

# Achromatopsia caused by novel missense mutations in the *CNGA3* gene

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## Abstract

• **AIM:** To identify the genetic defects in a Chinese family with achromatopsia.

• **METHODS:** A 2.5-year-old boy, who displayed nystagmus, photophobia, and hyperopia since early infancy, was clinically evaluated. To further confirm and localize the causative mutations in this family, targeted region capture and next-generation sequencing of candidate genes, such as *CNGA3*, *CNGB3*, *GNAT2*, *PDE6C*, and *PDE6H* were performed using a custom-made capture array.

• **RESULTS:** Slit-lamp examination showed no specific findings in the anterior segments. The optic discs and maculae were normal on funduscopy. The unaffected family members reported no ocular complaints. Clinical signs and symptoms were consistent with a clinical impression of autosomal recessive achromatopsia. The results of sequence analysis revealed two novel missense mutations in *CNGA3*, c.633T>A (p.D211E) and c.1006G>T (p.V336F), with an autosomal recessive mode of inheritance.

• **CONCLUSION:** Genetic analysis of a Chinese family confirmed the clinical diagnosis of achromatopsia. Two novel mutations were identified in *CNGA3*, which extended the mutation spectrum of this disorder.

• **KEYWORDS:** achromatopsia; genetic analysis; missense mutation

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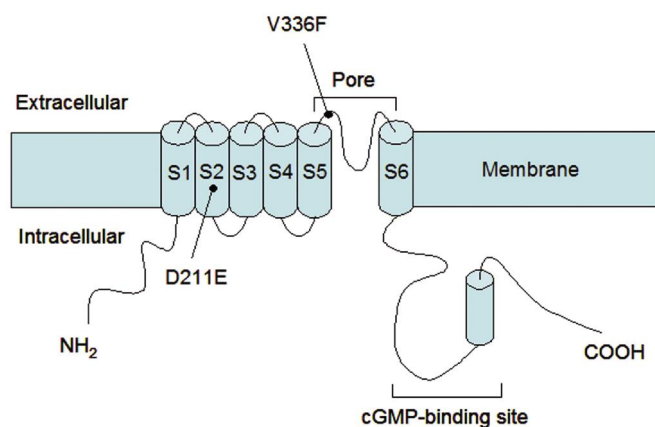
## INTRODUCTION

Achromatopsia (ACHM), also known as rod monochromatism, is a congenital, autosomal recessive inherited retinal disorder with varied prevalence in different regions<sup>[1]</sup>. Affected individuals show a loss of color discrimination, photophobia, pendular nystagmus, and reduced visual acuity<sup>[2]</sup>. Traditionally, ACHM was considered to be a stationary condition. Recent studies indicate that structural changes in the retina occur with time<sup>[3]</sup>, and recent retinal imaging data shows that a progressive degeneration can appear<sup>[4,5]</sup>.

ACHM is genetically heterogeneous. To date, mutations located in five genes have been identified as associated with ACHM in humans, including *CNGA3* [cyclic nucleotide-gated cation channel alpha-3 (MIM 600053)]<sup>[6]</sup>, *CNGB3* [cyclic nucleotide-gated cation channel beta-3 (MIM 605080)]<sup>[7,8]</sup>, *GNAT2* [guanine nucleotide-binding protein G (t) subunit alpha-2 (MIM 139340)]<sup>[9,10]</sup>, *PDE6C* [the catalytic alpha-subunit of the cone cyclic nucleotide phosphodiesterase (MIM 600827)]<sup>[11,12]</sup>, and *PDE6H* [the inhibitory gamma-subunit of the cone photoreceptor PDE (MIM 601190)]<sup>[13]</sup>. All these genes encode essential components of the cone-specific phototransduction cascade. At present, mutations in *CNGB3* account for 40%-50% of all ACHM cases, representing the most common cause of this disorder. The next most common reason is the mutations of *CNGA3* which exist in about 25% of the achromats. Mutations in *GNAT2*, *PDE6C*, and *PDE6H* are rare, and are responsible for fewer than 2% of all cases<sup>[9,14-16]</sup>.

