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ORIGINAL ARTICLE

Exome sequencing and RNA analysis identify two novel *CPLANE1* variants causing Joubert syndrome

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Abstract

Background: Joubert syndrome (JS) is a genetically heterogeneous disorder; its genetic etiology involves more than 35 genes, and a limited number of studies have investigated the pathogenic mechanism of variants in patients with JS. RNA splicing analysis is critical to determine the functional significance for noncanonical splicing variants.

Methods: Whole exome sequencing was performed to screen the causative gene variants in a JS family. Sanger sequencing was used to verify the variants. cDNA PCR products were analyzed and functional experiments were performed to determine the pathogenicity of the variants.

Results: The clinical phenotypes and *CPLANE1* variants in the JS patient were analyzed and proved consistent. We identified two novel heterozygous variants of *CPLANE1* in the proband first, including c.4459del (frameshift variant) and c.7534-14G > A (intronic variant). We analyzed the pathogenic consequences of the 2 variants and classified the c.4459del as likely pathogenic according to the ACMG/AMP guidelines; however, the pathogenic significance of c.7534-14G > A was uncertain. Furthermore, we performed RNA splicing analysis and revealed that the noncanonical splicing variant (c.7534-14G > A) caused aberrant exon 37 skipping. It produced an aberrant transcript that was predicted to encode a C-terminal truncated protein.

Conclusions: The genetic variation spectrum of JS caused by *CPLANE1* was updated. Two novel variants further deepened our insight into the disease's molecular mechanism and confirmed the significance of diagnostic whole-exome sequencing.

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KEYWORDS
aberrant splicing, *CPLANE1*, diagnostic whole exome sequencing, Joubert syndrome, novel variants

1 | INTRODUCTION

Joubert syndrome (JS; OMIM 213300) is a neurodevelopmental disorder caused by dysfunction of the primary cilia (Parisi et al., 2007). It is a rare autosomal recessive condition marked by a distinctive cerebellar and brainstem defect on cranial MRI known as the "molar tooth sign" (MTS), which is the key diagnostic feature (Maria et al., 1999). Several other more variable symptoms in JS include skeletal features such as polydactyly, retinal dystrophy and colobomas, cystic kidney disease, and congenital hepatic fibrosis; many of these are also shared by other ciliopathy conditions (Bachmann-Gagescu et al., 2015; Vilboux et al., 2017). It is estimated that the prevalence of JS was 1:80,000 to 1:100,000 (Kroes et al., 2016). JS is a genetically heterogeneous disorder, with the development of next generation sequencing; more than 35 genes had been reported associated with JS (Parisi, 2019; Vilboux et al., 2017).

CPLANE1 (OMIM 614571, also known as C5orf42) gene has been reported as an important causative gene of JS (Alazami et al., 2012; Radha Rama Devi et al., 2020; Srour et al., 2012); it encodes a transmembrane protein that contains two transmembrane domains, two predicted coiled coil domains, and a Joubert syndrome-associated domain (Romani et al., 2015). The variants in *CPLANE1* result in about 8–14% of JS patients (Kroes et al., 2016). Some researchers proved that *CPLANE1* may participate in the process of ciliogenesis (Hong et al., 2019).

Since strong genotype–phenotype correlations are known for JS, a definitive molecular diagnosis will help the JS family in preventing the birth of a JS child through prenatal or preimplantation genetic diagnosis. Whole exome sequencing (WES) is a more comprehensive way for JS molecular diagnosis (Tsurusaki et al., 2015), but sequencing data annotation often focuses on the exon and 10 bp of flanking intronic sequence. In our study, we considered the case where a couple had given birth to a JS baby that had undergone expanded carrier screening for recessive diseases, for which the result was negative; then, WES test in Canada by GeneDx company was also performed and only found one pathogenic variant (*CPLANE1*c.4459del) was found which could not explain the cause of JS, since *CPLANE1* is autosomal recessive inheritance pattern. To help the family, we attempt diagnostic WES and expanded the range of data interpretation to 30-bp of flanking intronic sequence; we finally identified the c.7534-14G > A variant and proved that it is a likely pathogenic variant through RNA analysis.

2 | METHODS

2.1 | Sample collection and genomic DNA extraction

One non-consanguineous Chinese family with JS was considered from the International Peace Maternity and Child Health Hospital. The proband (III-1) was a fetus that had been aborted. The JS diagnosis was produced according to the results of prenatal screening, obstetric examination, and autopsy report. Genomic DNA was extracted from the muscular tissue of the proband and peripheral blood of pedigree members. All subjects signed an informed consent form allowing anonymous use of their DNA samples and clinical data for research purposes.

