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Electrophysiological features and genetic diagnosis of a Chinese pedigree with Charcot-Marie-Tooth disease revealed by next-generation sequencing

Running title: Electrophysiological and genetic findings of a Chinese CMT pedigree

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Abstract

Background: Charcot-Marie-Tooth (CMT) disease refers to a group of clinically and genetically heterogeneous peripheral neuropathies. This study aimed to identify disease-causing gene in a Chinese family with CMT.

Results: In the present study, the proband and her father were preliminary diagnosed as CMT according to clinical manifestations, electrophysiology examination and family history. Twenty-seven known CMT disease-causing genes of the proband and her father were captured using a custom capture array followed by sequencing with massively parallel next-generation sequencing (NGS). The pathogenic variants were then confirmed by polymerase chain reaction (PCR) and Sanger sequencing.

The electrophysiology features indicated that the proband and her father showed multiple sensory and motor nerve fiber demyelination associated with axonal damage in the upper and lower limbs, accompanied with conduction block. The father was more severely affected than his daughter. Genetic analysis reveals that the gap junction protein β 1 gene (GJB1) c.44G>A mutation was identified in the two patients, with a heterozygote status in the proband and a hemizygote status in her father. Segregation analysis of the mutation in eight family members revealed that the mutation co-segregated with CMT.

Conclusions: GJB1 c.44G>A mutation was found to be the genetic cause of X-linked dominant CMT disease type 1 in the family by using the targeted NGS approach. The electrophysiology features of the proband and her father were reported in details for better understanding of electrophysiological characterization of the disease in women.

Keywords: Charcot-Marie-Tooth disease, CMT, electrophysiological features, genetic diagnosis, next-generation sequencing

Background

Charcot-Marie-Tooth diseases (CMT), also known as hereditary motor and sensory diseases, are the most common heritable disorder of the peripheral nervous system and affect approximately 1 in 1,200 individuals in Norway [1]. The prevalence of CMT in different regions of the world remains limited [2]. The main clinical manifestations of CMT include progressive wasting and weakness of distal muscle, impaired distal sensation, and loss of tendon reflex. CMT is classified into 4 main types: CMT1, CMT2, CMT4 and CMTX based on electrophysiological and pathological findings [3]. Each of these types can be further divided into subtypes according to different disease-causing genes or chromosome loci [4]. The majority of patients with CMT have autosomal dominant (AD) inheritance, although many will have forms with X-linked or autosomal recessive (AR) inheritance [5, 6]. Autosomal dominant CMT1A is caused by a peripheral myelin protein 22 (PMP22) mutation, and accounts for 40%~50% of CMT cases. X-linked Charcot-Marie-Tooth disease type 1 (CMT1X) is caused by mutations in the gap junction protein β 1 gene (GJB1) coding for connexin 32 (Cx32), and accounts for 7%~11% of total CMT as the second most type of CMT [7]. Until now, more than 80 genes have been identified to cause different types of CMT [8]. Due to phenotypic and genetic heterogeneity of CMT [9],

it is still a challenge to diagnose exact forms of CMT in a clinically feasible manner.

With the advent of next-generation sequencing (NGS), studies of hereditary diseases have been transformed [10]. The technology has enabled the identification of molecular characterization of hereditary traits, especially the identification of the underlying causative genes of hereditary disorders [11]. As many diseases present a broad spectrum of phenotypes, the advent of next-generation sequencing has greatly helped to clarify the diagnosis of these unidentified conditions [12, 13]. In this study, we performed gene capture combined with NGS in a CMT family and identified causative gene that associated with the disease.

Results

Clinical symptoms

The proband (IV11) was a 32 years old female who presented progressive limbs (lower limbs prominent) weakness and numb without apparent inducement around her 20 years old. Physical examination showed thenar muscle and dorsal interossei atrophy and weakness in both hands, anterior tibialis and gastrocnemius atrophy in the lower limbs, distal extremities hypoalgesia, and foot abduction. No pes cavus and paresthesia were observed in the proband. According to the complaint of the proband, the other 2 female patients (II4 and III5) had relatively mild symptoms, only showed mild anterior tibialis and gastrocnemius atrophy in the lower limbs, without obvious foot abduction. All the male patients except patient IV10, showed more severe symptoms than the proband. Normal myocardial enzyme levels were observed in the

proband (IV11) and her father (III7).

