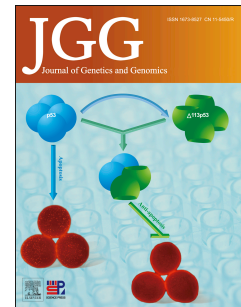


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Newborn screening with targeted sequencing: A multi-center investigation and a pilot clinical study in China

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ABSTRACT

Different newborn screening (NBS) programs have been practiced in many countries since the 1960s. It is of considerable interest whether next-generation sequencing is applicable in NBS. We have developed a panel of 465 causative genes for 596 early-onset, relatively high incidence and potentially actionable severe inherited diseases in our Newborn Screening with Targeted Sequencing (NESTS) program to screen 11,484 babies in eight Women and Children's hospitals nationwide in China retrospectively. The positive rate from preliminary screening of NESTS was 7.85% (902/11,484). With 45.89% (414/902) follow-up of preliminary positive cases, the overall clinically confirmative diagnosis rate of monogenic disorders was 12.07% (50/414), estimating an average of 0.95% ($7.85\% \times 12.07\%$) clinical diagnosis rate, suggesting that monogenic disorders account for a considerable proportion of birth defects. The disease/gene spectrum varied in different regions of China. NESTS was implemented in a hospital by screening 3,923 newborns to evaluate its clinical application. The turn-around time of a primary report, including the sequencing period less than 7 days, was within 11 days by our automatic interpretation pipeline. Our results suggest that NESTS is feasible and cost-effective as a first-tier NBS program, which will change the status of current clinical practice of NBS in China.

Keywords: Newborn screening; Next-generation sequencing; Targeted sequencing; monogenic disorders; Clinical practice

Introduction

Newborn screening (NBS) began in the 1960s to identify asymptomatic newborns affected by rare yet treatable inborn errors of metabolism (IEMs) such as phenylketonuria (PKU). It is now routinely and mandatorily performed on almost every infant in many countries. The application of tandem mass spectrometry (MS) has expanded the screening panel list to about 50 IEMs by identifying and quantitating a variety of metabolites simultaneously (Cdc, 2001; Rashed, 2001; Chace et al., 2002; Wilcken et al., 2003). The list of disorders varied from jurisdiction to jurisdiction, but mostly followed the principles for NBS programs set out by Wilson and Jungner in 1968 (Wilson and Jungner, 1968).

Next-generation sequencing (NGS) has been widely used in molecular diagnosis of inherited disorders or Mendelian traits (Yang et al., 2013; Posey et al., 2016; Retterer et al., 2016; Hu et al., 2018). More recently, the advances of sequencing technologies have raised the possibility of its application in NBS (Bailey and Gehtland, 2015; Almannai et al., 2016; Kingsmore, 2016; Luo et al., 2018). Newborn screening by NGS has great advantages in accuracy, high throughput and broad spectrum of disorders. The outputs reduce the diagnostic odyssey for ill newborns and provide opportunities for early intervention. NBS performed by NGS also identifies carrier status, which is informative for future reproductive planning for both parents and grown-up children. Most importantly, the cost of NGS is getting lower and lower with the development of new technologies.

Newborn screening by NGS has been evaluated through several programs in some aspects of technical, populational, clinical, social, economic and ethical issues (Beckmann, 2015;

Reinstein, 2015; Berg et al., 2017; Friedman et al., 2017; Ceyhan-Birsoy et al., 2019). The major challenges are: 1) scope of genomic information to interpret; 2) disease information returned to the counselees; 3) intervention of asymptomatic newborns; 4) reasonable cost and turn-around time (TAT); 5) mis-use of the newborn's genomic information. Genomic sequencing data may have potential impacts on perceived child vulnerability, parent-child bonding, and self or partner blame (Frankel et al., 2016).