2.2 | Ethical compliance

The study was approved by the ethics committee at International Peace Maternity and Child Health Hospital.

2.3 | Whole exome sequencing (WES) and variant screening

Whole-exome sequencing library construction and sequencing were performed using Illumina platform by Beijing Genomics Institute (BGI) according to the manufacturer's protocols. A sequencing library targeting all coding regions, canonical splice sites, and at least 30-bp of flanking intron sequences were generated.

Variants were filtered with a frequency less than 0.01 or 0.05 for dominant or recessive inherited JS, respectively, in the genome Aggregation Database (gnomAD). It is worth noting that synonymous variants and intron variants in 30-bp segments flanking the exons were also analyzed for impacts on splicing by prediction software of spliceAI (https://spliceailookup.broadinstitute.org/) and varSEAK (https://varseak.bio/).

2.4 | Sanger sequencing verification and pedigree segregation analysis

The nomenclature of variants is according to the Human Genome Variation Society (HGVS) standards (http://www.hgvs.org/mutnomen/). Two novel *CPLANE1* (NM_023073.3; ENST00000425232) variants of c. 4459del (GRCh37/hg19, chr5-37,184,810-T-) and c.7534-14G > A (GRCh37/hg19, chr5-37,164,443-C-T) were detected in the affected member. Variant validation was performed by direct Sanger sequencing, and segregation analysis of all available family members was also evaluated. Sequences of the primers used to confirm the c.4459del, p.(Ser1487Valfs*3) variant by Sanger sequencing was *CPLANE1*_4459-F: 5′-AGGAAATGATGTCTGTTGTC-3′ and *CPLANE1*_4459-R: 5′-TATTCTTATGAGTGGAGG GG-3′. Sequences of the primers used to confirm the c.7534-14G > A, p.(?) variant by sanger sequencing was *CPLANE1*_7534_14-F: 5′- CAAGAGCATGTTTAGAG GCA-3′ and *CPLANE1*_7534_14-R: 5′- TGGGCAGAAATG TAGGTAGC-3′.

2.5 | RNA extraction and splicing analysis

QIAamp RNA Blood Mini kit (Germany) was used for RNA extraction from the muscular tissue of the proband and whole blood cells of his family members. Then, total RNA was reverse transcribed using a reverse transcription system (Takara). The primer sequences used for cDNA amplification were 5′- GAA CAAGGTGATGCTGGACAC-3′ (forward) and 5′- CAGT CAAAGGCTCATGTTCTGG-3′ (reverse). The PCR amplification products were analyzed by agarose gel electrophoresis and Sanger sequencing.

TABLE 1 Clinical features of the patient with *CPLANE1* (NM_023073.3) variants

Patient ID	**III-1**	
Age	Termination of pregnancy at 22 weeks	
Sex	Male	
First trimester screening	Nuchal translucency (NT)	4.2 mm ↑
	PAPP-A	0.6 MoM ↑
	hcg	0.96 MoM ↑
	Risk for T21	1:7
Brain	Brain vermian hypoplasia (vermian craniocaudal diameter 9.4 mm at 22 weeks) Posterior fossa cyst Dandy–Walker malformation Mild ventriculomegaly (10.1–12.9 mm) Corpus callosum foreshortened Subependymal nodular heterotopia Agenesis olfactory bulbs and tracts Cerebral cortical maturation abnormality, widespread Hippocampal dysplasia Subcortical and periventricular neuronal hetertopias Hypothalamic hamartoma Fragmented cerebellar roof nuclei	
Face	Micrognathia Mild hypertelorism Short philtrum Cleft palate Lobulated tongue Synophrys Low nasal bridge	
Eye	Eyes posterior coloboma	
Hands and feet	Polydactyly involving all 4 extremities	
Urinary tract	Genitourinary tract echogenic kidneys, bilateral	
Endocrine	Focal cytomegalic change in adrenal	
Developmental delay	(−)	
Bone	Short long bones	

3 | RESULTS

3.1 | Clinical features

The proband (III-1) was a fetus that was diagnosed as JS according to routine obstetric examination and an autopsy report from Mount Sinai Hospital in Toronto, Canada. The proband showed typical clinical features of the hereditary JS, for example, brain vermian hypoplasia, hypothalamic hamartoma, lobulated tongue, eyes with posterior coloboma, polydactyly involving all 4 extremities, and long bone dysplasia. All clinical features and first trimester screening results are listed in Table 1.

