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Research article

Clinical characteristics and identification of novel *CNOT1* variants in three unrelated Chinese families with Vissers-Bodmer Syndrome

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ABSTRACT

Vissers-Bodmer Syndrome, an autosomal dominant disease, is a neurodevelopmental disorder characterized by global developmental delay, intellectual disability, hypotonia and autistic features with a highly variable phenotype. It is caused by variants in the CCR4-NOT transcription complex, subunit 1 gene (*CNOT1*). However, the pathophysiologic mechanism of the Vissers-Bodmer Syndrome remains unclear. Notably, this syndrome has not been previously reported in the Chinese. In this study, we utilized whole exome sequencing to identify three novel variants in the *CNOT1* gene, encompassing one frameshift variant and two missense variants, in three Chinese patients mainly presenting with developmental delay, intellectual disability and/or autism. Interestingly, three patients exhibited novel manifestations including spina bifida occulta, horse-shoe kidney and café-au-lait spot. The frameshift variant, p.Gly172Alafs*5, occurring *de novo*, leading to a premature stop codon in the protein, was classified into pathogenic. Two missense variants c.3451A > G (p.Asn1151Asp) and c.557C > T (p.Ser186Phe) were predicted to be deleterious by multiple prediction algorithms with high conservation among a variety of species. Additionally, three-dimensional structure modeling and predicting indicated the substitution of the mutated amino acids would decrease the stability of CNOT1 protein. Given that *CNOT1* is a relatively novel disease gene, we evaluated the gene-disease validity following ClinGen Standard Operating Procedure. The existing evidence substantiates a "Definitive" level of gene-disease relationship. The genetic findings provide a reliable basis for the genetic counseling of the family reproduction. Moreover, our results expand the genetic and phenotypic spectrum of *CNOT1*-related Vissers-Bodmer Syndrome.

1. Introduction

Vissers-Bodmer Syndrome is a neurodevelopmental disorder characterized by global developmental delay, impaired intellectual development, hypotonia, behavioral abnormalities, and autistic features with or without facial abnormalities (age of index subjects:

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newborn to 22 years). This autosomal dominant condition has been reported to be caused by truncating and missense variants in CCR4-NOT transcription complex, subunit 1 gene (*CNOT1*). The majority of the reported variants occurred *de novo*, while three of them were inherited from a mildly affected or asymptomatic mosaic mother [1]. *CNOT1* variants are likely haploinsufficient hypomorph or neomorph with no effect on CNOT1 expression or on composition of the CCR4-NOT complex. No significant genotype/phenotype correlation was observed between the patients carrying truncating and missense variants in *CNOT1*. As expected, most reported missense variants affected highly conserved residues in functional domains [1]. However, the underlying pathophysiological mechanism remains unknown and no Chinese patients have been reported so far.

The CCR4-NOT complex is an essential and conserved protein playing a prominent role in multiple aspects of gene expression especially in mRNA degradation which consists of eleven subunits [2–4]. CNOT1, as the largest subunit of the complex, serves as a scaffold protein which has 2376 residues with human ortholog [5]. It plays an important role in the stable expression of the complex and it positively regulates the deadenylase activity of the complex [6]. CNOT1 is composed of five domains respectively named HEAT, TTP, CAF1, DUF3819 and Not1 based on Pfam (http://pfam.xfam.org/search/sequence). The function of DUF3819 domain is poorly understood yet. A central MIF4G (middle domain of eukaryotic initiation factor 4G) domain in CNOT1 provides a platform for catalytic module CNOT6 and CNOT7 [5,7]. CNOT1 modulates the conformation of DDX6, a translational repressor and decapping activator, and then stimulates its ATPase activity [8]. The interaction between MIF4G domain and DDX6 plays an important role in miRNA-mediated repression. In addition, it was reported that Phe1101, Asn1105, Lys1114, Glu1142, Asn1144 and Phe1145 of CNOT1 located at RecA2-binding interface of DDX6. Notably, some structure-based variants on the concave surface of the MIF4G domain specifically weakened the interaction with DDX6 [9,10].

