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ORIGINAL ARTICLE

Identification of a novel *CACNA1F* mutation in a Chinese family with CORDX3

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Abstract

Background: X-linked cone-rod dystrophy (CORDX) is one form of inherited retinal disorders (IRDs) characterized by progressive dysfunction of photoreceptor. Three types of CORDX were reported and *CACNA1F* gene defect can cause CORDX3. The aim of this study was to investigate the pathogenic variant in a Chinese family with IRD.

Methods: The two affected subjects including the proband and his elder sister underwent ophthalmic examinations. Whole exome sequencing (WES) was performed in the proband at first, then co-segregation analysis was performed in the family by Sanger sequencing. Minigene approach was used to verify the effect of the mutation on the splicing of *CACNA1F*. X-chromosomal inactivation assay was performed to evaluate the inactivation patterns of the female carriers.

Results: The ophthalmic examination results of the proband fit the clinical description of CORDX3, and the female patient presented with only mild symptoms due to mildly skewed X-chromosomal inactivation (ratio 67: 33). Molecular genetic testing identified a novel splice-site mutation c.3847-2A > G in *CACNA1F* (NM_005183.4) gene in the patients, which inherited from their asymptomatic mother. Minigene approach confirmed that c.3847-2A > G could affect the splicing of *CACNA1F*.

Conclusion: Our study identified a novel splice-site mutation in the *CACNA1F* gene, which expanded the mutational spectrum of *CACNA1F*-releated diseases and demonstrated the importance of combining clinical and genetic testing in the diagnosis of IRDs.

KEYWORDS
CACNA1F, minigene, splice-site mutation, X-linked cone-rod dystrophy

1 | INTRODUCTION

X-linked cone-rod dystrophy (CORDX) is a rare, progressive retinal disease characterized by decreased visual acuity, myopia, abnormal color vision and cone or cone-rod dystrophy. CORDX is clinically and genetically heterogeneous (Jalkanen et al., 2006). The age of onset and severity of symptoms are variable expressivity, and three gene loci have been identified. CORDX1 (OMIM: 304020) is caused by *RPGR* defect (Demirci et al., 2002), which also causes retinitis pigmentosa 3 (OMIM: 300029) (Meindl et al., 1996) and macular degeneration (OMIM: 300834) (Ayyagari et al., 2002). Additional forms of CORDX include CORDX2 (OMIM: 300085) mapped to Xq27 (Bergen & Pinckers, 1997), and CORDX3 (OMIM: 300476) caused by *CACNA1F* mutation (Jalkanen et al., 2006; Mäntyjärvi et al., 2001). The *CACNA1F* gene defect is also responsible for Aland Island eye disease (AIED, OMIM:300600) (Jalkanen et al., 2007) and incomplete congenital stationary night blindness (icCSNB, OMIM: 300071) (Bech-Hansen et al., 1998; Strom et al., 1998). It comprises 48 exons and codes for the pore-forming α_{1F} subunit of the L-type voltage gated calcium channel (Cav1.4), which localized to rod and cone photoreceptor ribbon synapses and supported Ca^{2+} influx under relatively depolarized conditions (Abdelkader et al., 2018; Koschak et al., 2021).

Over 200 mutations in *CACNA1F* including missense, nonsense, splice-site variants, and indels have been reported (Mahmood et al., 2021).

Here, we describe the clinical phenotypes of two patients—a male patient and his elder sister—from a Chinese family with congenital visual impairment. A novel splice-site mutation c.3847-2A > G in the *CACNA1F* gene was identified and the deleterious effect was demonstrated by minigene approach. Combined with the ophthalmic examinations and genetic testing results, the family was finally diagnosed as CORDX3.

