Novel variants identified in a three-generation family with concomitant exotropia

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Received June 17, 2022; Accepted August 30, 2022

DOI: 10.3892/etm.2022.11624

Abstract. Concomitant exotropia is a condition where there is a misalignment between both eyes, which is more prevalent in Asians than in Caucasians. It is an eye disease related to the neural development of binocular vision and eye movement control. Studies have indicated that genetic factors contribute to the development of concomitant exotropia; however, the underlying mutations have not been thoroughly investigated to date. In the present study, whole-exome sequencing was performed in a three-generation family with concomitant exotropia. In the proband and the proband's father, bioinformatics analyses identified a duplication of the genomic region spanning genes *PCDHA1-7* and a heterozygous mutation c.3775G>A (p.A1259T) of the *COL3A1* gene, which is located in the conserved COLFI domain and leads to decreased stability of the encoded protein product. Furthermore, a deletion of amino acid S165 in the gene *NCOA7* was discovered in the family members, including the proband, the proband's mother and maternal grandfather. S165 was predicted to be a conserved phosphokinase site of CK1/VRK and CK1/CK1. The genes in which these variants reside are all involved in cortical neuronal development. The present study reveals novel variants of concomitant exotropia and suggests that aberrant cortical neuronal development may contribute to the origin of concomitant strabismus.

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Key words: concomitant exotropia, *PCDHA, COL3A1, NCOA7*, genetic variants

Introduction

Concomitant strabismus is a common eye disease among infants, children and adults, with a prevalence ranging from 2 to 6% (1,2). The prevalence of concomitant exotropia is higher in Asian populations (3,4), while concomitant esotropia is more likely to occur in Caucasians (2). Affected patients may suffer severe problems, such as binocular visual impairment, diplopia and social stigma, and may require multiple corrective procedures during their lifetime. However, the pathological mechanism of concomitant strabismus remains unknown. Family studies have demonstrated that genetic factors have a significant role in concomitant strabismus development (5). Hu (6) reported that the incidence of strabismus in first-, second- and third-degree relatives of 425 patients with exotropia was 9.0, 2.2 and 1.1%, respectively, and the heritability of exotropia was 81.3%. In two other studies, multivariate analyses revealed that children with a family history were at a significantly higher risk of strabismus than children without a family history (7,8). Furthermore, a study of strabismus in twins concluded that 67.3% of the strabismic phenotypes were concordant in 49 pairs of twins and the concordance rate for monozygotic twins (82.4%) was higher than that for multizygotic twins (47.6%) (9).

To date, only a few genes associated with concomitant strabismus have been reported in families from multiple countries. In 2003, Parikh *et al* (10) identified the first concomitant strabismus locus, *STBMS1* gene on chromosome 7p22.1 locus based on a model of recessive inheritance in one esotropia pedigree. Furthermore, it was suggested that *MGST2* on chromosome 4q28.3 and *WNT2* on chromosome 7q31.2 were both potential candidate genes for concomitant strabismus in 55 Japanese pedigrees (11,12). However, these genes have only been reported in concomitant esotropia or unspecified subtypes of concomitant strabismus. There has been no exact gene reported to be associated with concomitant exotropia.

Materials and methods

Whole-exome capture and sequencing. Genomic DNA was extracted from peripheral blood and exome capture was subsequently conducted using SureSelect Human All Exon V6 (Agilent Technologies, Inc.). Exome sequencing was performed on the Illumina Novaseq 6000 platform (Illumina, Inc.) according to the manufacturer's instructions.

Whole-exome sequencing (WES) data analyses. Fastq data were generated from raw sequencing files using Illumina bcl2fastq software (Illumina, Inc.), the adapter was removed and low-quality reads were removed. Subsequently, Burrows-Wheeler Aligner was used to align fastq data to the Hs37d5 reference human genome, while marking of duplicate reads was performed by sambamba tools (13) to generate 150 base pair (bp) paired-end reads.

The sequencing reads were aligned to the human reference genome hg19, single nucleotide variants (SNVs) and indels were called using Samtools, and variants were filtered according to the hard-filter criteria: i) Read depth, >4; ii) quality of variant, >20; iii) root-mean-square mapping quality >30. Variants were annotated with ANNOVAR (version 20180416) (14).

