Original Article
Whole exome sequencing facilitated the diagnosis in four Chinese pediatric cases of Joubert syndrome related disorders

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Abstract: Objectives: Joubert syndrome is a spectrum of rare genetic disorders, mainly characterized by a distinctive cerebellar and brain stem malformation called the “molar tooth sign” (MTS), hypotonia, and intellectual disability/developmental delay. Methods: In this study, 4 pediatric cases with developmental delay and oculomotor abnormalities were recruited, and submitted to a clinical evaluation and magnetic resonance imaging (MRI) examination. Afterwards, genetic detection with whole exome sequencing (WES) was conducted on the 4 patients. Results: Imaging results demonstrated cerebellar dysplasia in all probands, yet the MTS findings varied in severity. WES detected diagnostic variations in all four probands, which were distributed in four genes, namely CC2D2A, NPHP1, AHI1, and C5orf42. Two variants were newly identified, which were the CC2D2A: c.2444delC (p.P815fs*2) and the AHI1: exon (15-17) del. In silico analysis supported the pathogenicity of the variations in this study. Conclusions: Our findings expanded the mutation spectrum of Joubert syndrome related disorders, and provided solid evidence to the affected families for further genetic counseling and pregnancy guidance.

Keywords: Joubert syndrome, CC2D2A, NPHP1, AHI1, C5orf42

Introduction
Joubert syndrome (JBTS, MIM #213300), representing a spectrum of rare congenital conditions, was first described by Marie Joubert in 1969 [1] and is characterized mainly by hypotonia, intellectual disability/developmental delay, and a distinctive cerebellar and brain stem malformation called the “molar tooth sign” (MTS) [2]. Other additional features in JBTS patients consist of abnormal respiratory pattern and oculomotor apraxia [3]. In general, the breathing abnormalities improve with age, truncal ataxia develops over time, and acquisition of gross motor milestones is delayed; cognitive abilities are variable, ranging from severe intellectual disability to normal [2, 4]. Some JBTS patients may have multisystem organ involvement including retinal dystrophy, ocular colobomas, occipital encephalocoele, renal abnormality, hepatic fibrosis, oral hamartomas, polydactyly, and endocrine abnormalities [4, 5]. The term “Joubert syndrome and related disorders” (JSRD) was extensively used in recent years because of the strong clinical heterogeneity of these conditions [2].

Estimatedly, the incidence of JBTS ranges between 1/80,000 and 1/100,000 in live births [2]. So far, over 40 genes have been recognized to be responsible for JBTS, in which the vast majority conform to the autosomal recessive inheritance pattern, except for one (the OFD1 gene) which is X-linked [4, 6, 7]. All of the gene products localize in and around the primary cilium, rendering JBTS a canonical ciliopathy [6]. In about 62%-97% of individuals with clinical manifestations of JSRD, a molecular diagnosis can be established via analyzing these genes, depending on specific detection efficiency in various studies [4, 8, 9].
These pleiotropic features are typical of a number of disorders of the primary cilium, and make the identification of causative genes challenging given the significant overlap between JBTS and other ciliopathy conditions such as nephronophthisis and Meckel, Bardet-Biedl, and COACH syndromes [3]. Encountering such situations, molecular detection with next generation sequencing has shown robust capacity in the diagnosis of JSRD [7], while clinical and imaging profiling is still of importance to the analysis of subtle differences in phenotypes and their causes [10].

Here in the present study, four young patients with typical clinical and imaging indications of JSRD were recruited in the outpatient department of our center. A comprehensive clinical evaluation and genetic detection using whole exome sequencing (WES) was conducted to explore the causative variations.

Material and methods

This study was approved by the Ethics Committee of Shijiazhuang Obstetrics and Gynecology Hospital (approval No. 20210068), and written informed consent was obtained from all parents of the participants.

Subjects

Between Jun 2016 and Aug 2020, four young children with mental, motor developmental delay of unknown origin were referred to our center with their parents. We carried out a thorough clinical survey including cerebral magnetic resonance imaging (MRI). Subsequently, the peripheral blood samples of all four pedigrees were collected for the following genetic detection.