In this study, we aim to meet these challenges by performing rapid targeted sequencing of curated genes, which we called Newborn Screening with Targeted Sequencing (NESTS). After curation, we selected 465 causative genes for 596 early-onset, relatively high incidence and potentially actionable severe inherited diseases in our initiative panel (version 1.0) (**Table S1**). Our gene panel includes most of the disease causative genes of current NBS programs in China, such as the national NBS program for PKU and congenital hypothyroidism (CH), glucose-6-phosphate dehydrogenase (G6PD) deficiency and thalassemia in southern China, hearing loss (HL) and congenital adrenocortical hyperplasia (CAH) in some regions, as well as MS-targeted IEMs in many provinces. The concept of “actionable” here refers to relieving symptoms to some degree, prolonging life, or avoiding long diagnostic quests once symptoms appear (Bombard et al., 2009). Notably, it is always debating on which gene or disease should be included in the screening panel. This panel should be open for revision based on the practice depending on the conditions of the country or region. Integrating the technologies of sequencing with dried-blood spot DNA (Bassaganyas et al., 2018), full-automatic library construction and automatic interpretation pipeline, we were able to obtain the primary results from our workflow within 7 days and to issue the reports within 11 days in terms of TAT. Using

NESTS, we retrospectively screened 11,484 newborns in eight regions across China, and further evaluated the use of NESTS in a single hospital in Beijing by screening 3,923 newborns as a pilot study for its clinical practice. We here provide evidence that targeted sequencing is feasible and cost-effective as the first-tier NBS program in clinical practice, especially for those rare disorders that lack methods for conventional biochemical NBS. Likewise, for those disorders which are already in NBS programs, other methods such as tandem mass spectrometry screens can be a supplement of targeted sequencing for confirmation of diagnosis.

Results

Demographics of 11,484 babies screened in eight provinces of China

For the retrospective study, 11,484 babies were recruited from eight representative provinces in China for the screening of the 465-gene panel (**Table 1 and S1**). Male to female ratio was 1:0.93. The samples from Xinjiang were less than 100, in which the preliminary positive cases were not followed up and the ranking of disease distribution was not calculated. Unfortunately, we were not able to follow up the preliminary positive cases from Guizhou due to inaccessible personal data.

Positive rates of disorders with single gene mutations

In the retrospective study of 11,484 cases, after interpretation following the ACMG/AMP

guidelines (Richards et al., 2015), 902 cases (7.85%, 902/11,484) were classified as preliminary positive from screening by our definitive criteria shown in Methods. 414 of 902 cases (45.89%) were successfully followed up and 50 of 414 cases (12.07%) were clinically diagnosed, estimating an approx. 0.95% ($7.86\% \times 12.07\%$) clinical diagnosis rate in this cohort (**Table 1 and 2**). Since this retrospective study used archived residual dried blood spots for sequencing that was performed years after birth, the follow-up rate was relatively lower compared to our pilot clinical study (see below) as some parents changed their telephone numbers and could not be reached. The preliminary positive rates ranged from 5.74% to 10.08% in different regions (**Table 1**), in which the rate in Guangxi was the highest mainly due to the high prevalence of G6PD deficiency and thalassemia (Yan et al., 2006; Xiong et al., 2010; Lai et al., 2017; Fu et al., 2018). It must be noted that we did not include the cases with large deletion/duplication or copy number variation (CNV) of the disease causative genes in this panel. The positive cases could be underestimated especially in southern regions of China where deletional *HBA* is very common (Shang et al., 2017). A comprehensive study on the large deletion/duplication of these disease genes is undergoing.

Distribution of disorders and genes

In all preliminary positive cases, HL accounted for the largest fraction of 35.25% cases (318/902), ranking as the highest monogenic disorder in this screening (**Table 3**). This may be due to the high prevalence of HL in China (0.2%–0.3%) (Dai et al., 2019) and a relative high fraction of HL genes in this panel (105 genes, **Table S1**). The following disorders ranked by

preliminary positive rates were G6PD deficiency, cataract, cardiomyopathy, and others. The spectrum of the disorders differed in provinces apparently (**Table 3**). The most frequent affected gene was *G6PD*, followed by *MYO7A*, *COL4A3* and *GJB2* nationwide (**Fig. 1**). Again, this spectrum may vary when counting the CNVs in these genes, especially the deletional *HBA* in Southern China.

A pilot study for clinical practice of NESTS

To evaluate the feasibility of NESTS in clinical practice, we performed NESTS together with the traditional NBS programs in 3,923 newborns in Beijing Shunyi Women and Children's Hospital of Beijing Children's Hospital with our workflow (**Fig. 2**). We collected the dried-blood spots at Day 3 after birth before the newborns were discharged from the hospital by obtaining the consents of their parents. We were able to obtain the preliminary results within 7 days after the dried-blood spots were received by following our workflow as described in **Figs. S1 and S2**. Formal reports were issued within 11 days in terms of TAT before the regular newborn physical examination at Day 42 after birth when the parents routinely visit our genetic counseling clinic if necessary. Subsequent phone call follow-up was done at their ages ranged from 23 to 28 months in this cohort.