In this study, singleton or trio-based whole exome sequencing was performed on the patients in order to elucidate the genetic cause and provide the basis for genetic counseling. Three independent Chinese patients carrying heterozygous *CNOT1* variants were reported with genotype and phenotype information. The potential effect of *CNOT1* missense variants on protein structure was further investigated by structural modeling. Our results enrich the spectra of both phenotypes and mutations of the *CNOT1*-related disorder.

2. Materials and methods

2.1. Whole exome analysis and sanger sequencing confirmation

The patients were recruited from Shanghai Children's Hospital. Peripheral Blood was collected from patients and their parents. Genomic DNA was extracted from blood with QIAamp® DNA Blood Mini kit (QIAGEN, Germany) according to the instruction. Whole-exome sequencing was performed for the patients searching for phenotype-associated genes on Illumina HiSeq™ 2000 machine (USA). In the study, minor allele frequency (MAF), conservation, pathogenicity prediction algorithms and disease-association database (ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) were used to search for all possible disease-cause variants in each case. The variant pathogenicity was classified by the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines [11] and ClinGen specifications [12]. Sanger sequencing was performed to confirm the variants from WES (*CNOT1* for NM_016284.4). The primer sequences for amplification of the variants were *CNOT1*-E6-F (5′ TGCCAAACAGAACTATGCCCATCA3′) and *CNOT1*-E6-R (5′ TGTCATGCTCGCCTGATCTTCC3′), *CNOT1*-E25-F (5′GTCACGTATGGGTTGAGTTGTGA3′) and *CNOT1*-E25-R (5′ TACAGAGGGCGACACCATACA3′), *CNOT1*-E7-F (5′ TCTTCTGCGTTCTTACATTGAC3′) and *CNOT1*-E7-R (5′ TCTTCTGCGTTCTTACATTGAC3′). The PCR products were sequenced on the ABI 3500DX Genetic Analyzer (USA).

2.2. In silico analysis

Single nucleotide variants (SNVs), insertions variants, deletions variants, copy number variants (CNVs) and structural variants were filtered by frequency (MAF<1%) and variants previously reported as pathogenic or likely pathogenic in ClinVar or HGMD. The variant pathogenicity was predicted by prediction tools such as Rare Exome Variant Ensemble Learner (REVEL) (https://sites.google.com/site/revelgenomics/about), M-CAP (http://bejerano.stanford.edu/mcap/index.html) and Combined Annotation-Dependent Depletion (CADD) (https://cadd.gs.washington.edu). The protein stability was predicted by mCSM (http://biosig.unimelb.edu.au/mcsm/), MUpro (http://mupro.proteomics.ics.uci.edu/), i-Mutant (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi) and SAFFEC-SEQ (http://compbio.clemson.edu/SAAFEC-SEQ/index.php). MetaDome (https://stuart.radboudumc.nl/metadome/) was applied to analyze the mutation tolerance at each position in human protein using data from Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/) and ClinVar. The protein structure was obtained from SWISS-MODEL (https://swissmodel.expasy.org/interactive) and secondary structure model was displayed by HNN (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_hnn.pl).

3. Results

3.1. Clinical description

Patient 1 was a 9-year-old boy born at full term to non-consanguineous parents with no family history of neurological disease. He presented with global developmental delay before age 2. He was able to walk independently at 18 months and started speaking at 2 years. He presented being talkative, impulsive, aggressive, and diagnosed with attention deficit hyperactivity disorder. Interestingly, he showed some unique manifestations including spina bifida occulta and horse-shoe kidney which have not been previously reported

in other patients with Vissers-Bodmer Syndrome. Growth hormone stimulation test identified human growth hormone deficiency. The results of the brain magnetic resonance imaging (MRI) and facial feature were both normal. The boy had undergone growth hormone therapy and his height was within the normal range at 10 years old.

Patient 2 was a 12-year-old girl who was adopted after birth with unknown family history. She had a history of neonatal hypoxia. Not long after birth, developmental delay appeared gradually. She was not able to walk and speak until 2 years old. She exhibited intellectual disability, learning difficulties, slurred speech with no obvious facial abnormality. The brain MRI indicated normal findings.