3.2 | Pedigree analysis and genetic studies revealed 2 novel CPLANE1 variants

We performed WES on the proband and validated the variants in all family members by using Sanger sequencing to determine the genetic basis of JS in this family. The proband's father (II-1), mother (II-2), and the generation before them (I-1 to I-4) are healthy. The pedigree and Sanger sequencing results are shown in Figure.1a and Figure.1b.

Compound heterozygous variant NM_023073.3:c.[4459del];[7534-14G > A] in *CPLANE1* was found in the proband (Figure.1c). The frameshift variant, c.4459del (p.Ser1487Valfs*3), was inherited from his father and predicted to generate a truncated protein or undergo nonsense mediated mRNA decay (NMD). This variant is a novel variant that had never been reported as a causative of JS and will damage the coiled coil domains and Joubert syndrome-associated domain of *CPLANE1*. The c.4459del variant is extremely rare in gnomAD total population with an allele frequency of 0.000004019 (1/248824). Therefore, the novel c.4459del variant is classified as likely pathogenic (PVS1 + PM2_Supporting) according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines and ClinGen specifications (Zhang et al., 2020). The c.7534-14G > A was also a novel variant and inherited from the mother.

Significantly, the JS family had undergone a genetic test in Canada by GeneDx company and only found the c.4459del likely pathogenic variant; however, one likely pathogenic variant cannot explain the cause of JS, since *CPLANE1* is an autosomal recessive inheritance pattern. So, the family was referred to our hospital for genetic counseling. Through diagnostic WES, we found the c.7534-14G > A variant first; the variant was not present in the Human Gene Variant Database, but is it a pathogenic variant that can explain the causes of JS in this family? We needed more evidence.

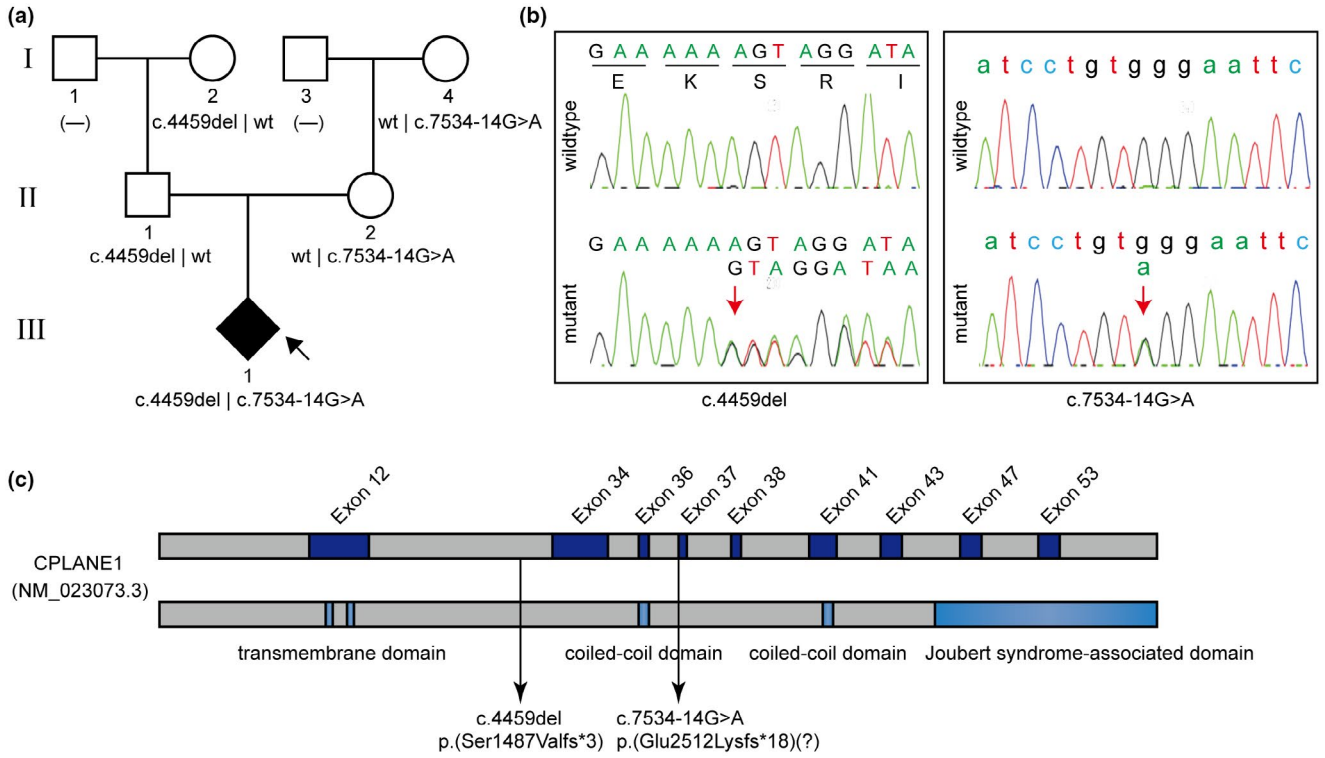

FIGURE 1 Pedigree, genetic findings, and functional domain of the variants. (a) The pedigree of the JS family shows two novel variants. (b) Sequence chromatograms exhibiting 2 heterozygous variations in *CPLANE1*. (c) CPLANE1 protein structure and the locations of 2 novel variants