Identification of deleterious variants. Possible deleterious genetic variants were required to meet the following criteria: i) SNVs and indels are located at exons or splicing sites. ii) Functional annotations are required to be nonsynonymous SNVs, stop loss, stop gain, frameshift indels or variants at splicing donor/recipient sites. iii) SIFT (15) and PolyPhen-2 (16) annotations are required to be '.' or 'D'. iv) The variant allele frequency in East Asian Population of the 1000G (17), ExAC (version 0.3) (18), gnomAD (version 2.1.1) (19) and ChinaMAP (20) databases are all <0.01.

Based on the kinship of the affected family, the likely inheritance pattern was tested for the possible pathogenetic variants. For an autosomal dominant inheritance model, the proband and the proband's parents should be heterozygous for the candidate mutation; for an autosomal recessive inheritance model, both the proband and one parent should be homozygous for the mutation and the other parent should be heterozygous. The possibility of parents carrying the same or different pathogenic genes was considered.

Gene prioritization based on relevant phenotypes was performed using Phenolyzer (21).

CNV analysis. CNVs were called from the WES data using CoNIFER (22) with the SVD threshold set as 10. CNV calls located in the segment duplication regions reported in the Database of Genomic Variants (DGV) (23) and those in the region of repeated sequence annotated with RepeatMasker were excluded from further analyses for being prone to be false positive. CNV calls located in scattered repeating sequence or low complexity sequence are prone to have alignment errors and were thus also excluded from further analyses.

CNV calls were further filtered based on the pathogenicity annotation. Those annotated as benign by StringentLib, InclusiveLib (24) and DGV GoldStandard (July 2015) were removed from analyses. CNVs were annotated as being of high priority if they had 50% overlap with those in the database of CNVD (25), which contains 212,277 CNV data records related to human diseases.

The quality of the CNVs was inspected using Integrative Genomics Viewer (version no. 2.11.0; software.broadinstitute.org/software/igv/home).

Quantitative (q)PCR validation of CNVs. For CNVs that passed IGV inspection, qPCR validation was performed. Genomic DNA (20 ng) was used in a final volume of 10 μl according to the recommended protocol provided by the manufacturer using the SYBR® Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.). The optimal reaction conditions are 45 cycles of two-step amplification at 95˚C for 12 sec and 62˚C for 45 sec. The GAPDH gene was used as an internal control. Primer sequences were as follows: STRCPl forward, 5'-AGC TCCAGCCATCTATCTGC-3' and reverse, 5'-GATCCTGCA GCTCGGTAGAC-3'; STRC forward, 5'-CCTGGGTCTCCT GCAAATAA-3' and reverse, 5'-GTGCAGATGTACGAG GGACA-3'; PCDHA6 forward, 5'-CGTGTACCTGATCAT CGCCA-3' and reverse, 5'-AGGACAAGGTGAAAGGCT GG-3'. GAPDH forward, 5'-CACCCGCCCCAGTCTCTG-3' and reverse, 5'-AACTCAAAGGGCAGGAGTAAAGG-3'.

Each sample was repeated three times independently. Changes in the expression of target genes were determined based on the relative values of $2^{-\Delta\Delta Cq}$ (26).

Plasmid construction and Sanger sequencing. For PCR amplification, genomic DNA (100 ng) was used in a final volume of 50 μl according to the recommended protocol provided by the manufacturer of the 2X Phanta® Flash Master Mix (Nanjing Vazyme Biotechnology Co., Ltd.). The reaction conditions were as follows: 95˚C for 3 min, followed by 30 cycles of 3-step PCR (95˚C for 15 sec, 56˚C for 15 sec and 72˚C for 1 min), 72˚C for 5 min and then hold at 4˚C. Oligonucleotide primers for PCR reactions of variants were designed by the website Primer 3 (bioinfo.ut.ee/primer3-0.4.0/) and the sequences are listed in Table SI. For indel variants, after PCR amplification, the two alleles were cloned into the *pEASY*-Blunt Zero Cloning vector, followed by transforming into *Trans*1-T1 competent cells (storage at -70˚C and incubation in LB medium containing ampicillin; Beijing Transgen Biotechnology Co., Ltd.) using the *pEASY*-Blunt Zero Cloning Kit (Beijing Transgen Biotechnology Co., Ltd.) according to the manufacturer's protocol. After incubation overnight, certain single clones were selected for sequencing.

