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Research article

Functional analysis of a novel intronic variant of *MCPH1* with autosomal recessive primary microcephaly

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ABSTRACT

Autosomal Recurrent Primary Microscopic (MCPH, OMIM: 251200) is a neurodevelopmental disorder that is characterized by a noticeable decrease in brain size, particularly in the cerebral cortex, but with a normal brain structure and a non-progressive intellectual disability. *MCPH1* has been identified as the gene that triggers primary microcephaly (MCPH1 , OMIM: 607117). Here we report a case of autosomal recessive primary microcephaly as caused by a novel variant in the *MCPH1* gene. Head circumference was measured by Magnetic Resonance Imaging (MRI), while the Wechsler Intelligence Scale was used to evaluate the intelligence of the individual being tested. B-ultrasound was used to assess gonadal development, and semen routine was used to assess sperm status. The whole-exome sequencing (WES) was performed on the proband. Sanger sequencing was conducted on the parents of the proband to determine if the novel variant in the MCPH1 gene was present. The effect of the mutation on the splicing of *MCPH1* was verified by minigene approach. It was observed that the proband had autosomal recessive primary microcephaly and azoospermatism. A novel splice-site homozygous mutation (c.233+2T > G) of the *MCPH1* gene was identified, which inherited from his parents. Minigene approach confirmed that c.233+2T > G could affect the splicing of *MCPH1*. Therefore, our findings contributed to the mutation spectrum of the *MCPH1* gene and may be useful in the diagnosis and gene therapy of MCPH.

1. Introduction

Autosomal recessive primary microcephaly (MCPH) is a rare neurogenic brain developmental disorder that exhibits genetic heterogeneity. It is characterized by a reduced head circumference at birth, minimal changes in brain structure, and varying degrees of cognitive impairment [1].People with MCPH are characterized by a congenital small cranium, as evidenced by an occipito-frontal head

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circumference (OFC) that is more than two standard deviations (SD) below the average for their age, sex, and ethnicity (severe microcephaly OFC <3 SD) [2]. This is accompanied by mild to moderate intellectual disability, mild seizures, simplified gyral pattern, periventricular neuronal heterotopias, polymicrogyria, speech delay, hyperactivity and attention deficit. MCPH is caused by mutations in several genes, such as *MCPH1, WDR62, CEP152, ASPM* and so on, which lead to a reduction in the number of cerebral cortex neurons formed during embryonic neurogenesis [3]. The *MCPH1* gene located 8p23.1 encodes a regulator of chromosome condensation. Mutations in the *MCPH1* gene have a pronounced influence on neural differentiation and the development of the brain, but its effects on other systems are rarely reported.

In our research, a novel homozygous mutation, c.233+2T > G, was discovered in the *MCPH1* gene of the proband, who presented with MCPH and azoospermatism. The deleterious effect of this mutation was further confirmed by minigene analysis. Our study broadened the mutation spectrum of the MCPH1 gene and may further contribute to the diagnosis and gene therapy of MCPH.

2. Materials and methods

2.1. Clinical evaluations

The proband was a 26-year-old man, who visited the Center of Reproductive Center, Guizhou Provincial People's Hospital in 2022, as a result of azoospermatism. Upon physical examination, it was observed that the proband had a small head circumference, a high ear position, a unibrow, and six deformed fingers on the right hand. Consequently, MRI, intelligence assessment, karyotype analysis and Whole Exome Sequencing (WES) were conducted. A written informed consent was obtained from the study participants before the study commenced. The study was conducted in accordance with the relevant regulations of Guizhou Provincial People's Hospital.

2.2. DNA extraction

The QIAamp DNA Blood Mini Kit (Qiagen, Germany) was utilized for extracting genomic DNA from the peripheral blood lymphocytes of the participants. Agarose gel electrophoresis was performed to assess the quality of the DNA, and the Qubit 2.0 fluorimeter (ThermoFisher Scientific) was used for quantifying the DNA.

