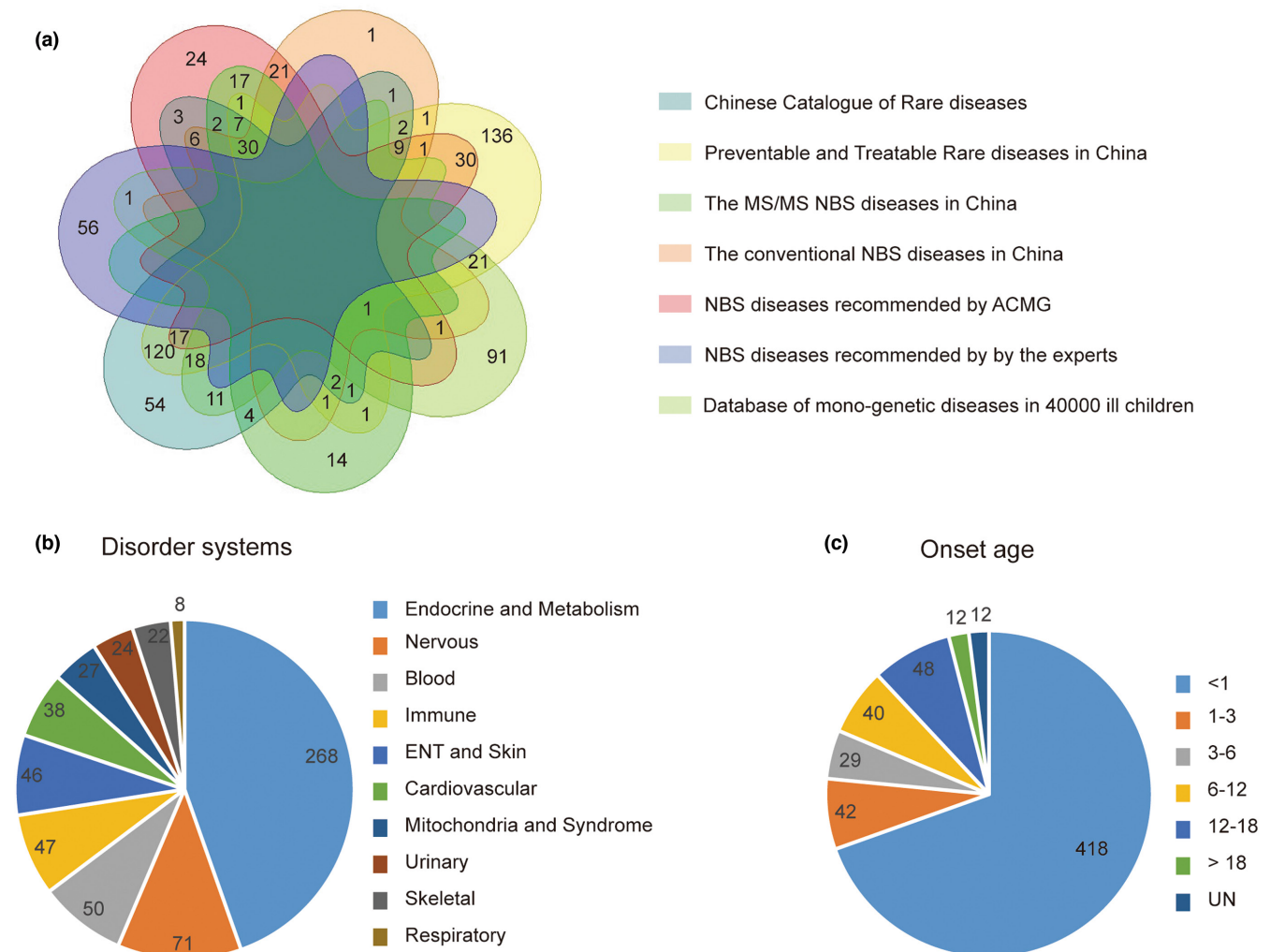
Firstly, we evaluated the performance of NeoEXOME by validating it with the data of unrelated 301 Chinese from

the “1000 Genomes Project”. Of those individuals, 72 were negative, 223 were carriers, and 23 were positive, with a positivity rate of 7.6%. The variants of *FLG* and *GJB2* account for the most frequently affected genes, at 47.8% (11/23) and 21.7% (5/23), respectively (Table S2).