2 | MATERIALS AND METHODS

2.1 | Clinical examinations

Four individuals including two patients and their parents were recruited in this study (Figure 1a), and ophthalmic examinations were performed on each of the two patients (II_2 and II_3). The ophthalmic examinations included best corrected visual acuity (BCVA), color vision test (pseudo-isochromatic plate tests), panoramic laser scanning fundus photography, and full field electroretinogram (ff-ERG) in accordance with International Society for
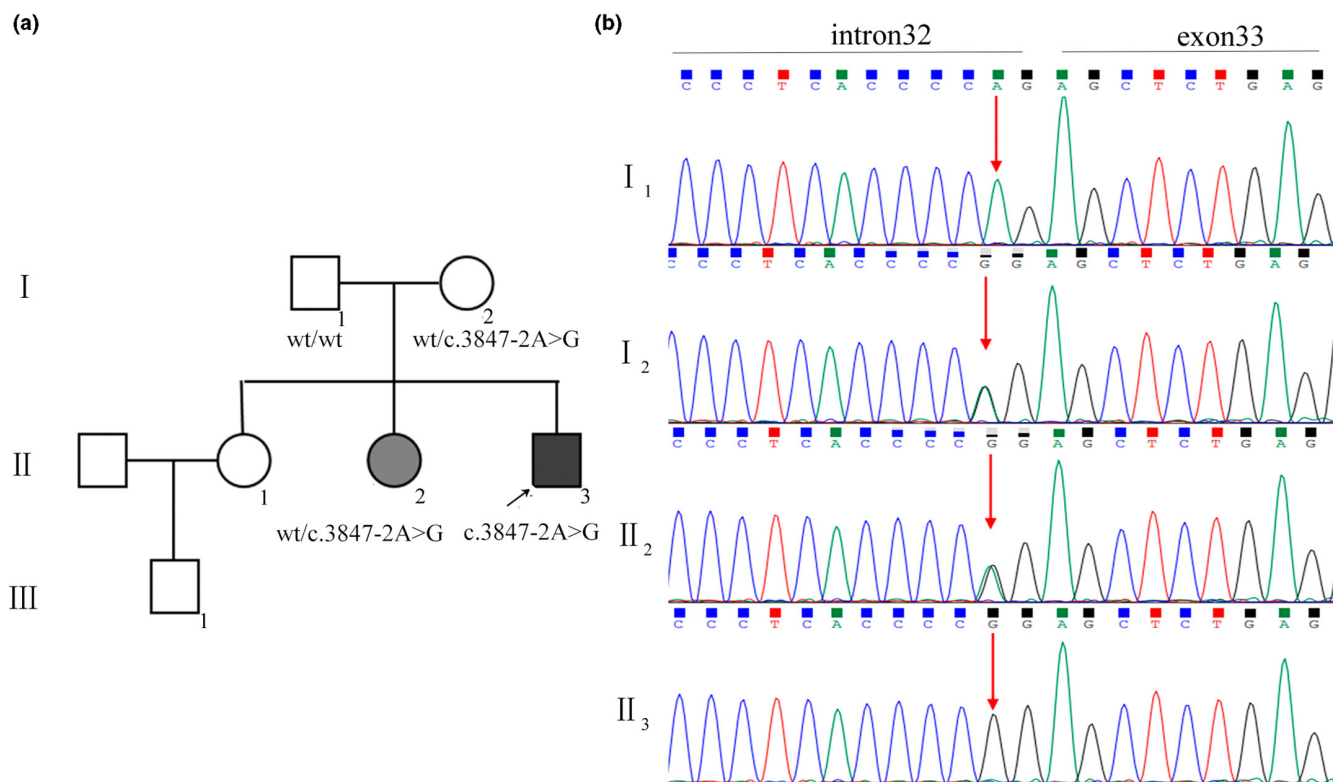

FIGURE 1 Identification of *CACNA1F* mutation. (a) Family pedigree. Black arrow represents the proband. Wt represents wild type. (b) Sanger sequencing of the *CACNA1F* (NM_005183.4) mutation c.3847-2A > G for the proband II_3 (mutant-type hemizygote), father I_1 (wild-type hemizygote), mother I_2 (heterozygote) and the elder sister II_2 (heterozygote)

Clinical Electrophysiology of Vision (ISCEV) guidelines (Hauke et al., 2013; Kimchi et al., 2019).