Results

Three-generation family with concomitant exotropia. The present study recruited a Chinese family with three generations and four members suffering from concomitant exotropia (Fig. 1A). The proband, an 11-year-old male, was found to have intermittent exotropia during the physical examination in November 2020 at Tianjin Eye Hospital, Tianjin, China. The patient had 35 PD of exotropia at near distance and 20 PD at far distance. There were no refraction errors with uncorrected visual acuities of 20/20 in both eyes. The parents and maternal grandfather of the proband had concomitant exotropia. The maternal grandmother had no presentation of exotropia. The clinical manifestations of the children were more severe than

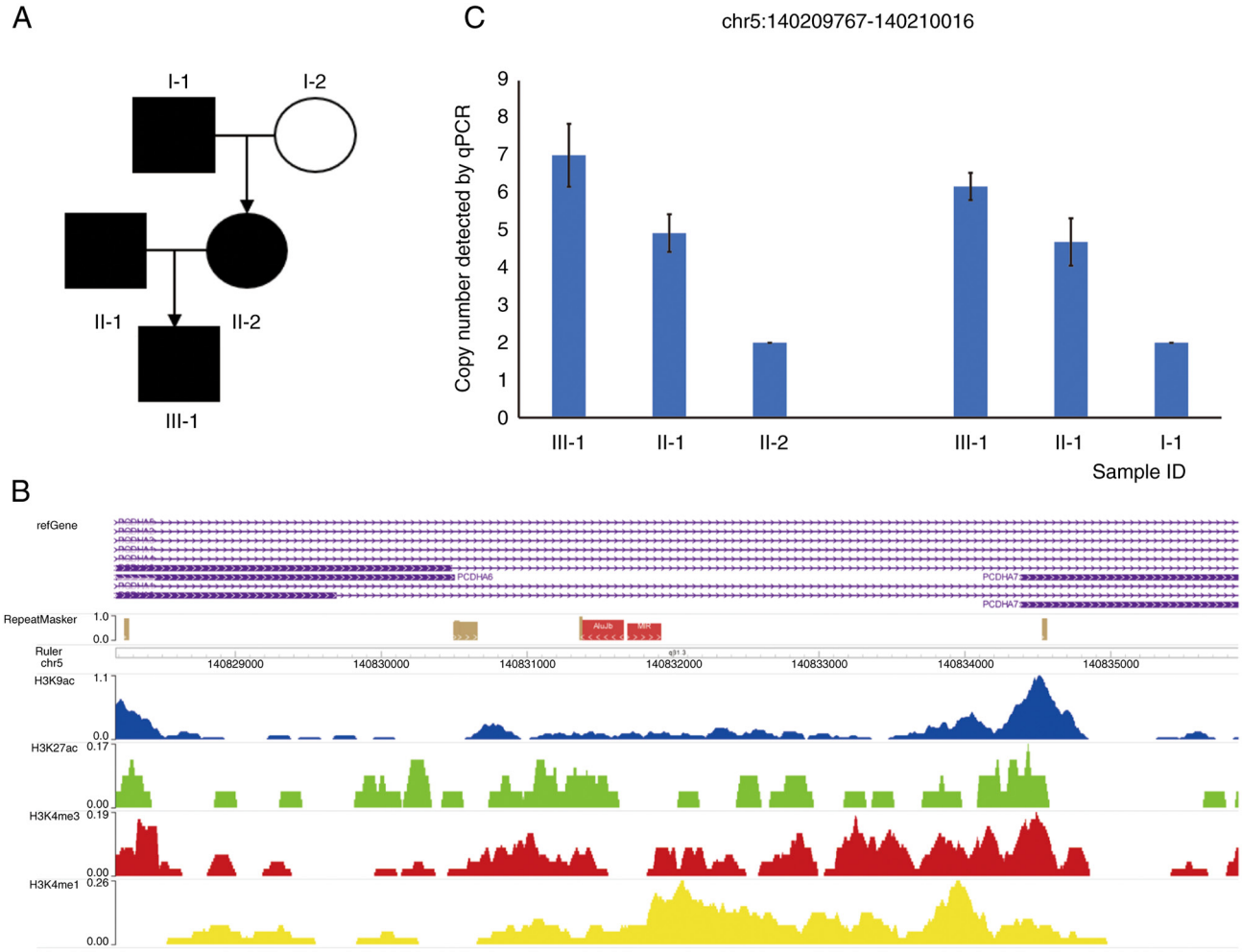

Figure 1. *PCDHA* mutation in a Chinese pedigree with concomitant exotropia. (A) Pedigree of the Chinese family with concomitant exotropia. (B) Histone modifications of CNV duplication region overlapping with *PCDHA* gene. The area presented is the predicted CNV duplication region (chr5:140207766-140215463). Histone modifications of neural progenitor cell transcription-activity related elements (H3K9ac, H3K27ac, H3K4me3 and H3K4me1) from ENCODE are described by histogram trajectories. (C) qPCR results of *PCDHA6* gene indicating the copy number variation in this region carried by individuals III-1 and II-1. CNV, copy number variation; qPCR, quantitative PCR; chr, chromosome.

those of the parents. All subjects underwent cover tests to check for strabismus conducted by a professional ophthalmologist. As the exotropic phenotype appears in three generations, it was speculated that the etiology of strabismus in this family may have a strong genetic component. Genomic DNA from all the individuals with concomitant exotropia in this family was analyzed by WES (Fig. 1A).

