A de novo Variant in CROCC identified in a Chinese family implies the potential association with Atlanto-occipital Fusion (AOF)

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Introduction Atlanto-occipital fusion (AOF), also known as “occipitalization of the atlas” or “atlas assimilation”, is one of the craniovertebral junction (CVJ) disease characterized with fusion of the atlas and the base of the occipital bone. Due to its rare incidence, asymptomatic onset and the limited techniques available, there are very few studies on AOF.
Although sequencing technique has been successfully applied in studying small sample size of many CVJ diseases, for instance, a homozygous mutation on gene MEOX1 was detected and proved to be one of the pathological mutations for type II Klippel-Feil syndrome using the whole-exome sequencing (WES) technique, there is not such a study on AOF so far.

Aim and methods In this study, we performed WES and SNP (Single-nucleotide Polymorphism) analysis on clinical samples from a Chinese Han family with AOF son to understand the genomic basis of AOF. This study has the ethics approval (No.2014S010) from the Beijing Institute of Genomics, Chinese Academy of Sciences. The clinical investigations were informed to all participants and written informed consents were received to allow the publication of this study. A total of 293 participants were selected for this study, including 220 healthy volunteers and 73 AOF patients who have been diagnosed in the outpatient department of the China PLA General hospital, in which included a family samples of one AOF patient and four healthy members. The peripheral blood samples were collected at the China PLA General hospital using standard operational protocol. The hospital medical staff provided all medical reports. Furthermore, the exome sequencing data were stored at the internal database of Beijing Institute of Genomics and it is available upon requests for research purposes only.
The peripheral bloods from all participants were collected for DNA isolation. Exome enrichment was conducted following the manufacturer’s protocol with Illumina Hiseq2500 sequencing technique. Primers for PCR and qPCR amplifications of all target variants were designed using the web-based Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3). All variants were compared among family subjects, including the proband, his parents and his two sisters. Genetic testing was then performed to inspect the candidate variant. The candidate variant was verified by conservation of species and Gene Ontology Consortium (GO) cluster. CRISPR-Cas9 was then applied to edit CROCC for its verification.

Results

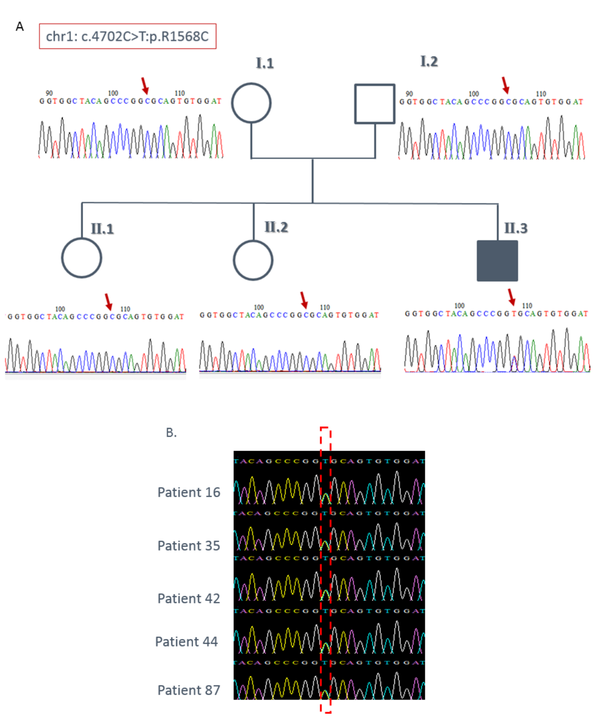


Figure 1. (A) The pedigree chart for the genotype of the proband family with mutation chr1: c.4702C>T:p.R1568C on gene CROCC. The Sanger sequencing results showed the proband with a mutated “T” which was different from the rest of the family. (B) Sanger sequencing results for the candidate variant in sporadic patients.

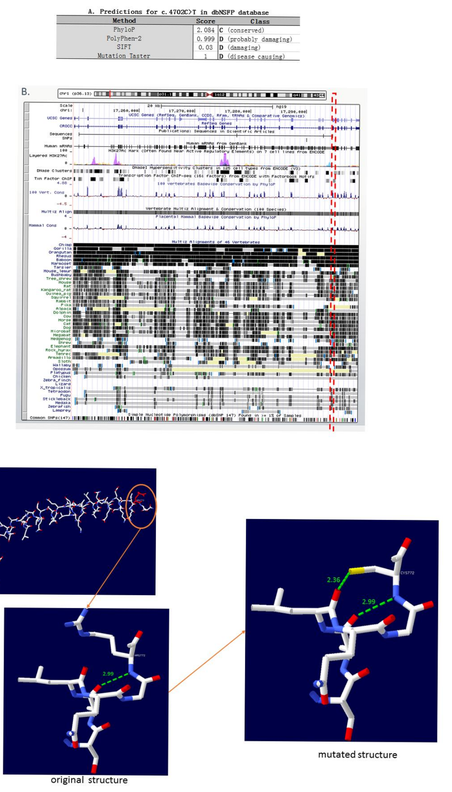


Figure 2. The allelic conservation (chr1: c.4702C>T: p.R1568C) and the structure simulations between wild type and mutated CROCC. (A) Pathogenicity prediction for c.4702C>T in dbNSFP database. (B) The amino acid conservation results on CROCC and the amino acid containing the candidate SNV showed highly conservation. (C) Simulations on the protein structure after the candidate SNP changing from “C” to “T” showed the amino acid changed from arginine to cysteine.