2.2 | DNA mutation detection

Peripheral blood DNA from the two patients and their parents was isolated using the Blood Genomic DNA Extraction kit (Solarbio, Beijing, China). Genomic DNA of the proband II_3 was analyzed by whole exome sequencing (WES) in MyGenostics (Beijing, China) using the XTen system (Illumina, San Diego, CA, USA). Sequenced reads were mapped against the human reference genome (GRCh37) with Burrows-Wheeler Aligner (BWA). Variant identification was performed with Genome Analysis Toolkit (GATK). The annotation of variants was performed by ANNOVAR. Variants identified in known genes associated with inherited retinal disorders (IRDs) were carefully prioritized by allele frequency in gnomAD and predicted results using bioinformatics software including SIFT (http://sift.bii.a-star.edu.sg), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutationtaster.org/), NNSplice (http://www.fruitfly.org/seq_tools/splice.html), and FSPLICE (http://www.softberry.com). A splice-site mutation in *CACNA1F* (NM_005183.4) was found as candidate variant. Co-segregation analysis was performed by Sanger sequencing (Sangon Biotech, Shanghai, China) after specific PCR using primer 1 h-CA-F/R (Table 1). The pathogenicity of the variant was assessed according to the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015).

2.3 | Minigene approach

Using the Minigene approach to verify the effect of mutations on gene splicing has been widely accepted (Nakajima et al., 2016). Fragments of *CACNA1F* gene spanned intron 30 to intron 35 were amplified from control and patient genomic DNA (I_1 and II_3) respectively using 2× Phanta® Master Mix (Vazyme, Nanjing, China) and primer 2 h-CA-F/R (Table 1, Figure 3a). The amplicons were cloned into pcDNA3.1(+) vector at the *Nhe* I and *Xho* I sites respectively, then transformed into Trans5α chemically competent cell (TransGen, Beijing, China). Positive clones were analyzed by Sanger sequencing (Figure S4). EndoFree Mini Plasmid Kit II (TianGen, Beijing, China) was used to extract the pcDNA3.1-CA-wt/mut plasmids. Transient transfection studies were performed in HEK293T cells in 6-well plates using LipoFiter (HANBIO, Shanghai, China), and total RNA was extracted by RNAsimple Total RNA Kit (TianGen, Beijing, China). Reverse transcription

was performed using HiScript®IIQ RT SuperMix for qPCR (Vazyme, Nanjing, China). To analyze the splicing products, PCR was performed using primer 3 h-CA-F/R present in exon 31 and 34 (Table 1, Figure 3a). Five-microliter PCR products were investigated by electrophoresis on a 2% agarose gel. Amplified bands were directly excised from the gel and purified using Gel Extraction Kit (CWBIO, Beijing, China), then analyzed by Sanger sequencing.

2.4 | X-chromosomal inactivation assay

X-chromosomal inactivation (XCI) patterns of II_2 and I_2 were analyzed according to Allen et al. (1992). In brief, genomic DNA was digested with methylation-sensitive restriction endonuclease *Hpa* II. The polymorphic repeat $(CAG)_n$ regions in *AR* gene exon 1 from digested and undigested DNA samples were amplified by PCR with primer AR-F/R (AR-F: 5-FAM-GCTGTGAAGGTTGCTGTTCCT CAT-3, AR-R: 5-TCCAGAATCTGTTCCAGAGCGTGC-3) (Daza-Cajigal et al., 2013), then the products were analyzed by ABI3500DX (ABI, USA). XCI ratio was calculated according to the following formula: $[(d1/u1)/(d1/u1+d2/u2)]$, where $d1$ = peak height of allele 1 (digested DNA), $d2$ = peak height of allele 2 (digested DNA), $u1$ = peak height of allele 1 (undigested DNA), and $u2$ = peak height of allele 2 (undigested DNA) (Butler et al., 2007). The XCI pattern was classified as random (ratio 50:50 ~ 59:41), mildly skewed (65:35 ~ 79:21), or severely skewed (≥80:20).