Mutations in PCDHA, COL3A1 (p.A1259T) and NCOA7 (p.S165del) detected by WES analysis. WES is an effective and efficient method for studying gene loci within the human genome. It has had great success in the genetic research of numerous complex diseases. In the present study, WES was used to identify a CNV duplication in *PCDHA*, a c.3775G>A (p.A1259T) mutation in *COL3A1* and a c.492CAGT>C (p.S165del) mutation in *NCOA7* considered as the likely causative genes in a Chinese pedigree with concomitant exotropia, including the proband, the proband's parents and maternal grandfather.

Identification of variants in PCDHA and COL3A1 (p.A1259T) carried by the proband and the proband's father. After CNV calling, CNVs carried by the proband and by the proband's father or mother were screened. Due to uncertainty in CNV calling, like DGV, CNVs of different individuals whose scope overlaps 70% or more were considered as shared CNVs (23). Based on genetic analysis and functional annotation of the CNVs, one CNV at the *PCDHA* gene cluster was found to be likely to be a pathogenic CNV carried by the proband (chr5:1402077661-40215463) and the proband's father (chr5:140207600-140216026). This CNV region is a duplication in the exon region of genes *PCDHA6* and *PCDHA7*, and intron region of genes *PCDHA1*, *PCDHA2*, *PCDHA3*, *PCDHA4* and *PCDHA5*. This region contains histone modification peaks of H3K4me1 and H3K4me3 indicative of active promoter and enhancer in neuronal progenitor cultured cells and different brain regions based on the Encode dataset (Fig. 1B). Previous studies indicated that *PCDHA* gene clusters encoding neurocadherin-like cell adhesion proteins have important roles in the establishment of specific cell-cell connections in the brain (27). In the present study, four family members were analyzed by qPCR to validate the presence of *PCDHA* CNVs. The qPCR amplification region is at the center of CNVs called by WES. The results suggested the increased copy number of *PCDHA* in the proband and the proband's