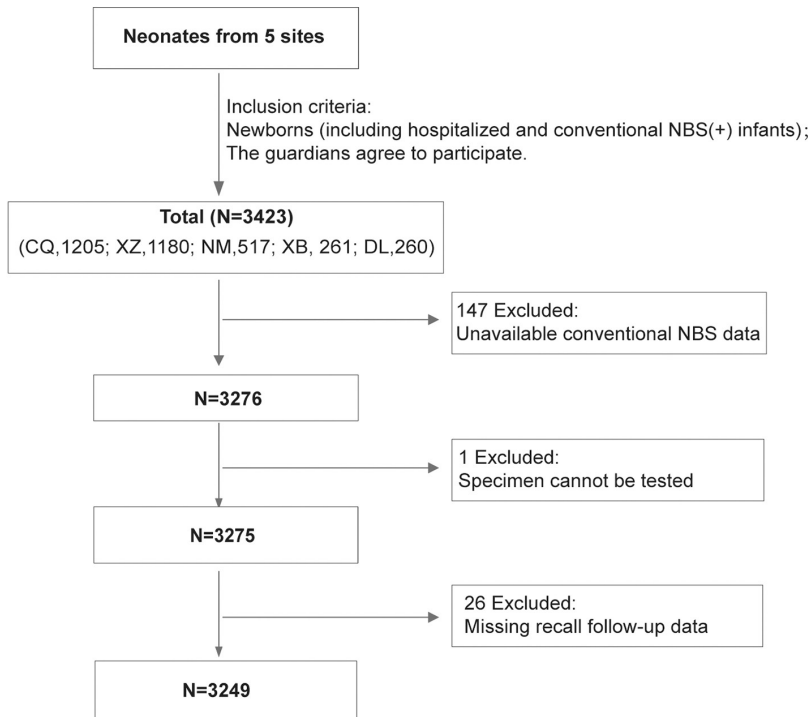
### 3.2.2 | A pilot study of the performance of NeoEXOME for NBS in China

We conducted a pilot, multicenter clinical trial to evaluate the performance of NeoEXOME. From October 2019 to September 2021, 3423 neonates were enrolled from five centers (Figure 2). DBS was collected within 3 days following birth, and NGS was performed based on our NeoEXOME panel.

Among the 3249 neonates eligible for the analysis, 934 were NGS negative, 389 were NGS positive (244 high



**FIGURE 1** Characteristics of 601 gene-diseases associations. (a) 7 categorizations of disease were collected and included to generate the gene panel. The disease system (b) and onset age (c) for those gene-disease associations were demonstrated. <1, <1 year of age (Infants); 1–3, 1–3 years of age (Toddlers); 3–6, 3–6 years of age (Preschoolers); 6–12, 6–12 years of age (Middle Childhood); 12–18, 12–18 years of age (Young Teens and Teenagers); >18, >18 years of age (Adulthood); UN, unknown.



### Data analysis and comparison between NGS and conventional NBS

risk, 4 moderate risk, 141 low risk), and 1926 were carriers. The NGS positivity rate was 12.0% with most neonates genetically susceptible to monogenic-related diseases (Figure 3a,b). Autosomal recessive inheritance was the most common inheritance pattern (Figure 3a,b). Endocrine and metabolism system disorders accounted for the highest proportion among neonates (Figure 3c). Most of the diseases detected were predicted to develop within the first year of life (Figure 3d). The variants of *DUOX2* accounted for the most frequent changed genes (18.3%, 131/716), followed by *UGT1A1*, *PAH*, *GJB2*, *FLG* (Figure 3e). The top related disorders were thyroid dyshormonogenesis, hyperbilirubinemia, phenylketonuria, deafness, hyperbilirubinemia, and ichthyosis vulgaris, with the frequency of variant 5.7%, 5.4%, 5.0%, 4.1%, respectively. Of the 1926 infants that were tested as carriers, 3462 variants were found, including 1224 pathogenic variants and 2238 likely pathogenic variants (Figure 4a). The variants of *GJB2* were the most common among all the centers, followed by *UGT1A1*, *DUOX2*, *FLG*, and *SLC25A13* (Figure 4b).