The overall preliminary positive rate was 9.73% (382/3,923). This rate is higher than the retrospective study in Beijing (**Table 1**), which could be attributable to the larger number of screened newborns. Parents of 3,394 newborns accomplished the follow-up and the rate was 86.5% (3,394/3,923). 415 had an invalid phone number or did not respond to three consecutive

phone calls, 114 declined the phone interviews. As for the preliminary positive cases, seven newborns were clinical diagnosed (including mutations in *SOX10*, *MYO6*, *PTPN11*, *ANK1*, *SLC2A9*, *COL4A5* and *PAH*, supplemental information), resulting in a clinically confirmation rate of 0.22% (7/3,394). However, the number of positive cases may be underestimated because some phenotypically normal newborns who carry pathogenic mutations in late childhood-onset disease genes are likely missed the diagnosis in their early ages.

For the 38.4% (1,505/3,923) parents who visited our clinic for genetic counseling, information such as inheritance, incidence, age of onset, recurrence risk, and intervention was delivered. For newborns with positive rate from preliminary screening, detailed clinical features and family history information were collected and evaluated. Parental Sanger verification and other specific clinical examination were offered if necessary. The carrier status was informed to the parents (counselees) for the awareness of risk for the next-born baby and next-generation baby. We developed a questionnaire (**Text S2; Table S2**) for those parents with their babies undergoing NESTS who visited our clinic for genetic counseling. 222 parents (15%) were included: 93.24% understood the significance and purpose of NESTS after the pre-test counseling session; 91.44% believed that they had benefited from NESTS; 64.41% have different levels of anxiety or psychological pressure after reading the report, but the percentage decreased significantly after counseling; 87.39% were satisfied with our genetic counseling service. Moreover, 97.2% respondents felt that the NESTS program should be included in the national health insurance or governmental free screening program (**Table S2**).

During this clinical application, we could confirm the diagnosis for patients in this cohort after genetic counseling and follow-up. The confirmed diagnostic results are directive of

clinical management and may have profound effects on management outcomes, including providing genetic counseling, referring for systematic evaluation and offering appropriate measures of treatment or prevention. Seven of diagnosed cases (including cases with incidental findings) with detailed clinical and management information were described in supplemental information and **Fig. S3**.

Discussion

It has been testified that NBS is an effective measure for birth defects prevention since Dr. Guthrie initiated PKU screening for newborns in 1963 (Guthrie and Susi, 1963; Guthrie, 1969). However, due to the differences in economic, social, cultural, epidemic conditions and incidence of diseases, NBS in different countries had diverse public health outcomes. NBS in China was initially carried out in 1980s (Chen et al., 1984; Chen et al., 1986; Liu and Zuo, 1986). In recent years, hundreds of NBS centers have been established throughout China supported by central or local government and charity organizations. At present, the screened disorders are mainly focused on PKU and CH (Chen et al., 1984). G6PD deficiency and thalassemia screening tests have been carried out in southern China (Lai et al., 2017; Fu et al., 2018). Screening for CAH has been carried out in some regions. HL with an incidence of 0.2%-0.3% was screened in most areas of China (Kennedy and McCann, 2004; Gelb, 2018; Dai et al., 2019). In 2002, since the application of tandem MS for NBS in China, the clinical screening and diagnosis of over 40 IEMs have also made significant progress (**Table S3**). However, the traditional MS screening method is limited due to the relatively high false positive rates and

total costs (Tarini et al., 2006; Mak et al., 2013; Lee et al., 2019). A possible explanation for high false positive rates is the lack of appropriate interpretation of the abnormal values in the MS report. In some MS screening programs, only real values were reported without an indicative diagnosis of IEMs. Therefore, for those disorders which are already in NBS programs, we recommend NESTS as a supplement of these methods for confirmative diagnosis at genomic level. A most recent research also had similar conclusion by comparing NGS and MS results from 1,190 cases (Adhikari et al., 2020). For those inherited disorders that lack methods on conventional biochemical NBS, NESTS can be considered as a first-tier screening strategy.