Genomic DNA extraction

Peripheral blood was collected from the patients and their parents. Genomic DNA was extracted using the QIAamp DNA Blood Mini-Kit (Qiagen Sciences, USA), and the DNA quality was validated by 1% agarose gels and Qbit DNA Assay Kit in Qubit 2.0 Flurometer (Life Technologies, CA, USA).

Whole-exome sequencing (WES)

WES was carried out as previously described [11]. Briefly, the enrichment of the exonic sequences was conducted by the Sure Select Human Exon Sequence Capture Kit (Agilent, USA). The sequencing libraries were quantified using the Illumina DNA Standards and Primer Premix Kit (Kapa Biosystems, USA), and were massively parallel-sequenced using the Illumina Novaseq6000 platform. After sequencing and filtering out the low-quality readings, the high-quality reads (with quality level Q30>89%) were compared to the human genome reference sequence [hg19]. The GATK software was used to identify suspected pathogenic variants (https://software.broadinstitute.org/gatk/). The CNV variants were called by a routine protocol (https://cnvkit.readthedocs.io/en/stable/pipeline.html). The variations were identified by sequence alignment with the NCBI Reference Sequence using Chromas v2.33. The pathogenicity of the identified variants was then assessed according to the common guidelines issued by the American Association of Medical Genetics and Genomics (ACMG) [12] referring to multiple databases (1000g2015aug_eas, https://www.internationalgenome.org/; ExAC_EAS, http://exac.broadinstitute.org; gnomAD_exome_EAS, http://gnomad.broadinstitute.org/); HGMD®: Human Gene Mutation Database (Professional Version 2019.4) with the Enliven® Variants Annotation Interpretation system (Berry Genomics, China).

Validation by Sanger sequencing and fluorescent quantitative PCR (qPCR)

The suspected diagnostic variant was validated by Sanger sequencing using ABI 3730 Automated Sequencer (Applied Biosystems, USA) according to the manufacturer’s protocol. qPCR was also carried out to verify the deletion variants identified in Case 2 and 3 (detailed methods in Supplementary Material 1).

Analysis of missense variants

The evolutionary conservatism of amino acids (AAs) residue affected by specific missense variants was analyzed using MEGA7 (http://www.megasoftware.net) with default parameters.

Results

Clinical manifestations

Patient 1, a boy first born to his family, was referred to our center due to a delayed mile-
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stone at 7 months (hypotonia; could not sit alone; poor head control). The general physical examination result was normal, except for a slight strabismus. Plasma biochemical test, screening for markers of genetic metabolic disease (GMD) and EEG showed no abnormalities. Cerebral MRI indicated that the vermis of cerebellum was smaller, the middle cerebellum fissure was deeper, and the extracerebral space of bilateral frontal part was wider (Figure 1A-C). Follow-up showed that the patient had learned to walk at 15 months, had normal gross motor and language development, had fair fine hand movements, and had the same social and adaptive abilities as children of the same age.

Patient 2, male, the second child in his family, was referred to our outpatient department due to hypotonia and motor retardation at 8 months. The general physical characterization was normal, yet his eyeballs were slightly cohesive. Biochemical, GMD, EEG results were all normal. MRI result generally suggested that the patient had cerebellar dysplasia (Figure 1D-F). Follow-up found that the boy learned to walk at 14 months, his motor and language development was fair, yet the ocular cohesiveness still existed.

Patient 3, male, was referred to our outpatient department due to hypotonia, with poor visual fixation and chasing ability at 2+ months. The general physical characterization was normal, except for the eye movements. Biochemical, GMD, EEG results were all normal. Follow-up found that the patient could only walk 5-6 meters independently, and only speak simple geminate words at 2.5 years old. MRI revealed the cerebellar dysplasia (Figure 1G-I).

Patient 4, male, referred to our outpatient department due to abnormal eye movement at 1 month. The general physical characterization was normal, except that his eyes squinted to one side when he looked at things. Biochemical, GMD, EEG results were all normal. Follow-up to the age of 3 revealed that strabismus was always present, and his motor and language development was slightly behind that of his peers. MRI revealed the cerebellar dysplasia (Figure 1J-L).

Genetic variations

The pedigree diagrams of the enrolled families were depicted in Figure 2 (A for Family 1; D for Family 2; F for Family 3; I for Family 4). Overall, all 4 probands had positive genetic results. Four variations composed by 7 variants were detected, confirming to an autosomal recessive inheritance pattern in all 4 patients. The details of all variants, including the ACMG pathogenic level, are demonstrated in Table 1.