To further validate the use of NGS in NBS, we collected conventional NBS results to compare the consistency of both approaches. Conventional NBS results of CH, PKU, hearing screening (HS), congenital adrenal hyperplasia (CAH), glucose-6-phosphate dehydrogenase (G6PD), and tandem MS detection were collected from the five centers (Table S3). However, CAH and G6PD detection were detected only in the Children's Hospital of Chongqing Medical University

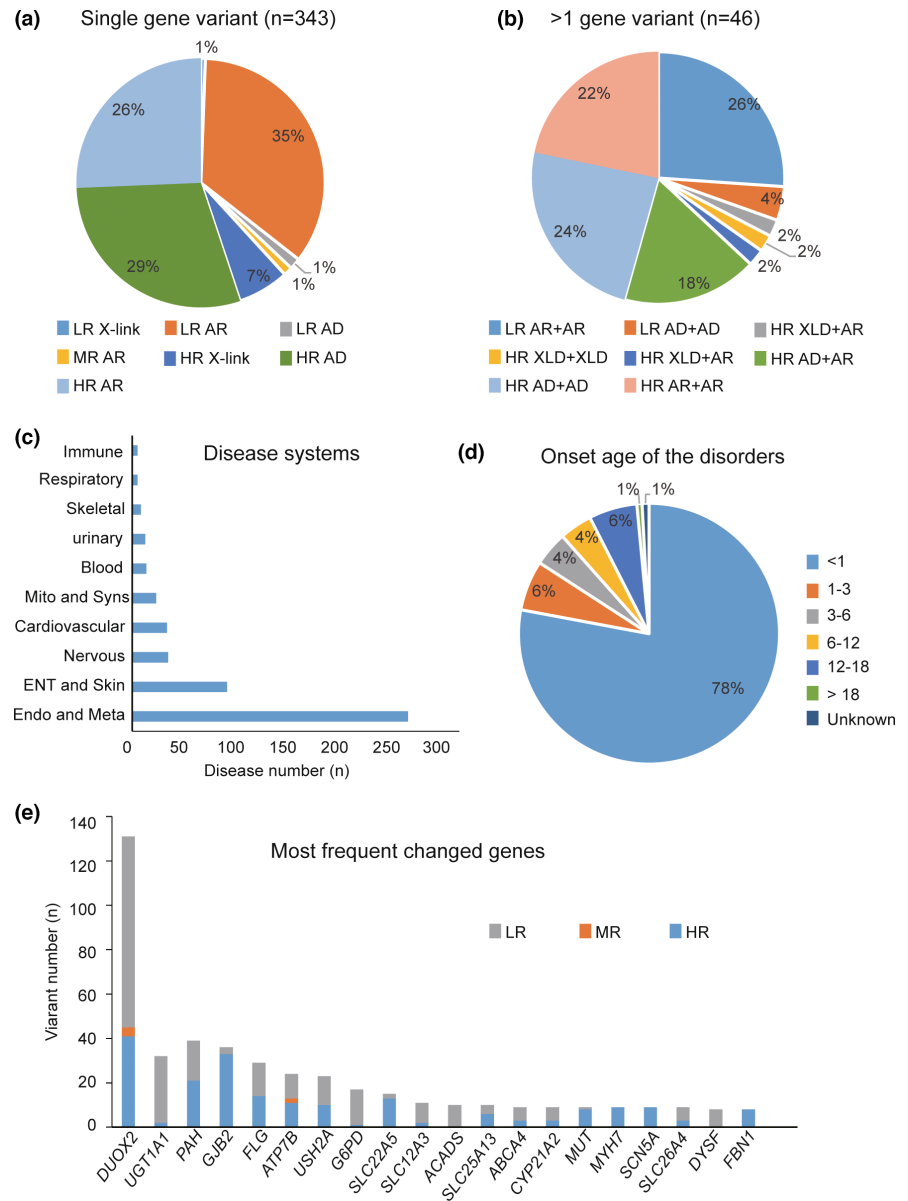
**FIGURE 2** The overall flowchart of pilot multicenter study. 3423 neonates meet the inclusion criteria were enrolled from five institutions. CQ, Children's Hospital of Chongqing Medical University; XZ, Xuzhou Maternal and Child Health Care Hospital; NM, Inner Mongolia Maternal and Child Health Hospital; XB, Northwest Women and Children's Medical Center; DL, Dalian Maternal and Child Health Hospital.

