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ORIGINAL ARTICLE

Novel and recurrent nuclear gene variations in a cohort of Chinese progressive external ophthalmoplegia patients with multiple mtDNA deletions

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Abstract

Objectives: This study aimed to investigate the clinical and genetic spectrum in Chinese patients with multiple mtDNA deletions presenting with autosomal-inherited mitochondrial progressive external ophthalmoplegia (PEO).

Methods: Long-range polymerase chain reaction and massively parallel sequencing of the mitochondrial genome were performed to detect deletions in muscle mtDNA of 274 unrelated families. Then, targeted next generation sequencing was used to detect nuclear gene variations in patients with multiple mtDNA deletions.

Results: A total of 40 Chinese PEO patients (10 males and 30 females) from 20 families were found to have multiple mtDNA deletions in this study, and the median age at onset was 35 (1–70) years. PEO and positive family history were the two prominent features of these patients, and ataxia, neuropathy, and hypogonadism were also present as onset symptoms in some patients. Fifteen of 20 probands with multiple mtDNA deletions were identified to carry nuclear gene variants; eight (40.0%) probands had variants within *POLG*, two (10.0%) within *TWNK*, two (10.0%) within *RRM2B*, two (10.0%) within *TK2*, and one (5.0%) within *POLG2*. A total of 24 variants were found in these five nuclear genes, of which 19 were novel. The causal nuclear genetic factors in five pedigrees remain undetermined.

Conclusions: The *POLG* gene is the most common disease-causing gene in this group of PEO patients with multiple mtDNA deletions. While inherited PEO is the most prominent symptoms in these patients, genotypic and phenotypic heterogeneity still exist, for example in onset age, initial symptoms, and accompanying manifestations.

KEYWORDS

progressive external ophthalmoplegia, mtDNA maintenance defect, multiple mtDNA deletion, *POLG*, *POLG2*, *RRM2B*, *TK2*, *TWNK*

Yue Hou and Xutong Zhao contributed equally as co-first authors.

1 | INTRODUCTION

Progressive external ophthalmoplegia (PEO) is a clinically diverse mitochondrial disorder that is characterized by ptosis or weakness of extraocular muscles and often co-occurs with multisystem involvement. The underlying genetic defect can be in either a mitochondrial or nuclear gene, thus explaining the variable inheritance pattern of PEO, which can exhibit maternal, Mendelian, and sporadic inheritance (Viscomi & Zeviani, 2017). Mitochondrial defects are associated with either primary mtDNA mutations (i.e., point mutations or single large-scale rearrangements) or secondary mutations in nuclear genes responsible for maintaining the integrity and stability of mtDNA, (i.e., multiple deletions or depletion of mtDNA) (El-Hattab et al., 2017). Autosomal-inherited PEO is a typical manifestation of multiple mtDNA deletions, and the genes most commonly responsible for the accumulation of these deletions include those that encode for the catalytic subunit of mitochondrial DNA polymerase γ (*POLG*), the accessory subunit of mitochondrial DNA polymerase γ (*POLG2*), mitochondrial DNA helicase (*TWNK* or *C10orf2*), thymidine kinase 2 (*TK2*), and ribonucleotide reductase regulatory TP53-inducible subunit M2B (*RRM2B*) (Viscomi & Zeviani, 2017).

To date there have been many reports about the association of autosomal dominant or recessive PEO with nuclear gene mutations (Zhu et al., 2017). To the best of our knowledge, however, screening of Chinese patients has not been done. Here, we performed comprehensive mutation analysis of mtDNA and nuclear DNA and examined the genotype–phenotype correlation in a large cohort of Chinese patients presenting with PEO.

2 | PATIENTS AND METHODS

2.1 | Patients/subjects

In the retrospective study, data from a total of 274 probands with a diagnosis of mitochondrial PEO were collected from the Department of Neurology at Peking University First Hospital between January 1999 and December 2020. Clinical follow-up of some patients was conducted face-to-face and by telephone to assess the new clinical manifestations. All patients manifested with ptosis or external ophthalmoplegia, associated with some degree of multiple system involvement, and were diagnosed by myopathological examination and gene testing. Twenty relatives from eight unrelated families presented with autosomal-inherited PEO were also included in our study, which comprised 294 patients in total.

2.2 | Pathological analysis

All 274 probands received muscle biopsies. The muscle specimens were frozen in isopentane, cooled in liquid nitrogen, and then stored at −80°C. Routine histological and histochemical staining, using techniques involving hematoxylin and eosin (H&E), modified Gomori trichrome (MGT), Succinate dehydrogenase (SDH) staining, cytochrome oxidase (COX), and cytochrome oxidase/succinate dehydrogenase (COX/SDH) staining.