In cone photoreceptors, cyclic nucleotide-gated (CNG) channels are integral heterotetrameric cell membrane proteins composed of either two A3 and two B3 subunits, or three A3 and one B3 subunit<sup>[14,17,18]</sup>. The A3 subunit is topologically composed of six transmembrane helices (S1-S6), a pore region between S5 and S6, a cyclic nucleotide-binding domain (CNBD), and a C-linker between S6 and CNBD (Figure 1)<sup>[19,20]</sup>. Previous studies have revealed 82 disease-causing mutations in the *CNGA3* gene, the majority of which are missense mutations, implying that *CNGA3* peptides are evolutionarily conserved and its function has little tolerance for amino acid variation<sup>[2,4,6,15,21-32]</sup>.

In this study, we describe the clinical characteristics and genetic analysis of a Chinese family, and report two novel amino acid substitutions (D211E, V336F) in *CNGA3* implicated in ACHM.



**Figure 1** A schematic model of the CNGA3 polypeptide. Cylinders imply helical structures in the protein; S1 to S6 denote transmembrane helices; dots represent the novel mutations located in functionally important domains.

## SUBJECTS AND METHODS

**Clinical Assessment and Blood Sampling** The patient was a 2.5-year-old boy presenting with pendular nystagmus, severe photophobia, and hyperopia shortly after birth. There was no family history of ophthalmic disease and the parents were not affected. After a full medical and ophthalmic history was acquired, an ophthalmological examination was performed, including external examination, slit-lamp and fundoscopic examination. Cross-sectional retinal images were recorded horizontally through the fovea (transverse width 12 mm) using optical coherence tomography (OCT) (3D-OCT 2000, Topcon, Tokyo, Japan). The best-corrected visual acuity (BCVA), color vision, visual field, and spectral sensitivity were difficult to assess as the patient was too young. Since electroretinogram (ERG) is the golden standard for the diagnosis of ACHM, we strongly recommended the patient to perform full-field ERG. But his parents refused to do ERG examination under anesthetics, and preferred to do genetic analysis to help with the diagnosis.

After informed consent was obtained, blood samples, used for genetic analysis, were taken from the patient and his parents followed the standard procedure to ensure his safety.

### Molecular Genetic Analysis

**Targeted region capture and next-generation sequencing** A custom-made capture array (NimbleGen, Madison, WI, USA) was designed to capture the 1.5 Mb target region corresponding to the coding exons including 10 bp flanking intron sequence of 283 retinal disease genes (gene list not shown), which included 5 disease-causing genes of ACHM, *CNGA3*, *CNGB3*, *GNAT2*, *PDE6C*, and *PDE6H*. Total genomic DNA of the proband and his parents were extracted from peripheral blood according to the manufacturer's standard procedure using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The genomic DNA was fragmented by Covaris S2 (Massachusetts, USA)

to generate paired-end libraries (200-300 bp). The libraries were pooled and hybridized to the custom-made capture array for 72h at 47°C. After hybridization, the array was washed and eluted according to the manufacturer's instructions (Roche NimbleGen, Inc.). The captured library was sequenced on Illumina HiSeq2000 Analyzers for 90 cycles per read to generate paired-end reads (following the manufacturer's standard sequencing protocols). Image analysis and base calling were performed using the Illumina Pipeline to generate raw data.

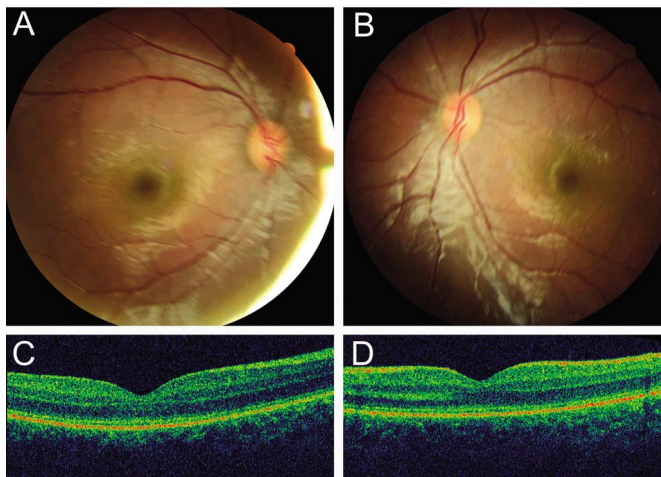
**Variants identification and validation** To detect potential variants of the patient, we performed filtering criteria to generate clean reads (with a length of 90 bp) for further analysis, and then aligned the clean reads against the human genome reference from the NCBI database (NCBI build 37.1) using the BWA (Burrows Wheeler Aligner) Multi-Vision software package. Single-nucleotide variants (SNVs) and Indels were identified using SOAPsnp software and Samtools, respectively. All SNVs and Indels were verified using the NCBI dbSNP, HapMap project, 1000 Genome Project and the database of 100 healthy Chinese adults from BGI to exclude common variants. The novel candidate variations in known *ACHM* genes were validated by polymerase chain reaction (PCR) and Sanger sequencing. PCR primer sets were designed *via* Primer6.0 and products were sequenced using a Bigdye terminator v3.1 cycle sequencing kit (ABI, Foster City, CA, USA) and analyzed on an ABI 3700XL Genetic Analyzer.