and Xuzhou Maternal and Child Health Care Hospital. In the 200 neonates that had positive results of conventional NBS, 118 were NGS positive (64 high risk, 2 moderate risk, 52 low risk), 14 were NGS negative, and 68 were NGS carriers (Table 1). Of the 118 neonates that were positive for both conventional NBS and NGS, 69 (58.5%) had consistent results with conventional NBS and NGS (Table S4). In 3049 neonates that had negative results of conventional NBS, 271 (8.9%) were NGS positive (180 high risk, 2 moderate risk, 89 low risk), 920 were NGS negative, and 1858 were NGS carriers (Table 1). Among the 271 neonates that had positive results of NGS, and negative results of conventional NBS, 168 were clinically followed up, and 9 of them (including genes of *DUXO2*, *PAH*, *MUT*, *WAS*, and *SLC22A5*) were clinically diagnosed with diseases.

### 3.3 | Comparison of NeoEXOME with other NBS panels

Finally, we compared our NeoEXOME panel with other NGS panels and whole genome sequencing (WGS) gene-disease lists globally in terms of gene-disease system and the disease onset age. The gene-disease information from "BabySeq" (Ceyhan-Birsoy et al., 2017), "NC NEXUS" (Milko et al., 2019), and "NESTS" (Luo et al., 2020) projects were collected. There were 414 common genes in BabySeq and our panel (Figure 5a), most of which were

**FIGURE 3** NeoEXOME detection results of positive cases. (a) Gene variants interpretation (risk grade and inheritance patterns) of neonates with mono-gene change. (b) Gene variants interpretation (risk grade and genetic patterns) of neonates with multi-genes change. (c) Disease system distribution of neonates that were NGS positive. (d) Gene–disease associations' onset age of neonates that were NGS positive. (e) Top 20 genes distribution of neonates with NGS positive cases. LR, Low risk; MR, Moderate risk; HR, High risk; AD, Autosomal dominant; AR, Autosomal recessive; XLD, X-linked dominant; Mito, Mitochondrial; Syns, Syndrome; ENT, ear, nose, and throat; Endo, Endocrine; Meta, metabolism.