2.3 | Molecular genetic analyses

Total genomic DNA was extracted/isolated from skeletal muscle biopsies and whole blood samples of the probands and all available family members, using standard procedures (Leng et al., 2015). A long-range polymerase chain reaction (LR-PCR) assay or massively parallel sequencing were used to detect mtDNA deletions in skeletal muscle DNA using a protocol described previously (Grady et al., 2014). Then, for patients with multiple mtDNA deletions or with clear family histories, targeted next generation sequencing (NGS) was used to detect nuclear gene variations. A neuromuscular disease panel (Agilent, USA) was designed to capture a 1.77-Mb region containing 6242 exons (including the 10 bp flanking on either side) of 509 nuclear genes known to be associated with common inherited mitochondrial diseases (Table S2). Samples with index tags were pair-end sequenced simultaneously on an Illumina HiSeq 2500 Sequencer (Illumina, San Diego, CA) for 150 cycles. FASTQ sequencing files were aligned with the human genome assembly GRCh38 using Burrows-Wheeler Aligner (Li & Durbin, 2009), processed using Picard, and jointly called with 2000 control samples from an in-house Chinese cohort using the GATK 3.0 Haplotype Caller. An average read depth of more than 200-fold coverage was obtained for more than 98% of the targeted regions. Sanger sequencing was performed using specific primers to confirm the variants detected by NGS.

Each novel sequence variant of a nuclear gene was classified according to the rules specified in the 2019 American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG-AMP) guidelines (Brandt et al., 2020). The genetic alleles were annotated in reference to NM_002693.2 (*POLG*), NM_007215.3 (*POLG2*), NM_021830.4 (*TWNK*), NM_004614.4 (*TK2*), and NM_015713.4 (*RRM2B*) (Brandt et al., 2020).

When assessing the frequencies of variants in large populations, the Genome Aggregation Database (gnomAD), NHLBI Exome Sequencing Project (ESP6500), 1000 Genomes Project (TGP), and Exome Aggregation Consortium (ExAC) Cohort, 100 healthy control subjects

of Chinese origin (100HC) were used as reference population data (Xie et al., 2019). Amino acid conservation across species was analyzed using the UCSC (University of California, Santa Cruz) genome browser. In silico analyses using Mutation Taster, PolyPhen-2, and SIFT were performed to predict the potential functional impacts of variants. Pedigree analysis was performed for the available family members of eight patients.

3 | RESULTS

3.1 | Myopathological changes in PEO patients

We carefully studied the pathological changes of biopsied muscle from PEO patients. HE staining showed basophilic changes in a few muscle fibers (Figure 1a, arrow); MGT staining showed ragged red fibers (Figure 1b, arrow); SDH staining showed ragged blue fibers (Figure 1c, arrow); SDH/COX staining showed more COX-negative muscle fibers (Figure 1d).

3.2 | Genetic results

LR-PCR revealed multiple mtDNA deletions in 7.3% (20/274) of the pedigrees; 75.0% (15/20) of probands were identified to have possible pathogenic nuclear mutations, including in *POLG* (8, 40.0%), *POLG2* (1, 5.0%), *TWNK* (2, 10%), *RRM2B* (2, 10%), and *TK2* (2, 10%). No clear nuclear mutations were found for the other five probands (Figure 2). A total of 24 variants were identified, of which five were previously reported as pathogenic and 19 were novel. Ten patients were found to harbor biallelic mutations in *POLG* ($n = 6$), *TK2* ($n = 2$), or *RRM2B* ($n = 2$), and five patients were found to have one mutant allele of *POLG* ($n = 2$), *POLG2* ($n = 1$), or *TWNK* ($n = 2$). All these novel candidate variants were absent from controls and population databases.

3.2.1 | *POLG* gene

In the eight patients with *POLG* mutations, 13 variants were identified, namely 12 missense mutations and one frameshift mutation. In addition to c.1880G>A/p.R627Q, c.2591A>G/p.N864S, c.2864A>G/p.Y955C, and c.3287G>A/p.R1096H, which have been previously reported as pathogenic mutations (Horvath et al., 2006; Luoma et al., 2005; Siibak et al., 2017; Van Goethem et al., 2003), nine novel mutations were found, namely one frameshift mutation (c.3002delG/p.G1001fs) and eight missense mutations (c.668G>C/p.W223S, c.703T>C/p.W235R, c.914G>A/p.S305N, c.923A>G/p.Q308R, c.924G>T/p.Q308H, c.1790G>A/p.R597Q, c.1832C>T/p.P611L, and c.2245T>G/p.F749V).