## RESULTS

**Clinical Features** Subjectively, the affected individual had a history of nystagmus, photophobia, and hyperopia since early infancy. He was noted to be captivated by bright lights but behaved poorly in a bright environment, and appeared to perform better in the dark. The latest examination at 2.5 years of age revealed an absence of fixation, presenting a small amplitude fast pendular nystagmus. The cycloplegic refraction was +4.50 dioptic sphere OU. Slit-lamp examination showed no specific findings in the anterior segments. The optic discs and maculae were normal on funduscopy. Meanwhile, the cross-sectional OCT images showed neither atrophic changes nor cystoid-like lesions in the maculae (Figure 2). The unaffected family members reported no ocular complaints.

All in all, despite the inaccessible examinations due to patient non-cooperation, a clinical impression of ACHM was made based on the clinical history.

**Genetic Findings** Using the capture panel described in experimental section, an average of 258× depth in target region was achieved. The 94.15% and 92.51% of design target regions were covered by at least 1× and 20×, respectively. All eight exons of *CNGA3* were covered by 100%, the mean sequence depth was approximately 361×, and the similar



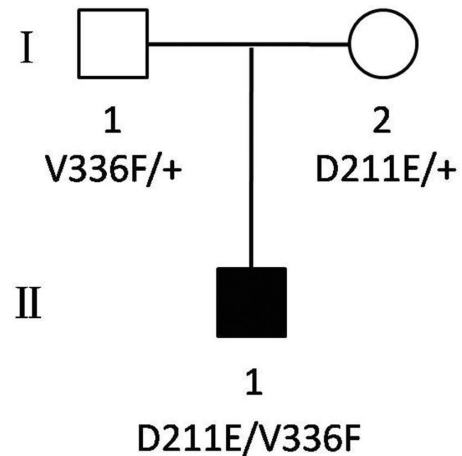
**Figure 2** Color fundus photographs of the posterior pole of the affected patient A: The right eye; B: The left eye. No specific findings are seen in the optic disks and maculae. OCT images through the fovea (C: Temporal to nasal in the right eye; D: Nasal to temporal in the left eye) show neither macular atrophy nor cystoid lesions.

quality data were obtained in the other four known ACHM genes, *CNGB3*, *GNAT2*, *PDE6C*, and *PDE6H*, demonstrating that a sufficient data quality was achieved for variants detection. Forty-eight variants were detected in coding regions and adjacent intronic regions of the five known ACHM genes, and only four variants were found in *CNGA3*. Thirty-nine of these variants have been reported for frequency >0.01 in 1000 genome database, which were considered to be common variants (data not shown). And in the nine rare variants, six variants were all in introns of *PDE6C* while one variant was in intron of *CNGB3*, which seem not to cause disease.

Among the eight coding exons of *CNGA3*, mutation analysis revealed two missense variants (c.633T>A and c.1006G>T) in a compound heterozygous state. These two nucleotide variants, in exons 6 and 7, resulted in changes from aspartic acid (GAT) to glutamic acid (GAA) at amino acid position 211, and from valine (GTC) to phenylalanine (TTC) at amino acid position 336, respectively. Both of the p.D211E and the p.V336F mutations have not been reported previously (Figure 3). Segregation analysis was performed. The father (I-1) was shown to be heterozygous for the mutation p.V336F and the mother (I-2) carried the heterozygous mutation p.D211E. The genealogical tree of this family suggested that the disorder followed an autosomal recessive mode of inheritance (Figure 4).