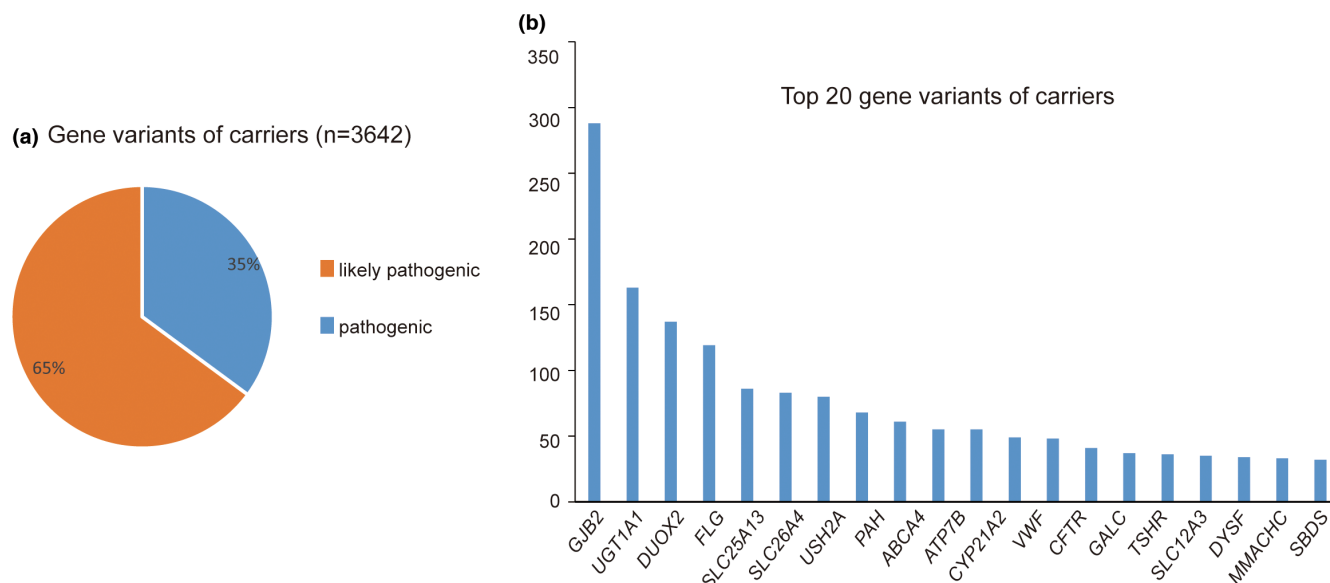


endocrine and metabolism system disease-genes. Nervous (*SLC12A6* and *GFAP*) system, ear, nose, throat (ENT) (*AMELX* and *DFNA5*) system, and syndromes (*CUL7*, *ERCC6*) disease-genes were higher in the BabySeq panel. Also, the BabySeq panel included gene–disease pairs of the digestive system and tumors (such as *SLC26A3*, *APC*, *MSH2*, *SMAD4*, *PTEN*, and *TP53*), which was not covered in our panel. There were 268 common genes in the NC NEXUS project and our panel (Figure 5b). ENT (*USH1E*, *EPS8L2*) system, respiratory (*ABCA3*, *SFTPB*) system, and syndromes (*ALMS1*, *CACNB2*) disease-genes were higher in the NC NEXUS panel. Similar to the BabySeq panel, NC NEXUS also covered gene–disease pairs of the digestive system and tumor (such as *CDH1*, *GIF*, *POLE*, and *PDX1*). We compared our panel with the “NESTS” panel for Chinese newborns recently reported by Dr. Li. There were 191 common genes in the two panels (Figure 5c).

Deafness-related genes (*KCNQ4*, *PTPRQ*) dominated the gene list of the NESTS panel. Genes related to the respiratory system (*RSPH4A* and *DNAAF1*) were higher in the NESTS panel. Two genes *MUTYH* and *APC* that were related to multiple colorectal adenomas and adenomatous polyposis coli were also included in the NESTS panel. However, the NESTS panel did not include genes of *DUOX2*, *UGT1A1*, *FLG*, and *ATP7B*, which showed a high positive rate in our NeoEXOME panel. In general, the gene–disease association list designed by different projects vary in terms of focus and characteristics.

## 4 | DISCUSSION

NGS may play an important role in the detection of congenital anomalies. Carrier screening is a useful tool for



**FIGURE 4** NGS detection results of carrier. (a) Gene variants characteristics of neonates that were carrier. (b) Top 20 genes distribution of neonates that were carrier.

**TABLE 1** Comparison between NGS and conventional newborn screening.

	NeoEXOME+			Carriers	NeoEXOME-	Total
	High risk	Moderate risk	Low risk			
Conventional NBS+	64	2	52	68	14	200
Conventional NBS-	180	2	89	1858	920	3049
Total	244	4	141	1926	934	3249

Abbreviation: NBS, newborn screening; NGS, next-generation sequencing.

