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ORIGINAL ARTICLE

Whole exome sequencing is an alternative method in the diagnosis of mitochondrial DNA diseases

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Abstract

Background: Mitochondrial disease (MD) is genetically a heterogeneous group of disorders with impairment in respiratory chain complexes or pathways associated with the mitochondrial function. Nowadays, it is still a challenge for the genetic screening of MD due to heteroplasmy of mitochondrial genome and the complex model of inheritance. This study was designed to investigate the feasibility of whole exome sequencing (WES)-based testing as an alternative option for the diagnosis of MD.

Methods: A Chinese Han cohort of 48 patients with suspect MD features was tested using nanoWES, which was a self-designed WES technique that covered the complete mtDNA genome and 21,019 nuclear genes. Fourteen patients were identified with a single genetic variant and three with single deletion in mtDNA.

Results: The heteroplasmy levels of variants in mitochondrial genome range from 11% to 100%. NanoWES failed to identify multiple deletions in mtDNA compared with long range PCR and massively parallel sequencing (LR-PCR/MPS). However, our testing showed obvious advantages in identifying variations in nuclear DNA. Based on nanoWES, we identified two patients with nuclear DNA variation. One of them showed Xp22.33-q28 duplication, which indicated a possibility of Klinefelter syndrome.

Conclusion: NanoWES yielded a diagnostic rate of 35.4% for MD. With the rapid advances of next generation sequencing technique and decrease in cost, we recommend the usage of nanoWES as a first-line method in clinical diagnosis.

KEYWORDS

genetic diagnosis, mitochondrial disease, next generation sequencing, whole exome sequencing

Chong Sun and Shengyang Wu contributed equally to this work.

1 | INTRODUCTION

Mitochondria are one of the major ancient endomembrane systems in eukaryotic cells containing their own genetic system (Couser & Gucsavas-Calikoglu, 2017). Human mitochondrial DNA (mtDNA) is a circular molecule of 16.5 kb, encoding 13 polypeptides, 22 mitochondrial transfer RNAs (mt-tRNAs), and 2 ribosomal RNAs (12S and 16S ribosomal RNAs) (Couser & Gucsavas-Calikoglu, 2017; McCormick et al., 2018; Riley et al., 2020). The structure and function of mitochondrion are under dual genetic control. The majority of mitochondrial proteins are encoded by nuclear DNA (nDNA), with merely 13 proteins in oxidative phosphorylation (OXPHOS) pathways encoded by mtDNA (Cooper & Davies, 2000; McCormick et al., 2018). Most human nucleated cells have 500 ~ 2000 mitochondria, and there are multiple copies of mtDNA in each mitochondrion (Couser & Gucsavas-Calikoglu, 2017). In contrast to nDNA, mtDNA is maternally inherited. In addition, the heteroplasmy of mitochondrial genomes with a specific variant may differ greatly between tissues. Heteroplasmy is defined as presence of two or more types of mtDNA in an individual, which is caused by somatic mutation and maternal cytoplasmic inheritance. This characteristic led to presence of heteroplasmy in the majority of causative mtDNA variants. Mitochondrial diseases (MDs) have revealed dramatic variability in the phenotype even when patients harbor the same variant. The dosage of mutated variants affected the type and severity of symptoms in MD patients. A theory called threshold effect (Gorman et al., 2016) was previously affirmed that even a causative variant would not trigger the MD disease unless the proportion of mutated mtDNA co-existing with wild type mtDNA reaching a certain threshold. In addition, an increased proportion would alternate the phenotype (Rossignol et al., 2003). These features lead to the complexity of clinical presentations in MD patients (Koopman et al., 2012).

MD can occur at any age with an estimated prevalence of 1:5000 (Falk & Sondheimer, 2010; Kerr et al., 2020; Skladal et al., 2003), typically those with hypotonia, cardiomyopathy, lactic acidosis, hearing loss, isolated vision loss, and seizure (Chinnery & Hudson, 2013). Primary MD results from impairment in respiratory chain complexes. Besides, mitochondrial dysfunction could be secondary to other genetic syndromes (Niyazov et al., 2016; Valenti et al., 2014). Therefore, it is still a challenge to confirm the MD due to genotypic heteroplasmy of pathogenic mtDNA variations, and its broad clinical phenotypes.