To be specific, Patient 1 carried a compound heterozygous variation in the CC2D2A gene consisting of 2 variants, c.2444delC (p.P815fs*2) and c.4238G>A (p.C1413Y) (NM_001080522) (Figure 2B, 2C); Patient 2 harbored a homozygous deletion in the NPHP1 gene (exon 1-20) (Figure 2E); Patient 3 had a compound heterozygous variation in AHI1 gene consisting of c.910dupA (p.T304Nfs*5) (NM_017651) and AHI1: exon (15-17) del (Chr6: 135751004-135754394) (Figure 2G, 2H); while Patient 4 had a compound heterozygous variation in the C5orf42 gene consisting of 2 variants, c.4006C>T (p.R1336W) and c.3551G>A (p.R1184H) (NM_023073) (Figure 2J, 2K). Two variants were newly identified in this study, namely the CC2D2A: c.2444delC (p.P815fs*2) and the AHI1: exon (15-17) del.

Validation with Sanger sequencing and qPCR demonstrated that the variants these patient carried were all inherited from their asymptomatic heterozygous carrier parents, as demonstrated in pedigree diagrams. The detailed results of qPCR validation were included in Supplementary Material 1.

Conservatism analysis of missense variants

In this study, three missense variants were detected, which were CC2D2A: c.4238G>A (p.C1413Y), C5orf42: c.4006C>T (p.R1336W) and C5orf42: c.3551G>A (p.R1184H). We analyzed the AAs they affected. Results indicated that all 3 AAs maintained evolutionary conservatism among species (Figure 3).

Discussion

Early clinical differential diagnosis of Joubert syndrome (JBTS) requires profound pediatric experience, as JBTS has overlapping phenotypes with many primary cilium disorders, such as nephronophthisis, Meckel, Bardet-Biedl, and COACH syndromes [13-17]. In this study, 4 patients were all younger than 12 months old with the preliminary and developing indications, which added to the difficulty of
Figure 1. MRI results of the 4 patients. A-C. Patient 1. A. T2WI coronal plane: line-like cerebrospinal fluid between the bilateral cerebellar hemispheres with long T2 signal “midline fissure sign”, the upper part of the fourth ventricle “bat wing”. B. T1WI coronal plane: the midbrain and the enlarged upper cerebellar peduncle form a “grinding sign”. C. T1WI sagittal plane: absence of inferior cerebellar vermis. D-F. Patient 2. D. T2WI coronal plane: line-like cerebrospinal fluid between the bilateral cerebellar hemispheres with long T2 signal “midline fissure sign”, the upper part of the fourth ventricle “bat wing”. E. T1WI coronal plane: the midbrain and the enlarged upper cerebellar peduncle form a “grinding sign”. F. T1WI sagittal plane: absence of inferior cerebellar vermis. G-I. Patient 3. G. T2WI coronal plane: the upper part of the fourth ventricle is “bat wing-shaped”, and the middle part expands into a triangle. H.
T1WI coronal plane: the midbrain and the enlarged upper cerebellar peduncle form a “grinding sign”. I. T1WI sagittal plane: the enlarged upper cerebellar peduncle is almost perpendicular to the midbrain. J-L. Patient 4. J. T2WI coronal plane: line-like cerebrospinal fluid between the bilateral cerebellar hemispheres with long T2 signal “midline fissure sign”, the upper part of the fourth ventricle “bat wing”. K. T1WI coronal plane: the midbrain and the enlarged upper cerebellar peduncle form a “grinding sign”. L. T1WI sagittal plane: the enlarged upper cerebellar peduncle is almost perpendicular to the midbrain.

