·Basic Research·

A novel *CRX* mutation by whole-exome sequencing in an autosomal dominant cone-rod dystrophy pedigree

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Abstract

• AIM: To identify the disease-causing gene mutation in a Chinese pedigree with autosomal dominant cone-rod dystrophy (adCORD).

• METHODS: A southern Chinese adCORD pedigree including 9 affected individuals was studied. Whole-exome sequencing (WES), coupling the Agilent whole-exome capture system to the Illumina HiSeq 2000 DNA sequencing platform was used to search the specific gene mutation in 3 affected family members and 1 unaffected member. After a suggested variant was found through the data analysis, the putative mutation was validated by Sanger DNA sequencing of samples from all available family members.

• RESULTS: The results of both WES and Sanger sequencing revealed a novel nonsense mutation c.C766T (p.Q256X) within exon 5 of *CRX* gene which was pathogenic for adCORD in this family. The mutation could affect photoreceptor-specific gene expression with a dominant-negative effect and resulted in loss of the OTX tail, thus the mutant protein occupies the CRX-binding site in target promoters without establishing an interaction and, consequently, may block transactivation.

• CONCLUSION: All modes of Mendelian inheritance in CORD have been observed, and genetic heterogeneity is a hallmark of CORD. Therefore, conventional genetic diagnosis of CORD would be time-consuming and labor-intensive. Our study indicated the robustness and cost-effectiveness of WES in the genetic diagnosis of CORD.

• KEYWORDS: cone-rod dystrophy; autosomal dominant cone-rod dystrophy; whole-exome sequencing; Sanger sequencing; *CRX* gene; mutation

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INTRODUCTION

Cone-rod dystrophies (CORDs; prevalence, 1/40 000) are progressive inherited retinal disorders characterized predominantly by cone dysfunction in the early stage and subsequent rod degeneration[1]. The clinical manifestations of CORDs include photophobia, reduced visual acuity, color vision defects, and central scotoma. Nystagmus may present in some cases. Absent or severely impaired cone function on electroretinography (ERG) is the typical sign of CORDs[2]. Impairment of rod function is frequently observed soon after significant cone dysfunction [1]. In extreme cases, these progressive symptoms are accompanied by widespread, advancing retinal pigmentation along with central and peripheral chorioretinal atrophy. At the advanced stage, CORDs can be difficult to differentiate from retinitis pigmentosa based on the clinical signs alone[3,4].

CORDs are exceptionally heterogeneous, both genetically and phenotypically [3-5]. Not only is the diagnosis of some of these diseases difficult because of overlapping phenotypes, but mutations within a single gene can cause very different phenotypes [3-5]. CORD may be transmitted as an autosomal dominant (adCORD), autosomal recessive (arCORD), or X-linked trait (xlCORD). To date, mutations in at least 26

genes have been reported to be associated with different forms of CORDs. Of these, 10 genes with mutations responsible for adCORD are: *AIPL1*[6], *CRX*[7], *GUCA1A*[8], *GUCY2D*[9], *PITPNM3*[10], *PROM1*[11], *PRPH2*[12], *RIMS1*[13], *SEMA4A*[14], and *UNC119*[15]. The next 13 for arCORD are: *ABCA4*, *ADAM9*, *C8ORF37*, *CACNA2D4*, *CDHR1*, *CERKL*, *CNGB3*, *CNNM4*, *KCNV2*, *PDE6C*, *RAX2*, *RDH5*, and *RPGRIP1*[15]. Other mapped loci or chromosomal regions for arCORD are: *NF1*, 18q21.1-q21.3, 1q12-q24, and 10q26[16]. xlCORD is caused by mutation in an alternative terminal exon 15 (ORF15) of the *RPGR* gene, which maps to chromosome Xp21.1[17]. Additional forms of xlCORD are Xq27.2-q28 and the *CACNA1F* gene on chromosome Xp11.23[18].

Whole-exome sequencing (WES) is a direct, reproducible, and robust method for the confirmation of novel pathogenic genes and the genetic diagnosis of both Mendelian and complex diseases[19]. Protein-coding genes constitute only about 1% of the human genome but harbor nearly 85% of the disease-causing mutations at individual Mendelian loci[20]. Therefore, selectively sequencing complete coding regions can serve as a genome-wide scan for pathogenic genes[20]. Moreover, WES may be a better choice for some diseases with overlapping symptoms that might be ambiguously associated with many pathogenic genes, in which event the detection of mutation by Sanger sequencing could be time-consuming and labor-intensive[19,21].