Patient 3 was a 3-year-old boy born to non-consanguineous parents. His birth weight was 3,500 g. Generally he presented with developmental delay especially in language. Prior to 18 months old, he achieved typical developmental milestones, including head control at 4 months, rolling over at 5 months, sitting independently at 7 months, standing with support at 12 months, walking unassisted at 13 months, and speaking consciously at 18 months. Then language regression was observed with slurred speech. His motor coordination was poor with mild hypotonia. He was noticed to have a vacant stare frequently. He exhibited social isolation and

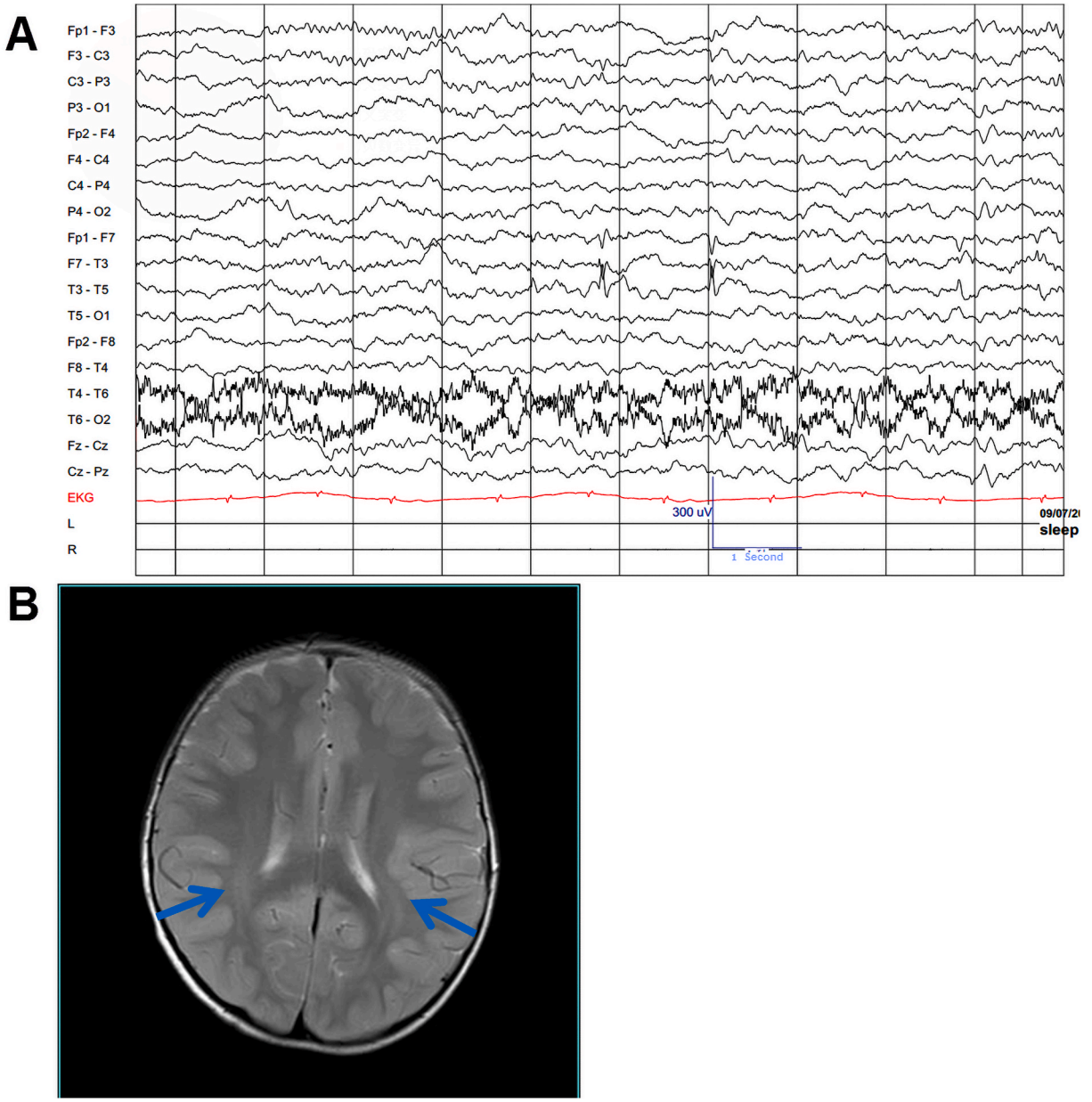

Fig. 1. The electroencephalogram and brain magnetic resonance imaging of patient 3
A The EEG showed abnormality with some cusp wave and slow spike-wave discharges on the left side during sleep; **B** MRI showed abnormal signals in bilateral posterior horns of lateral ventricles with hyperintensities on T2-weighted and fluid-attenuated inversion recovery imaging (FLAIR).