2.3. Whole exsome sequencing and variant analysis

The proband was subjected to Whole Exon Sequencing (WES). Fragments of 100–700 bp were generated through the use of an ultrasonic disruptor (Covris-S220, USA), and a MyGenostics Standard Library Construction Kit (MyGenostics, Beijing, China) was then employed to construct the DNA library. Utilizing an Illumina Nextseq 500 sequencer (Illumina, San Diego, CA, USA), next-generation sequencing was executed. The sequenced reads were then mapped to the human reference genome (GRCh37) through the Burrows-Wheeler Aligner (BWA).

Subsequently, the pathogenicity of each genetic variant was evaluated using the Online Mendelian Inheritance in Man (OMIM) (https://www.omim.org/), the Human Genetic Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php), the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), and the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) guidelines.

2.4. Sanger sequencing

The specific PCR primers for the *MCPH1* mutation were designed by the Primer 5.0 software and synthesized by Sangon Biotech. The primers' sequences for NM_024596.5: c.233+2T > G were F:5′–GAATTGCTGGGGTAGAGGTTTT–3'; R:5′–AGTCCACCTTGCATACCCAG–3'. The amplified products were sequenced using an ABI 3730 Genetic Analyzer (Foster City, CA, USA). The sequence chromatograms were compared and visualized by the TBtools software.

2.5. Minigene approach

Genomic DNA of the proband and the control were used to amplify the minigene regions spanning MCPH1 exon 2–4 and intron 2–3 of the *MCPH1* gene respectively, employing primers with *Bam*HI/*Xho*I restriction sites (Fig. 3A). The amplified products were cloned into the pMini-CopGFP vector (Beijing Hitrobio Biotechnology Co., Ltd.) by using ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). The wild-type plasmid and the mutant plasmid were validated respectively by Sanger sequencing. The selected plasmids were prepared for further transfection. HEK293T cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (HyClone) and held in an environment of 37 °C and 5 % CO_2. HEK293T cells were transfected with recombinant plasmids using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Total RNA was extracted from cells cultured for 48 h with TRIzol reagent (Cowin Biotech Co.). Reverse transcription-polymerase chain reaction (RT-PCR) was conducted with a pair primer of MiniRT-F (5′-GGCTAACTAGAGAACCCACTGCTTA-3′) and MiniRT-R (5′- TAATTAGGCTTGATAAGTGTTCATTC-3′). PCR fragments were evaluated by agarose gel electrophoresis, and isoforms were identified through Sanger sequencing.

3. Results

3.1. Clinical features of the patient

On physical examination, the patient with azoospermatism was found to have a small head circumference, low hairline, high ear position, conjoined eyebrows, and deformity of six fingers of the right hand (Fig. 1A). Brain MRI with 3D-T1WI, T2WI, and T2WI-FLAIR sequence were performed. No significant abnormal signal was found in the brain MR imaging. The values of the brain main radius showed that the brain biparietal diameter (Fig. 1B), the occipital-frontal (Fig. 1C) and the head circumference (Fig. 1D) were 86.0 mm, 114.2 mm and 521.1 mm respectively. Brain segmentation software was used to calculate the volume of the key brain regions (Fig. 1E), and accounting for the percentage of total brain as follows: hippocampus 4.83 cm^3(0.75 %), frontal lobe 67.00 cm^3(10.42 %), temporal lobe 50.60 cm^3 (7.87 %), cerebral ventricle 7.94 cm^3(1.23 %), and basal ganglia 16.69 cm^3 (2.59 %). The proportion of the frontal lobe is very small, and the occipital-frontal head circumference is smaller than 2 standard deviations of the average expected for age, gender and population. Those radius and volume of the small frontal lobe confirmed that the patient is a microencephaly. The chromosome karyotype of the patient's peripheral blood was 46,X,Yqh-, but Y-chromosomal microdeletions were not observed. Follicle-stimulating hormone is elevated. The result of the semen examination revealed no sperm present. The superficial B-ultrasound of the scrotum revealed varying sizes for the left and right testicles (2.3 × 1.5 cm, 3.4 × 1.7 cm), with a regular shape and parenchymal echo, along with bilateral Varicocele (Fig. 1F). The Adult Wechsler Intelligence Scale revealed that the proband's IQ score was 68, indicating mild developmental delay.