With our multi-center retrospective study, we were able to briefly depict the birth defects spectrum and mutation carrier status nationwide. Our clinical confirmative diagnosis rate by NESTS was estimated as 0.95%. Considering that the birth defect rate in China was estimated as 5.6% in 2012 (Qi et al., 2017), monogenic disorders account for a relatively large proportion of birth defects. However, this estimated rate may be underestimated because some disorders that we identified in preliminary positive cases will be in late childhood onset and that CNVs in this disease gene panel were not included. NGS-based genetic screening for newborns has been recommended by panels of experts since 2012 (Goldenberg and Sharp, 2012; Tarini and Goldenberg, 2012; Landau et al., 2014). Although debates on applying NGS in NBS have been existing for many years by raising a number of issues on technical, clinical, economic, psychological, and ethical aspects (Roberts et al., 2014; Beckmann, 2015; Reinstein, 2015; Berg et al., 2017; Friedman et al., 2017; Pereira et al., 2019; VanNoy et al., 2019), advances in genome sequencing have opened the door to accessible NGS-based NBS. In this study, we

mainly focused on technical feasibility, clinical application and outputs. Our experience has provided an example for the successful application of NGS in NBS in China.

Previous studies showed some concerns on returning too much information (especially carrier information) uncovered by NGS which may increase parental anxiety (Bell et al., 2011; Ceyhan-Birsoy et al., 2019; Pereira et al., 2019; VanNoy et al., 2019). Instead of whole genome sequencing, we only sequenced selected curated genes. There is no consensus on whether to return carrier information or not (Bell et al., 2011; Friedman et al., 2017; Ceyhan-Birsoy et al., 2019; VanNoy et al., 2019). On average, each person carries 2.8 pathogenic variants in recessive disease genes (Bell et al., 2011). In our experience, reporting carrier status is informative for the follow-up, as shown in the last case (N7) we documented in **Fig. S3** and **Text S1**. By analyzing variants on sex chromosome, we also incidentally identified newborns with Klinefelter syndrome. After consulting pediatric endocrinologists, we decided to report this disorder to the parents. It is beneficial to the children's gender correction and the improvement of accompanying symptoms. Some endocrinologists even suggested that all newborns should be screened for sex chromosome abnormalities routinely.

Awareness of carrier status of newborns is indicative when these newborns will be having their own offspring. Likewise, carrier status of newborns is informative for risk evaluation to their parents and families. Furthermore, for some certain diseases (especially metabolic diseases), carrier may also manifest mild symptoms. From the survey, more than half of the respondents have different levels of anxiety or psychological pressure before counseling. 87.4% respondents were satisfied with our genetic counseling and considered helpful to understand the results. 95.2% respondents think their anxiety or psychological pressure was relieved after

counseling (**Table S2**), indicating that medical services are required for supporting NBS programs.

Admittedly, our current NBS program had some limitations. For a NBS program, short TAT and low cost are critical factors for clinical application. In our study, the primary interpretation period, including the sequencing period of less than 7 days, was within 11 days with the implementation of an automatic interpretation pipeline (**Figs. S1 and S2**). As the sequencing costs of price and time are continuously declining together with the application of artificial intelligence (AI)-assisted interpretation, we predict that the TAT and cost of NESTS will be dramatically decreased. Moreover, as a sequencing-based NBS program, the 465-gene should be open for revision based on our screening results of the numbers of the newborns carrying mutations in each gene (**Table S1**). Other genes are likely to be included to the list as more known birth defect genes call for our attention. The probes also required further optimization to detect potential CNV in each gene.

This study provided unbiased information on the technical feasibility, clinical application and public recognition on NESTS. NESTS is directive to medical intervention for cases with confirmed diagnosis. Our results reveal a relatively high birth defect rate (approx. 1%) with monogenic diseases. NESTS is to put the targeted genes or diseases into “nests” for intervention, and to “nest” different NBS programs in one program. With the increase of screened newborns in the in-house database, the gene list will be further optimized based on our clinical practice, and interpretation of pathogenic alleles will be more accurate.

Materials and methods

Detailed materials and methods are described in supplemental information.