engaged in self-talk. Subsequently, the electroencephalogram (EEG) showed abnormality with some cusp wave and slow spike-wave discharges on the left side during sleep (Fig. 1A). The video EEG showed epileptic discharge in the left anterior middle temporal region during sleep. Brain MRI showed abnormal signals in bilateral posterior horns of lateral ventricles with dysmyelination of white matter (Fig. 1B). Oral levetiracetam and rehabilitation treatment were beneficial for him to improve the verbal expression and motor coordination. And there was no convulsion during the course of disease. At the age of 3 years and 8 months old, his height measured 101 cm, weight was 18 kg and the head circumference was 49 cm, all falling within the normal range. His mother was reported to suffer from mild autism when she was a child.

The detailed clinical phenotypes of three patients and the phenotypic frequency of previously reported patients were shown in Table 1.

3.2. Genetic analysis

The coverage of targeted regions with depths greater than 50 × reads is more than 92%. Three novel variants were identified in the heterozygous state in each patient.

A novel *CNOT1* frameshift variant (c.513del, p.Gly172Alafs*5), occurring *de novo*, was identified in patient 1 (Fig. 2A). This one-nucleotide deletion, was predicted to result in a frame-shift transcript and a truncated protein product. Though this variant itself has not been previously reported, multiple downstream truncating variants of this gene have been reported in numerous patients with neurodevelopmental delay (9), indicating the intolerance of truncating variants of *CNOT1*. This variant was not recorded in gnomAD, supporting its possibility of disease-causing. According to the variant classification guidelines of ACMG and ClinGen [11,12], it was categorized as a pathogenic variant (PVS1+PS2_moderate + PM2_supporting).

Two novel missense variants c.3451A > G (p.Asn1151Asp) and c.557C > T (p.Ser186Phe) were detected in patient 2 and patient 3, respectively (Fig. 2B and C). The origin of c.3451A > G was unknown and c.557C > T was inherited from his mildly affected mother.

Table 1
Summary of clinical and genetic characteristics with three patients and previously reported patients [1].

	Patient 1	Patient 2	Patient 3	Vissers et al. (39 patients) (Phenotype frequency)
Age	9-year-old	12-year-old	3-year-old	Newborn to 22 years
Gender	Male	Female	Male	Male:20 Female:19
Birth history	Normal	Neonatal hypoxia	N/A	Abnormal birth weight (8/35) Abnormal head circumference at birth (4/17)
Height	Short stature Growth hormone deficiency	Normal	Short stature	Short stature (18/33)
Neurologic history	Developmental delay	Developmental delay	Developmental delay	Developmental delay (34/37)
Intellectual disability	Mental retardation	Low IQ; Learning difficulty	Mental retardation	23/32
Speech delay	First speak at 2 years; Talkative	First speak at 2.5 years; Glossolalia	First speak at 18 months; Slurred	29/35
Motor delay	First walk at 18 months	First walk at 20 months	First walk at 13.5 months	29/35
Behavioral disturbances	Attention Deficit and Hyperactivity Disorder (ADHD); impulsive; aggressive	N/A	Soliloquy and often in a trance	20/32
Genetic analysis	c.513del (p.Gly172Alafs*5)	c.3451A > G (p.Asn1151Asp)	c.557C > T (p.Ser186Phe)	Shown in Fig. 4
Brain MRI	Normal	Normal	Abnormal signals in bilateral posterior horns of lateral ventricles with dysmyelination of white matter	Abnormal MRI (20/31)
EEG	N/A	N/A	Abnormality with some cusp wave and slow spike-wave discharges on the left side during sleep	N/A
Video EEG	N/A	N/A	Epileptic discharge in the left anterior middle temporal region during sleep	N/A
Unique characteristics unreported in other patients	Spina bifida occulta Horse-shoe kidney	N/A	Café-au-lait spot on the trunk	N/A
Family history	No	No	His mother had mild autism when she was a child.	N/A

N/A: not available.