The proband had difficulty learning and only completed primary school education. The parents do not exhibit any relevant clinical manifestations. The mother has given birth twice, with the first child being a female twin. Unfortunately, the sister of the twins died at the age of 1 and the reason remains unknown. The surviving twin sister is in good health (Fig. 2A).

3.2. Identification of a novel mutation in MCPH1

To identify the genetic cause of microcephaly and azoospermatism, WES was performed on the proband. WES detected a novel

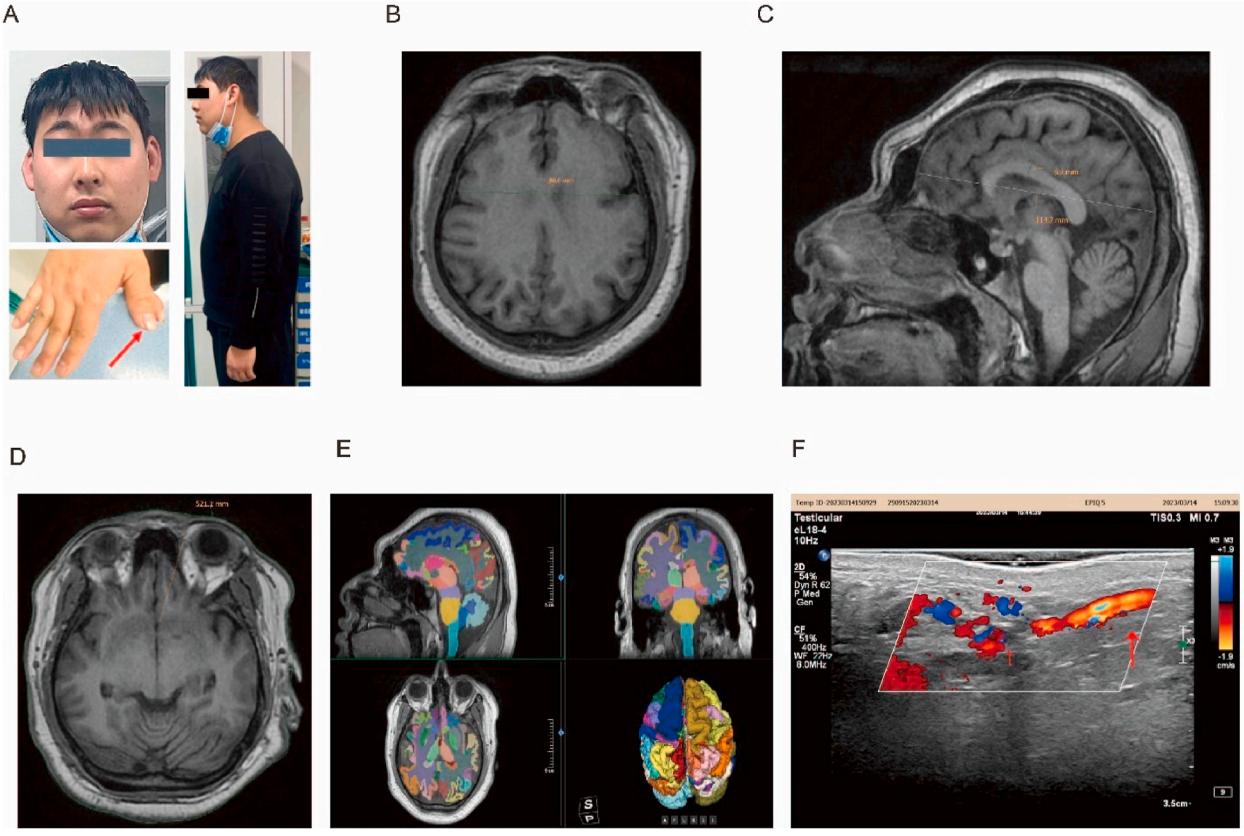

Fig. 1. Clinical presentation of the proband with microcephaly. A, physical examination of the proband. The red arrows indicate the probands with deformity of six