3 | RESULTS

3.1 | Clinical characteristics

There are two affected patients in this family (Figure 1a). The parents denied consanguinity. The proband (II_3) is 31 years old. He had severe myopia, amblyopia since childhood, and nystagmus was present since the first weeks of life. BCVA was 0.25 and 0.15 in the right and left eye, respectively. Panoramic laser scanning fundus photography revealed typical fundus features of high myopia in both eyes, including peripapillary atrophy, peripheral retinal degeneration, and chorioretinal atrophy (Figure 2a). ff-ERG showed severely decreased scotopic and photopic responses with almost extinguished a- and b- waves in rod-specific, combined rod-cone, and cone-specific responses. Oscillatory potentials were markedly reduced and the 30-Hz flicker responses were extinguished in both eyes (Figure 2b). Pseudo-isochromatic plate tests indicated that he had red-green color defect. II_2 is a 33-year-old female who presented with hyperopia and astigmatism. BCVA was 0.4 and 0.5 in the right and left

TABLE 1 Primers for co-segregation analysis and minigene approach

Primer name	Sequence	Restriction enzymes
1 h-CA-F	5-TAATGACCCCACCATCACCC-3	–
1 h-CA-R	5-TCTCCAAAGTGCCAAGGACT-3	–
2 h-CA-F	5-CTAGCTAGCGCCCATCGTGTG ATCCAA-3	Nhe I
2 h-CA-R	5-CCGCTCGAGGCCAGGAAACAT CTCGGG-3	Xho I
3 h-CA-F	5-GAACACGTTTGACGCTCT TATT-3	–
3 h-CA-R	5-AATGACGGCATAGATGAA GAATA-3	–
GAPDH-F	5-AGAAGGCTGGGGCTCATTTG-3	–
GAPDH-R	5-AGGGGCCATCCACAGTCTTC-3	–

Note: The underlined sequence is the restriction endonuclease recognition site.

eye, respectively. Fundus photograph revealed normal. ff-ERG in both eyes showed reduced rod-specific responses and oscillatory potentials. The rod-cone and cone responses were electronegative. The 30-Hz flicker responses were mildly reduced (Figure S1).

3.2 | A novel splice-site mutation was identified in *CACNA1F*

We first performed WES in the proband II$_3$, and identified two nucleotide variants in the *CACNA1F* (NM_005183.4) gene: c.3847-2A > G and c.3940C > T (p.Arg1314Cys). Co-segregation analysis showed that both of the two mutations inherited from the asymptomatic mother I$_2$. The father I$_1$ did not carry any of them (Figure 1b, Figure S2). There are three male individuals reported to carry the variant c.3940C > T in gnomAD. The mutation c.3847-2A > G has not been reported in databases including gnomAD, dbSNP, ClinVar and 1000 Genomes before. Several software including NNsplice, MutationTaster and FSPLICE predicted that c.3847-2A > G abolished the normal splice-acceptor site of intron 32 and would result in aberrant splicing (Figure S3). Based on this information, c.3847-2A > G was assessed as a pathogenic (PVS1 + PM2 + PP3) variant according to ACMG guidelines.

3.3 | Effect of the splice-site mutation c.3847-2A > G in *CACNA1F*

To investigate the effect of c.3847-2A > G, we performed a minigene approach. RT-PCR analysis of the

pcDNA3.1-CA-wt transcript showed two different bands (Figure 3b). The longer band was 250 bp and the other one was 121 bp. In contrast, the pcDNA3.1-CA-mut RT-PCR products revealed only one band of 121 bp with the 250 bp band missing (Figure 3b). Sanger sequencing confirmed that the 250 bp band corresponded to the transcript with skipping of exon 32, contained exons 31, 33 and 34 of *CACNA1F*. The 121 bp band contained exon 31 and 34 (Figure 3c). These results suggested that c.3847-2A > G resulted in aberrant splicing of *CACNA1F* exon 33, although neither the wild-type nor mutant minigene constructs corresponded to the canonical transcript of *CACNA1F* including exon 32. The in-frame deletion of exon 32 and 33 was predicted to lead to the absence of transmembrane segment IVS4 and nearby extracellular loops (DeepTMHMM, https://dtu.biolib.com/DeepTMHMM) (Figure S5).