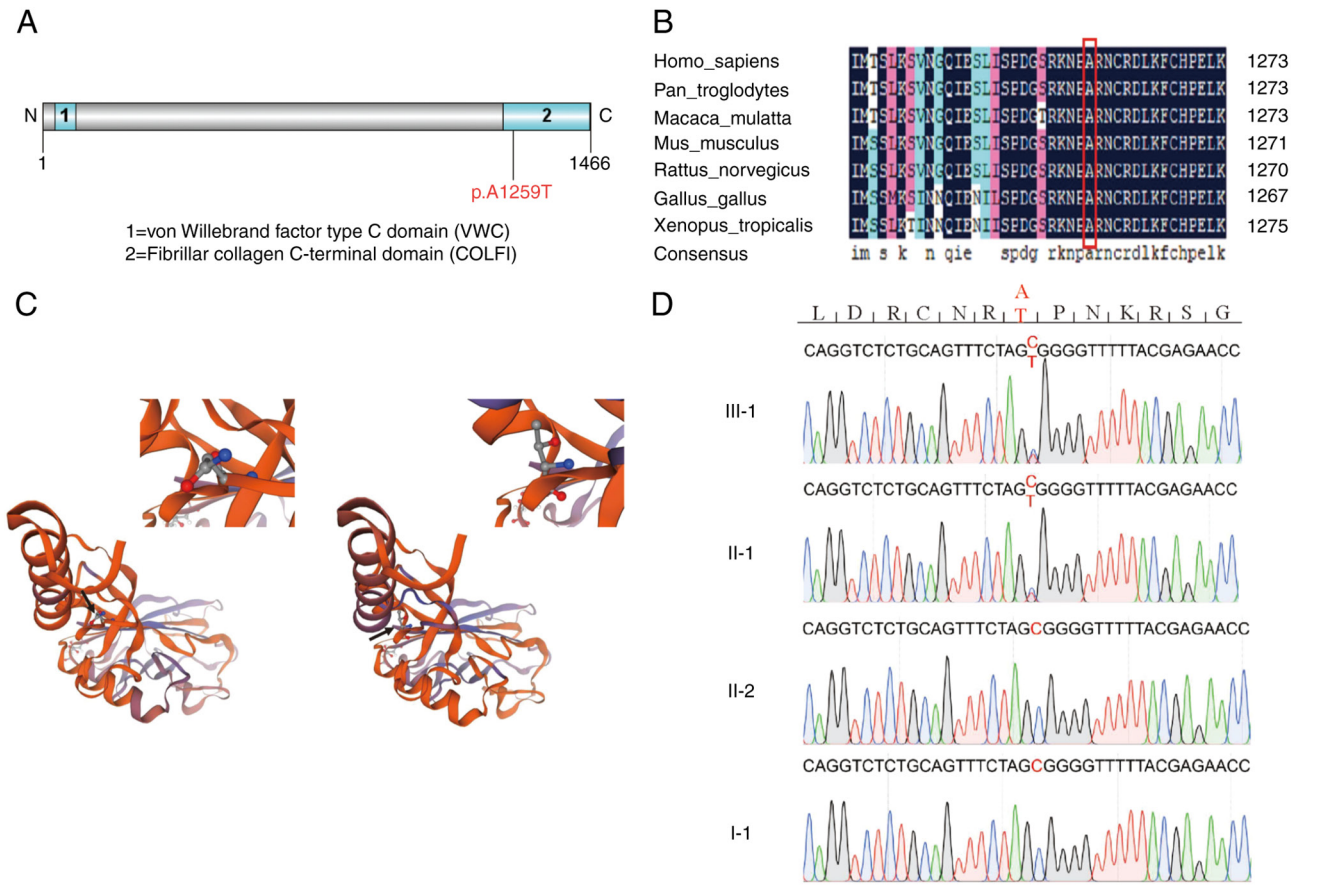

Figure 2. *COL3A1* mutation in a Chinese pedigree with concomitant exotropia. (A) Protein domains in COL3A1. The mutation c.3775G>A (p.A1259T) was located in the COLFI domain of COL3A1 protein. (B) Amino acid residue A1259 (red frame) of COL3A1 protein is highly conserved across species. (C) Three-dimensional structure of wild-type (left) and mutant (right) COL3A1 protein. The mutation c.3775G>A (p.A1259T) caused the positional change of the amino acid side chain and decreased predicted stability. (D) Sanger sequencing of *COL3A1* gene indicating the heterozygous mutation c.3775G>A in the affected individuals III-1 and II-1.

father compared to that of the mother or the maternal grandfather (Fig. 1C).

After variant filtering based on calling quality, functional annotation and inheritance mode, it was indicated that the proband and the proband's father carry a heterozygous mutation in gene *COL3A1* (chr2: 189873899, p.A1259T) which fits into a dominant inheritance mode. There are no recessive SNVs passing all the filtering criteria. The *COL3A1* gene is the most relevant gene according to Phenolyzer (21) and the mutation is predicted to be 'dangerous' by SIFT, PolyPhen2 and PROVEAN_pred. The pro-alpha 1 chain of type III collagen encoded by the *COL3A1* gene is involved in regulating the integrity of the pial basement membrane and cortical laminate in the brain, which is critical for neuronal migration (28). The mutation site c.3775G>A (p.A1259T) is located in the COLFI conserved domain (Fig. 2A) and the altered amino acid residue is highly conserved across species (Fig. 2B). In addition, the three-dimensional structure of the mutant COL3A1 protein is predicted to be destabilized (Fig. 2C). Sanger sequencing validated the identification of the *COL3A1*(p.A1259T) mutation (Fig. 2D).

Considering that concomitant strabismus is an eye disease related to neural development, the mutation in *COL3A1* (p.A1259T) and large CNV covering gene cluster *PCDHA1-7* are likely to contribute to its pathogenesis. The known functions of these genes are summarized in Table SII. Knockout mice of *PCDHA* or *COL3A1* exhibited defects in brain morphology or muscle morphology (Table SIII).