Figure 2. Genetic variants in the 4 patients. A-C. Patient 1. A. The pedigree diagram; B. Sanger sequencing result for CC2D2A: c.2444delC heterozygous variant; C. Sanger sequencing result for CC2D2A: c.4238G>A heterozygous variant. D, E. Patient 2. D. The pedigree diagram; E. The homozygous deletion of NPHP1 exon (1-20) by the schematic of capture efficiency. F-H. Patient 3. F. The pedigree diagram; G. Sanger sequencing result for AHI1: c.910dupA (p.T304Nfs*5) heterozygous variant; H. The heterozygous deletion of AHI1 exon (15-17) by the schematic of capture efficiency. I-K. Patient 4. I. The pedigree diagram; J. Sanger sequencing result for C5orf42: c.3551G>A (p.R1184H) heterozygous variant; K. Sanger sequencing result for C5orf42: c.4006C>T heterozygous variant.

diagnosis. Furthermore, the core appearance indication of all 4 patients was oculomotor abnormity. Their MRI results showed typical cerebellar dysplasia, but the MTS varied. This may be because the MTS finding is itself a spectrum and can have milder types [18], such
## Table 1. Information of the variants identified in this study

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Variant No.</th>
<th>Gene (transcript)</th>
<th>Genomic alteration</th>
<th>Protein alteration</th>
<th>Frequencies in 3 databases*</th>
<th>Revel score*</th>
<th>HGMD* level (PMID*)</th>
<th>ACMG* level (evidence)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>CC2D2A (NM_001080522)</td>
<td>c.2444delC</td>
<td>p.P815fs*2</td>
<td>0; 0; 0</td>
<td>/</td>
<td>/</td>
<td>Likely pathogenic (PVS1+PM2)</td>
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<tr>
<td>2</td>
<td>2</td>
<td>NPHP1</td>
<td>del exon (1-20)</td>
<td>null</td>
<td>/</td>
<td>0.306</td>
<td>DM (27491411)</td>
<td>Likely pathogenic (PM3.Strong+PM3+BP4)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>AHII (NM_017651)</td>
<td>c.910dupA</td>
<td>p.T304Nfs*5</td>
<td>0; 0; 0</td>
<td>/</td>
<td>DM (multi studies)</td>
<td>Likely pathogenic (PVS1+PM2)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>AHI1 (NM_017651)</td>
<td>del exon (15-17)</td>
<td>uncertain</td>
<td>/</td>
<td>/</td>
<td>DM (16453322)</td>
<td>Pathogenic (PVS+PS1+PM2)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>C5orf42 (NM_023073)</td>
<td>c.4006C&gt;T</td>
<td>p.R1336W</td>
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<td>0.273</td>
<td>DM (22425360)</td>
<td>Likely pathogenic (PM2+PM3)</td>
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<tr>
<td>6</td>
<td>6</td>
<td>C5orf42 (NM_023073)</td>
<td>c.3551G&gt;A</td>
<td>p.R1184H</td>
<td>0; 0; 0</td>
<td>0.797</td>
<td>DM (25407461)</td>
<td>Likely pathogenic (PM2+PM3+PM5+PP3)</td>
</tr>
</tbody>
</table>

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Figure 3. Evolutionary conservatism of 3 amino acids affected by the missense variants in this study. A. The C1413 residue of CC2D2A protein. B. The R1184 residue of C5orf42 protein. C. The R1336 residue of C5orf42 protein.

as those caused by mutations in the C5orf42 [19], which is consistent with the situation in our study (Case 4). In other cases, owing to the lack of examination on multiple MRI cuts for subtle findings of MTS and vermis hypoplasia, even experienced neuroradiologists may miss this hallmark [4].