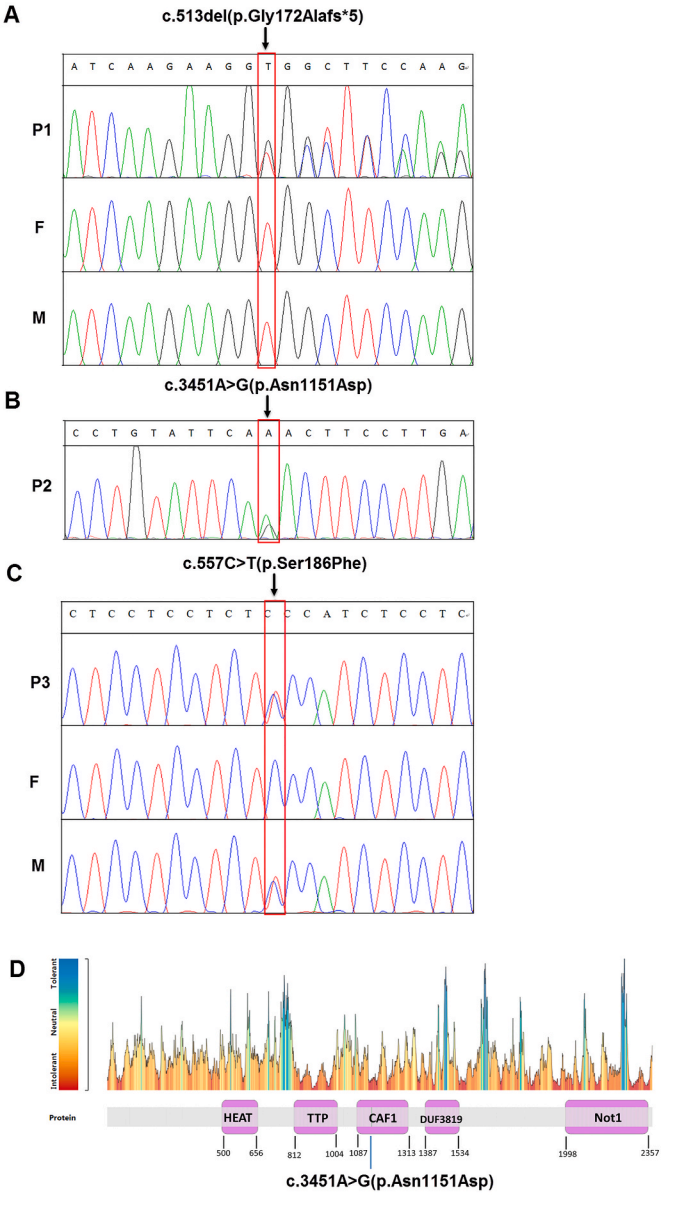

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Fig. 2. The genetic information of three patients
A Sanger sequencing of family 1, P1: Patient 1; F: Father; M: Mother; **B** Sanger sequencing of patient 2; P2: Patient 2; **C** Sanger sequencing of family 3, P3: Patient 3; F: Father; M: Mother; **D** Meta-domain information showed that the variant located in the conserved CAF1 domain is highly intolerant (score: 0.13) (red means intolerant, blue means tolerant); **E-F** The position of 1151 and 186 are highly conserved among multiple species by Cluster Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Asterisks mean completely conserved residues; red frame represents evolutionary conservation of asparagine and serine at the position of 1151 and 186 in CNOT1, respectively.

The position of 1151 located in the MIF4G domain and the position of 186 near HEAT domain were highly conserved among a variety of species (Fig. 2E and F). Both missense variants were predicted to be deleterious by multiple prediction tools (Table 2). In addition, the c.3451A > G variant located in the conserved CAF1 domain showed highly intolerant by Meta-domain information (Fig. 2D). The three-dimensional structure modeling has visually demonstrated that one or more hydrogen bonds (H-bonds) disappear between mutant amino acids and neighbors compared to wild-type, reducing the protein stability (Fig. 3). Moreover, the impact of the two variants on protein thermodynamic stability was determined by measuring the changes in the Gibbs free energy of unfolding (ΔΔG) between mutant and wild-type protein. Based on the results obtained from mCSM, MUpro, i-Mutant and SAFFEC-SEQ tools, ΔΔG values predicted that the variants would decrease the stability of CNOT1 (Table 3).