3.3 | A novel splicing variant in CPLANE1 causes exon skipping

Computationally assisted analyses with the spliceAI tool and varSEAK tool revealed that c.7534-14G > A variant in *CPLANE1* had a moderate and strong impact on splicing, respectively (Figure 2a). Since in silico tools cannot always predict splicing defects correctly, RNA analysis was performed to confirm the predictions. To evaluate the effect of c.7534-14G > A in *CPLANE1*, the spanning exon cDNA amplification product was analyzed by agarose gel electrophoresis. Two transcripts were observed in cDNA PCR products from patient I-4, while one transcript of that was from patient I-3 and the healthy control (Figure 2b). Direct sequencing of cDNA PCR products revealed that the c.7534-14G > A variant caused skipping of exon 37 (55 bp), which would introduce a premature termination codon (Figure 2c). Exon 37 skipping would lead to either nonsense-mediated decay (NMD) or early translational termination of *CPLANE1* (p.Glu2512Lysfs*18). Then, the variant will destroy the coiled coil domain and Joubert syndrome-associated domain of *CPLANE1*. In Figure 2d, we showed the effect of c.7534-14G > A variant on exon 37 skipping. According to our research, the novel c.7534-14G > A variant is classified as likely pathogenic (PS3 + PM2_Supporting+PM3 + PP3).