Identification of variant in NCOA7 (p. S165Δ) carried by the proband and the proband's mother. Similar variant and CNV analyses on the proband's maternal side yielded the identification of a three-bp in-frame deletion in the gene *NCOA7*. The *NCOA7* gene encodes an important V-ATPase regulatory protein in the brain that modulates lysosomal function, neuronal connectivity and behavior. This indel results in the deletion of S165, which is highly conserved across species (Fig. 3A) and S165 is predicted to be a phosphorylation site of serine/threonine kinase vaccina related kinase (VRK) of the casein kinase 1 (CK1) family (Fig. 3B). The details of the candidate causative variant are presented in Table I. PCR amplification of the 563 bp genomic region surrounding *NCOA7*(s165Δ) was performed and the PCR products were cloned into the *pEASY*-Blunt Zero Cloning vector. Sanger sequencing of individual clones confirmed the presence of the heterozygous *NCOA7*(s165Δ) mutation (Fig. 3C). The functions of the *NCOA7* gene are summarized in Table SII. Previous research indicated that *NCOA7*$^{del/del}$ mice displayed abnormal brain and synapse morphology (Table SIII).

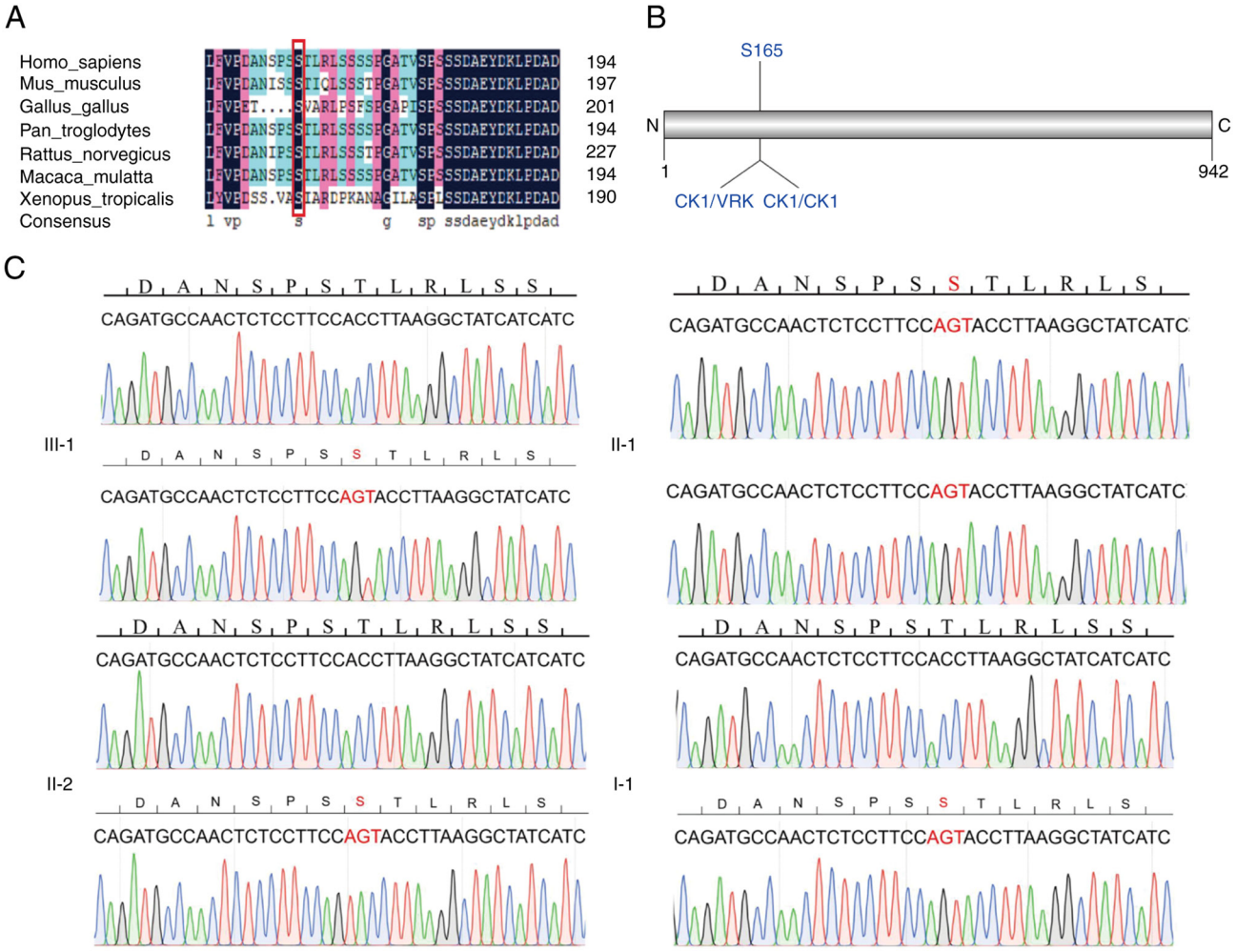

Figure 3. *NCOA7* mutation in a Chinese pedigree with concomitant exotropia. (A) Deleted amino acid residue S165 (red frame) of NCOA7 protein is highly conserved across species. (B) Deleted amino acid residue S165 of NCOA7 protein was located in the CK1/VRK and CK1/CK1 kinase-specific phosphorylation site. (C) Sanger sequencing of *NCOA7* gene indicated the heterozygous mutation c.492CAGT>C in affected individuals III-1, II-2 and I-1.