Subsequently, the impact of amino acid alteration on domain structure was evaluated by comparing secondary structure between mutant and wild-type sequences. The results pointed the mutated structure p.Asn1151Asp was predicted to consist of many alpha helixes that are 1242 (52.27%), followed by 940 random coils (39.56%), and 194 extended strands (8.16%), compared to 1245 (52.40%), followed by 939 (39.52%), and 192 (8.08%) for WT secondary structure. Additionally, the two variants were not present in the control population based on the gnomAD database. According to ACMG and ClinGen guidelines, the two missense variants were classified into variant of unknown significance (VUS) (PM2_supporting + PP2).

4. Discussion

In our study, three novel variants, including one frameshift variant and two missense variants in *CNOT1* were first discovered in three independent Chinese patients. Our patients presented with typical clinical features of the disease and some unique characteristics including spina bifida occulta, horseshoe kidney and café-au-lait spots, which have not been observed in the previously reported patients with Vissers-Bodmer Syndrome.

CNOT1 was first linked with Vissers-Bodmer Syndrome based on heterozygous variants in *CNOT1* identified in 39 patients with neurodevelopmental delay in a recent study. Three nonsense variants, five splicing variants, five frameshift variants, sixteen missense variants, one intragenic deletion (exon 17–21) and one whole gene deletion of *CNOT1* were reported. Combined with three novel variants discovered in this study, a schematic diagram illustrating the overall mutant spectrum of *CNOT1* gene is presented in Fig. 4. The majority of 39 patients occurred *de novo* with two inherited from mildly affected mother and one from mosaic mother. This study also pointed out that there were no significant differences in clinical phenotypes among patients between loss-of-function variants and missense variants [1], indicating the potentially identical pathophysiologic mechanism underlying the disease. Besides, the pLI (probability of Loss of function Intolerance) and Z scores reported for *CNOT1* are 1.0 and 7.25, respectively, indicative of intolerance of both loss of function variants and most missense variants.

Since Vissers-Bodmer syndrome caused by heterozygous variants in *CNOT1* is a relative new disease-gene relationship and only a limited number of studies have been reported, we performed a gene curation based on the ClinGen protocol [13]. On the basis of previously published patients and three Chinese patients in the study, the total clinical validity score reaches 16 points (12 from genetic evidence and 4 from experimental evidence), which is sufficient to support a “Strong” gene-disease relationship. It has been over three years since the first case was reported [1]. In summary, a “Definitive” gene-disease relationship can be concluded which can provide a strong basis for molecular diagnosis.

The frameshift variant c.513del was expected to result in an early termination codon and then a truncated protein lacking important domains including MIF4G and DUF3819. Asn1151 is located in the MIF4G domain and the mutant aspartatic acid is expected to disturb the alpha helix which may have severe effects on the structure of CNOT1. Additionally, the variant will possibly affect the interaction with DDX6 inducing the inhibition of its ATPase activity and finally affect the miRNA pathway. Ser186 before HEAT domain is highly conserved among multiple species. Although the mutant serine is predicted no change on the secondary structure by HNN tool, two of H-bonds disappeared between mutant amino acids and neighbors compared to wild-type. Missense variants before position 186 have not been reported before. However, patient 2 and patient 3 cannot reach a definite diagnosis of Vissers-Bodmer Syndrome due to the unknown clinical significance of the two missense variants detected. Functional research is needed to further

Table 2
In silico pathogenicity prediction and classification of two missense variants in *CNOT1*.

Variant	REVEL	M-CAP	CADD	Classification based on ACMG and ClinGen guidelines
c.3451A > G (p.Asn1151Asp)	0.227	0.039, PP	23.8, P	VUS (PM2_supporting + PP2)
c.557C > T (p.Ser186Phe)	0.478	0.072, PP	24.5, P	VUS (PM2_supporting + PP2)

PP: Possibly Pathogenic; P: Pathogenic.

