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Whole-exome sequencing as a powerful tool for identifying genetic causes in a patient with POLG-related disorders and phenylketonuria

Lin Li[1], Jin-Qi Zhao[2], Chengrong Wang[1], Nan Yang[2], Li-Fei Gong[2], Hai-He Yang[2], Chenghong Yin[1] and Yuan-Yuan Kong[2]

Abstract

Objective: This study's aim was to identify the genetic causes in a patient with phenylketonuria and hearing loss, liver disease, developmental and mental retardation, hypotonia, and external ophthalmoplegia.

Methods: Whole-exome sequencing and Sanger sequencing analysis were used to determine the genetic causes of manifestations in a young boy with hearing loss, liver disease, develop-mental and mental retardation, hypotonia, and external ophthalmoplegia.

Results: We found that the child harbored polymerase gamma (*POLG)* compound heterozygous mutations, c.2617G>A (p.E873K) and c.3550G>A (p.D1184N), and phenylalanine hydroxylase (*PAH)* compound heterozygous mutations, c.721C>T (p.R241C) and c.728G>A (p.R243Q). Among them, the *POLG* p.E873K mutation is a novel mutation and is not present in the Exome Aggregation Consortium database, Genome Aggregation database, and 1000 Genomes database. The two heterozygous mutations were each inherited from both of the child's parents. This finding suggested that the phenotype and the genotype were segregated.

Conclusion: Using whole-exome sequencing, we not only identified *PAH* mutations causing phenylketonuria, but also identified the genetic cause of the mitochondrial disease and found a novel *POLG* mutation. Our findings could be useful in helping future parents obtain healthy embryos through assisted reproductive technology.

[1]Central Laboratory, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Chaoyang, Beijing, China
[2]Department of Newborn Screening, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Chaoyang, Beijing, China

Corresponding author:
Yuan-Yuan Kong, Department of Newborn Screening, Beijing Obstetrics and Gynecology Hospital, and Beijing Maternal and Child Health Care Hospital, Capital Medical University, Chaoyang, Beijing 100026, China.
Email: kongyuanyuan1971@163.com

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Introduction

The polymerase gamma (*POLG*) gene is involved in replication and repair of mitochondrial DNA (mtDNA). Mutations in *POLG* cause mtDNA instability and lead to syndromes related to mtDNA abnormalities, such as Alpers–Huttenlocher syndrome, childhood myocerebrohepatopathy spectrum, myoclonic epilepsy myopathy sensory ataxia, ataxia neuropathy spectrum, progressive external ophthalmoplegia (PEO), and ovarian dysfunction.[1–6] *POLG*-related disorders exhibit overlapping phenotypes, and most patients with *POLG* mutations have some, but not all, of the clinical features of a certain syndrome. The first identified *POLG* mutation was found in a patient suffering from progressive external ophthalmoplegia in 2001.[3] To date, more than 250 different mutations in *POLG* have been identified and deposited in the Human DNA Polymerase Gamma Mutation Database (https://tools.niehs.nih.gov/polg/). However, an increasing number of novel *POLG* mutations are being identified because of the increasing use of high-throughput sequencing technology over recent years to identify the genetic causes of diseases.[7] This is especially beneficial for the diagnosis of *POLG*-related disorders, which were clinically defined a long time before molecular genetic analysis could be performed.[8]

We report a patient with phenylketonuria and hearing loss, liver disease, developmental and mental retardation, hypotonia, and external ophthalmoplegia. To diagnose this disease and its causes, whole-exome sequencing (WES) was performed in this patient, and compound heterozygous mutations in *POLG* were identified, one of which was novel. Therefore, WES could be a powerful method for identifying *POLG* mutations and also for helping clinicians diagnose precise disorders.

Materials and methods

Participants

The patient was from a non-consanguineous Chinese family. The patient's father and mother were healthy and did not have any abnormalities that the patient had. This study was approved by the Ethics Committee of Beijing Obstetrics and Gynecology Hospital, and was conducted in accordance with the 1964 Helsinki declaration and its later amendments. The participants signed informed consent for this study. A volume of 5 mL of peripheral blood was collected from the patient and his parents.