Nowadays, extensive methods have been utilized in the diagnosis of MD, including magnetic resonance imaging (MRI), biopsy, and biochemical tissue analysis. Genetic screening is only performed for the selection of gene mutations (Wortmann et al., 2015). To date, several high-throughput methods have been used for the genetic screening of MD. For instance, long-range PCR and massively parallel sequencing (LR-PCR/MPS) has been validated as a valuable method for accurate quantification of nucleotide heteroplasmy. Besides, it is also sensitive to detect heteroplasmy as low as 1.5% at every single-nucleotide position of the entire mitochondrial genome (Cui et al., 2013). However, it is still a difficult task to detect the variation in ~1300 nuclear genes involved in the pathogenesis of MD (Alston et al., 2017). Next generation sequencing (NGS), especially the extensive utility of whole exome sequencing (WES), is widespread in screening nDNA variations. Several studies have focused on the usage of panel-based ES in screening mtDNA and nDNA variants in specific diseases (Abicht et al., 2018; Levy et al., 2021; Puusepp et al., 2018; Schoonen et al., 2019). Nevertheless, rare attention has been paid to the WES in genetic diagnosis of complex diseases such as MD. In a previous study, Griffin et al. (2014) focused on the efficiency of three "off-the-shelf" exome capture kits in the identification of pathogenic point mutations in MD patients, compared with the Sanger sequencing. This study expanded our vision that exome sequence held its utility in a diagnostic setting to screen both protein-coding nuclear genes and mtDNA. Quality control, ethnic origin, and maternal ancestry were also able to acquire when performing ES rather than conventional Sanger sequencing (Griffin et al., 2014). These results indicated that with a basic minimum base coverage, whole-exome capture would reliably detect the mtDNA variations effectively. However, nuclear mitochondrial DNA sequences (NUMTs) are reported to trigger mis-mapping causing false positive and negative in NGS test covering both genomic and mitochondrial genes. In a recent study, Singh et al. (2021) focused on the classifier algorithm of alignments of NUMTs and authentic mtDNA. MitoScape algorithm, based on random forest systematically, contributed to solve the misalignment caused by NUMTs and decreased false positives to zero (Singh et al., 2021). The two-step NGS approach was a considerable strategy to detect MD pathogenesis. The strategy was based on sequential combined single long-range PCR on mtDNA and homozygosity mapping and WES on nuclear genes. MtDNA analysis yield a 20% diagnostic rate, while a subsequent WES yield an additional 49% diagnostic rate in nuclear genes. This strategy was a comprehensive and unbiased approach for the genetic diagnosis among MD patients (Theunissen et al., 2018). However, clinical evaluation of a parallel sequencing which simultaneous detects mtDNA and nDNA in the clinical setting to diagnosis of MD is still not elucidated.

This retrospective study was conducted to investigate the efficiency of a self-designed exome capture kit

(designated as nanoWES) covering the complete mtDNA genome and 21,019 nuclear genes based on a Chinese cohort of 48 patients with suspect MD features.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

The protocols of this study were approved by the Ethics Committee of Huashan Hospital, Fudan University. Written informed consent was obtained from each subject.

2.2 | Patients

The retrospective study enrolled 48 patients with suspected MD from 2018 to 2019 who underwent genetic tests in the Department of Neurology, Huashan Hospital. Patients who were positive for nuclear gene variants in the previous tests were excluded from this study. All patients were Han Chinese, and the demographic features of these cases were provided in Table S1.

2.3 | Sample preparation

Sample DNA was extracted from whole peripheral blood or skeletal muscle tissues as previously described (van der Walt et al., 2012). Target-enriched capture was performed with nanoWES kit v1.0 (Berry Genomics, Beijing, China). The nanoWES probe could cover 56.7 Mb DNA, including whole exons and exon-intron boundaries of 21,019 nuclear genes. The probe also covers 16.5 Kb mtDNA which is the complete mitochondrial genome. The nanoWES probes covered 93.0% genes in OMIM database and 98.6% genes in HGMD database. In brief, DNA was first fragmented to ~200 bp. Then, repair end, 3′-dA overhang and adaptor ligation were performed on the fragmented DNA. Purified ligation product was then hybrid with nanoWES probe to enrich target genes without PCR amplification. Exome library was then obtained by removal of un-ligated adaptor, PCR amplification and purification. Exome library was quantified by Real-Time RCR, followed by sequencing on Illumina Novaseq6000 (Illumina, CA, USA).