fingers of the right hand; B, The brain biparietal diameter of the proband in brain MR imaging; C, The brain occipital-frontal diameter of the proband in brain MR imaging; D, The head circumference of the proband in brain MR imaging; E, Calculation of the volume of the key brain regions by brain segmentation software. F, The proband with superficial B-ultrasound of the scrotum.

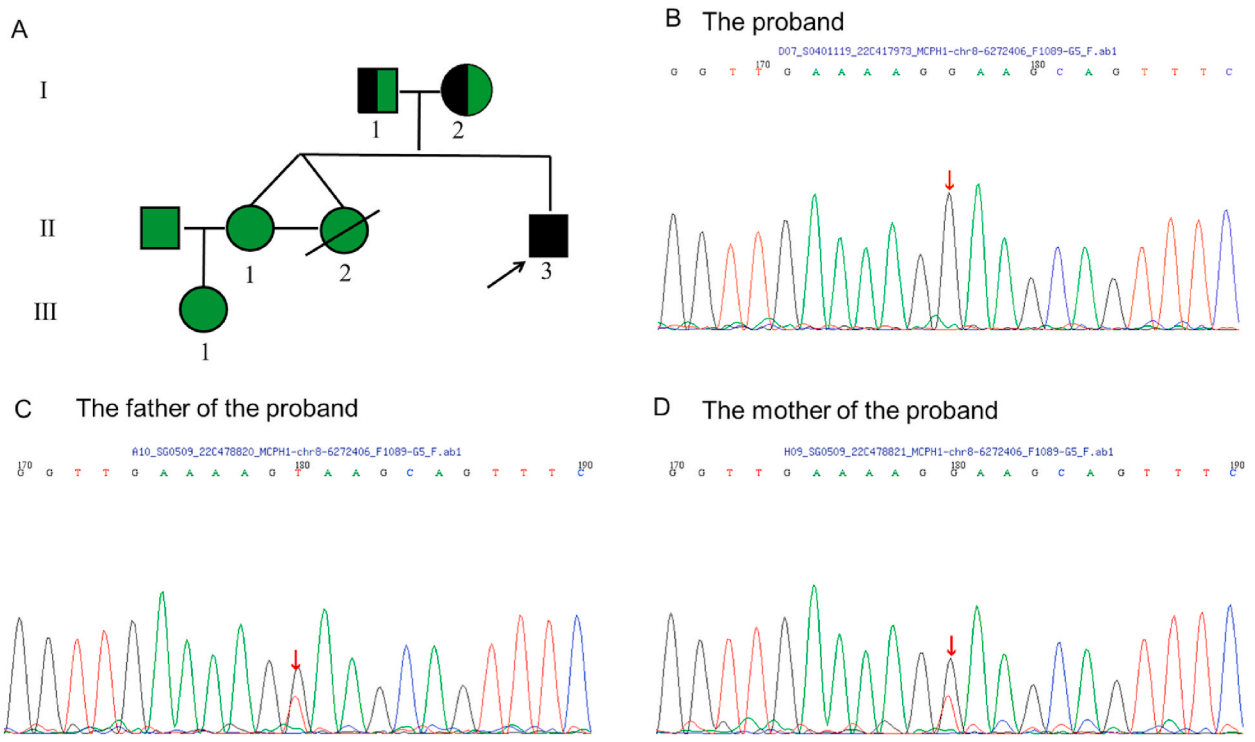

Fig. 2. Pedigree and sequence analysis of the proband. A, The pedigree of the Family. The arrows indicate the probands; B,C,D,The mutation c.233+2T > G of *MCPH1* was identified in the proband (II: 3), his father (I: 1), and mother (I: 2). The proband has a genotype of GG, while both his father and mother have a genotype of TG.

