Novel *CC2D2A* compound heterozygous mutations cause Joubert syndrome

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Abstract. Joubert syndrome (JS) is an autosomal recessive disorder, which is characterized by hypotonia, ataxia, psychomotor delay, and variable occurrences of oculomotor apraxia and neonatal breathing abnormalities. JS is clinically and genetically heterogeneous. The present study investigated a typical JS family. The 'molar tooth sign' was observed in the proband through magnetic resonance imaging. Other symptoms of JS include cerebellar vermis hypoplasia/dysplasia, oculomotor apraxia and intellectual disability. High-throughput sequencing revealed that JS was caused by coiled-coil and C2 domain containing 2A (*CC2D2A*) compound heterozygous mutations. One *CC2D2A* allele was affected with a missense mutation, c.2581G>A, which led to a p.Asp861Asn amino acid replacement. The other allele was affected with a c.2848C>T nonsense mutation, which resulted in a truncated CC2D2A protein (p.Arg950Ter). Both of these alterations are novel. Further investigation indicated that the proband's father was the c.2581G>A carrier, whereas the mother was the c.2848C>T carrier. These results indicated that JS in the proband was caused by novel compound heterozygous mutations in *CC2D2A*, which were inherited from both parents. These findings may be used to establish prenatal molecular diagnostic criteria, which may be beneficial in future pregnancies.

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Introduction

Joubert syndrome (JS) is a recessive or X-linked genetic disorder, which is characterized by hypotonia, apnea/hyperpnea in infancy, oculomotor apraxia, psychomotor delay/mental retardation, and cerebellar vermis hypoplasia and dysplasia. JS is accompanied by brainstem abnormalities, which result in radiological detection of the 'molar tooth sign' (1-6). JS can be classified into six phenotypic subtypes, based on other clinical abnormalities detected alongside JS, including occipital encephalocele, polymicrogyria, kidney lesions, polydactyly, hepatic fibrosis and ocular coloboma (6). JS has an incidence of 1 in 80,000-100,000 individuals in the United States (6). Several of the JS phenotypes are similar to those associated with Meckel syndrome (MKS) (7-9), which is a lethal recessive disorder that is characterized by renal cystic dysplasia; occipital encephalocele, or other central nervous system phenotypes; polydactyly and hepatic fibrosis. Therefore, some researchers consider JS and MKS to be different phenotypes of the same disease. JS is clinically and genetically heterogeneous, and overlaps with several other ciliopathies, including nephronophthisis, Senior-Loken syndrome and MKS (10). To date, >30 causative genes (11,12) have been identified in JS.

Coiled-coil and C2 domain containing 2A (*CC2D2A*) was initially described in JS and MKS in 2008, and further investigations have elucidated the role of *CC2D2A* in ciliary function (13-19). Previous studies have linked JS, and other syndromic disorders, to defective cilia (14,16,20-22). The present study identified a JS family with novel compound heterozygous mutations in *CC2D2A*.

Case report

Ethical permission for genetic analysis and collection of test data in the present study was granted by the Research Ethics Committee at Zunyi Medical University (Zunyi, China). Written informed consent was obtained from the participants or participants' family. The proband was an 8-month-old male who was diagnosed with JS before being subjected to cerebral magnetic resonance imaging (MRI) using a 1.5 Tesla MRI scanner (Siemens AG, Munich, Germany). The patient matched the JS diagnotic criteria: Cranial MRI demonstrated brainstem 'molar tooth sign' (Fig. 1) (6,23). Further

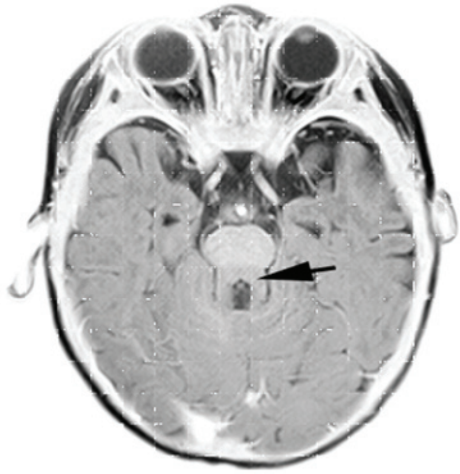

Figure 1. 'Molar tooth sign' was observed, indicating brainstem abnormalities caused by cerebellar vermis hypoplasia, in the proband through magnetic resonance imaging. The 'molar tooth sign' is indicated by an arrow.