#### DISCUSSION

Mutations of the *CNGA3* gene have been shown to be responsible for both congenital ACHM and progressive cone dystrophy [15,21,22]. Recently, Li *et al* [32] have reported *CNGA3* mutations in 46 Chinese families, describing an additional 26 new *CNGA3* mutations. In our study, the clinical features of

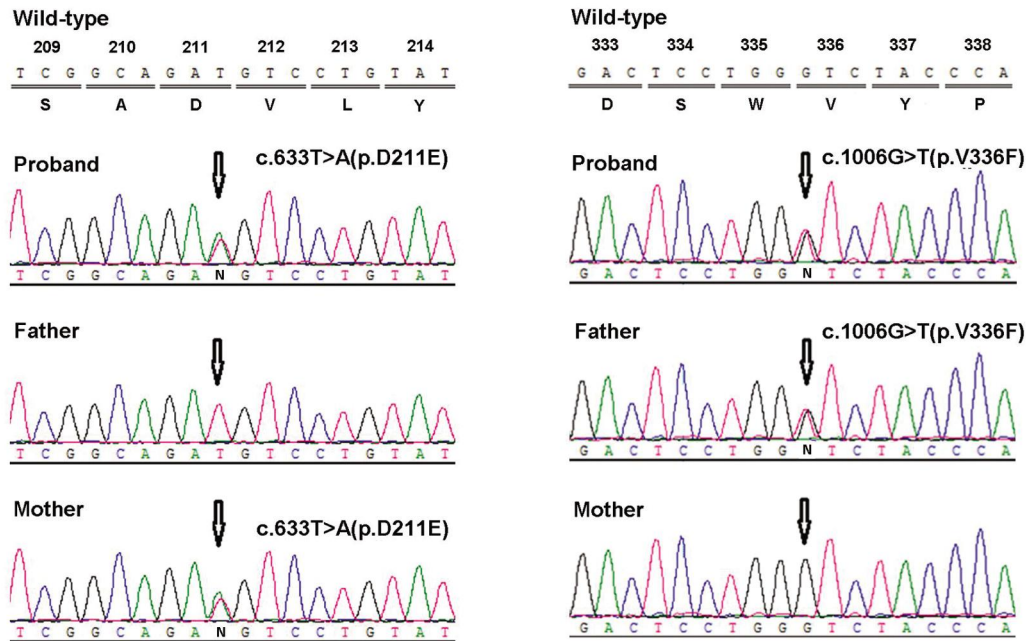


**Figure 3** The pedigree of the family with autosomal recessive ACHM The solid square indicates the proband who carries the two compound heterozygous mutations, p.D211E and p.V336F. The unaffected parents are heterozygous carriers of either the V336F allele or the D211E allele. Accordingly, 100% concordance of genotype and phenotype was observed.

a Chinese patient with ACHM were investigated and the two missense mutations p.D211E and p.V336F were identified as underlying genetic causes in this patient. Neither mutation has been reported previously.

Recent molecular screening studies involving the *CNGA3* gene resulted in the identification of more than 80 different mutations. The majority were missense mutations that altered highly conserved amino acid residues. *CNGA3* mutations were mainly confined to functionally and structurally important regions, including the six transmembrane helices (S1-S6), the ion pore, the C-linker region, and the cGMP binding site. In the present study, we identified the compound heterozygous *CNGA3* mutations p.D211E and p.V336F located in the S2 segment and the pore region, respectively (Figure 1). Theoretically, expression and function of the channels need to be assessed *in vitro* to determine whether the mutations have a pathogenic effect on the patient. Regarding to the phenotype of the patient, it indicated that the variants could be pathogenic.