Since the first gene for JSRD, NPHP1, was identified in 2004 [18], over 40 genes have been implicated in its causation accounting for 65%-75% cases [3, 4, 7]. All of the genes identified so far are localized to or play a role in the function of the sub-cellular structure, the primary cilium, especially at the transition zone of the cilia where it joins the plasma membrane [20]. In our study, 4 patients were positive with diagnostic variations in different genes, reflecting the strong genetic heterogeneity of JBTS. Patient 1 had a compound heterozygous variation in CC2D2A, which encodes a component of a protein complex in the basal body, a ring-like structure that functions in the transition zone at the base of cilia and acts as a barrier to restrict protein diffusion between the plasma and ciliary membranes [21]. In this variation, the c.4238G>A (p.C1413Y) variant was reported to be associated with nephronophthisis-related ciliopathy [22], and indexed as “disease causing” in HGMD. The patient described by Kang et al. did not have extra-renal phenotype [22], which was quite different from Patient 1 in this study. This phenotypic difference was probably owing to the novel variant, c.2444delC (p.P815fs*2), on the other allele. As for Patient 2, the homozygous NPHP1 deletion he carried was considered as the most frequent genetic defect for nephronophthisis, particularly with neurologic involvement [23]. Therefore, attention should be paid to the renal manifestations in cases like Patient 2 in future management. Like CC2D2A, AHI is also a component of the protein complex in the basal body, a ring-like structure that functions in the transition zone at the base of cilia [21]. The variation caused the symptoms of Patient 3 consisted of a sequence variant and an exonic deletion, which were inherited from the parents, respectively. The AHI: c.910dupA (p.T304Nfs+5) variant was reported to cause J BTS [24]; in terms of the micro deletion containing exon 15-17, although it has not been identified before, another ~3.4 kb deletion covering the exon 14-16 of AHI1 was reported.
to cause JBTS [25], which supports the pathogenicity of the variant in the present study. The 2 variants detected in Patient 4, c.4006C>T (p.R1336W) and c.3551G>A (p.R1184H) in C5orf42 gene, have both been reported, yet with different conditions. The c.4006C>T variant was identified in a French Canadian case of JBTS [26], while the c.3551G>A was associated with a much rarer JBTS subtype, the oral-facial-digital type VI syndrome, characterized by preaxial and mesoaxial polydactyly, hypothalamic hamartoma and other congenital defects along with neurological indications [27]. Using a mouse model, Damerla et al. found that C5orf42, which they called Jbts17, colocalized with Nphp1 in the ciliary transition zone [28]. In C5orf42-knocked-down Xenopus embryos, Toriyama et al. observed ciliopathy-related developmental defects, including failure of neural tube closure, defective Hedgehog signaling, and defective left-right patterning [29]. Moreover, Hong et al. found that human C5orf42 was required for cilia assembly in ciliated cells [30]. Since in all cases, the genes involved in the diagnostic variations fit an autosomal recessive inheritance pattern, those couples still have a 25% risk of having a future pregnancy affected. Therefore, more preventive management, such as prenatal diagnosis or pre-implantation diagnosis, should be recommended [5, 31]. The main limitation of this study was the small sample size included, which made it impossible to trace the association between genotype and phenotype. In addition, necessary functional experiments or biophysical analyses may help to clarify the impact of specific variants.

In conclusion, we identified the causative variations, which were distributed in various genes including CC2D2A, NPHP1, AH1, and C5orf42, in 4 cases of Joubert syndrome related disorders. Our findings not only extended the spectrum of JSRD mutations, but also highlighted the capability of WES to identify different types of variants.

Acknowledgements

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Disclosure of conflict of interest

None.

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## Contents of qPCR validation

### Methods

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<th>Table S1. PCR Primers for qPCR</th>
</tr>
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<tbody>
<tr>
<td><strong>Primers</strong></td>
</tr>
<tr>
<td>NPHP1-exon5-F</td>
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<tr>
<td>NPHP1-exon5-R</td>
</tr>
<tr>
<td>NPHP1-exon11-F</td>
</tr>
<tr>
<td>NPHP1-exon11-R</td>
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<tr>
<td>AHI1-exon15-F</td>
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<tr>
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</tr>
<tr>
<td>AHI1-exon16-F</td>
</tr>
<tr>
<td>AHI1-exon16-R</td>
</tr>
<tr>
<td>β-globin-QF</td>
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<tr>
<td>β-globin-QR</td>
</tr>
</tbody>
</table>

**Reaction reagents and condition**

The expression level was assessed by real-time Quantitative Fluorescence PCR using SYBR Premis Ex Taq II (Perfect Real Time) (Takara) with ABI 7500 system. Data are presented as mean ± standard deviation of three independent real-time PCR experiments. The PCR cycle was as follows: 10 min 95°C, 1 cycle; 10 s 95°C, 30 s 60°C + fluorescence acquisition, 55 cycles. Values for each gene were normalized to expression level of beta-actin gene (ACTB) via the 2-ΔΔCT method.

**Results**

As demonstrated in the figures below, qPCR verified the CNV calling by WES.

**Figure S1.** The qPCR results for exon5 and 11 in the NPHP1 gene on the samples from members of Case 2 and controls.
Figure S2. The qPCR results for exon15 and 16 in the AHI1 gene on the samples from members of Case 3 and controls.