NGS panel design

When designing the panel, the priority was given to genes with early-onset, relatively high incidence and potentially actionable severe diseases. Gene curation was performed according to ClinGen Gene-Disease Validity Standard Operating Procedure, version 7 (clinicalgenome.org/curation-activities). After curation, 465 disease genes that are related to 596 OMIM phenotypes were included in our NESTS panel. For those genes with Gene-Disease relationship classification level lower than “strong”, reasons for the inclusion were also provided (**Table S1**). Detailed description is shown in supplemental information.

Newborn recruitment

Newborns were recruited from eight Women and Children’s hospitals (see authors’ affiliations b–i) representing different regions of China with written informed consent from parents. The blood spots of 11,484 newborns were collected at Day 3 after birth and stored in cold-room after traditional PKU/CH screening. When this study was initiated, their ages at sequencing tests ranged from 2 months to 29 months. For the clinical practice study, 3923 newborns were recruited from Shunyi Women and Children’s Healthcare Hospital of Beijing Children's Hospital from October 2018 to June 2019, and the blood spots were collected at Day 3 after birth. This study was approved by the institutional review board (2017-K-39).

Variants sequencing and interpretation pipelines

Variants were sequenced and filtered according to the criteria in supplemental information. The overall workflow and quality control data are shown in **Fig. S1** and **Table S4**. The automatic interpretation pipeline is shown in **Fig. S2**. Reports with the following three types of variants were considered as preliminary positive: 1) heterozygous/hemizygous pathogenic or likely pathogenic variants detected in dominant genes; 2) homozygous/hemizygous pathogenic or likely pathogenic variants detected in recessive genes; 3) two or more variants in the same recessive gene are detected, and at least one is pathogenic or likely pathogenic. Sanger sequencing to confirm the variants and their origins from the parents were performed when necessary.

Clinical applications

After a retrospective study, clinical application was performed at Shunyi Women and Children's Healthcare Hospital of Beijing Children's Hospital, Beijing. The pregnant women were recruited from October 2018 to June 2019. They received pre-test counseling and participated in our test autonomously. Three categories of variants were referred to manual review (**Fig. S1**). The overall workflow is shown in **Fig. 2**. Genetic counseling was provided to the parents according to the reports. Phone call follow-up was done for the parents of tested newborns during February 2020 to May 2021.

CRediT authorship contribution statement

Chanjuan Hao: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Methodology, Supervision, Writing - review & editing. **Ruolan Guo:** Conceptualization, Data curation, Formal analysis, Methodology, Writing - original draft. **Xuyun Hu:** Data curation, Formal analysis, Methodology, Writing - original draft. **Zhan Qi:** Data curation, Formal analysis, Methodology. **Qi Guo:** Data curation, Formal analysis, Methodology, Software. **Xuanshi Liu:** Data curation, Formal analysis, Methodology. **Yuanhu Liu:** Project administration, Supervision. **Yanhua Sun:** Resources. **Xiaofen Zhang:** Formal analysis, Methodology. **Feng Jin:** Data curation, Methodology. **Xiujie Wu:** Resources. **Ren Cai:** Resources. **Dingyuan Zeng:** Resources. **Xijiang Hu:** Resources. **Xiaohua Wang:** Resources. **Xiaoping Ji:** Resources. **Wenjie Li:** Resources. **Quansheng Xing:** Resources. **Lanfang Mu:** Resources. **Xiulian Jiang:** Resources. **Xue Yang:** Resources. **Weimin Yang:** Resources. **Yan Zhang:** Resources. **Qianli Yin:** Resources. **Xin Ni:** Conceptualization, Funding acquisition, Project administration, Supervision. **Wei Li:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing - review & editing.

Conflict of interest

The authors declare no competing financial interest.

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Supplementary data

Supplementary data including supplementary text 1-3, figure 1-3 and table 1-4 to this article can be found online at xxx.

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Figure legends

Fig. 1. Gene distribution of preliminary positive cases.

Top 40 genes identified in preliminary positive cases and their distribution in each province, gene ranking represents the national-wide ranking. IM, Inner Mongolia; QH, Qinghai; HB, Hubei; SD, Shandong; BJ, Beijing; GZ, Guizhou; GX, Guangxi.

Fig. 2. The overall workflow of NESTS in clinical applications.