Electrophysiological examination characteristics

The proband and her father received nerve electrophysiological examination, and the results are shown in Table 1 and Table 2. The proband (IV11) exhibited the decreased compound muscle action potential (CMAP) amplitudes of right median, bilateral ulnar, bilateral peroneal and bilateral tibial nerves; the prolonged distal motor latency (DML) of CMAPs; the decreased motor nerve conduction velocity (MNCV) of bilateral fibular head-medial malleolus; the decreased sensory nerve active potential (SNAP) amplitudes of bilateral median, bilateral ulnar and left tibial nerves; and the decreased sensory nerve conduction velocity (SNCV) of bilateral median, bilateral ulnar and bilateral tibial nerves. The SNAPs of bilateral peroneal nerves were not elicited. These results showed multiple sensory and motor nerve fiber demyelination associated with axonal damage in the upper and lower limbs of the proband.

The proband's father (III7) exhibited the decreased CMAP amplitudes and the prolonged DML of bilateral median, bilateral ulnar nerves. The CMAPs of bilateral peroneal and bilateral tibial nerves were not elicited. The SNAPs of left median, bilateral ulnar, bilateral tibial and left peroneal nerves were not elicited. The SNAP amplitudes of right median nerve were normal. The SNCV of right median nerve was decreased. Needle electrode electromyography (NEMG) showed recruitment potentials of left anterior tibial muscle, abductor digiti minimi and gastrocnemius were absent; spontaneous potentials of gastrocnemius were absent. These results

showed the father was more severely affected with multiple peripheral nerve damage than his daughter in the upper and lower limbs.

Exon capture NGS and bioinformatics analysis

The whole coding exons plus 100 bp exon-intron splice junctions fragment of 27 previously reported CMT disease-causing genes (PMP22, MPZ, LITAF, EGR2, NEFL, KIF1B, MFN2, RAB7A, LMNA, MED25, TRPV4, GARS, HSPB1, HSPB8, AARS, GDAP1, DNM2, YARS, MTMR2, SBF2, SH3TC2, NDRG1, PRX, FGD4, FIG4, GJB1, PRPS1) were captured and analyzed. On average 99.9% of base pairs with >100× coverage were successfully detected. As shown in Figure 2, the average of sequencing depth approximates to the sequencing depth median of all exons for the GJB1 gene, which means good randomicity of sequencing.

To find potential diseasing-causing mutations in the 27 known CMT disease genes, the NGS data were filtered through the following steps: (1) exclusion of variants with >0.05 frequency in four databases (dbSNP, HapMap, 1000 Genomes Project and 100 control healthy Chinese individuals), (2) exclusion of variants that were not in the coding regions or in the splice sites, (3) exclusion of synonymous variants. After these steps, a reported mutation c.44G>A (p.Arg15Gln) was found in the GJB1 (NM_001097642) gene by the targeted NGS approach. The missense mutation has been reported in Inherited Peripheral Neuropathies Mutation Database (http://www.molgen.ua.ac.be/CMTMutations/). Furthermore, we applied in silico studies to predict function of the mutated protein by using the SIFT and PolyPhen

software, result indicated that the c.44G>A (p.Arg15Gln) mutation was likely to damage the structure and function of GJB1.

Sanger sequencing

In order to verify the pathogenic variants identified by target capture NGS, Sanger sequencing was performed to analyze the point mutation of GJB1 gene. Sequencing result revealed that the proband (IV11) was heterozygous for GJB1 c.44G>A mutation and her father (III7) was hemizygous for the mutation (Figure 3). The other female patient III5 was heterozygote, and the other two male patients III11 and IV10 were hemizygotes. The mutation was not present in three asymptomatic family members (III1, III3 and IV12). Segregation analysis of the mutation in the eight family members revealed that the mutation co-segregated with CMT.

Discussion

Currently, the diagnosis of CMT is mainly based on patient's clinical characteristics, electrophysiology and neuropathology characteristics. Due to the complexity of clinical phenotype, the accurate diagnosis of CMT is very difficult. In this research, the CMT family exhibits a dominant inheritance pattern, and only one father (patient III9) didn't pass the condition to his son (IV12). Therefore, we couldn't make sure absence of male-to-male transmission and consider X-linked inheritance in the family. The proband and her father's electrophysiology features showed multiple sensory and motor nerve fiber damage combined with demyelination and axonal damage of upper

and lower limb, indicating a clinical impression of demyelinating and axonal form of CMT. Thus, we focused on the disease-causing genes and performed the targeted NGS approach. Our result showed that the proband and her father were found to have the c.44G>A mutation in exon 2 of GJB1 that resulted in a missense mutation (p.Arg15Gln) in the N-terminal of Cx32 protein.