3.4 | X-chromosomal inactivation patterns of the female carriers

Two *AR* gene amplified products were found before digestion of the genomic DNA from patient II$_2$. The 287 bp (allele 1) and 293 bp (allele 2) fragments were inherited from her father I$_1$ and mother I$_2$, respectively. Peak height of the 293 bp fragment was significantly reduced than that of the 287 bp band after *Hpa* II digestion (peak height: d1 = 2398, u1 = 2804; d2 = 889, u2 = 2137). Accordingly, the paternal X-chromosome was mildly skewed inactivation in patient II$_2$ with an XCI ratio of 67: 33 (Figure S6). XCI assay of the mother I$_2$ showed a random inactivation pattern (ratio 52: 48, peak height: d1 = 898, u1 = 438, d2 = 653, u2 = 346) (Figure S6).

4 | DISCUSSION

We performed ophthalmic examinations and genetic testing on the proband II$_3$ and his sister II$_2$ and identified a novel splice-site mutation c.3847-2A > G in *CACNA1F*, which was inherited from the mother I$_2$. Minigene showed that c.3847-2A > G resulted in aberrant splicing of *CACNA1F* exon 33. However, since the short band was also detected in the wild-type transcript, it was inadequate to determine whether the short band detected in the mutant transcript was due to the splice-site variant or minigene approach (Zeitz et al., 2019). The mutation may result in exon 33 skipping to produce a truncated protein, or potential splice-sites lead to the presence of termination codon, which in turn triggers nonsense-mediated mRNA degradation (NMD). As for the absence of exon 32, a systematical screen for alternatively spliced exons in Cav1.4 transcripts from the human retina cDNA revealed that the
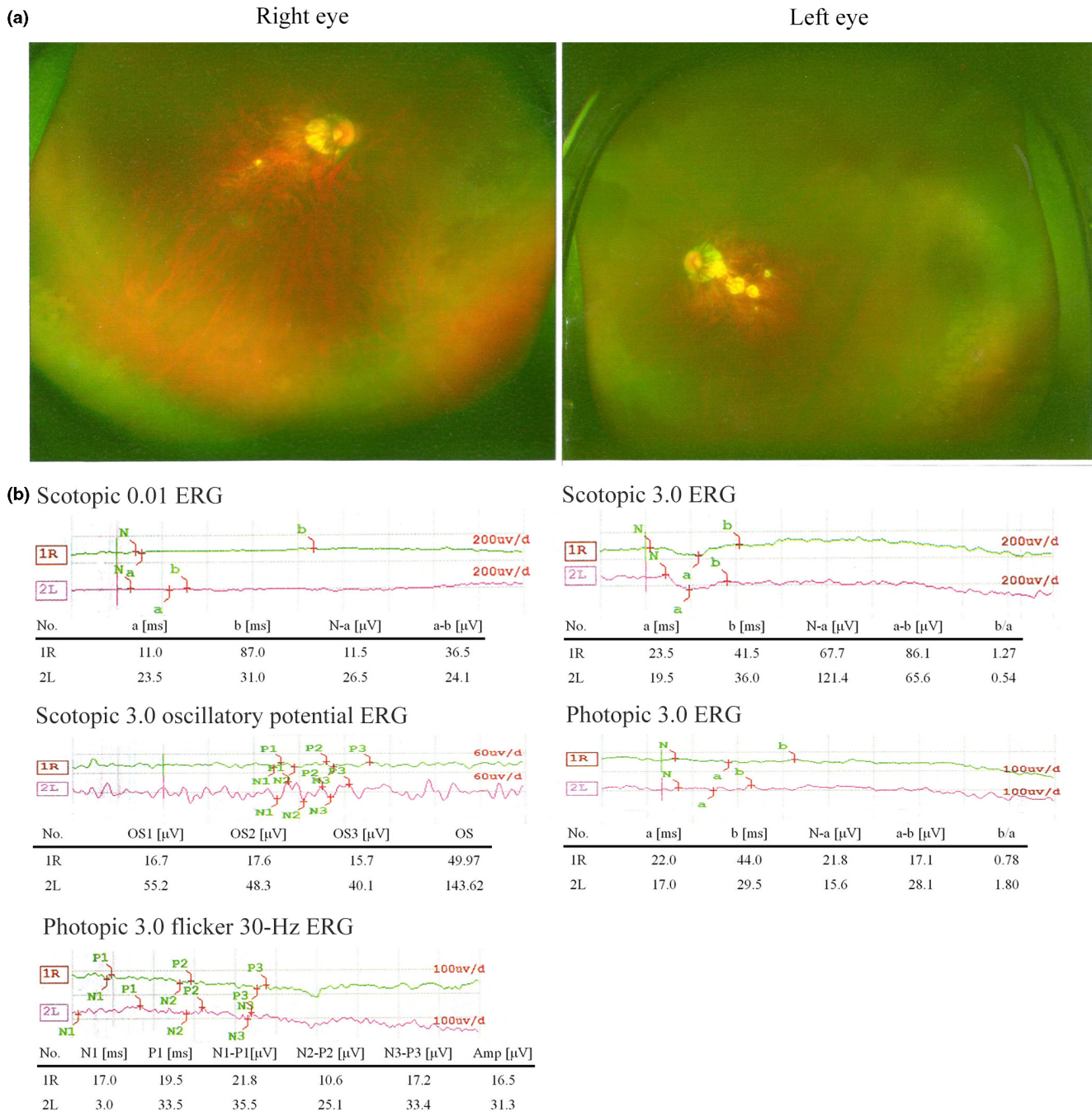
(a)