2.4 | Data analysis

Workflow of bioinformatical analysis was shown in Figure S1. Briefly, the sequencing reads were mapped to human reference genome (GRCh38) using BWA software (Burrows-Wheeler Aligner) (Li & Durbin, 2010). PCR duplicates were removed by using Picard v2.10.7 (http://picard.sourceforge.net/). Samtools, sambamba, bedtools, GATK, and in-house software VeritaTrekker® (Berry Genomics, Beijing, China) were used in further data processing and variants calling (Li et al., 2009; Quinlan & Hall, 2010; Tarasov et al., 2015). Mutserver was employed for mitochondrial variants calling (Weissensteiner et al., 2016). Lumpy tools were used for CNV identification in mtDNA (Layer et al., 2014). Variant annotation and interpretation in nuclear genome were conducted by ANNOVAR (Wang et al., 2010) and Enliven® in-house software (Berry Genomics, Beijing, China). MITOMAP and ClinVar databases were used in variant annotation and interpretation in mtDNA (Brandon et al., 2005; Landrum et al., 2014). Single nucleotide variant (SNV) was confirmed by Sanger sequencing, and was classified according to the American College of Medical Genetics and Genomics guidelines (Abou Tayoun et al., 2018; Biesecker et al., 2018; Ghosh et al., 2018; McCormick et al., 2020; Richards et al., 2015).

3 | RESULTS

In total, 48 patients (male: 27; female: 21; age: 5–76 yrs) with suspected clinical MD symptoms were enrolled in this retrospective study. Patients positive for nuclear gene variants previously or confirmed with pathogenic nuclear gene variants were excluded. Eight patients showed muscle weakness (Pt. 1/9/13/19/25/27/28/37). Eleven patients showed obvious ptosis (Pt. 1/3/6/10/11/29/34/43/47/48/52). Three patients presented external ophthalmoplegia (Pt. 21/47/52). Four patients (Pt. 9/12/31/47) showed hearing impairment. Ten patients showed visual impairment or blurred vision (Pt. 5/9/14/15/18/24/26/30/44/46). Five patients showed memory impairment (Pt. 2/12/17/44/49). Epilepsy was diagnosed in seven patients (Pt. 23/31/33/37/39/41/51). Two patients exhibited respiratory failure (Pt. 27/32).

Twelve patients (Pt. 1/6/13/16/19/20/21/25/26/32/37/50) underwent electromyogram and showed myogenic damages. Three cases (Pt. 6/13/50) received determination of cytochrome oxidase (COX) analysis and the results were normal. Two patients (Pt. 12/39) showed brain atrophy after MRI. Seven cases (Pt. 6/13/26/27/28/37/50) underwent Gomori trichrome staining. All of them showed characteristic ragged-red fibers under the microscope, which implied the accumulation of abnormal mitochondria in muscle fibers (Table S1).

All the 48 patients received WES analysis. The workflow of analysis was shown in Figure S1. On average, the nuclear genome was covered to 90× depth, and mtDNA was covered to ~5000× depth. The boxplot of depth of

nuclear genes and the nuclear encoded MitoCarta genes was shown in Figure S2. A considerable data quality of both nuclear and mitochondrial genes was revealed. Disease-causing gene variants in mtDNA and nDNA were confirmed by a multidisciplinary team, and the outcome of analysis was listed in Tables 1–3. Ten patients were identified to present with causative mitochondrial variants in mtDNA (Table 1). The heteroplasmy of these variants was in a range of 11% to 100%. SNV m.3243A > G was the most common mutation in *MT-TL1* with a mean heteroplasmy of 14.3% (11.1%–89%), which encoded a mitochondrial tRNA leucine. CNVs in mtDNA were inspected by Lumpy, and then were manually reviewed by Integrative Genomics Viewer (IGV) (Robinson et al., 2017). Single deletion was identified in four patients (Pt. 1/6/16/52), and multi-deletion was detected in one case (Pt. 3), with the heteroplasmy in a range of 13% to 81% (Table 2 and Figure S3). We also adopted *mity* tool, a sensitive analysis pipeline in identifying low-level heteroplasmic mitochondrial SNV and INDEL (Puttick et al., 2019), which showed that no further variants were identified. We then further compared the heteroplasmy detected using our test with that of LR-PCR/MPS, the mean and standard deviation of the difference was $0.183 \pm 1.654\%$, which revealed a qualified performance from our test on heteroplasmy.