Patients with CMT1X clinically present with slowly progressive distal muscle atrophy and weakness, variable sensory loss and areflexia. Heterozygous women are less severely affected than men at all stages of CMT1X [14, 15]. Electrophysiologic and pathologic studies in men with GJB1 mutations typically show a mixture of demyelinating and axonal features, and intermediates lowing of nerve conduction velocities is characteristic [16, 17]. The Peripheral nerve electrophysiological characteristics of CMT1X men can not only show demyelination of nerve fibers (lower MNCV, SNCV), but also show axonal lesion (CMAP, SNAP decrease), in addition, demyelination and secondary axonal degeneration will be aggravated with age [18, 19]. However, the electrophysiological features of women with CMT1X are dissimilar from those in men [16]. In addition, there are a few females with no symptoms of neuropathy. It has been postulated to be due to random X-chromosome inactivation in schwann cell of female peripheral nerve system [20]. In this study, the electrophysiology features of the proband and her father were reported in details for better understanding of electrophysiological characterization of CMTIX in women.

The disease-causing GJB1 gene encodes protein Cx32, is expressed in Schwann cells of peripheral nerve system as well as oligodendrocytes or neurons of central

nerve system [17, 21]. Cx32 hexamer is formed on the cell membrane, it interact with the adjacent gap junction protein hexamer to form a complete gap junction channels, allowing intercellular ions, small molecules to diffuse across cell membrane, which plays an important role in the transmission of information and chemical material [22]. So far, approximately 400 GJB1 mutations have been reported to be able to affect GJB1 protein function [17, 23]. The Cx32 protein is consist of 4 transmembrane domains, 2 extracellular loops, 1 cytoplasmic loop, cytoplasmic C-terminal and N-terminal [24]. The N-terminal of Cx32 cooperate with transmembrane domain 1 to control voltage-gating of the channel. In addition, there is evidence that N-terminal domain participate the transport process of precursor protein into endoplasmic reticulum [25-27]. A study reported that the p.Arg15Gln mutant protein could not form functional gap junction although it can be transported to the cell membrane [28]. The Cx32 p.Arg15Gln mutation is the genetic entity of our patients.

Conclusions

The Cx32 p.Arg15Gln (GJB1 c.44G>A) mutation was identified in the Chinese family with the aid of NGS, and it may account for the genetic cause of the CMT disease. The electrophysiology features of the proband and her father were reported in details for better understanding of electrophysiological characterization of CMTIX in women.

Materials and methods

Sample collection and characterization

We studied a 5 generations Chinese family consisting of 11 affected (2 of them deceased) and 24 unaffected members (1 of them deceased) aged from 21 to 74 years old. The age of onset of the symptoms was 18 to 30 years old and the proband was a 32 years old female (Figure 1). The proband and her father clinically received neurological examination and nerve electrophysiological examination.

Targeted sequence capture and next-generation sequencing

The sequencing target was all coding regions plus 100 bp exon-intron splice junctions fragment of 27 known CMT disease-causing genes (PMP22, MPZ, LITAF, EGR2, NEFL, KIF1B, MFN2, RAB7A, LMNA, MED25, TRPV4, GARS, HSPB1, HSPB8, AARS, GDAP1, DNM2, YARS, MTMR2, SBF2, SH3TC2, NDRG1, PRX, FGD4, FIG4, GJB1, PRPS1) and additional 179 genes. Genomic DNA was extracted from blood samples using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's standard procedure. The qualified genomic DNA sample was randomly fragmented by Covaris followed by library preparation by standard Illumina protocols. The adapter-ligated templates were purified by the Agencourt AMPure SPRI beads. Purified DNA was then amplified by ligation-mediated PCR (LM-PCR) on non-captured samples, purified, and hybridized to a customized solution-based capture system from Roche NimbleGen (Madison, USA) for 68-72 hours, captured fragments were bound to the strepavidin beads whereas non-hybridized fragments were washed out. Captured LM-PCR was then performed

to generate libraries for further analysis. Captured LM-PCR products were subjected to Agilent 2100 Bioanalyzer and ABI StepOne to estimate the magnitude of enrichment. After quality control, sequencing was then performed with the HiSeq2000 (Illumina, San Diego, USA) to produce paired-end reads (approximately 90 bp at each end) according to the manufacturer's instructions.