Right eye	Left eye

(b) Scotopic 0.01 ERG

No.	a [ms]	b [ms]	N-a [μV]	a-b [μV]
1R	11.0	87.0	11.5	36.5
2L	23.5	31.0	26.5	24.1

Scotopic 3.0 ERG

No.	a [ms]	b [ms]	N-a [μV]	a-b [μV]	b/a
1R	23.5	41.5	67.7	86.1	1.27
2L	19.5	36.0	121.4	65.6	0.54

Scotopic 3.0 oscillatory potential ERG

No.	OS1 [μV]	OS2 [μV]	OS3 [μV]	OS
1R	16.7	17.6	15.7	49.97
2L	55.2	48.3	40.1	143.62

Photopic 3.0 ERG

No.	a [ms]	b [ms]	N-a [μV]	a-b [μV]	b/a
1R	22.0	44.0	21.8	17.1	0.78
2L	17.0	29.5	15.6	28.1	1.80

Photopic 3.0 flicker 30-Hz ERG

No.	N1 [ms]	P1 [ms]	N1-P1[μV]	N2-P2 [μV]	N3-P3 [μV]	Amp [μV]
1R	17.0	19.5	21.8	10.6	17.2	16.5
2L	3.0	33.5	35.5	25.1	33.4	31.3

FIGURE 2 (a) Panoramic laser scanning fundus photography of patient II_3 revealed typical fundus features of high myopia with peripapillary atrophy, peripheral retinal degeneration and chorioretinal atrophy in both eyes. (b) ff-ERG of patient II_3 showed severely decreased scotopic and photopic responses with almost extinguished a- and b- waves in rod-specific, combined rod-cone and cone-specific responses. Oscillatory potentials were also markedly reduced and the 30-Hz flicker responses were extinguished in both eyes. 1R-right eye, 2 L-left eye

most abundant productive splice variant was Δ32 (exon 32 skipping) (Tan et al., 2012). Although more experiments on protein level are needed to verify the function of the splice-site mutation, we can confirm that it does lead to the loss of the normal transcript in the patients.