Here, we used WES, coupling the Agilent whole-exome capture system to the Illumina HiSeq 2000 DNA sequencing platform, to identify a Chinese pedigree with adCORD.

SUBJECTS AND METHODS

Participants and Examinations A five-generation Chinese family including 9 affected individuals was investigated in this study(Figure 1). Ophthalmic examination and diagnostic testing was based on color vision testing of three color axes using the Hardy-Rand-Rittler tests (HRR), full-field ERG, Goldmann perimetry, optical coherence tomography (OCT) scans and retinal thickness measurements. One hundred unrelated healthy matched controls were included. This study was conducted in conformity with the Declaration of Helsinki and was approved by the Ethics Committee of Zhejiang University. Written informed consent was given by all participants.

Exome Sequencing and the Data Filtering and Analysis Pipeline Peripheral blood genomic DNA samples from each of 4 members of the family, III-4, III-10, III-12 and IV-7, were prepared for WES (Figure 1). The whole-exome capture array design, library construction, next-generation sequencing, data filtering, and analysis pipeline followed protocols described previously[21].

Linkage Analysis MERLIN (Multipoint Engine for Rapid Likelihood Inference) software was used to analyze the compiled pedigree structures of the WES dataset[22]. The filter threshold as a minimum LOD score of 6.0 was set for further analysis.

Prediction of Functional Impact In an effort to assess the functional significance of the gene variations identified in this study, we used Sorting Intolerant from Tolerant (SIFT; http://sift.bii.a-star.edu.sg/) to predict the functional effect of non-synonymous single-nucleotide polymorphisms (SNPs)[23].

Sanger Sequencing The putative mutations were validated by Sanger DNA sequencing of samples from available family members (Figure 1).

RESULTS

Clinical Examination The proband (Figure 1, III-12) was a 50-year-old male, who had been diagnosed with progressively reduced visual acuity and mild color vision abnormalities when he was 40 years old. Fundus photographs indicated a 2PD gold-foil-like reflection of the macula, diffuse hypopigmentation. The peripheral retina exhibited bone spicule-like hyperpigmentation with attenuation of the retinal arteries (Figure 2A). OCT revealed retinal thinning in the macular region (Figure 2B). Fundus autofluorescence showed hyperfluorescence in the macula without peripheral choroidal atrophy (Figure 2C). ERG revealed mildly reduced cone responses and normal rod responses. The other affected members exhibited a similar clinical phenotype, and showed a clear autosomal dominant pattern of inheritance. Therefore, a diagnosis of CORD was made based on the published criteria[2,3,24,25].

Exome Sequencing Identified 4 Candidate Pathogenic Genes We generated an average of about 4.5 billion bases of sequence per affected individual as paired-end, 90 bp reads, and the fraction of effective bases on-target was about 95% with a minimum 20-fold average sequencing depth on-target. At this depth of coverage, >99% of the targeted bases were sufficiently covered to pass our thresholds for variant-calling (Tables 1, 2). We focused on non-synonymous (NS) variants, splice acceptor, and donor site mutations (SS), anticipating that synonymous variants would be far less likely to be pathogenic. Filtering against public SNP databases, the 1000 Genome Project, eight HapMap exomes, the in-house exome database provided by BGI and one normal individual from the family, 26 genes harboring 26 different NS/SS were shared by the three patients (Table 1).

Functional Significance of Gene Variations c.C766T (p.Q256X) in *CRX* was considered to be a definite pathogenic mutation due to the creation of a novel upstream stop-site which truncates the normal protein. The pathogenicity of the other 25 variants was excluded by SIFT (Table 1) and linkage analysis.

Validation of the *CRX* Germline Mutation The Sanger DNA sequencing scan of the whole *CRX* gene was completely consistent with WES. c.C766T (p.Q256X) within exon 5 of *CRX* was found in the affected family members