Among those patients harboring no causative variants or CNVs on mtDNA, variants and CNVs on nDNA were identified and reviewed. Two patients (Pt. 23/46) were identified with causative variation in nDNA. For the clinical symptoms, one (Pt. 23) showed recurrent epileptic seizure, migraine headache, and nausea. Genetic screening indicated that the patient was identified with duplication in Xp22.33-q28 and a nonsynonymous SNV NM_000742.4: c.1397T > A:p.M466K on *CHRNA2* (MIM No.: 118502). The other case (Pt. 46) showed papilledema in right eye, visual loss for 5 months, and optic atrophy. Genetic screening indicated 240 bp deletion of *OPN1LW* gene (MIM No.: 300822) on chromosome 8 and nonsynonymous SNV NM_001077182.3: c.548G > C:p.R183P in *FSCN2* (MIM No.: 607643) on chromosome 17. According to the previous description (Liu et al., 2020), these two CNVs were confirmed as disease-causing, and the two SNVs were classified as variants of unknown significance (VUS) using REDBot according to ACMG guideline.

LR-PCR/MPS-based approach was also used to confirm the variants detected by nanoWES in mtDNA (Cui et al., 2013). Similar results were obtained in SNV identification (Tables 1–3). Besides the four cases with single deletion in mtDNA mentioned above, two cases (Pt. 21/47) were identified with single deletion and three cases (Pt. 28/36/50) with multi-deletions by LR-PCR/MPS (Table S2). However, we carefully inspected the corresponding nanoWES bam files in IGV, and no noticeable change was found in our data (data not shown).

In total, ten mitochondrial SNV, five mitochondrial deletions, and two nuclear variants were identified by nanoWES in the cohort. The diagnosis rate was 35.4% (Tables 1–3).

4 | DISCUSSION

In this study, a Chinese cohort with suspect MD was genetically detected by nanoWES, which resulted in a diagnosis rate of 35.4%. Based on the broad coverage spectrum of nanoWES probes covering complete mtDNA genome and more than 20,000 nDNA genes, we identified 15 patients with mtDNA variations and two patients with nDNA variations. LR-PCR/MPS was also adopted to verify the variations in mtDNA. Compared with LR-PCR/MPS, nanoWES yielded almost equivalent efficiency in detecting SNV in mtDNA, but its efficiency in detecting CNV was lower compared with LR-PCR/MPS (Tables 1 and 2).

The clinical phenotypes of MD are rather complicated. In a previous study, the diagnostic rate of cohorts yielded by WES ranged from 35% to 59% (Puusepp et al., 2018; Riley et al., 2020). Our nanoWES yielded a diagnostic rate of 35.4% for the screening of MD. The diagnostic yield was considered to be associated with the following aspects: MD scoring systems were adopted to make a solid clinical diagnosis. Up to now, several scoring systems have been proposed to evaluate the probability of MD, which take the clinical features, brain imaging, and pathomorphological results into consideration (Morava et al., 2006; Nissenkorn et al., 1999; Walker et al., 1996). For instance, based on an MD severity score method designated as Morava et al. (2006), Riley et al. (2020) reported a diagnostic rate of 67% for MD. It has been reported that trio analysis on other family members based on WES would increase the diagnostic rate. For example, Strande and Berg (2016) showed that trio sequencing could increase the diagnostic rate by 8%–17% in WES analysis. Furthermore, we used the samples derived from peripheral blood and skeletal muscle tissues, as sample type was also crucial in genetic diagnosis. Indeed, genetic diagnosis merely based on peripheral blood is not sufficient to identify the tissue specific variants.

Other genetic syndromes (e.g., neuromuscular metabolic disorders) show phenotypes of secondary mitochondrial dysfunction, which may present overlapped phenotypes with MD (Niyazov et al., 2016). This may lead to misdiagnosis of genetic syndrome into MD. Panel-based ES for MD has apparent shortcomings in such application scenarios and may ignore variants causing primary