Discussion

Concomitant exotropia is an eye disease with genetic heterogeneity. In the present study, WES was performed on a three-generation Chinese strabismus pedigree involving an affected male pediatric patient, as well as the patient's parents and maternal grandfather. A CNV involving *PCDHA1-7* and a heterozygous mutation c.3775G>A (p.A1259T) in the *COL3A1* gene were identified in the proband and the proband's father. Furthermore, a deletion of one amino acid (S165) in NCOA7 was detected in family members over three generations, including the proband as well as the proband's mother and maternal grandfather.

Both the visual and oculomotor systems are comprised of neural networks that link numerous structures from the eyes to the brain. All of these structures must be functionally coordinated and work properly in order to achieve normal visual function, including normal binocular vision with a normal alignment of the eyes. The cause of strabismus is generally an abnormal or immature development of the neural networks caused by genetic or acquired factors. For instance, a primary lack of innervation of extraocular muscle from deficient, absent, or misguided cranial nerves causes various forms of complex incomitant strabismus (29-32). Certain gene mutations have been identified for these special types of strabismus, such as *KIF21A* mutations for congenital fibrosis of the extraocular muscles type 1 (CFEOM 1), *PHOX2A* mutations for CFEOM 2, *TUBB3* mutations for CFEOM 3, *CHN1* mutations for Duane retraction syndrome type 2 and *ROBO3* mutations for horizontal gaze palsy with progressive scoliosis (33), all of which are now known as congenital cranial dysinnervation disorders. However, the pathogenesis of concomitant strabismus is much more complex. To date, it has not been attributed to a specific gene. Previous studies support a hypothesis that any insult or injury during the normal processes of neurogenesis, neuronal migration, axonal growth, synaptogenesis and myelination may potentially lead to strabismus (34). For instance, when cutting the cortical-cortical connections of cats, they rapidly exhibit misaligned eyes and strabismus (35,36). Strabismus may also be caused by abnormal inputs from cortical structures, such as the frontal eye field, supplementary eye field and parietal eye field, all of which have a critical role in controlling eye movements (34). The gray matter volume of the cortical areas of the eyes of patients with strabismus is always abnormal, either larger or smaller, in neuroimaging studies (37). It remains unknown,

Table I. Detailed information of the variants *COL3A1* (p.A1259T) and *NCOA7* (p. S165Δ).

Carrier	Chr	Pos (GRCh37)	Ref	Alt	Gene	AD	ChinaMap	ExAC	GnomAD	1000G	SIFT	Polyphen2	PROVEAN_pred
III-1 and II-1	2	189873899	G	A	COL3A1	III-1: 45,55; II-1: 34,32	0.000756	0.0012	0.0012	-	D	D	D
III-1, II-2 and I-1	6	126202268	CAGT	C	NCOA7	III-1: 49,42; II-2: 15,14; I-1: 43,32	0.00298	0.0027	0.0027	-	-	-	-

CPolyphen2 specifically points to Polyphen2_HDIV. Carrier, family member who carries mutations; ChinaMap, mbiobank.com/_(20); Chr, chromosome; Pos, position on chromosome; Ref, reference base; Alt, alternate base; AD, allele depth; ExAC, Exome Aggregation Consortium; SIFT, sorting intolerant from tolerant; Polyphen2, polymorphism phenotyping v2; PROVEAN, protein variation effect analyzer.

however, whether genetic influences have a substantial role in all these processes of brain development (38).