Multiple in silico analysis models predict that the variants c.668G>C/p.W223S, c.923A>G/p.Q308R, c.924G>T/p.Q308H, c.1832C>T/p.P611L, and c.2245T>G/p.F749V are probably damaging to the protein structure or function (*probable deleterious effects on POLG structure, function, or protein–protein interaction*). Algorithms did not agree on the potential impact of the missense changes c.1790G>A/p.R597Q, c.914G>A/p.S305N, and c.703T>C/p.W235R. All of these novel mutations were absent in large population cohorts and occurred at positions that are conserved across species (Table 1, Tables S1 and S3).

The frameshift mutation c.3002delG causes a frameshift starting with the codon Glycine 1001, which is predicted to cause loss of normal protein function (*disrupts gene function or product by shifting the way the sequence is*

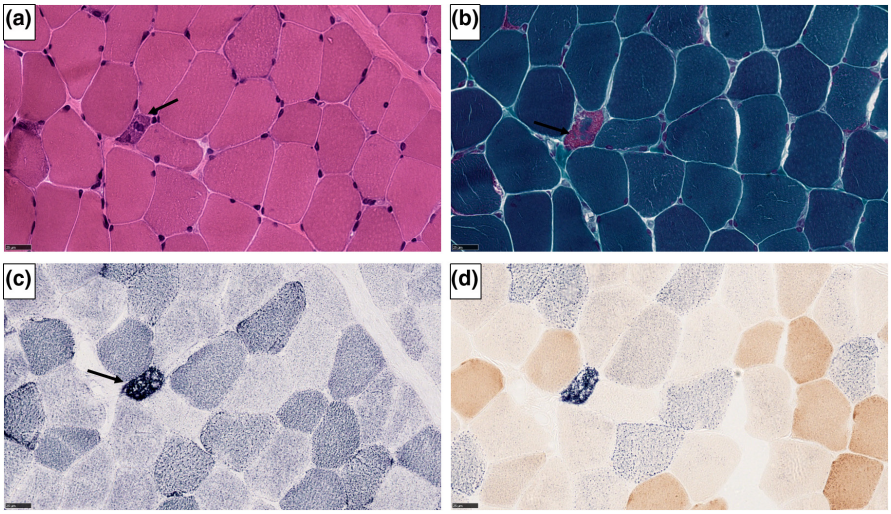

FIGURE 1 Myopathological changes in this group of patients (a–d). (a) HE staining showed basophilic changes in a few muscle fibers (arrow); (b) MGT staining showed ragged red fibers (arrow); (c) SDH staining showed ragged blue fibers (arrow); (d) SDH/COX staining showed more COX-negative muscle fibers (blue muscle fibers)