WES

WES was performed as previously described.[9] Briefly, genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). The whole exome was captured using a TruSeq Exome kit (Illumina, San Diego, CA, USA). The enriched products were then sequenced using the HiSeq2500 platform (Illumina). Raw sequencing data for

the patient were mapped to the human reference genome hg19 using Burrows–Wheeler Aligner (http://bio-bwa.sourceforge.net/). Variants were called using the software package Genome Analysis Toolkit (https://software.broadinstitute.org/gatk/). Functional annotations of all variants were assigned using ANNOVAR software (http://annovar.openbioinformatics.org/en/latest/).

Sanger sequencing confirmation

Sanger sequencing was used to validate the mutations in the *POLG* gene in the patient and his parents. For the one variant in *POLG* (c.2617G>A: p.E873K), the forward primer was 5′-AGATAGGACCGTGTAG GTGAGGG GT-3′ and the reverse primer was 5′-GACAAGCAGGAGTGAGAAAA GCAGC-3′ for PCR amplification and Sanger sequencing. For another *POLG* variant (c.3550G>A: p.D1184N), the forward primer was 5′-GAAAACAGTGCTGGAC CTTCACC-3′ and the reverse primer was 5′-AGCCTGAGTCAAGAGTGGA TTCTCT-3′.

Protein sequence alignment

Protein sequence alignment was performed as previously described.[10] Protein accession numbers for each species were as follows: human, NP_002684; Rhesus monkey, XP_014998464; cattle, XP_010815288; Norway rat, NP_445980; house mouse, NP_059490; chicken, XP_015147533; and zebrafish, XP_001921130.

Results

Detailed clinical features of the patient

The male patient was born prematurely on March 11, 2011 after 30 weeks' gestation through normal delivery with a birth weight of 1640 g. The patient was later diagnosed with phenylketonuria by neonatal screening, with a blood phenylalanine concentration of 7.3 mg/dL. Phenylalanine hydroxylase (*PAH*) gene testing was performed, and c.721C>T (p.R241C) and c.728G>A (p.R243Q) mutations were identified. Thereafter, low phenylalanine dietary treatment was followed. Blood phenylalanine concentrations were maintained from 2 to 6 mg/dL. During the same period, repeated hearing tests were performed, and the patient failed the initial screening and re-screening.

At the age of 6 months (4 months as corrected on the basis of gestational age), the patient was admitted to hospital because of fever and poor weight gain. A routine examination showed an elevated alanine aminotransferase (ALT) concentration (179 U/L). During re-examination at 7 months and 8 months, the ALT concentration was 41 U/L. During re-examination at 9 months, the ALT concentration had increased to 80 U/L. At 10 months, the ALT concentration was 74 U/L. An electroencephalogram, electrocardiogram, cranial B-mode ultrasonography, magnetic resonance imaging, and ultrasound cardiography, which were performed at 7 and 10 months, did not show any major abnormalities. At 11 months, mitochondrial enzyme activity was examined, and mitochondrial complexes I and V were found to be defective, and levels of complexes I to III were low. We wanted to quantify the mtDNA amount in the liver, but tissue samples were not available. Therefore, this condition was clinically considered to be mitochondrial liver disease. Mitochondria DNA testing showed no mutations. Hearing problems were diagnosed at 8 months. There was no response to a brainstem auditory evoked potential (ABR100db). A multiple-frequency auditory steady-state response examination showed that the hearing loss in both ears was above moderate-to-severe. Therefore, the patient was initially diagnosed with auditory neuropathy. During and after this period, the child's feet

were pronated and slight tiptoeing was observed. The patient had low muscle tone, retarded development, mild mental retardation, and extreme gross motor retardation (unable to sit stably, crawl, stand, or walk). We also found later that the child had external ophthalmoplegia. The child died at the age of 6 years. The patient was difficult to diagnose on the basis of his clinical features. Therefore, we attempted to use genetic analysis to assist the diagnosis.