In the present study, a heredity analysis of a Chinese strabismus family was performed and *PCDHA* mutations that may cause genetic susceptibility to strabismus were discovered. The protocadherin alpha gene cluster is one of three related clusters connected in tandem on chromosome 5. The alpha gene cluster is made up of 15 cadherin superfamily genes, including 13 highly similar and 2 more distantly related coding sequences. The most likely function of the protocadherin alpha gene encoding neurocadherin-like cell adhesion proteins is to participate in the establishment of specific cell-cell connections in the brain (27). Compared with wild-type mice, a mouse mutant ($PCDHA^{\Delta CR/\Delta CR}$) exhibited abnormal serotonergic fibers in the cerebral cortex, hippocampus, basal ganglia and thalamus. Serotonergic fibers gather around the dorsal lateral geniculate nucleus and the medial geniculate nucleus but are scarce in the central regions of these nuclei (Table SIII) (39). In *PCDHA* knockout mice, huge aggregates, formed by the terminals of retinal ganglion cells projecting to the dorsal lateral geniculate nucleus, contribute to vision loss (40).

In addition, a *COL3A1* mutation was discovered in the present study. The *COL3A1* gene is located on chromosome 2q32.2 and encodes the pro-alpha 1 chain of type III collagen, which is used as a ligand for the adhesion receptor GPR56 (ADGRG1). This interaction regulates the integrity of the pial basement membrane and cortical laminate in the brain, which is critical for neuronal migration (28). *COL3A1* is related to Ehlers-Danlos syndrome (EDS), vascular type and polymicrogyria with or without vascular-type EDS. $Col3a1^{-/-}$ mice have cobblestone-like cortical malformations with breakdown of the pial basement membrane and marginal zone heterotopias. There was also neuronal overmigration and radial glial detachment (41) (Table SIII). Research has indicated that collagen III has a critical role in the development of the brain (41). In the results of the present study, the missense mutation c.3775G>A caused the changed amino acid residue (p.A1259T) located in the conserved COLFI superfamily domain of COL3A1.

A novel *NCOA7* variant was also discovered in the present study. The *NCOA7* gene, mapped to chromosome 6q22.33, contains 15 exons and spans ~150 kb of genomic DNA. The protein containing 942 amino acids encoded by *NCOA7* is also known as ERAP140 (42). In neuroblastoma-derived RTBM1 cells, the expression level of ERAP140/NBLA10993 was increased at the mRNA level during the process of neuronal differentiation mediated by all-trans retinoic acid (43). NCOA7 is an important V-ATPase regulatory protein in the brain that modulates lysosomal function, neuronal connectivity and behavior. $NCOA7^{del/del}$ mice exhibit a larger number of proximal neurites on cortical neuronal processes, a reduced number of calbindin (CB)-positive interneurons in the somatosensory and visual cortex, and reduced inhibitory contacts on cortical and somatosensory cortex neurons (44) (Table SIII). In the case of the present study, the deletion of three adjacent nucleotides resulted in the loss of serine, which is a highly conserved amino acid throughout evolution. Besides, this amino acid site is located in the CK1/VRK and CK1/CK1 kinase-specific phosphorylation site. These findings support that the novel *NCOA7* variants,

as a possible pathogenic mutation, may have a potential role in the pathogenesis of strabismus.

In conclusion, the present results indicated that a CNV duplication variant in *PCDHA*, c.3775G>A (p.A1259T) mutation in *COL3A1* and c.492CAGT>C (p.S165del) mutation in *NCOA7* may have contributed to the susceptibility to concomitant exotropia in the Chinese family examined by an additive effect and suggested that aberrant cortical neuronal development may contribute to the origin of concomitant strabismus.

Acknowledgements

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (grant nos. 81770956 and 81371049), the Science Fund for Distinguished Young Scholars of Tianjin (grant no. 17JCJQJC46000), Project of Tianjin 131 Innovative Talent Team (grant no. 201936), Jinmen Medical Talent Project of Tianjin, the Science and Technology Planning Project of Tianjin (grant no. 21JCYBJC00780) and Tianjin Key Medical Discipline (Specialty) Construction Project (grant no. TJYXZDXK-016A).

Availability of data and materials

The datasets analyzed during the current study are not publicly available due to privacy or ethical restrictions but are available from the corresponding author on reasonable request.

Authors' contributions

JXL collected the clinical samples, performed the experiments, analyzed and interpreted the data and wrote the manuscript. YM and WZ analyzed and interpreted the data and edited the manuscript. WL and LW collected the clinical samples and performed the experiments. SM analyzed the data. JL and XS conceptualized and designed the study, reviewed the manuscript and confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study followed the Declaration of Helsinki. Written informed consent regarding genetic testing was provided by all participants or the legal guardian. The study was approved by the Ethics Committee of Tianjin Eye Hospital (Tianjin, China; no. 202015).

Patient consent for publication

The subjects or the legal guardian provided written consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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