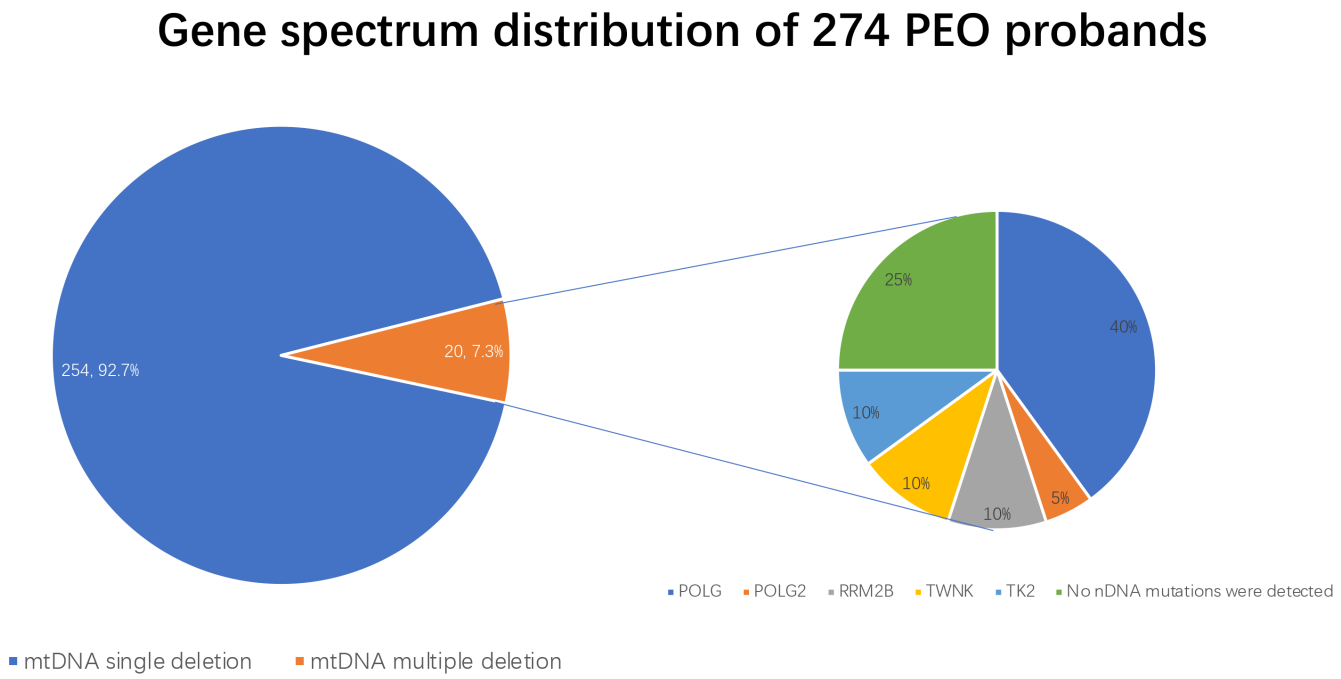

FIGURE 2 Gene spectrum distribution of 274 PEO probands. LR-PCR revealed multiple mtDNA deletions in 7.3% (20/274) of the pedigrees; 75.0% of probands were identified to have possible pathogenic nuclear mutations, including in *POLG*, *POLG2*, *TWNK*, *RRM2B*, and *TK2*. No clear nuclear mutations were found for the other five probands

read), which can be regarded as very strong evidence of pathogenicity (Richards et al., 2015).

Testing of parental samples revealed that c.914G>A and c.2864A>G were present in the heterozygous state in patient P7 and P8, and their family histories were consistent with dominant transmission. The compound heterozygous missense mutations in P1 were inherited from the parents. In P2, the compound heterozygous frameshift mutation c.3002delG was de novo, and the missense mutation c.924G>T was inherited from the mother. The other four patients carried two *POLG* genes, but further linkage analyses were not performed.

3.2.2 | *POLG2* gene

The novel heterozygous missense variation c.285C>A, a stop-gain variant predicted to cause premature termination of the gene product was identified in P9. This variant was not present in the population databases. Paternal testing was not performed because the parents of P9 had passed away. The patient had two asymptomatic offspring who did not carry this variant.

3.2.3 | *TWNK* gene

We detected two single heterozygous mutations in *TWNK* in P10 and P11. The heterozygous missense variation c.1121G>A, which results in an amino acid change of the highly conserved Arg-374 to Gln (p.R374Q), has been reported as pathogenic (Baloh et al., 2007; Martin-Negrier et al., 2011). SIFT software prediction of functional consequences described c.1369A>C/p.T457P as "Tolerated", but another missense mutation, c.1370C>T/T457I, at the same codon has been reported to be pathogenic (Sarzi et al., 2007), highlighting the importance of this codon. The parents of P11 were unaffected, and testing of parental samples revealed that the mutation c.1369A>C was de novo.

3.2.4 | *TK2* gene

A total of four novel missense mutations were identified in the *TK2* gene: c.551C>T/p.T184I in exon 8 and c.161G>A/p.C54Y in exon 3 from P12 and c.659T>C/p.L220P in exon 9 and c.367C>G/p.R123G in exon 5 from P13. Furthermore, the substrate binding site (encoded by exon 5) and active site (encoded by exon 8) are recognized as mutation hotspots for the *TK2* gene (Garone et al., 2018). Computational predictions as to whether or not the c.367C>G variant is damaging to the protein structure or function were inconsistent. The other three variants were predicted to be probably damaging to the protein. These variants were not observed in the large population cohorts and occurred at positions that are conserved across species. Pedigree analysis suggested that the