WES analysis

Because of the complex phenotype of the patient and unexplored reasons for causing his disease, we decided to use WES to identify the underlying genetic causes for this abnormality. By analyzing the pedigree, we speculated that the recessive mode of inheritance was most likely. Therefore, we focused on homozygous or compound heterozygous variants (non-synonymous, frameshift, stop gained, and splice site) when analyzing the WES data. We also filtered out variants with a minor allele frequency of more than 1% in the databases, including Exome Aggregation Consortium (http://exac.broadinstitute.org/), Genome Aggregation database (http://gnomad.broadinstitute.org/), 1000 Genomes (1000G, http://browser.1000genomes.org/index.html), Exome Variant Server (ESP6500, http://evs.gs.washington.edu/EVS/), and the Short Genetic Variations database (dbSNP, http://www.ncbi.nlm.nih.gov/snp/). We first identified compound heterozygous mutations in the *PAH* gene (PAH: NM_000277: exon7: c.721C>T: p.R241C and c.728G>A: p.R243Q) that accounted for phenylketonuria in this patient. We then attempted to identify the mutations associated with mitochondrial liver disease. We identified two compound heterozygous mutations in *POLG* (POLG: NM_002693: exon17: c.2617G>A: p.E873K and exon22: c.3550G>A: p.D1184N).

Sanger sequencing validation

We then used the Sanger sequencing method to validate the mutations in the family. Consistent with the exome sequencing result, the affected boy harbored two mutations of which c.2617G>A was inherited from his father and c.3550G>A was inherited from his mother (Figure 1b).

Bioinformatic analysis of the mutations

We performed bioinformatic analyses to demonstrate pathogenicity of the two mutations We found that six online prediction tools, including Polymorphism Phenotyping v2 (Polyphen-2), Sorting Intolerant from Tolerant (SIFT), Protein Variation Effect Analyzer (PROVEAN), Mutation Taster, Single Nucleotide Polymorphisms and Gene Ontology (SNPs&GO), and Predictor of Human Deleterious Single Nucleotide Polymorphisms (PhD-SNP), predicted both of the mutations as disease-causing/damaging mutations (Table 1). The prediction results were also supported by the extremely low allele frequencies of the two mutations (Table 1). Additionally, we analyzed conservation of the two mutations. Both of the mutations were located in the polymerase domain of POLG protein (Figure 2a). Using protein sequence alignment analysis, we found that the mutations were 100% conserved from human to zebrafish (Figure 2b), suggesting an intolerant feature of the mutation sites. Therefore, based on the clinical and genetic features of the patient, we considered that he should be diagnosed with childhood myocerebrohepatopathy spectrum.

Discussion

In this study, we performed WES in a boy with hearing loss, liver disease, developmental and mental retardation, hypotonia, and external ophthalmoplegia. We identified compound heterozygous mutations in