detecting severe genetic defects. It involves the screening of both prospective parents to determine whether they carry any harmful genetic mutations that could be transmitted to their offspring. NGS-based carrier screening allows to detect multiple variants simultaneously, providing a more comprehensive screening approach (Gregg et al., 2021). As the second-step prevention of congenital anomalies, peri-conception screening using NGS can detect chromosomal abnormalities in fetal free DNA of the maternal blood. The chromosomal abnormalities mainly involve Trisomy 21, 18, 13 syndromes (Breveglieri et al., 2019). The peri-conception screening offers a non-invasive and reliable alternative to invasive prenatal diagnostic testing, such as amniocentesis or chorionic villus sampling, reducing the risk of miscarriage. NBS using NGS is a critical tool for detecting severe, actionable, and early-onset monogenic inherited diseases in newborns (Zhou & Zhao, 2021). In our study, we aimed to provide suitable technology, a set of rational diseases, and a gene-disease association list for NBS. We designed a NeoEXOME panel covering diseases that are severe and actionable for the Chinese population and verified it through a multicenter neonate study. We also compared our NeoEXOME panel

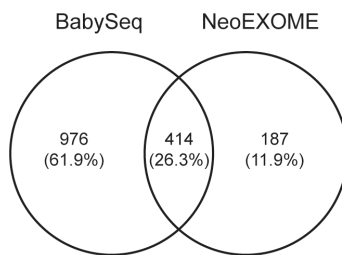
with other NGS-based NBS panels to illustrate the respective characteristics.

The gene-disease list is critical in the design of the NGS panel. The BabySeq project conducted a study to curate a catalog of gene-disease pairs based on the ACMG, the ClinGen clinical validity classification framework criteria, penetrance, and age of onset. After screening, they identified 954 genes that met their inclusion criteria (Ceyhan-Birsoy et al., 2017). The NC NEXUS project developed an age-based framework to assess the gene-disease list and categorized 822 gene-disease pairs into four groups (Milko et al., 2019). In China, several NGS screening studies for NBS have been conducted, including the panel designed by Dr. Yu (573 genes) (Luo et al., 2020), Dr. Li (465 genes) (Hao et al., 2022), Dr. Zhao (134 genes) (Huang et al., 2022), and Dr. Xu (164 genes; Tong et al., 2022; Wang et al., 2022). In our study, we integrated the diseases catalog of Rare Diseases in China, routine NBS diseases in China, ACMG and mainland experts' recommendation, and the database of genetic diseases in 40,000 ill-children to generate a comprehensive list of 601 genes for the NeoEXOME panel for NBS.

The positivity rate of our NGS panel in the "1000 Genomes Project" was 7.6%, higher than that of previous

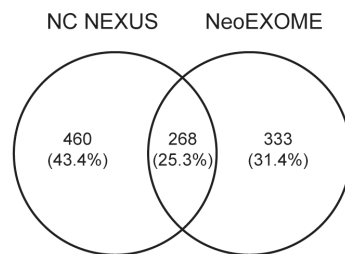


(a)



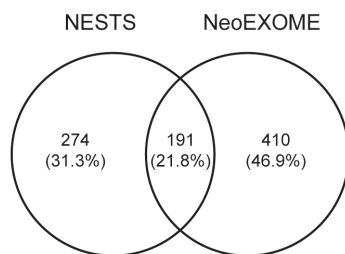
	BabySeq	NeoEXOME	Commo n genes	Only in BabySeq	Only in NeoEXOME
<b>Gene-Number</b>	1390	601	414	976	187
<b>Disease systems, n (%)</b>					
Endocrine and Metabolism	359 (25.83)	268(44.59)	204	155	64
Nervous	291 (20.94)	71(11.81)	42	249	29
Blood	62 (4.46)	50(8.32)	33	20	17
Immune	38 (2.73)	47(7.82)	24	14	23
ENT and Skin	167 (12.01)	46(7.65)	33	134	13
Cardiovascular	111 (7.99)	38(6.32)	27	84	11
Mitochondria and Syndrome	164 (11.80)	27(4.49)	13	151	14
Urinary	47 (3.38)	24(3.99)	19	28	5
Skeletal	65 (4.68)	22(3.66)	12	53	10
Respiratory	40 (2.88)	8(1.33)	7	33	1
Digestive	23 (1.65)	0	0	0	23
Tumor and others	23 (1.65)	0	0	0	23
<b>Onset age, n (%)</b>					
<18 years	1309(94.17)	577(96.00)	407	902	170
>18 years	81(5.83)	24(4.00)	7	74	17
<b>BabySeq Category</b>					
A	818		310		
B	55		29		
C	517		75		