TABLE 1 Summary of genetic data in *POLG*, *POLG2*, *TK2*, *RRM2B*, and *TWNK*

Patient	Gene	cDNA position	Exon	Amino acid changes	Type of variants	Parental derivation
P1	*POLG*	c.1832C>T	exon10	p.P611L	Missense	Maternal
		c.924G>T	exon4	p.Q308H	Missense	Paternal
P2	*POLG*	c.3002delG	exon19	p.G1001fs	Frameshift	De novo
		c.924G>T	exon4	p.Q308H	Missense	Maternal
P3	*POLG*	c.703T>C	exon3	p.W235R	Missense	NA
		c.668G>C	exon3	p.W223S	Missense	NA
P4	*POLG*	c.3287G>A[a]	exon21	p.R1096H	Missense	NA
		c.1880G>A[a]	exon10	p.R627Q	Missense	NA
P5	*POLG*	c.2591A>G[a]	exon16	p.N864S	Missense	NA
		c.1790G>A	exon10	p.R597Q	Missense	NA
P6	*POLG*	c.2245T>G	exon13	p.F749V	Missense	NA
		c.923A>G	exon14	p.Q308R	Missense	NA
P7	*POLG*	c.914G>A	exon4	p.S305N	Missense	Maternal
P8	*POLG*	c.2864A>G[a]	exon18	p.Y955C	Missense	Maternal
P9	*POLG2*	c.285C>A	exon1	p.C95*	Nonsense	NA
P10	*TWNK*	c.1121G>A[a]	exon1	p.R374Q	Missense	NA
P11	*TWNK*	c.1369A>C	exon2	p.T457P	Missense	De novo
P12	*TK2*	c.551C>T	exon8	p.T184I	Missense	Maternal
		c.161G>A	exon3	p.C54Y	Missense	Paternal
P13	*TK2*	c.659T>C	exon9	p.L220P	Missense	Maternal
		c.367C>G	exon5	p.R123G	Missense	Paternal
P14	*RRM2B*	c.175G>C	exon2	p.A59P	Missense	NA
		c.1010T>G	exon9	p.M337R	Missense	NA
P15	*RRM2B*	c.811C>T	exon8	p.P271S	Missense	Maternal
		c.1055A>T	exon9	p.*352L*9	Stop loss	Paternal

Abbreviation: NA, not available.

[a]The variants has been previously reported.

variants segregated with disease in both of the patients' families and that the asymptomatic offspring of these patients did not carry the variants.

3.2.5 | *RRM2B* gene

We identified four novel *RRM2B* variants, namely three missense variants and one stop-loss variant. The c.175G>C, c.1010T>G, and c.811C>T mutations occurred at positions that were conserved across species, and in silico analyses predicted that these variants are probably damaging to protein structure or function. The c.1055A>T mutation altered the stop codon, resulting in the extension of the gene product, which is considered moderate evidence of pathogenicity. The mutations c.1010T>G and c.1055A>T are located in exon 9, which has been shown to be a mutation hotspot in mtDNA maintenance disorders (Fratter et al., 2011). Analyses of the two pedigrees suggested that the four heterozygous variants were inherited from the patients. For P15, the same two allelic mutations were also found in her affected sister, and her daughter, who only carried c.811C>T, was asymptomatic.

3.3 | Clinical data

The clinical manifestations varied among our group of autosomal-inherited PEO patients, while the skeletal muscle was predominantly affected. A summary of the clinical phenotypes is given in Table 2. The median age of disease onset was 35 years (1–70), the median disease duration was 16 (8–22) years. Among the 40 patients, 77.5% had ptosis or restriction of eye movement as the initial symptoms, and other initial symptoms were limb muscle weakness (6/40), neuropathy (1/40), ataxia (1/54),

TABLE 2 Clinical features of patients with multiple mtDNA deletions

Gene	Patients	Inheritance	Sex	Age (y)	Age at onset (y)	Symptoms at onset	Other major symptoms and signs
POLG	P1	Ar	M	34	15	PEO	Impaired glucose tolerance
	P2	Ar	F	30	23	PEO	Weakness
	P3	Ar	F	48	28	PEO	Weakness, migraine
	P4	Ar	F	56	45	Ataxia	PEO, weakness, dysphagia, neuropathy, cognitive decline
	P5	Ar	M	57	42	Neuropathy	PEO, weakness, ataxia, WPW
	P6	Ar	F	55	37	PEO	Exercise intolerance
	P7	Ad	M	50	37	Weakness	PEO, dysarthria, dysphagia
	P7's mother	Ad	F	75	55	Weakness	PEO
	P7's uncle	Ad	M	64	50	Weakness	PEO
	P7's sister	Ad	F	45	40	PEO	Exercise intolerance
	P8	Ad	F	35	30	Hypogonadism	PEO
	P8's mother	Ad	F	63	35	PEO	Weakness, dysphagia
POLG2	P9	Ad	F	74	52	PEO	Exercise intolerance, neuropathy, parkinsonism, cataract, diabetes
	P9's mother	Ad	F	D(92)	70	PEO	Parkinsonism
TWNK	P10	Ad	F	43	39	PEO	Weakness
	P11	Ad	M	63	41	PEO	Weakness, dysdipsia, sensorineural hypoacusis, neuropathy
TK2	P12	Ar	F	38	1	PEO	Weakness, respiratory insufficiency
	P13	Ar	F	43	32	PEO	Exercise intolerance, weakness
RRM2B	P14	Ar	F	20	13	PEO	Weakness, leukoencephalopathy, sensorineural hypoacusis
	P15	Ar	F	44	24	Weakness	PEO, dysphagia, dysarthria, respiratory insufficiency, neuropathy, leukoencephalopathy, sensorineural hypoacusis
	P15'sister	Ar	F	38	21	Weakness	PEO, dysphagia, dysarthria, respiratory insufficiency, neuropathy, leukoencephalopathy, sensorineural hypoacusis
Undetermined	P16		F	21	15	PEO	Weakness, dysphagia
	P17		M	44	39	PEO	Weakness, dysphagia, dysarthria, diabetes
	P17'sister		F	49	42	Weakness	PEO
	P18		F	45	40	PEO	Weakness
	P18's mother		F	68	57	PEO	Weakness
	P18's sister		F	44	42	PEO	Exercise intolerance
	P19		F	48	33	PEO	