In fact, D211E and V336 are phylogenetically conserved not only in other mammalian orthologs but also in chicken *CNGA3* (Figure 5), which suggests that a slight variation can result in loss of function. Although mutations within the S2 segment of *CNGA3* have not been reported previously, a recent study involving a missense mutation D262N at S2 of *CNGA3* confirmed that variations in a conserved region can lead to cone dysfunction [33]. The other mutation identified in our study was located in the pore-forming region, a loop between S5 and S6 responsible for ion permeation and gating. According to previously reported results, this mutation (V336F) might result in one or more of the following possible consequences: 1) reduced protein expression and/or



**Figure 4** Sequence analysis and identification of the novel *CNGA3* mutation in the affected Chinese family Wild-type sequence of part of exon 6 (left panel) and exon 7 (right panel) in *CNGA3* gene (top row) and corresponding sequence of the affected individual (the second row), and his parents (the third and bottom rows) are shown. The arrows indicate the sites of nucleotide substitution. In the proband, two heterozygous variations, c.633T>A and c.1006G>T, are revealed, resulted in the p.D211E and p.V336F mutations. While the parents are obligate carriers of either of the two variations.

A		211	
NP_001289.1	201	MLWLVLDSADVLYVLDVLV	220
XP_001156943.1	201	MLWLVLDSADVLYVLDVLV	220
XP_001101944.2	201	MLWLLLDYSADVLYALDALV	220
NP_776704.1	220	MLWLVLDSADILYGMMLV	239
NP_034048.1	142	TLWLVLDSADVLYVLDMLV	161
NP_445947.1	122	TLWLVLDSADALYVVDMLV	141
NP_990552.1	243	KLWLFLDYCSDIYVDFMFV	262
B		336	
NP_001289.1	326	KFIGFGTDSWVYPNISIPEH	345
XP_001156943.1	326	KFIGFGTDSWVYPNISIPEH	345
XP_001101944.2	326	KFIGFGTDSWVYPNISIPEH	345
NP_776704.1	345	KFIGFGTDSWVYPNVSNPY	364
NP_034048.1	267	KFIGFGTDSWVYPNTSKPEY	286
NP_445947.1	247	KFIGFGTDSWVYPNTSKPEY	266
NP_990552.1	368	KVIGFGTDSWVYPNVSIPY	387

**Figure 5** Multiple protein sequence alignment of *CNGA3* in different species Arrows indicate the mutated aspartic acid 211 (A) and valine 336 (B) residues which are evolutionarily highly conserved. The polypeptide sequences are NP\_001289.1 *CNGA3*, Homo sapiens; XP\_001156943.1 *CNGA3*, Pan troglodytes; XP\_001101944.2 *CNGA3*, Macaca mulatta; NP\_776704.1 *CNGA3*, Bos taurus; NP\_034048.1 *CNGA3*, Mus musculus; NP\_445947.1 *CNGA3*, Rattus norvegicus; NP\_990552.1 *CNGA3*, Gallus gallus.

pathway; 3) loss of interactions between subunits for gating; 4) interference of pore helix rotation during gating<sup>[28]</sup>.

Vision is one of the most important sources of information, and cone vision determines the visual quality in the light environment. ACHM causes dysfunction and degeneration of the cone photoreceptor, which severely influences the quality of a patient's life. Currently, this disease is untreatable, but it has been shown that using red contact lenses or wearing red-tinted glasses can help to alleviate photophobia in ACHM patients<sup>[34]</sup>.

Recently, more and more studies have focused on gene replacement therapy for retinal channelopathies, such as ACHM<sup>[35-38]</sup>. In 2010, Michalakis *et al*<sup>[37]</sup> succeeded in the restoration of cone function in the *CNGA3* knockout mouse model. In 2012, Pang *et al*<sup>[39]</sup> showed that adeno-associated viral 5 (AAV5) vector could prevent cone photoreceptor from degeneration and restored cone function. The therapeutic effect lasted more than 5mo in mice with *CNGA3* mutations. In 2013, Komaromy *et al*<sup>[40]</sup> found that a combination of the gene therapeutic technique with intravitreal injection of ciliary neurotrophic factor (CNTF) could effectively rescue cone-mediated function in older *CNGB3* mutant dogs. All these proof-of-concept studies have established the foundation for future gene therapy trials for ACHM in humans.

In conclusion, our study identified a Chinese patient with ACHM due to two compound heterozygous *CNGA3* mutations (p.D211E and p.V336F). To our knowledge,

impaired function; 2) alteration of the ion permeation

neither of the mutations has been reported previously, therefore extending the mutation spectrum of this disorder.

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**Conflicts of Interest:** **Chen XT**, None; **Huang H**, None; **Chen YH**, None; **Dong LJ**, None; **Li XR**, None; **Zhang XM**, None.

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