(b)



	NC NEXUS	NeoEXOME	Commo n genes	Only in NC NEXUS	Only in NeoEXOME
<b>Gene-Number</b>	728	601	268	460	333
<b>Disease systems, n (%)</b>					
Endocrine and Metabolism	197(27.06)	268(44.59)	159	38	109
Nervous	34(4.67)	71(11.81)	17	17	54
Blood	79(10.85)	50(8.32)	19	60	31
Immune	33(4.53)	47(7.82)	16	17	31
ENT and Skin	121(16.62)	46(7.65)	15	106	31
Cardiovascular	73(10.03)	38(6.32)	20	53	18
Mitochondria and Syndrome	103(14.15)	27(4.49)	9	94	18
Urinary	11(1.51)	24(3.99)	3	8	21
Skeletal	12(1.65)	22(3.66)	4	8	18
Respiratory	54(7.42)	8(1.33)	6	48	2
Digestive	9(1.24)	0	0	0	9
Tumor and others	2(0.27)	0	0	0	2
<b>Onset age, n (%)</b>					
Birth	384(52.75)	418(69.55)	191	193	227
Childhood	114(15.66)	111(18.47)	53	61	58
Adolescent	20(2.75)	48(7.99)	15	5	33
Adulthood	81(11.13)	12(2.00)	9	72	3
Variable	107(14.70)	0	0	107	0
UN	22(3.02)	12(2.00)	0	22	12
<b>NC NEXUS Category</b>					
1	420		186		
2	214		73		
3	18		2		
4	17		4		
NR	59		5		

(c)



	NESTS	NeoEXOME	Commo n genes	Only in NESTS	Only in NeoEXOME
<b>Gene-Number</b>	465	601	191	274	410
<b>Disease systems, n (%)</b>					
Endocrine and Metabolism	120(25.81)	268(44.59)	94	26	174
Nervous	20(4.30)	71(11.81)	10	10	61
Blood	22(4.73)	50(8.32)	11	11	39
Immune	25(5.38)	47(7.82)	11	14	36
ENT and Skin	138(29.68)	46(7.65)	19	119	27
Cardiovascular	18(3.87)	38(6.32)	13	5	25
Mitochondria and Syndrome	42(9.03)	27(4.49)	10	32	17
Urinary	33(7.10)	24(3.99)	11	22	13
Skeletal	16(3.44)	22(3.66)	10	6	12
Respiratory	29(6.24)	8(1.33)	2	27	6
Digestive	2(0.43)	0	0	0	2
<b>Onset age, n (%)</b>					
<1	335(72.04)	420(69.88)	153	182	267
1-3	39(8.39)	43(7.15)	11	28	32
3-12	73(15.70)	74(12.31)	24	49	50
12-18	18(3.87)	49(8.15)	3	15	34
≥18	0	15(2.50)	0	0	15

**FIGURE 5** Comparison of NeoEXOME with other NBS panel. (a) NeoEXOME and BabySeq. (b) NeoEXOME and NC NEXUS. (c) NeoEXOME and NESTS.

studies (Zhang et al., 2015). The most frequently changed genes in our analysis were *FLG* and *GJB2*. *FLG* is a pathogenic gene for ichthyosis vulgaris, which encodes filaggrin and plays a key role in epidermal terminal differentiation and skin barrier formation. The proportion of *FLG* gene variation in ichthyosis vulgaris was reported to be 55.6%, and patients having ichthyosis vulgaris with *FLG* gene variant experienced more severe diseases (Qian et al., 2015). However, *FLG* also has high variation in the normal population, Palmer and colleagues carried out *FLG* analysis with 1008 people of the European origin and found that the functional deletion variants of the *FLG* gene were approximately 9% (Palmer et al., 2006). We believed that the *FLG* gene should not be included in our panel. Therefore, we recalculate NGS positivity rate excluding the *FLG* variants, 376 infants were NGS positive (11.57%) among the 3249 neonates eligible for the analysis. In addition, *GJB2* gene variation is generally considered to be a common cause of non-syndromic deafness (Koohiyan et al., 2020). Our analysis identified 5 *GJB2* variants, four of which were c.109G>A variant (Table S2), a pathogenic variant with incomplete penetrance and having a high carrier rate among Asians (Shen et al., 2019). Our study highlights the importance of genetic counseling for such genetic variations.