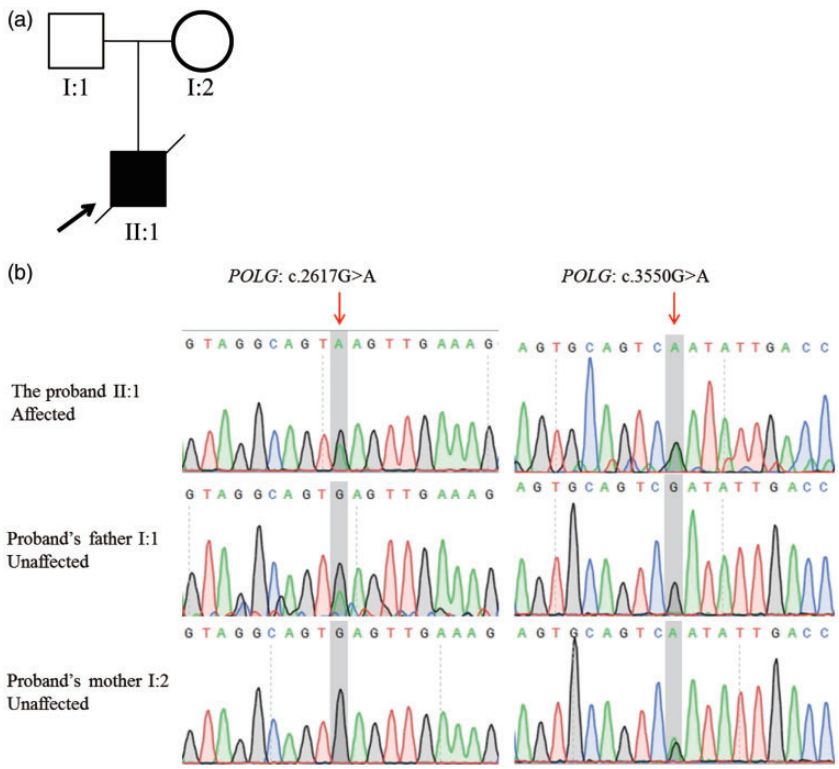

Figure 1. *POLG* mutations in the patient. (a) The family tree shows a patient from a non-consanguineous family. (b) Sanger sequencing confirmed compound heterozygous *POLG* mutations in the patient. The patient's father and mother carry different heterozygous mutations. The red arrow points to the mutation sites. *POLG*, polymerase gamma.

POLG. c.2617G>A (p.E873K), which was inherited from his father, and c.3550G>A (p.D1184N), which was inherited from his mother.

The c.2617G>A (p.E873K) mutation has not been described previously. This mutation was not found in Exome Aggregation Consortium, the Genome Aggregation database, and the 1000 Genomes database, suggesting that this mutation is extremely rare in the human population. The c.2617G>A (p.E873K) mutation is also predicted as a pathogenic mutation by all of the prediction tools that we used, which suggested that the prediction result was accurate. The E873 site is highly conserved and is located in the polymerase domain of *POLG*. More than one third of mutations in *POLG* occur in the polymerase domain, of which most mutations are associated with PEO and Alpers syndrome, myocerebrohepatopathy, and other infantile hepatocerebral syndromes (https://tools.niehs.nih.gov/polg/).

Another mutation, c.3550G>A (p.D1184N), has been reported in several studies. One study reported that two brothers harbored the compound heterozygous mutations R227W and D1184N.[11] One of the brothers died at 26 months old and the other died at 43 months old. Both of the brothers experienced failure to thrive, retardation, hypotonia, and hearing loss,[11] similar to our patient. Another study reported a girl who died at 3 years old. She carried

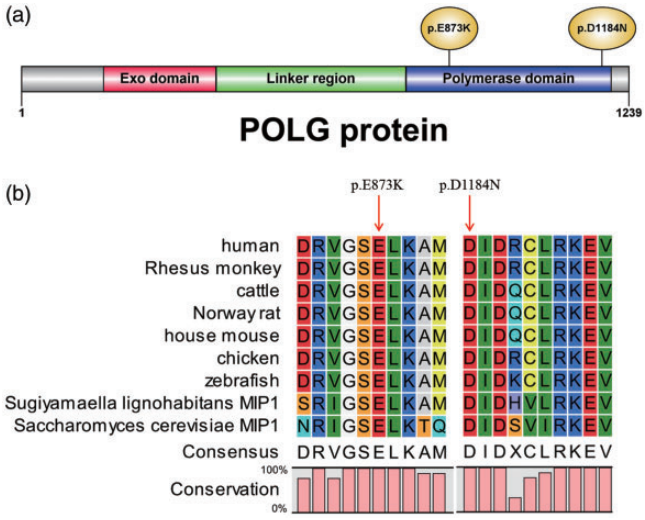

Figure 2. *POLG* mutation sites were conserved. (a) Domains and mutation sites in the POLG protein. The full-length protein is 1239 amino acids. A 3′-5′ proofreading exonuclease (Exo) domain (red box), linker region (green box), and polymerase domain (blue box) are shown. (b) Sequence alignment of POLG proteins in different species. The red arrows indicate the mutational sites. POLG, polymerase gamma; MIP1, DNA-directed DNA polymerase gamma MIP1.