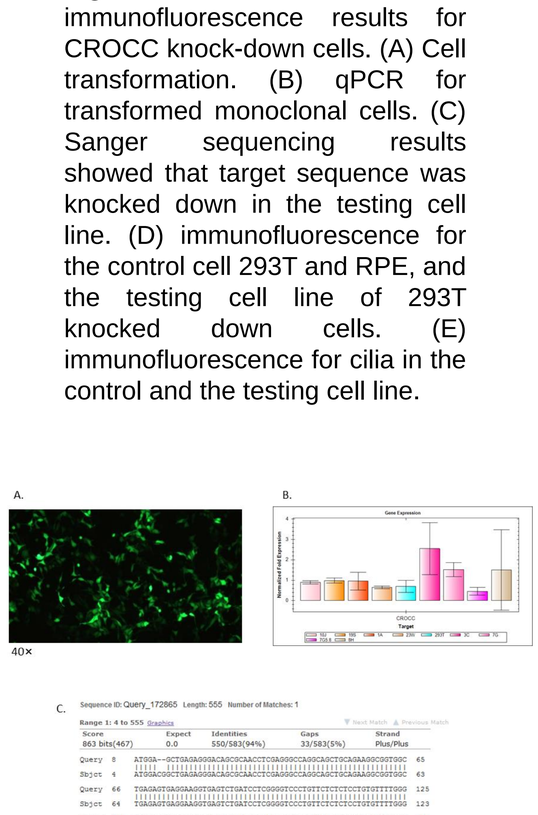


Figure 5. CRISPR-Cas9 and immunofluorescence results for CROCC knock-down cells. (A) Cell transformation. (B) qPCR for transformed monoclonal cells. (C) Sanger sequencing results showed that target sequence was knocked down in the testing cell line. (D) immunofluorescence for the control cell 293T and RPE, and the testing cell line of 293T knocked down cells. (E) immunofluorescence for cilia in the control and the testing cell line.

Figure 3. Location of the genes (IHH, Smoothened, PTCH1, GLI1, GPC3, PTHrp) in the hedgehog signaling pathway. This pathway can be affected by the candidate SNP mutation of CROCC and the genes activation.

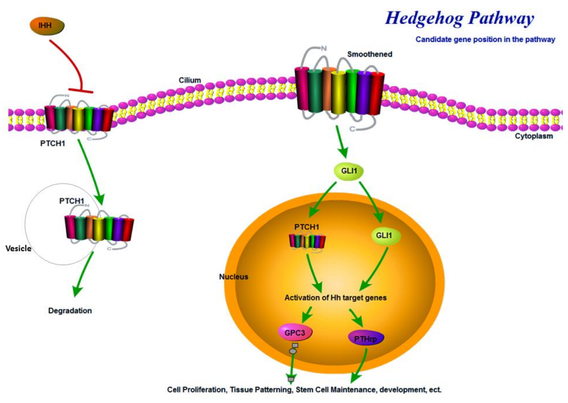
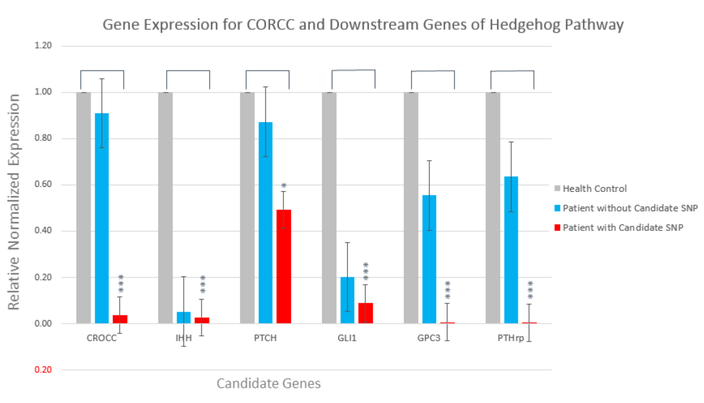


Figure 4. The real-time PCR results for CROCC, IHH, PTCH1, GLI1, GPC3, PTHrp. The red column was the gene expression for the patients with candidate SNP, the gray column for the health donors’ gene expression, and the blue column for the patients without candidate SNP. Each bar in this chart is the average cq values of real-time PCR. The gene expression level (CROCC and downstream genes including GLI1, GPC3, PTHrp) of AOF patients with candidate SNP (red bar) were reduced compared with those of healthy samples (blue bar).



Conclusion A novel variant (chr1: c.4702C>T: p.R1568C) was identified for the first time on gene ciliary rootlet coiled-coil (CROCC) (MIM# 615776) based on comparison of the family WES sequencing data and analysis of experimental verifications of 220 healthy volunteers, 68 non-mutated and 5 mutated AOF patient samples. The SNP (chr1: c.4702C>T: p.R1568C) was found rare population frequency and could change the protein CROCC structure that will reduce the expression of CROCC, and then directly affect the biological signaling transfer of hedgehog pathway. Our results suggested that the candidate SNP (chr1: c.4702C>T: p.R1568C) could be one of the potential causes of the development of AOF (or CVJ).

References

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