TABLE 2 (Continued)

Gene	Patients	Inheritance	Sex	Age (y)	Age at onset (y)	Symptoms at onset	Other major symptoms and signs
	P19's father		M	70	45	PEO	
	P19's uncle		M	65	42	PEO	Exercise intolerance
	P19'sister		F	42	30	PEO	Exercise intolerance
	P20		M	37	7	PEO	Weakness, WPW
	P20's grandma		F	66	35	PEO	
	P20's mother		F	D(62)	30	PEO	Respiratory insufficiency
	P20's uncle		M	65	34	PEO	
	P20's uncle		M	D(45)	23	PEO	Respiratory insufficiency
	P20's aunt		F	D(66)	40	PEO	
	P20's aunt		F	43	30	PEO	Weakness
	P20's aunt		F	41	32	PEO	Weakness
	P20'sister		F	36	18	PEO	

Abbreviations: Ad, autosomal dominant; Ar, autosomal recessive; D, death; F, female; M, male; P, patient; PEO, progressive external ophthalmoplegia; WPW, Wolff–Parkinson–White syndrome; y, years.

and premature ovarian failure (1/40). At the time of last follow-up, all patients presented with external ophthalmoplegia, and other frequent clinical features included exercise intolerance or limb muscle weakness (26/40), bulbar dysfunction (8/40), peripheral neuropathy (6/40), diabetes or impaired glucose tolerance (3/40). Relatively less common clinical findings were sensorineural hearing loss (4/40), respiratory insufficiency (3/40), leukoencephalopathy (3/40), parkinsonism (2/40), ataxia (2/40), preexcitation syndrome(2/40), migraine (1/40), cognitive impairment (1/40), hypogonadism (1/40), and cataract(1/40).

Cases were classified as "pure PEO" ($n = 6$), and "PEO plus" ($n = 34$). Within "PEO plus", two subgroups were differentiated according to the main associated symptoms: (1) myopathy ($n = 22$), including exercise intolerance, muscle weakness, bulbar symptoms, and respiratory insufficiency; (2) other features ($n = 12$), including endocrine system abnormalities, central nervous involvement, parkinsonism, and peripheral nervous involvement.

3.4 | The clinical features of different genotypes

3.4.1 | *POLG*

Median age of initial symptom in the cohort was 37 years (range, 15–55 years). All patients presented with either ptosis or weakness of extraocular muscles. The clinical phenotype was also characterized by proximal muscle weakness or exercise intolerance (10/12), bulbar involvement (3/12), and peripheral neuropathy (2/12). Additional clinical features included ataxia (2/12), cognitive decline (2/12), pre-excitation syndrome (1/12), and premature ovarian failure (1/12), migraine (1/12), impaired glucose tolerance (1/12).

3.4.2 | *POLG2*

P9 was a 74-year-old woman presenting with gradually progressing eyelid ptosis and restriction of eye movement since age 52, accompanied by exercise intolerance. When she was 60 years old, she developed slowness of movement, decreased facial expression, and a resting tremor most frequently occurring in the right leg. She also had cataracts and diabetes requiring treatment with hypoglycemic drugs in her late sixties. Serum CK was mildly elevated and lactate level was normal. Nerve conduction velocity (NCV) measurement indicated sensory peripheral neuropathy. Cranial MRI did not reveal obvious abnormalities. Her mother presented with PEO at 70 years old, and limb resting tremors starting at around 75 years of age, and she passed away at the age of 92. Her two daughters who did not carry the pathogenic variant were asymptomatic.

3.4.3 | *TWNK*

The two *TWNK* patients had adPEO. Both the 43-year-old female (P10) and 63-year-old male (P11) had PEO as initial symptom at around age 40 and also had exercise

intolerance and weakness. The weakness in P11 was more severe; he had difficulty in climbing stairs and lifting heavy weights, and also occasionally experienced coughing while drinking water. Electro-neurophysiology was normal in P10 but indicated sensory peripheral neuropathy in P11. Audiological evaluation showed high frequency sensorineural hypoacusis in P11. Both patients had normal brain imaging results.