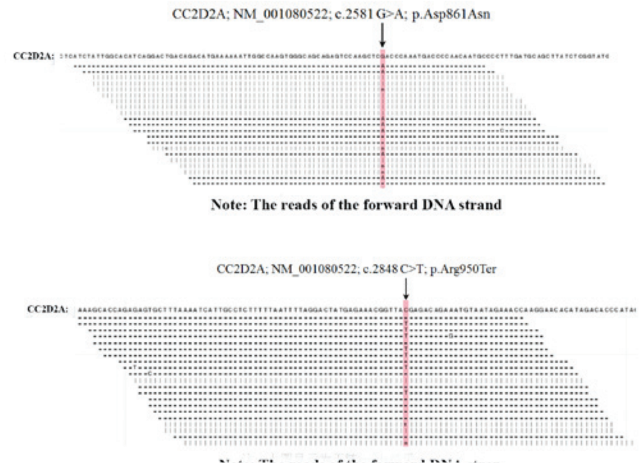

Figure 2. Compound heterozygous mutations identified in DNA isolated from blood samples collected from the proband through high-throughput exome sequencing. (A) One allele of the *CC2D2A* gene was mutated with c.2581G>A, which results in the replacement of Asp with Asn. (B) The other allele of *CC2D2A* has a c.2848C>T mutation, which generates an immediate stop code and leads to a truncated CC2D2A protein. *CC2D2A*, coiled-coil and C2 domain containing 2A; Asp, aspartic acid; Asn, asparagine.

examinations indicated that the proband exhibited hypotonia, psychomotor delay/intellectual disability, nystagmus and jerky eye movements. Other tests, including complete blood count, blood biochemistry, serum lactate, urine ammonia, plasma and urine amino acids, and metabolic screening of blood, were conducted in our laboratory.

Upon JS diagnosis, 10 μg DNA was extracted from a blood sample collected from the proband according to the protocol of the Invitrogen genomic DNA extraction kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), in order to determine what genetic alterations caused the disease. High-throughput exome sequencing was performed on the DNA samples obtained from the proband by Shenzhen BGI Diagnosis Technology Co., Ltd. (Shenzhen, China). The sequencing results revealed that the proband possessed *CC2D2A* compound heterozygous mutations (c.2581G>A/c.2848C>T), and demonstrated that two *CC2D2A* alleles were mutated (Fig. 2). The c.2581G>A mutation is located on exon 21, whereas the c.2848C>T alteration is situated on exon 23 of the gene. The c.2581G>A mutation results in an amino acid replacement (p.Asp861Asn), whereas the c.2848C>T mutation leads to an immediate stop code (p.Arg950Ter), which results in a truncated form of the *CC2D2A* protein. To determine the source of the mutations, the proband's parents and grandparents were recruited for genetic examination. Furthermore, the mother was pregnant (6 months) and also decided to screen the fetus. An MRI analysis indicated that the female fetus displayed molar tooth sign. Blood samples were collected for DNA extraction from the proband's parents, grandparents, and the proband. The present study screened for mutations in the samples obtained from the proband's available parents and grandparents using routine polymerase chain reaction (PCR) and DNA sequencing on exons 21 and 23. The PCR primers were designed and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). PCR was performed according to the manufacturer's protocol [Thermo Scientific Dream Taq Green PCR Master Mix (2X); #K1081; Thermo Fisher Scientific, Inc.]. Briefly, 50 pg patient DNA was mixed with 1 μM PCR primers and 25 μl Taq Green PCR Master

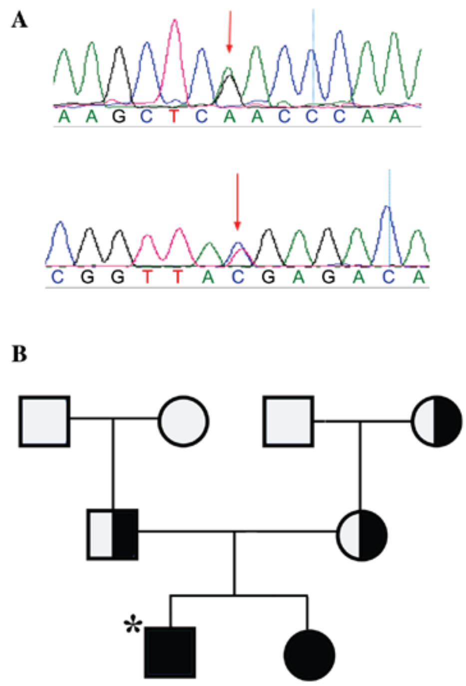

Figure 3. c.2581G>A and c.2848C>T mutations are inherited in the JS family. (A) The c.2581G>A mutation was detected in the father's DNA, whereas the c.2848C>T mutation was identified in the mother's and grandmother's DNA. (B) Pedigree of the JS family. *, proband; square, male; circle, female; full black square and circle, affected individual; half black square and circle, carriers; blank square and circle, normal individuals.