The relationship between genotype and phenotype of *CACNA1F* associated diseases is unclear. The pathogenic variants were distributed throughout the whole gene, and

no mutation hotspots were found. About 230 variants were reported to be associated with icCSNB, and only a few variants with CORDX3 were reported (Mahmood et al., 2021). The variant c.3847-2A > G identified in this study is located in intron 32, next to the splice-site variant c.3847-1G > A which is reported to be associated with icCSNB (Zeitz et al., 2019). However, we preferred to describe the family in our research as CORDX3 for
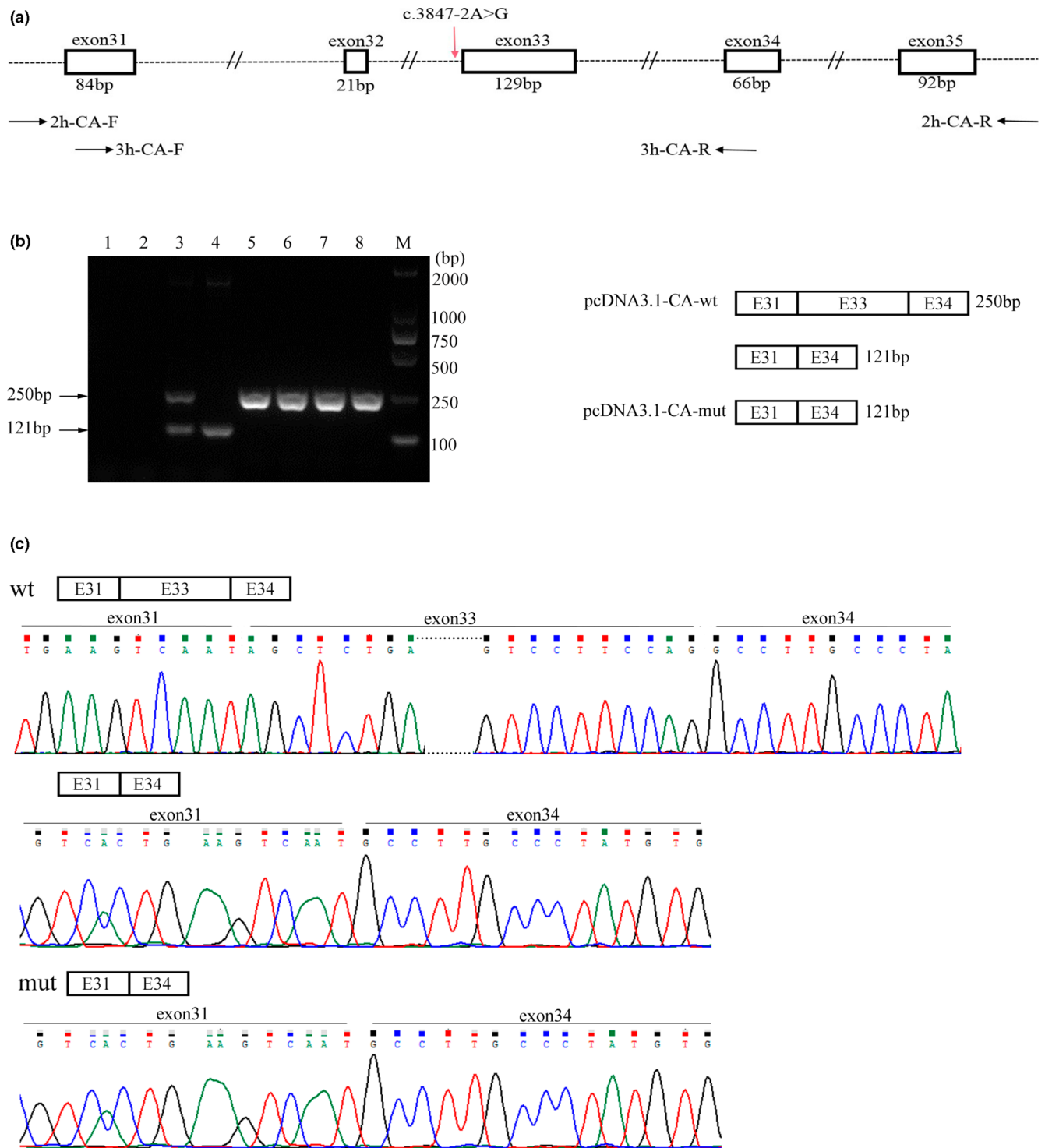
(a)