Data filtering, mapping and variant detection

When the entire run was completed, image analyses, error estimation and base calling were performed using the Illumina Pipeline (version 1.3.4). After removing the low quality reads, the remaining clean reads were aligned against the reference human genome database (NCBI37/hg19) using the BWA (Burrows Wheeler Aligner) software. SNPs and indels were identified using SOAPsnp software (http://soap.genomics.org.cn/) and the GATK Indel Genotyper (http://www.broadinstitute.org/gatk/), respectively. Previously identified SNPs were determined using the NCBI dbSNP or HapMap databases. Known disease-causing mutations were identified from the Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff (HGMD, http://www.hgmd.cf.ac.uk/), Leiden Open Variation Database (LOVD, http://www.lovd.nl/), Inherited Peripheral Neuropathies Mutation Database (http://www.molgen.ua.ac.be/CMTMutations/) or from previous literatures. All the variants were analyzed to obtain a frequency present in dbSNP, HapMap, 1000 Genomes Project and 100 control healthy Chinese individuals (local frequency). Novel missense variants were predicted to be damaging or not by SIFT

Genome tool (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). All the reference sequences in this study were based on the GRCh37/hg19 assembly of the human genome. Targeted sequence capture, next-generation sequencing and bioinformatics were completed by clinical laboratory of BGI-Shenzhen (Beijing Genomics Institute at Shenzhen, China).

Sanger sequencing

The potential mutated base and flanking sequence of exon 2 of GJB1 gene were amplified by polymerase chain reaction (PCR) and sequenced by Sanger sequencing. The sequencing results were compared with annotated GJB1 gene reference sequence (NG_008357.1) to confirm the candidate nucleotide variants. Nucleotide positions were determined according to the standard GJB1 gene reference sequence (GenBank accession number NM_001097642).

Abbreviations

CMT: Charcot-Marie-Tooth; NGS: next-generation sequencing; PCR: polymerase chain reaction; GJB1: gap junction protein β 1; CMT1X: X-linked Charcot-Marie-Tooth disease type 1; Cx32: connexin 32; CMAP: compound muscle action potential; DML: prolonged distal motor latency; MNCV: motor nerve conduction velocity; SNAP: sensory nerve active potential; SNCV: sensory nerve conduction velocity; NEMG: needle electrode electromyography

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Availability of data and materials

The datasets supporting the conclusions of this study are included within the paper. Using biological samples may be subject to further review by the ethics committee and may also be subject to individual participant consent.

Authors' contributions

DM and NZ performed the experiments, interpreted the data and drafted the paper. JC, CL and JZ recruited the family and provided genetics counseling. ZH, BD, WD, QY and NQ performed the experiments and analyzed the data. ZX designed the study and supervised the experiments. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was conducted with approval from the Ethics Committee of Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University. Written informed consent was obtained from participants.

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Figure legends

Figure 1 The pedigree of the Chinese family with CMT. Arrows indicates the proband.

Figure 2 Graph of the mean depth, median depth and sequencing coverage for the GJB1 gene.

A: The proband; B: The proband's father.

Figure 3 Visualization of c.44G>A mutation in GJB1 gene. The black arrows indicated the mutation.

A: A heterozygous mutation of c.44G>A in proband detected using Sanger sequencing;

B: A heterozygous mutation of c.44G>A in proband detected using NGS;

C: A hemizygous mutation of c.44G>A in the proband's father detected using Sanger sequencing;

D: A hemizygous mutation of c.44G>A in the proband's father detected using NGS.

Table 1 Motor nerve conduction studies in the proband and her father.

Motor nerve	The proband						The proband's father					
	Lat (ms)		Amp (mV)		CV (m/s)		Lat (ms)		Amp (mV)		CV (m/s)	
	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Medianus												
Wrist-APB	4.25	3.70	1.76	9.30			5.35	5.25	0.16	0.59		
Ulnaris												
Wrist-ADM	4.42	4.04	1.07	2.30			5.25	5.82	0.26	0.50		
Tibialis												
Ankle-Abd hal	7.27	6.94	0.86	0.54					NR			

Peroneus								
Ankle-EDB	5.17	5.47	1.70	0.19				NR
Fib head-Ankle	13.40	14.60	2.00	0.16	34.0	29.0		NR

Note: Lat, Latency; Amp, Amplitude; CV, Conduction velocity; APB, Abductor polllicis brevis; ADM, Abductor digiti minimi; Abd hal, Abductor hallucis; EDB, Extensor digitorum brevis; Fib head, Fibular head; NR, Not recordable.