3.4.4 | *TK2*

P12 was a 38-year-old female and had restriction of eye movement and droopy eyelids since childhood. The level of CK was elevated mildly and lactic acid was normal. NCV and cranial MRI were normal. At the age of 35, she had difficulty breathing and needed to wear a ventilator at night. P13 was 43-year-old female who reported having eyelid ptosis and ophthalmoplegia since she was 32 years old, accompanied by fatigue. EMG tests showed myogenic changes. Findings from nerve conduction studies, pulmonary function testing, and cranial MRI were normal.

3.4.5 | *RRM2B*

Three females in two unrelated families were diagnosed with arPEO; two of the patients were siblings. The median age at onset was 21 (13, 24), and the median duration of disease was 17 years (7, 20). P14 presented with ptosis and PEO when she was 13 years old and developed limb weakness at 16 years of age.

Two siblings in P15's family presented with limb weakness at 20 years of age and gradually developed ptosis after 5–6 years, had dysphagia and dysarthria at age 30, and developed respiratory insufficiency at age 40.

All three patients had high frequency sensorineural hypoacusis and leukoencephalopathy. Laboratory tests showed elevated serum CK and lactic acid levels. EMG tests showed myogenic changes in P14 and NCV showed sensory axonal peripheral neuropathy in P15 and her sister. Pulmonary function tests showed restrictive hypoventilation in P15.

4 | DISCUSSION

In this study, we conducted an extensive screen for mutations in nuclear and mitochondrial genes in a large cohort of Chinese patients with PEO, and we identified 20 individuals carrying multiple mtDNA deletions. Moreover, we identified 13, 1, 2, 4, and 4 nuclear gene variants in *POLG*, *POLG2*, *TWNK, RRM2B*, and *TK2*, respectively. However, target NGS did not identify the responsible pathogenic mutations in five pedigrees.

TWNK was first identified in adPEO, which is characterized by the presence of multiple deletions of mtDNA in patient tissues, and *POLG*, *POLG2*, *TK2*, and *RRM2B*ere subsequently identified in the same group of patients (Finsterer & Zarrouk-Mahjoub, 2018; Rahman & Copeland, 2019; Sommerville et al., 2014; Wang et al., 2018). These results opened up a new field for research on intergenomic signaling between the nuclear and mitochondrial genomes.

The *POLG* gene was the most common autosomal causative gene in our cohort of Mendelian PEO patients with multiple mtDNA deletions, which is consistent with data obtained from studies of the London-Oxford cohort and Italian cohort (Bugiardini et al., 2017; Woodbridge et al., 2013). The four variants (c.1880G>A, c.2591A>G, c.2864A>G, and c.3287G>A) identified in our arPEO patients were also prevalent in European patients (Farnum et al., 2014; Sohl et al., 2013; Van Goethem et al., 2003; Vogel et al., 2017). In addition, the compound heterozygous mutation c.3287G>A, c.1880G>A has been observed in individuals exhibiting diverse phenotypes including Alpers syndrome, early-onset cerebellar ataxia with PEO, and focal nocturnal motor epilepsy with status epilepticus at 1 month postpartum (Savard et al., 2013; Schicks et al., 2010; Schulte et al., 2009; Stumpf et al., 2013). Furthermore, we identified nine novel mutations. A different missense substitution at the same position caused the same amino acid change (p.Q308H), which has been identified in a patient with arPEO disorder (Horvath et al., 2006). Different amino acid changes at the same codons (c.915C>G/S305R, c.1970G>A/p.R597W, c.2246T>C/F749S) have been observed in individuals with features suggestive of POLG deficiency (Bostan et al., 2012; Nesbitt et al., 2014; Qian et al., 2015; Saneto et al., 2010).

We found a heterozygous *POLG2* missense mutation, c.285C>A, predicted to cause loss of normal protein function through premature protein truncation. Mutation of *POLG2* is not a common cause of mitochondrial disease, and this is the first Asian case reported. Nonallelic pathogenic variants of *POLG2* cause haplotype insufficiency or heterodimerization of the mutated and wild-type proteins, which is typically associated with multiple mtDNA deletions (El-Hattab et al., 2017; Longley et al., 2006). Homozygous mutation of *POLG2* resulting in mitochondrial depletion and the clinical presentation of severe hepatic failure was also reported (Varma et al., 2016). In addition to missense mutations, a heterozygous *POLG2* splice-acceptor variant (c.970-1G>C) and a 24-bp insertion variant (c.1207_1208ins24) were also detected in Belgium and Germany (Van Maldergem et al., 2017; Walter et al., 2010).