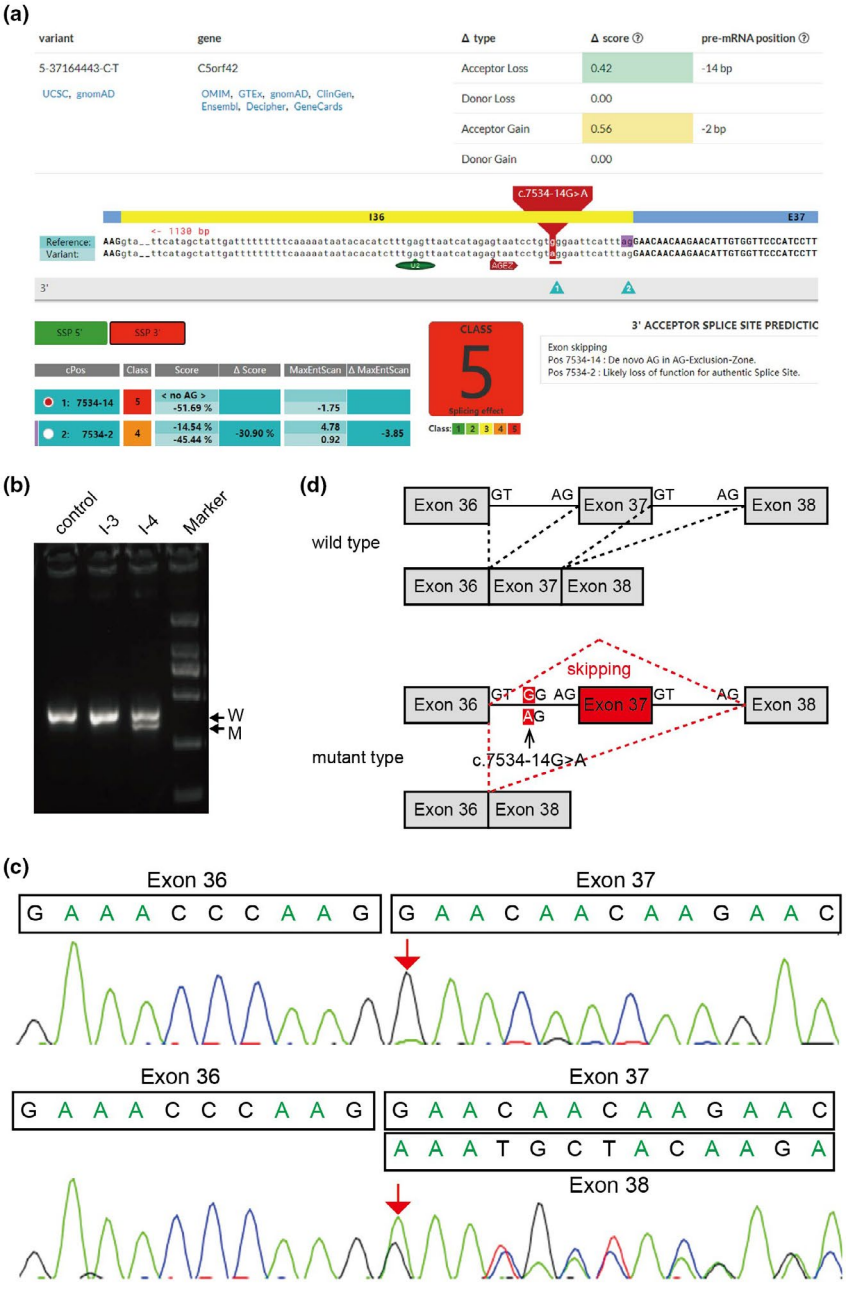

FIGURE 2 The c.7534-14G > A variant is associated with aberrant splicing of *CPLANE1* exon 37. (a) In silico bioinformatics tools of spliceAI and varSEAK predicted that the effect of c.7534-14G > A variant on splicing was moderate and strong, respectively. (b) Agarose gel electrophoresis image of cDNA PCR products from healthy control, patient I-3 and patient I-4. W: Wild transcript. M: Mutated transcript. (c) Sequencing result of cDNA PCR products from healthy control (upper) and patient I-4 (lower). (d) Schematic representation of aberrant splicing and skipping of exon 37 due to variant in the c.7534-14G > A site

4 | DISCUSSION

Since 2012, more and more variants of *CPLANE1* have been identified in JS families (Liu et al., 2020; Zhu et al., 2021). WES has proved to be a powerful tool for the identification of novel variants in JS (Koyama et al., 2017). In our study, the JS family had undergone expanded carrier screening for recessive diseases, the result was negative, and routine WES also could not explain the cause of JS. Eventually, diagnostic WES shed light on molecular pathogenesis. We can take the cue from the process.

First, expanded carrier screening for recessive diseases is not suitable for families with a history of the genetic disease (Kraft et al., 2019). Most current guidelines recommend only offering it to healthy women and their partners who are planning a pregnancy (Chokoshvili et al., 2018). Since expanded carrier screening panels often focus on childhood-onset diseases that are likely to have a significant impact on the child's quality of life (Committee Opinion No, 2017; Henneman et al., 2017), it is not comprehensive for families with a history of genetic diseases.

Second, routine WES often focuses on exons and 10-bp flanking sequences;, maybe it is not enough nowadays. Multiple researchers have proved that variation in deep intronic sequences can cause monogenic disorders through influence splicing (Torrado et al., 2018; Wang et al., 2020). To minimize the loss of detectable splice donor and acceptor variants, 30-bp of flanking sequences may be taken into consideration in sequencing data analysis.

Third, c.7534-14G > A variant that we screened out first is a variant of uncertain significance (VUS) in clinical. This may be the reason that the variant is undetected and unreported during the previous detecting. Although the fact that VUS represent a challenge in current genetic testing and genetic counseling, additional in-depth research may be needed to confirm the VUS's pathogenicity (Oulas et al., 2019). There is a pressing need for more accurate ways to identify the VUS that are truly pathogenic (Pottinger et al., 2020). Despite the fact that it is controversial whether VUS should be returned to patients, we thought it is necessary, at least in families with a strong family history of genetic diseases. (Fatkin & Johnson, 2020).

Interestingly, we hypothesized that the c.7534-14G > A variant will induce a nonsense-mediated decay (NMD) pathway, because it caused skipping of exon 37 (55 bp) and led to early translational termination of *CPLANE1* [p.(Glu2512Lysfs*18)]. However, the functional experiments proved that NMD had not happened. Our findings imply that the mechanism of the NMD pathway is complex (Hug et al., 2016); and that more research is required to fully elucidate it.

5 | CONCLUSIONS

We are the first to report 2 novel variants in *CPLANE1* . The c.4459del variant is extremely rare in the gnomAD. The c.7534-14G > A variant has not been reported in public databases. The functional experiment revealed that the c.7534-14G > A variant would destroy the coiled coil domains and Joubert syndrome-associated domain of CPLANE1.

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Not applicable.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Junyu Zhang designed this study. Hongjun Fei is responsible for data collection and statistical analysis. Yi Wu and Yanlin Wang paticipated in sample collection and clinical counseling. Junyu Zhang and Hongjun Fei drafted this paper, revised the manuscript, and supervised all the work.

CONSENT FOR PUBLICATION

Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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