TABLE 1 SNV of MD variants identified in mtDNA by WES and LR-PCR/MPS

Pt no.	NanoWES	Comparative analysis (LR-PCR/MPS)	Gene	Signs/symptoms	Associated phenotype/disease (Mitomap)
9	m.3243A > G, 25%	m.3243A > G, 24.5%	*MT-TL1*	Visual impairment; hearing impairment; muscle weakness	MELAS; Leigh Syndrome; DMDF; MIDD; SNHL; CPEO; MM; FSGS; ASD; cardiac + multi-organ dysfunction
13	m.11778G > A, 34%	Multiple deletion	*MT-ND4*	Muscle weakness; increased endomysial connective tissue	LHON; progressive dystonia
15	m.11778G > A, 100%	m.11778G > A, 99.7%	*MT-ND4*	Blurred vision	LHON; progressive dystonia
26	m.3243A > G, 89%	m.3243A > G, 86.9%	*MT-TL1*	Blurred vision	MELAS; Leigh Syndrome; DMDF; MIDD; SNHL; CPEO; MM; FSGS; ASD; cardiac + multi-organ dysfunction
27	m.3243A > G, 83%	m.3243A > G, 86.8%	*MT-TL1*	Muscle weakness; generalized edema; respiratory failure; pleural effusion; pneumonia; atelectasis	MELAS; Leigh Syndrome; DMDF; MIDD; SNHL; CPEO; MM; FSGS; ASD; cardiac + multi-organ dysfunction
30	m.11778G > A, 100%	m.11778G > A, 99.7%	*MT-ND4*	Optic neuritis; blurred vision	LHON; progressive dystonia
31	m.3243A > G, 23%	m.3243A > G, 24.6%	*MT-TL1*	Stroke; diabetes mellitus; seizure; hearing impairment	MELAS; Leigh Syndrome; DMDF; MIDD; SNHL; CPEO; MM; FSGS; ASD; cardiac + multi-organ dysfunction
32	m.8344A > G, 96%,	m.8344A > G, 96.9%	*MT-TK*	Neuromyelitis optica spectrum disorder; pneumonia; respiratory failure; hypertension; preexcitation syndrome	MERRF; depressive mood disorder; leukoencephalopathy
33	m.3243A > G, 33%	m.3243A > G, 31.4%	*MT-TL1*	Seizure; headache; nausea	MELAS; Leigh Syndrome; DMDF; MIDD; SNHL; CPEO; MM; FSGS; ASD; cardiac + multi-organ dysfunction
40	m.3243A > G, 11%	m.3243A > G, 11.1%	*MT-TL1*	Syncope; incomprehensible speech	MELAS; Leigh Syndrome; DMDF; MIDD; SNHL; CPEO; MM; FSGS; ASD; cardiac + multi-organ dysfunction

Abbreviations: ASD, autism spectrum disorder; CPEO, chronic progressive external ophthalmoplegia; DMDF, maternally inherited diabetes with or without deafness; FSGS, focal segmental glomerulosclerosis; ID, intellectual disability; LHON, leber's hereditary opticneuropathy; MELAS, mitochondrial encephalomyopadromethy lactic acidosis, and stroke-like episodes syndrome; MERRF, myoclonus epilepsy and ragged red fibers syndrome; MIDD, maternally inherited diabetes and deafness; MM, mitochondrial myopathy; MS, multiple sclerosis; Pt No., patient number; SNHL, sensorineural hearing loss.

TABLE 2 Single deletion of MD variants identified in mtDNA by nanoWES and LR-PCR/MPS

Pt No.	NanoWES	Comparative analysis (LR-PCR/MPS)	Gene	Signs/symptoms	Associated phenotype/disease
1	m.8483-13459 del, 72%	~m.8502-13402 del	*MT-ATP6; MT-ATP8; MT-COX3; MT-ND3; MT-ND4; MT-ND4L; MT-ND5*	Ptosis; Muscle weakness	Kearns-Sayre syndrome
3	m.4351-13922 del; m.8481-13446 del, 18%	Multi-deletion	*MT-TQ;MT-TM;MT-ND2;MT-TW;MT-TA;MT-TN;MT-TC;MT-TY;MT-CO1;MT-TS1;MT-TD;MT-CO2;MT-TK;MT-ATP8;MT-ATP6;MT-CO3;MT-TG;MT-ND3;MT-TR;MT-ND4L;MT-ND4;MT-TH;MT-TS2;MT-TL2;MT-ND5*	Vertigo; Abnormality of eye movement; Ptosis; Decreased body weight	–
6	m.10050-14598 del, 81%	~m.10100-14550 del	*MT-ND3; MT-ND4; MT-ND4L; MT-ND5; MT-ND6*	Ptosis	–
16	m.10847-14359 del, 45%	~m.10877- 14327 del	*MT-ND4; MT-ND5; MT-ND6*	Headache	–
52	m.8475-13451 del, 13%	~m.8052-13402 del	*MT-ATP6; MT-ATP8; MT-COX3; MT-ND3; MT-ND4; MT-ND4L; MT-ND5*	Ptosis; Easy fatigability; External ophthalmoplegia	–

Abbreviation: Pt No., patient number.