TWNK represents the second most common cause of adult Mendelian PEO associated with multiple mtDNA deletions. The most reported *TWNK* mutations are dominant mutations with multiple mtDNA deletions, which are associated with mitochondrial myopathy and adPEO (Fratter et al., 2010), as seen in our patients in whom heterozygous c.1121G>A and c.1369A>C mutations were detected. Recessive *TWNK* mutations were also reported in a few patients with phenotypes such as infantile-onset spinocerebellar ataxia and hepatocerebral form of mtDNA depletion syndrome (Hakonen et al., 2007). Three other families in France and North America also had the c.1121G>G/R374Q variant; however, related symptoms including, respiratory insufficiency, and parkinsonism were not observed in our patient. The novel heterozygous missense variant c.1369A>C replaced proline with threonine at codon 457 (p.T457P) of the *TWNK* protein in our cohort. One previous study in France identified the homozygous missense variant c.1370C>T, which results in a different amino acid substitution at the same codon (p.T457I), in a patient with a hepatocerebral form of mtDNA depletion syndrome.

We found four novel missense variants of *TK2* in two unrelated Chinese families. The most prevalent type of mutation in *TK2* is missense, which accounted for well over half of all variants (65.9%, 31/47) in a review study (Wang et al., 2018). Moreover, c.367C>G and c.551C>T, which encode the substrate binding site and active site, are recognized as mutation hotspots for the *TK2* gene. Biallelic pathogenic variants in the *TK2* gene cause a myopathic form of mitochondrial DNA maintenance defect (Wang et al., 2018). Infantile-onset myopathy is typically associated with severe neurological involvement, rapid progression to early mortality, and mtDNA depletion, and multiple mtDNA deletions are generally associated with late onset (Garone et al., 2018). However, our patient harboring the c.551C>T and c.161G>T variants along with multiple mtDNA deletions had manifested weakness of the extraocular muscle since infancy, but slow progression to survive to adulthood, expanding the natural history and prognosis of *TK2* deficiency.

Both recessive and dominant mutations in the *RRM2B* gene that cause the accumulation of multiple mtDNA deletions have been described. All four novel missense mutations found in our cohort were in the compound heterozygous form. Finsterer et al. found that the mean age at onset among those with multiple mtDNA deletions secondary to the *RRM2B* gene was 39.7 years old (Finsterer & Zarrouk-Mahjoub, 2018). The reason why the age at onset in our three patients was earlier than the mean age is that recessively inherited compound heterozygous mutations have an earlier age of disease onset compared with dominantly inherited heterozygous mutations (Pitceathly et al., 2012).

There were no visual abnormalities or retinal fundus changes in our group of patients with nuclear variants. However, recent studies suggest that mitochondrial retinopathy has been shown to present with a spectrum of distinct phenotypes. The retinal phenotype also may be the first indication of a mitochondrial disease. Specific retinal changes may be a characteristic finding in a variety of mitochondrial diseases and have been reported in a PEO with RRM2B mutation, indicating that it is crucial for achieving the systemic diagnosis (Birtel et al., 2021). Therefore, the further clinical follow-up for these patients is important.

LR-PCR of skeletal muscle DNA from all the patients clearly revealed multiple deletions of mtDNA, confirming a disorder of mtDNA maintenance. LR-PCR showed that only 7.3% of the patients in this group had multiple mtDNA deletions, which may be related to PEO was the main symptom in our inclusion criteria, so this ratio may be underestimated to some extent. Since there was still a relatively large number (20.0%) of patients with multiple mtDNA deletions in whom clear nuclear mutations were not identified, other nuclear genetic defects or pathophysiological mechanisms that lead to multiple mtDNA deletions should be studied in the future. In addition, further functional confirmation is needed for novel variants.

5 | CONCLUSION

Inherited PEO is the most prominent feature in this group of patients with multiple mtDNA deletions associated with mutations in five known nuclear genes, *POLG*, *POLG2*, *TK2*, *RRM2B,* and *TWNK*, while genotypic and phenotypic heterogeneity still exist because of a wide range of ages at onset, variable initial symptoms, and accompanying manifestation. The identification of previously unreported mutations may provide useful data for structure–function relationship analyses.

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AUTHOR CONTRIBUTIONS

ZXW and YH conceived the idea, designed studies. HY, XTZ and ZYX designed and carried out experiments, analyzed data. MY, WZ, HL,YY, and ZXW contributed to the clinical diagnosis of CPEO patients. YH, and XTZ wrote

and edited the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

No competing financial interest exist.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Peking University First Hospital (2019–181). Written informed consent was obtained from all participants or their legal guardians.

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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