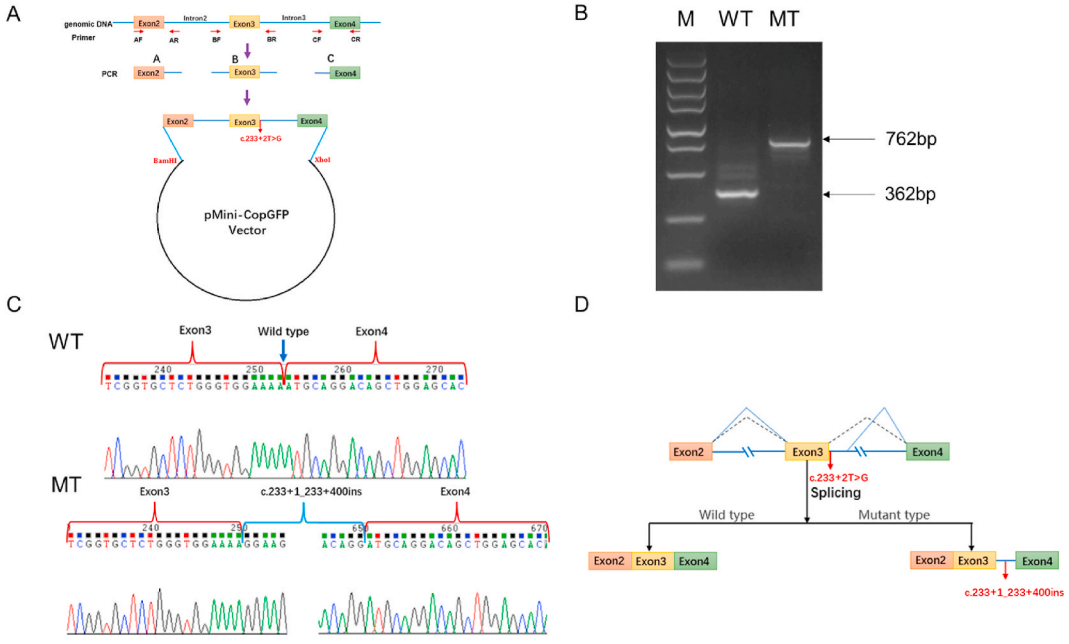

Fig. 3. Minigene assay for *MCPH1* c.233+2T > G mutation and schematic diagram of the splicing pattern. A, The construction of a Minigene trapping vector. B, Results from gel electrophoresis of RT-PCR demonstrated the presence of bands for wild-type and mutant-type. The agarose gel electrophoresis results showed that the PCR product of pMini-CopGFP-WT exhibited a band at 362 bp, whereas pMini-CopGFP-MT displayed a band at 762 bp. C, Analysis of the minigene product through sequencing showed that the wild-type minigene formed a normal mRNA, but the c.233+2T > G substitution of *MCPH1* caused a splicing abnormality, which eliminated the Intron 3 canonical splice site, leading to a 400bp insertion. D, The schematic diagram showed the splicing pattern of wild-type and mutant-type.

potential causative variant (c.233+2T > G) of the *MCPH1* gene. The parents of the proband were verified by sanger sequencing, and all of them were heterozygous variants of the sites (Fig. 2B,C,2D). No record of this mutation was found in the clinical disease-related database (ClinVar and HGMD) and it was not observed in the East Asian population of ExAC, 1000 Genomes, and the GnomAD database. This mutation was a classical splice mutation, which could lead to a decrease in the gene's functioning. The mutation was determined to be a likely pathogenic mutation according to the ACMG/AMP guideline based on the evidence of PVS1+PM2_Supporting + PM3_Supporting(hom).