Table 2 Sensory nerve conduction studies in the proband and her father.

Sensory nerve	The proband						The proband's father					
	Peak lat (ms)		Amp (μV)		CV (m/s)		Peak lat (ms)		Amp (μV)		CV (m/s)	
	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Medianus												
Dig I-Wrist	4.27	4.08	4.00	2.90	35.3	38.2	NR					
Dig III-Wrist	4.84	5.79	1.81	1.37	36.6	32.6	5.22	NR	2.1	NR	38.3	NR
Ulnaris												
Dig V-Wrist	4.43	4.21	2.70	1.72	30.3	36.1			NR			
Tibialis												

Dig I-Med mal	6.42	5.69	1.72	0.73	26.7	31.1	NR
Suralis							
Mid lower leg-Lat mal	NR						

Note: Peak lat, Peak Latency; Amp, Amplitude; CV, Conduction velocity; Dig, Digitorum; Med mal, Medial malleolus; Mid lower leg, Middle lower leg; Lat mal, Lateral malleolus; NR, Not recordable.

Figure 1

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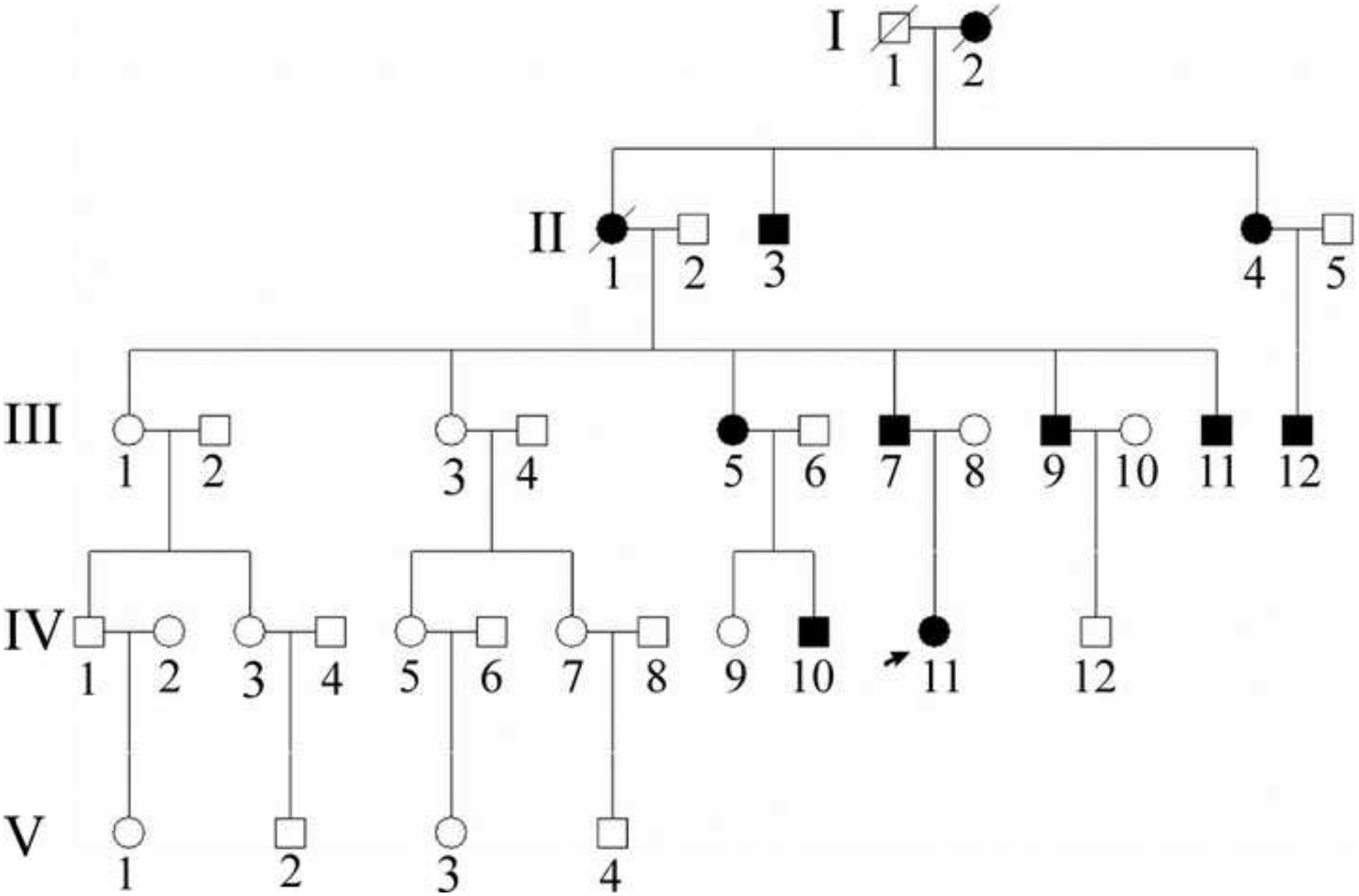