Moreover, it is also necessary to consider whether genes linked to adult-onset diseases should be detected during the neonatal stages. In the previous study conducted by the BabySeq project, a *BRCA2* pathogenic variant was discovered in an infant, which increases the risk of breast cancer by 45%, and ovarian cancer by 11% in women (Ceyhan-Birsoy et al., 2019). The researchers faced moral distress and proposed returning the adult-onset genetic variants (Holm et al., 2019). However, this sparked a heated ethical debate: Lainie FR published an article arguing that researchers should avoid identifying adult-onset genetic variants as it could have a psychological impact on the child and their parents and deprive the child of the right to an open future (Ross & Clayton, 2019). Our current panel included 12 adult-onset genes and 12 genes with unknown onset age which we plan to exclude in the next round of study.

Our study has several limitations. The primary disadvantage is the lack of complete follow-up data for some participants in our validation cohort. Our study indicated low concordance between NGS and conventional NBS (Table 1), which was also reported in the BabySeq project (Wojcik et al., 2021). One of the reasons for this is the incomplete conventional NBS data collected due to the varying detection indexes in different regions. Additionally, sequencing cannot fully represent phenotypes, and some participants may be gene-positive, but negative for the clinical phenotype (Hao et al., 2022). This could be due to the differences in the time of disease onset or the low

penetrance of the gene variant. Once the penetrance of a gene variant is low, the individual with this variant has a low chance to be symptomatic (Seaby & Ennis, 2020). This is also one limitation in our study. We did not include penetrance in our report interpretation category. Our report category needs modification, and we must strengthen our follow-up in the subsequent study.

Although studies have shown that NGS may play a role as second-tier screening in NBS and cannot replace MS, we believe that sequencing can be performed simultaneously with conventional NBS if the cost is reasonable. Sequencing could enable the early detection of diseases that have no biochemical markers, such as spinal muscular atrophy (SMA), Duchenne's muscular dystrophy (DMD), and cardiovascular diseases. Therefore, we proposed that the NeoEXOME panel may have the following applications in the clinical practice: (1) as a first-tier NBS for genetic diseases with no biochemical markers; (2) as an adjunct diagnostic tool for monogenic inherited diseases with obvious abnormal phenotype; (3) as a second-tier NBS in combination with conventional NBS to enhance diagnostic accuracy. The application of sequencing in healthy infants with negative conventional NBS results can help to determine the gene variants in the early stage of their lifetime to prevent mortality; (4) as an exclusion (quasi-first-line) screening for infants with low phenotypic specificity (jaundice, etc.). Further clinical trials are needed to validate the use of NeoEXOME in clinical practice.

In conclusion, we designed a promising targeted-sequencing panel for NBS in China and evaluated this approach in a multicenter pilot study. As the technology continues to evolve, NGS is expected to increasingly contribute to improving our ability to prevent and manage congenital anomalies.

## AUTHOR CONTRIBUTIONS

Conceptualization, LZ, GJY, and JW.; Investigation, ZYC, XYH, DJW, MSG, FS, RQ, RXZ, CRS, XHW, BZ, DHC, HHY, YPQ, GSS, JW, and PPW; Validation, ZYC, JXW, HYZ, ZJY, and LZ; Writing—Original Draft Preparation, ZYC and LZ; Writing—Review & Editing, ZYC and LZ; Visualization, ZYC and PPW; Supervision, LZ, and GJY; Funding acquisition, LZ, and GJY; All authors have read and agreed to the published version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

All data are available in the main text or the Supplementary Materials.

## ORCID

Lin Zou  <https://orcid.org/0000-0002-6265-6801>

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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