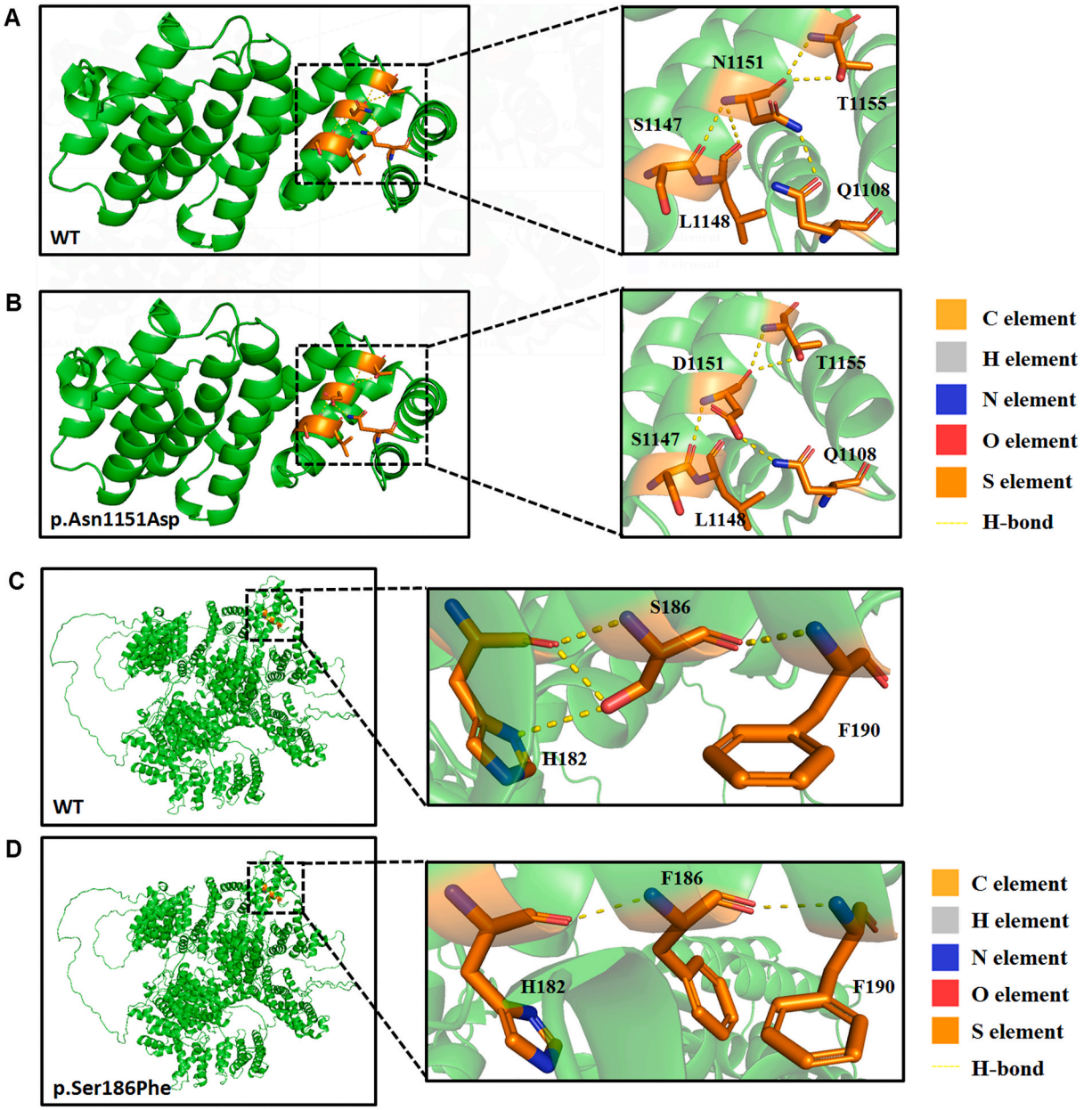

Fig. 3. Prediction of the three-dimensional structure of CNOT1 protein
A The three-dimensional structure of wild-type CNOT1 protein: the 1151th amino acid is Asn; **B** The 3D structure of mutant CNOT1 protein: the1151th amino acid is mutated to Asp; **C** The 3D structure of the wild type CNOT1 protein: the 186th amino acid is Ser; **D** The 3D structure of mutant CNOT1 protein: the186th amino acid is mutated to Phe.