3.3. *Effect of the splice-site mutation c.233+2T > G in MCPH1*

To investigate the effect of c.233+2T > G, we performed a minigene approach (Fig. 3A). Agarose gel electrophoresis indicated that the PCR product of pMini-CopGFP-WT had an amplified band at 362 bp, while pMini-CopGFP-MT had an amplified band at 762 bp (Fig. 3B).Sanger sequencing confirmed that the sequence of the wild plasmid conforms contains full exon2, exon3, and exon4. In comparison to the wild type, the mRNA product of the mutant plasmid is 400 bp longer in intron 3 (Fig. 3C). These results suggest that c.233+2T > G leads to abnormal splicing of *MCPH1* introns, resulting in the retention of 400bp sequences in mature mRNA (Fig. 3D).

3.4. *Bioinformatic analysis*

The location of the *MCPH1* c.233+2T > G mutation and the protein region where it is located are shown (Fig. 4A). To explore whether the random 400 bp insertion were associated with abnormal MCPH1, we performed 3D protein structure prediction on the wild-type and mutant-type amino acid sequences. The results showed that the mutant-type protein was shortened compared to the wild-type protein (Fig. 4B,C). We speculate that the mutation leads to premature termination of translation, resulting in protein truncation. the exact mechanism underlying the mutation requires further investigation.

4. Discussion

The incidence of MCPH varies from 1:30,000 to 1:250,000 depending on the population. The dramatic reduction in the size of the brain, which has an IQ of 30–80 percent of the average normal person, mainly affects the prefrontal cortex, which otherwise displays normal brain structure [4,5].

The cause of MCPH is the deletion or mutation of proteins involved in the formation of the centrosome or spindle. This disruption affects the cell cycle, hinders DNA replication, impairs cell proliferation, interferes with cell differentiation, and triggers apoptosis in neural precursor cells. As a consequence, the number of neurons is reduced, leading to a smaller brain size [6]. At last, microcephaly is manifested. The symptoms exhibited by the proband in this study were in line with those commonly observed in patients with MCPH. These symptoms included a smaller brain size, a simplified pattern of gyrus morphology, and an IQ of 68, which indicated mild

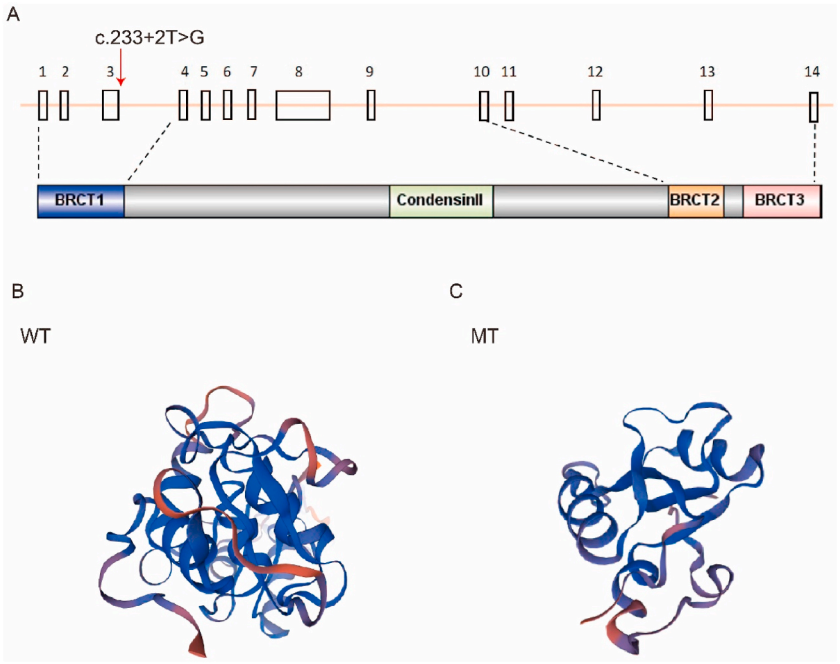

Fig. 4. The *MCPH1* c.233+2T > G mutation resulting in truncated proteins. A, Locations of c.233+2T > G in the *MCPH1* gene and protein structure. Red arrows indicate the positions of the mutation. B, Predicted wild-type protein; C, Predicted mutant-type protein.

intellectual disability.