TABLE 3 Nuclear DNA variants identified by NanoWES

Pt No.	Variants	Gene	Signs/symptoms	Associated phenotype/disease
23	Xp22.33-q28 duplication	–	Seizure; diabetes mellitus; stroke; lactic acidosis	Intellectual disability; Dysmorphic facial features (PMID: 26997944)
	NM_000742.4: c.1397T > A:p.M466K	*CHRNA2*		Epilepsy, nocturnal frontal lobe, type 4 (OMIM: 118502)
46	chrX:154156293-154156533_del	*OPN1LW*	Papilledema and visual impairment	Blue Cone Monochromacy and Colorblindness, Partial, Protan Series (OMIM: 300822)
	NM_001077182.3: c.548G > C:p.R183P	*FSCN2*		Retinitis pigmentosa 30 (OMIM: 607643)

Abbreviation: Pt No., patient number.

genetic disorders of the complex phenotype. Thus, WES product with a wide coverage spectrum on both mtDNA and nDNA would greatly benefit genetic diagnosis. In this study, we identified two patients with nDNA variants (Table 3). Moreover, one patient (Pt. 23) showed duplication in Xp22.33-q28 (156.04 Mb) (pathogenetic variants according to ACMG guideline), which implied that the patient may suffer from Klinefelter syndrome (El-Hattab et al., 1993). Thus, genetic diagnosis of either MD or other complex diseases would greatly benefit from wide usage of WES. A previous study attempted to integrate traditional WES probe from Agilent and unique mtDNA probe (Falk & Sondheimer, 2010). The coverage and stability generated in our study were similar with them. However, we aimed at more MitoCarta genes (>12 genes) in previous published work and designed probe to detect boundaries of exons and introns and flank regions, which would provide more information to interpretation and diagnosis (Falk et al., 2012).

Despite a genetic diagnosis rate of 35.4% in this study, 31 cases remain unresolved by WES. Five patients were detected with single or multi-deletions in mtDNA by LR-PCR/MPS. However, no obvious changes were found by manually inspecting corresponding bam files in IGV (data not shown). The possible reason is that LR-PCR/MPS can enrich the entire mitochondrial genome by single-amplicon long range PCR, which greatly improves the detection sensitivity and accuracy of multiple mtDNA deletions with unequivocally mapped break points. No causal variants were detected in the other 26 patients by both nanoWES and LR-PCR/MPS. One possible explanation is protein-coding variants, covered by whole exome region, could only account for around 85% of Mendelian disorders (Kaname et al., 2014; Majewski et al., 2011). Due to the limitation of whole exome sequencing method, other type of variants, like splicing form of genes, structural variants, and variants in intergenic regions, cannot be identified. Other high-throughput methods could be adopted as complementation of WES in further, such as whole genome sequencing, RNA-seq, and third-generation sequencing techniques. Kremer et al. (2017) reported the successful usage of RNA-seq in identifying causal variants in *TIMMDC1* gene. Whole genome sequencing (WGS) would be another strategy for MD diseases. However, there are additional turnaround time, cost and the burden of genetic counseling resulting from extra unknown significant variants when WGS performing on MD diseases. On this basis, a technical improvement of WGS is urgent in MD disease.

Thus, with the decrease in cost and rapid advance of NGS technique, WES is suitable as the first-tier method in clinical diagnostic applications. In addition, WES with broader coverage in mtDNA genome and nuclear genes would greatly facilitate genetic diagnosis.

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CONFLICT OF INTEREST

All authors except Chong Sun are employees of Berry Genomics. None of the authors hold stocks or bonds. Chong Sun declares no conflict of interest.

AUTHOR CONTRIBUTIONS

Yuezhen Li designed and supervised the project. Shengyang Wu, Ruiguo Chen, Chong Sun, and Yuezhen Li wrote and revised the manuscript. Chong Sun provided clinical data. Junwu Liu, Jiasen Wang, Yanyun Ma, and Zhulin Yuan performed WES data analysis and variants interpretation. All authors read and approved the final manuscript.

CONSENT FOR PUBLICATION

Written informed consent was obtained from the participants for publication of this article and any accompanying tables/images.

ETHICAL APPROVAL

The protocols of this study were approved by the Ethics committee of Huashan Hospital, Fudan University (Shanghai, China).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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