the *POLG* compound heterozygous mutations S1095R and D1184N.[12] The prominent clinical features of this girl were failure to thrive, congenital deafness, impaired liver function, and feeding problems.[12] D1184N was also found to cause PEO in trans with N468D in a 32-year-old patient[13] or to cause chronic PEO in heterozygous state in patients older than 70 years.[14]

Phenylketonuria and POLG-related disorders are rare diseases. Therefore, the presence of both of these diseases in this patient is even rarer. Our patient was diagnosed with phenylketonuria at birth, and we subsequently found genetic mutations in the *PAH* gene. However, many manifestations appeared later in the patient, including liver disease, hypotonia, and growth retardation, which were different from phenylketonuria. These clinical manifestations could not be explained by the *PAH* gene mutations, and could not be precisely diagnosed. Therefore, we used WES to assist in diagnosing the patient. Genetic analysis is important, especially for mitochondrial diseases. Many disorders related to mitochondrial abnormalities have common clinical manifestations, which is why these disorders are clinically defined a long time before molecular genetic analysis can be performed. If parents want a second child after the patient's death, they need to know the genetic causes leading to the disease. Identifying the genetic factors can prevent the second child from suffering from the same disease by using assisted reproductive technology. The parents of our patient now know that they each carry one mutant allele. Therefore, clinicians and scientists can help them screen embryos, using assisted reproductive technology and preimplantation genetic diagnosis, without passing on the mutations to healthy offspring. Therefore, WES technology is a powerful and vital tool for diagnosing disorders related to mitochondrial abnormalities, identifying genetic causes, and planning strategies to obtain healthy offspring.

Table 1. Bioinformatic analysis of the *POLG* variants.

Variant	Amino acid change	Polyphen-2[a]	SIFT[b]	PROVEAN[c]	Mutation Taster[d]	SNPs&GO[e]	PhD-SNP[f]	ExAC (total)[g]	ExAC (East Asian)[h]	1000 Genomes[i]	gnomAD (total)[j]
c.2617G>A	p.E873K	Probably damaging (0.999)	Damaging (0.000)	Deleterious (−3.87)	Disease causing (0.9999)	Disease (0.902)	Disease (0.672)	0	0	0	0
c.3550G>A	p.D1184N	Probably damaging (1.000)	Damaging (0.004)	Deleterious (−3.20)	Disease causing (0.9999)	Disease (0.806)	Disease (0.703)	0	0	0	8.121e-6

[a]For Polyphen-2, prediction scores range from 0 to 1, with high scores indicating probably or possibly damaging.
[b]For SIFT, scores vary between 0 and 1. Variants with scores close or equal to 0 are predicted to be damaging.
[c]For PROVEAN, variants with scores lower than −2.5 (cutoff) are predicted to be deleterious.
[d]For Mutation Taster, the probability value is the probability of the prediction (i.e., a value close to 1 indicates a high "security" of the prediction).
[e]For SNPs&GO, probability: disease probability (if > 0.5, mutation is predicted disease).
[f]For PhD-SNP, probability: disease probability (if > 0.5, mutation is predicted disease).
[g]Allele frequency of variation in the total ExAC database.
[h]Allele frequency of variation in the East Asian population of the ExAC database.
[i]Allele frequency of variation in the 1000 Genomes database.
[j]Allele frequency of variation in the total gnomAD (a large database containing 123,136 exome sequences and 15,496 whole-genome sequences).
Polyphen-2, Polymorphism Phenotyping v2; SIFT, Sorting Intolerant from Tolerant; PROVEAN, Protein Variation Effect Analyzer; SNPs&GO; Single Nucleotide Polymorphisms and Gene Ontology; PhD-SNP, Predictor of Human Deleterious Single Nucleotide Polymorphisms; ExAC, Exome Aggregation Consortium, gnomAD, Genome Aggregation database.

In summary, this study not only identified a novel *POLG* mutation, but also demonstrated that WES can assist in diagnosing mitochondrial disease.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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ORCID iD

Lin Li http://orcid.org/0000-0002-3597-3350

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