Figure 2

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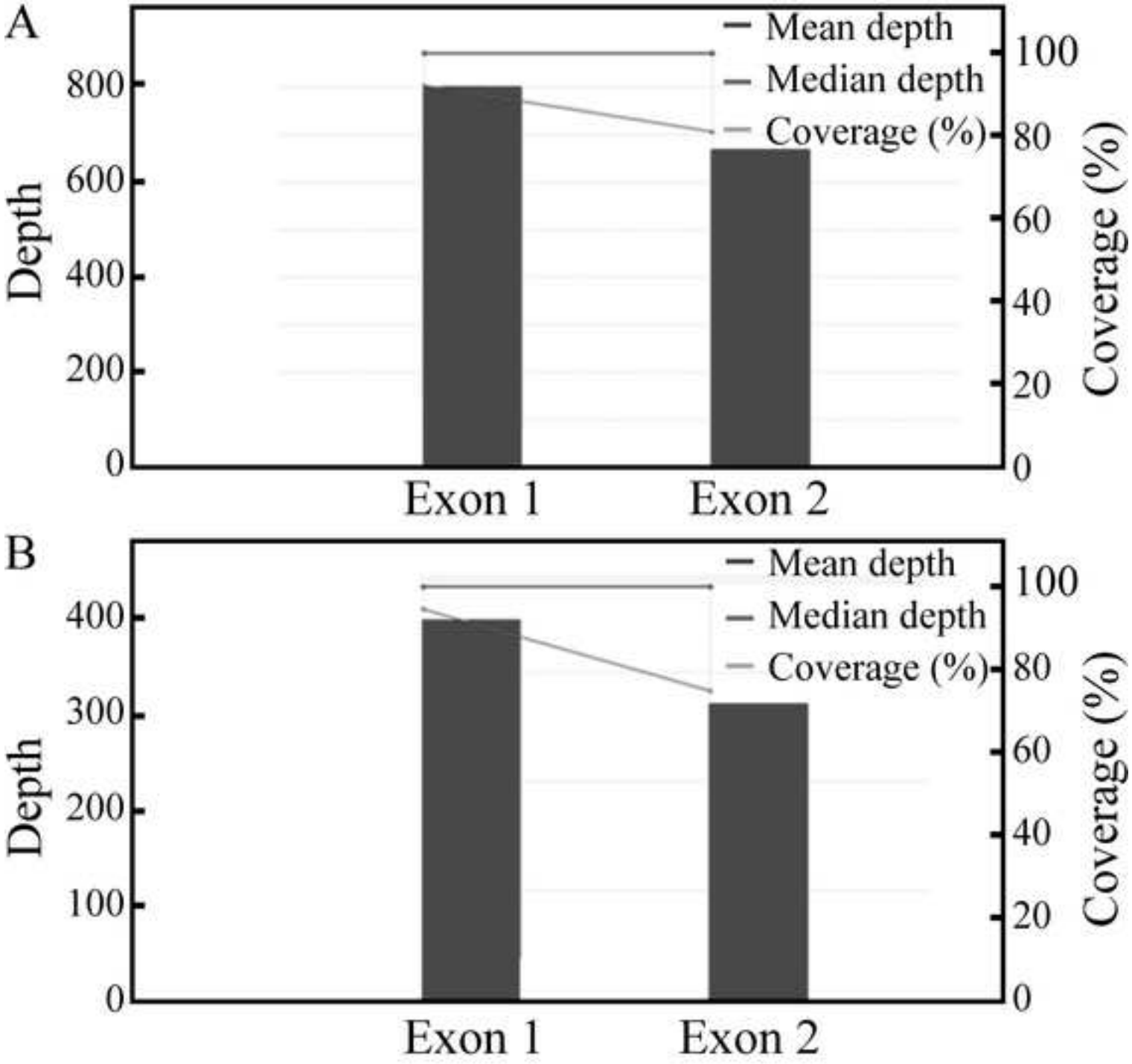

Figure 3

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