(b)

(c)

FIGURE 3 Analysis of the c.3847-2A>G mutation using minigene approach. (a) The *CACNA1F* gene fragment selected for minigene. The horizontal arrows showed binding sites of primer 2 h-CA-F/R used for expression vector constructing, and primer 3 h-CA-F/R used for RT-PCR analysis. The mutation c.3847-2A>G was marked by vertical arrow. (b) Electrophoresis of RT-PCR products of the *CACNA1F* transcripts obtained from HEK293T cells transfected with pcDNA3.1-CA-wt and pcDNA3.1-CA-mut. 1-HEK293T, 2-HEK293T + pcDNA3.1 (empty vector), 3-HEK293T + pcDNA3.1-CA-wt, 4-HEK293T + pcDNA3.1-CA-mut, 5~8-*GAPDH* PCR products of cDNA. (c) Directly Sanger sequencing of the RT-PCR products from the wild-type and the mutant minigene

the almost extinguished photopic and scotopic function, color vision defect, and the progressively decreased visual acuity described by the two patients. The occurrence of nystagmus and astigmatism presented in the family is atypical, but can be seen in some patients with CORDX3. Variable clinical features observed in CORDX3, AIED and

icCSNB are not explainable by different *CACNA1F* mutations (Hauke et al., 2013; Mahmood et al., 2021). Even, the same mutation in *CACNA1F* in one family can cause icCSNB and AIED in different members at the same time (Boycott et al., 2000; Vincent et al., 2011). Given the significant phenotype overlapping of CORDX3 and its allelic disorders icCSNB and AIED, to provide a more precise diagnosis researchers proposed to classify the diseases by genotype, calling them *CACNA1F*-associated retinal dystrophy (Men et al., 2017; Mihalich et al., 2022).

In general, most X-linked recessive genetic disease do not lead to clinical symptoms in carrier females. However, skewed X-chromosomal inactivation or homozygous mutations can lead to female involvement, and this phenomenon is not rare in retinal diseases (Kimchi et al., 2019; Michalakis et al., 2014). In our study, the mother in the family was asymptomatic and the daughter II_2 presented mild manifestation and therefore, we performed the XCI assay. The results suggested that mildly skewed XCI was responsible for the clinical presentation of II_2. Preising et al. reported two female siblings from a consanguineous Pakistani family showing high myopia and reduced visual acuity due to a novel homozygous splice-site mutation in *CANCA1F* (Preising et al., 2018). Therefore, females with visual impairment should be also concerned about whether they have X linked hereditary eye diseases.

IRDs are clinically and genetically heterogeneous diseases, and the early accurate diagnosis is difficult to achieve. Birtel et al. reported one pedigree with four IRDs in which the diagnosis was confirmed by combining in-depth retinal phenotyping and molecular genetic testing (Birtel et al., 2020). It is not rare for icCSNB patients to be misdiagnosed with other eye conditions before genetic testing (Men et al., 2017; Pasutto et al., 2018). In our research, the proband II_3 was diagnosed as high myopia and amblyopia when he was about 13 years old, and II_2 had never had an ophthalmic examination until they were found to carry a splice-site mutation in *CACNA1F* gene recently, which facilitated the clinical diagnosis in this family. Therefore, the combination of clinical examinations and genetic testing in IRDs patients is needed for precise diagnosis, even potential therapeutic options.

5 | CONCLUSION

In summary, we identified a novel variant in the *CACNA1F* gene causing CORDX3. This finding further expands the mutational spectrum and our knowledge of *CACNA1F* related disease, and highlight the importance of combining clinical examination with genetic testing for patients with IRDs.

CONFLICT OF 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.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The research was approved by the Ethical Committee of Shijiazhuang Second Hospital and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants.

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SUPPORTING INFORMATION

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