MCPH is a disorder that is caused by a variety of genes, making it genetically heterogeneous. Considering the various molecular mechanisms of MCPH, it has been divided into 1–30 subtypes. Various subtypes are characterized by different pathogenic genes. Thirty OMIM genes related to MCPH have been identified (Supplementary Table 1). These genes encode proteins that are indispensable for neurogenic programming, as they are responsible for cell cycle checkpoints and cell signaling. Approximately 40 % of mutations are located in the *ASPM* gene, and these mutations, which are either nonsense or frameshift in nature, lead to a non-functional protein and consequently MCPH5 [7]. Also the gene *MCPH1* is one of the most commonly mutated genes and was the first locus identified as being responsible for MCPH1 [8].The gene *MCPH1*, with total length of 241 kb, is located on chromosome 8 (8p23.1) and consists of 14 exons. It contains 835 amino acids with ~110 kDa molecular weight and exists in two major subtypes whose were similar in fetal tissue without cell type specificity, but both were subject to antagonistic regulation during the cell cycle [9]. MCPH1 is composed of three BRCT domains, located at the N- terminal domain (1–93 aa), and at the C- terminal domain (672–730 aa) and (751–833 aa) [10,11]. The BRCT domain is a conserved region which interacts with phosphopeptides and is commonly found in proteins associated with DNA damage response signaling and cell cycle regulation. The DNA damage response is a critical factor for the successful progression of brain development and the prevention of age-related neuropathy. In the event of damage to the BRCT domain, the corresponding clinical manifestations will be present.

A significant amount of data has been previously reported on pathogenic variants associated with MCPH1 in microcephaly, including missense, nonsense, and frameshift mutations. But there are only two cases of splicing in MCPH1 with MCPH in the literature. Reports of affected individuals associated with MCPH1 mutation are compiled in a single list (Table 1). Most of mutations are located in Exon 1–6, especially missense mutations of MCPH1 are located in Exon 2 and 3, which encode the N-terminal BRCT domain, indicating that this domain is essential for brain development [12]. But Naseer [13] reported that two new variants of the *MCPH1* gene (c.982G > A and c.1273T > A) were discovered in individuals with microcephaly in exon 8. Papoulidis [14] observed two sequential pregnancies that were complicated by extreme microcephaly due to a homozygous mutation of the *MCPH1* gene which were c.348del heterozygous from parents. The phenylalanine amino acid at position 116 of the protein is replaced by the leucine amino acid due to this mutation, which changes the reading frame and leads to an early stop codon at position 145 of the protein. Subsequently, the protein forms are rendered unable to execute their function. Two different splicing variations of MCPH1 have been identified to date: c.436+1G > T and c.322-2A > T, both of which result in severe MCPH. In our study, the c.233+2T > G gene mutation in MCPH1 was identified, which caused a 400 bp sequence of Intron 3 to be retained in the mature mRNA, resulting in a truncated protein. It is speculated that this mutation causes the breakdown of the N-terminal BRCT domain, leading to functional impairments. The impaired MCPH1 gene leads to a lack of neural production, resulting in a diagnosis of the proband with microcephaly.