Mix; nuclease-free water was added to ensure the final volume was 50 μl. The mixture was initially denatured for 3 min at 95˚C; PCR was then conducted for 35 cycles at 95˚C for 30 sec, 58˚C for 30 sec and 72˚C for 1 min; finally, the PCR products underwent extension at 72˚C for 5 min. DNA sequencing was carried out by Shenzhen BGI Diagnosis Technology Co., Ltd. The PCR primer sequences were as follows: Exon 21, forward

5'-GCACTTTTATGCACTGGACAGA-3', reverse 5'-CAG CACCCATGTTCTTAAACAG-3' (496 bp); exon 23, forward 5'-TGTGGCCTCTATTTCTTCCTGA-3'; reverse, 5'-GCT AAGTGGCTTGCATAAGCTC-3' (470 bp). PCR sequencing indicated that both parents were *CC2D2A* mutation heterozygotes: The father carried the c.2581G>A mutation, whereas the mother carried the c.2848C>T mutation (Fig. 3). The proband's parents displayed normal phenotypes due to the heterogeneity of the mutations. In addition, the proband's maternal grandmother was also a carrier of the c.2848C>T mutation.

The mother's pregnancy was terminated following detection of the molar tooth sign *in utero*. Fetal brain tissue was collected post-termination for genetic and histological analysis. Similar to the proband, the female fetus was demonstrated to possess the *CC2D2A* compound heterozygous mutations through PCR and DNA sequencing using post-termination brain tissue. With the exception of the molar tooth sign, no observable abnormalities were detected in the fetal cerebellar vermis, based on routine hematoxylin-eosin staining, probably due to the young age of the fetus (data not shown). In some cases, JS is accompanied with defects in other organs, such as the kidney and liver. The present study conducted histological and immunohistochemical (IHC) analyses using CC2D2A antibody (cat. no. 22293-1-AP; ProteinTech Group, Inc., Rosemont, IL, USA) on these fetal tissues post-termination. Briefly, collected tissues were fixed in 4% paraformaldehyde for 24 h. The tissues were then embedded in paraffin, according to the embedding machine manufacturer's protocol. Each paraffin block was cut into 5 μm sections for hematoxylin-eosin (H&E) staining and IHC analyses. Paraffin sections were deparaffinized and hydrated using xylene and a graded series of alcohol. For H&E staining, the tissue sections were stained with 0.5% hematoxylin and 0.5% eosin. For IHC, tissue section antigen retrieval was conducted using a citrate-based Antigen Unmasking Solution (cat. no. H-3300; Vector Laboratories, Inc., Burlingame, CA, USA). IHC was conducted using the Vectastain Universal Elite ABC kit (cat. no. PK-6200; Vector Laboratories, Inc.) according to the manufacturer's protocol. CC2D2A antibody was used at a 1:200 dilution, and tissues were incubated with it for 30 min at room temperature, following antigen retrieval and blocking with normal horse serum for 1 h. Subsequently, tissue sections were incubated with diluted anti-rabbit biotinylated secondary antibody (provided in the ABC kit) for 30 min. All the operations were performed at room temperature. In the fetal kidney samples, besides increased chronic inflammation and abnormal glomeruli, no renal cysts were observed. In addition, hepatic fibrosis was not observed in the affected fetal liver samples. Renal and hepatic involvement in JS is a progressive condition, which can be completely asymptomatic for several years; therefore, renal cysts/hepatic fibrosis may not appear at all while the patients develop this progressive disease. Therefore, in the fetus, it is unsurprising that no JS-related defects were observed in the kidney and liver at the age of 6 months.

As aforementioned, routine IHC analysis was used to examine CC2D2A protein expression in these tissues, and all tissues were found to be CC2D2A positive, thus indicating that abnormal/truncated CC2D2A proteins are produced by mutated *CC2D2A* alleles (data not shown).

Discussion

The present study identified a JS family with novel compound heterozygous mutations in *CC2D2A*. Both mutations detected are not present in the 1000 Genomes database (1000genomes.org) or the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/), thus indicating that they are novel *CC2D2A* mutations. Since the proband's parents are mutation carriers and not affected, both mutations are causative based on the recessive genetic two-hit theory.

In the present family, JS was caused by novel compound heterozygous mutations in *CC2D2A* inherited from the parents. These findings may be used to establish prenatal molecular diagnostic criteria, and may be beneficial in future pregnancies. In the present study, the proband's mother was pregnant and was subject to prenatal diagnosis based on the family history. The pregnancy was terminated at the parents' choice due to the confirmed compound heterozygous *CC2D2A* mutations and phenotypic defects.

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