After pre-test counseling, the pregnant women participated in this study autonomously. Sequencing was performed as described above after dried blood spot specimens of newborns were collected. Traditional newborn screening was performed simultaneously. After review and re-classification, reports were issued within 11 days before the infants' routine follow-up for physical examination at Day 42 after birth. Post-test counseling was provided for further follow-up. CH, congenital hypothyroidism; CNVseq, copy number variants sequencing; HL, hearing loss; IEMs, inborn errors of metabolism; MS/MS, tandem mass spectrometry; NESTS, Newborn Screening with Targeted Sequencing; PKU, phenylketonuria.

Table 1. Newborn numbers screened and the preliminary positive rates in different regions.

Region	Newborn number (Female + Male)	Preliminary positive case (rate, A%)	Follow-up case (rate)	Clinical confirmed case (rate, B%)	Estimated clinical diagnosis rate*
Inner Mongolia	2240 (1086+1154)	166(7.41%)	124(74.70%)	1(0.81%)	0.06%
Guizhou	2194 (1045+1149)	179(8.16%)	NA	NA	NA
Guangxi	2093 (1001+1092)	211(10.08%)	109(51.66%)	43(39.45%)	3.98%
Shandong	2023 (956+1067)	144(7.12%)	80(55.56%)	0(0.00%)	0.00%
Hubei	1328 (690+638)	79(5.95%)	40(50.63%)	3(7.50%)	0.45%
Qinghai	1010 (480+530)	86(8.51%)	43(50.00%)	2(4.65%)	0.40%
Beijing	505 (235+270)	29(5.74%)	18(64.86%)	1(5.56%)	0.32%
Xinjiang	91 (37+54)	8(8.79%)	NA	NA	NA
Total	11,484 (5530+5954)	902(7.85%)	414(45.89%)	50(12.08%)	0.95%

* Estimated clinical positive rate ($A\% \times B\%$) is defined as the result of clinically confirmative diagnosis rate (B%) of followed-up preliminary positive cases $\times$ the preliminary positive rate (A%) of all screened newborns. NA, not applicable.

Table 2. Follow-up of 902 preliminary positive cases.

Category	Cases number
Cases missed follow-up	488
Cases had no obvious related phenotypes during follow-up	364
Cases were clinically confirmed as positive after follow-up	50
<i>G6PD</i> - <i>G6PD</i> deficiency	41
<i>PAH</i> -phenylketonuria	2
<i>HBA</i> - α thalassemia*	1
<i>HBA</i> - α thalassemia* + <i>G6PD</i> - <i>G6PD</i> deficiency	4
<i>GJB2</i> -hearing loss	1
<i>SLC26A4</i> -hearing loss	1

* Only newborns with *HBA* point mutation were included.

Table 3. Top five disorders in seven different provinces with screened newborns > 100 (Xinjiang not included).

	Disorder	Preliminary positive cases
Inner Mongolia	Hearing loss	72
	Cataract	13
	Cystinuria	7
	HCM	7
	Long-QT syndrome	6
Guizhou	Hearing loss	56
	G6PD deficiency	41
	Cataract	8
	Stickler syndrome	8
	HCM	7
Guangxi	G6PD deficiency	114
	Hearing loss	47
	Kallman syndrome	6
	Cataract	4
	EB	4
Shandong	Hearing loss	65
	MED	10
	Alport syndrome	8
	HCM	8
	EB	8
Hubei	Hearing loss	31
	G6PD deficiency	4
	EB	4
	Nephrotic syndrome	4
	PCD	3
Qinghai	Hearing loss	29
	Alport syndrome	6
	CVID	6
	CMT	6
	DCM	4
Beijing	Hearing loss	13

	PCD	3
	Long-QT syndrome	2
	G6PD deficiency	2
	HCM	2
Overall	Hearing loss	318
	G6PD deficiency	161
	Cataract	25
	HCM	24
	EB	16

CMT, Charcot-Marie-Tooth disease; CVID, common variable immunodeficiency; DCM, dilated cardiomyopathy; EB, epidermolysis bullosa; G6PD, glucose-6-phosphate dehydrogenase; HCM, hypertrophic cardiomyopathy; MED, multiple epiphyseal dysplasia; PCD, primary ciliary dyskinesia.