Table 3
The Protein Stability Prediction of two missense variants in CNOT1.

Variant	mCSM (ΔΔG)	MUpro (ΔΔG)	i-Mutant (ΔΔG)	SAFFEC-SEQ (ΔΔG)	Predicted stability change
c.3451A > G (p.Asn1151Asp)	−1.28 Kcal/mol	−2.03 Kcal/mol	−1.38 Kcal/mol	−0.70 Kcal/mol	Decreased
c.557C > T (p.Ser186Phe)	N/A	−0.81 Kcal/mol	−0.09 Kcal/mol	−0.79 Kcal/mol	Decreased

N/A: not available.
ΔΔG<0: destabilizing variants; ΔΔG>0: stabilizing variants.

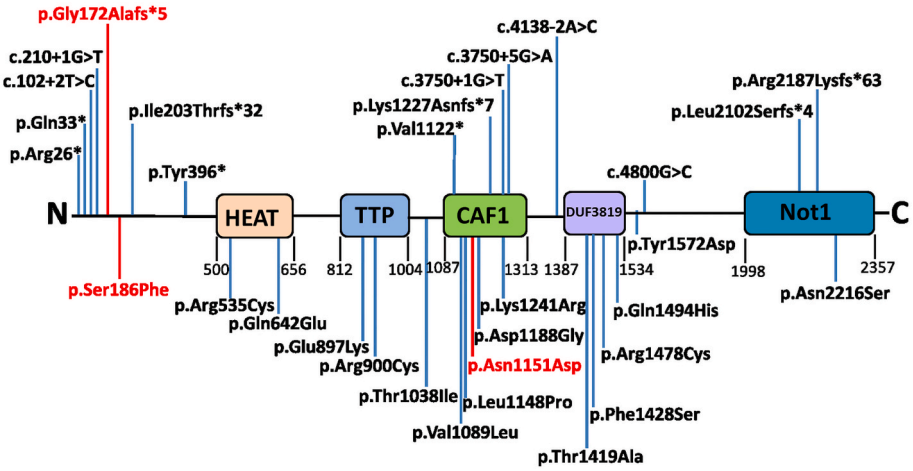

Fig. 4. A schematic diagram of the mutant spectrum of *CNOT1* gene
The schematic diagram showing each domain of CNOT1 and all the identified variants from the previously reported patients [1] and this study, nonsense, frameshift and splicing variants are showed above the line, missense variants are showed below the line, variants showed in red are novel variants reported in this study.

clarify the pathogenicity and a reclassification will be performed based on more information.

In conclusion, we first report three isolated Chinese patients carrying novel heterozygous variants including one frameshift variant and two missense variants in *CNOT1*. This study expands the mutation spectrum of *CNOT1* which contributes to the families getting effective genetic information for reproduction, and extends the phenotype spectrum of Vissers-Bodmer Syndrome with some new manifestations including spina bifida occulta, horse-shoe kidney and café-au-lait spot which is conducive to understanding the previous conclusion of no recognizable "CNOT1-phenotype" [1]. Moreover, we concluded a "Definitive" level of CNOT1-Vissers-Bodmer Syndrome relationship based on the ClinGen SOP which is beneficial for molecular diagnosis.

Ethics approval and consent to participate

This study has been approved by the patients' parents and the Ethical Committee of Shanghai Children's Hospital (Approval No. 2020R007-F01). Written consent forms were signed by the probands' parents.

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Data availability statement

All datasets for this study are avaiable in the article.

CRediT authorship contribution statement

Xiaojun Tang: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Xiaoping Lan:** Formal analysis. **Xiaozhen Song:** Formal analysis. **Wuhen Xu:** Validation. **Yuanfeng Zhang:** Data curation. **Simei Wang:** Data curation. **Man Xiao:** Validation. **Yongchen Yang:** Validation. **Hong Zhang:** Project administration, Funding acquisition. **Shengnan Wu:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, 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.

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