Moreover, the proband complained of infertility in the study. A semen analysis revealed azoospermia, and a B-ultrasound showed bilateral varicocele. It has been reported that MPCH1 plays an important role in gonad development. MCPH1 is expressed in high levels in the testes, which are responsible for the initial production of numerous spermatogonial stem cells during sperm production. Mutations in *MCPH1* may have an influence on fertility [28]. It was determined that the central domain of MCPH1 is imperative for gonad development in mammals, and it also suppresses microcephaly [28]. Liu [9] generated a mouse model with a deletion of the domain (Mcph1-ΔBR1 mice). Primary microcephaly was observed in mutant mice, and their reproductive organs were blocked, with testis atrophy and the absence of ovaries being the defining characteristics. Due to impaired MCPH1, male mutant mice display a condition characterized by thinning of testicular tubules and loss of spermatocytes, resulting in chromosome synapse failure, meiosis arrest, and apoptosis. It was established that the N-terminal BRCT domain plays an essential role in the functioning of MCPH1, which is responsible for the regulation of brain size, gonad development, and other cellular processes. However there has been limited research on MCPH1 with azoospermia. Our research has uncovered that the c.233+2T > G mutation in MCPH1 is responsible for the retention of a 400 bp sequence of Intron 3 in the mature mRNA. This aberration may result in the disruption of the N-terminal BRCT domain, potentially explaining the occurrence of azoospermia in our patient. While there have been no previous reports documenting azoospermia in individuals with MCPH1, further investigation is warranted to gain a more comprehensive understanding of this issue.

5. Conclusion

Our research ultimately showed that the novel splice-site mutation (c.233+2T > G) in MCPH1 was the likely pathogenic variant in the proband, as it was observed to be present in homozygosity and associated with microcephaly and azoospermia. In a word our findings contributed to expanding the mutation spectrum of the MCPH gene and may be useful in the diagnosis and gene therapy of MCPH.

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Guizhou Provincial People's Hospital.

Funding

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Table 1
Mutations in the MCPH1 gene with MCPH.

Base change	Location	Predicted protein or splicing alteration	References
c.74C > G	Exon 2	Ser25Term	[15]
c.136C > T	Exon 3	Gln46Term	[16]
c.302C > G	Exon 4	Ser101Term	[17]
c.1651G > T	Exon 8	Glu551Term	[18]
c.80C > G	Exon 2	Thr27Arg	[19]
c.215C > T	Exon 3	Ser72Leu	[17]
c.147C > G	Exon 2	His49Gln	[17]
c.149T > G	Exon 2	Val50Gly	[17]
c.223T > C	Exon 3	Trp75Arg	[17]
c.64G > A	Exon 2	Glu22Lys	[20]
c.2180C > T	Exon 12	Pro727Leu	[5]
c.1349A > C	Exon 8	Lys450Thr	[21]
c.151A > G	Exon 3	Ile51Val	[17]
c.664T > C	Exon 7	Cys222Arg	[22]
c.928G > A	Exon 8	Val310Ile	[13]
c.877A > G	Exon 8	Ser293Gly	[23]
c.1273T > A	Exon 8	Tyr425Asn	[13]
c.236G > T	Exon 4	Cys79Phe	[24]
c.436+1G > T	Intron5	Donor splice site	[17]
c.322-2A > T	Intron4	Acceptor splice site	[25]
c.1869_1870delAT	Exon 9	Cys624Ter	[23]
c.566dupA	Exon 6	sn189LysfsTer15	[17]
c.1254delT	Exon 8	Asp419IlefsTer24	[3]
c.321dupA	Exon 4	Arg108ThrfsTer2	[20]
c.1179delG	Exon 8	Arg393SerfsTer50	[26]
c.348delT	Exon 5	Phe116LeufsTer30	[14]
c.427dupA	Exon 5	Thr143AsnfsTer5	[17]
c.586delC	Exon 7	Gln196LysfsTer2	[27]

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Shulin Luo: Software, Resources, Data curation. **Lingyan Ren:** Writing – review & editing, Writing – original draft, Validation. **Rongping Wang:** Data curation. **Jianxin Hu:** Formal analysis, Data curation. **Wei Wei:** Formal analysis, Data curation. **Yurong Feng:** Supervision, Conceptualization. **Shengwen Huang:** Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30285.

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