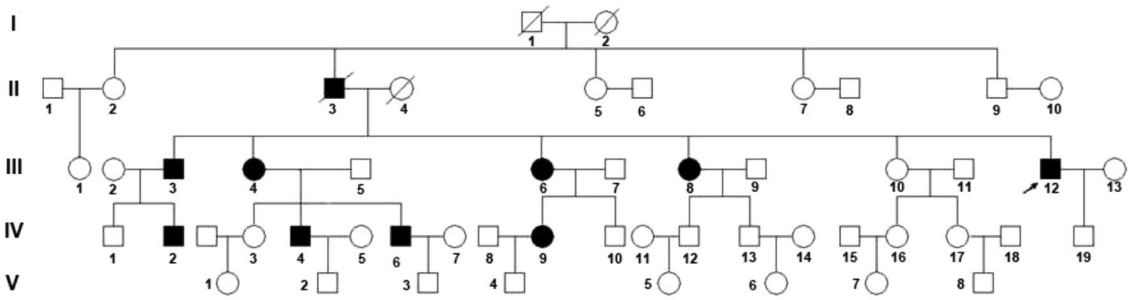

Figure 1 Pedigree of the family investigated Arrow indicates the proband (III-12).

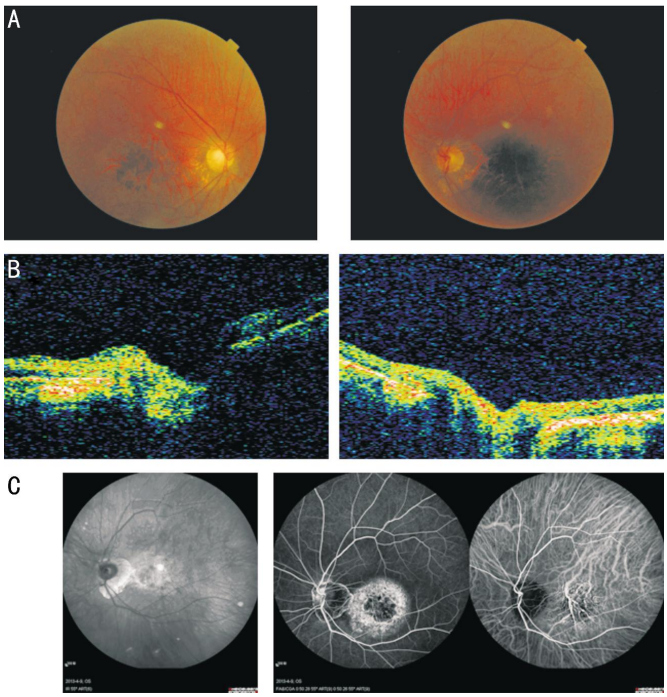

Figure 2 Ophthalmic examination results of the proband (III-12) A: Fundus photographs of the proband showed diffuse hypopigmentation and reduced sensitivity in a central scotoma accompanied by maculopathy. The peripheral retina exhibited bone spicule-like hyperpigmentation with attenuation of retinal arteries. B: OCT of the proband revealed retinal thinning in the macular region. C: Fundus autofluorescence of the proband showed hyperfluorescence in the macula without peripheral choroidal atrophy.

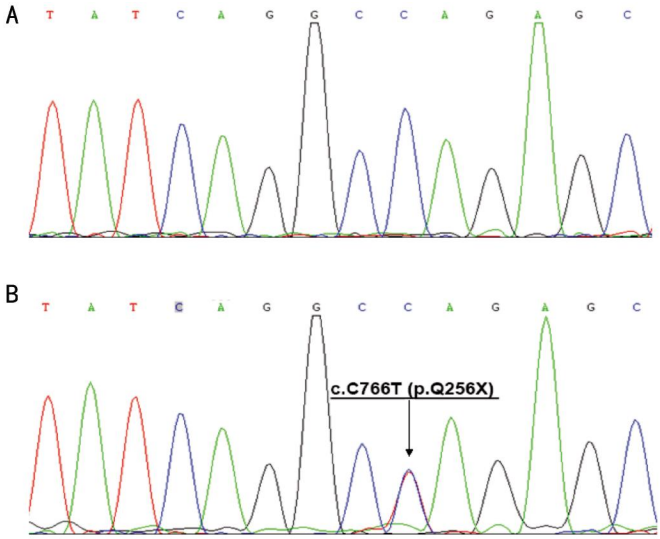

Figure 3 Results of nucleotide sequencing analysis A: Sequence analysis of the *CRX* coding region in an unrelated normal individual was reference homozygote. B: The proband (III-12) showed a heterozygous c.C766T (p.Q256X) mutation. Arrow indicates the position of the mutation.

but absent from the unaffected members and 100 normal controls (Figure 3).

DISCUSSION

In this study, we used WES to identify a novel nonsense mutation c.C766T (p.Q256X) within exon 5 of *CRX* in a Chinese adCRD family. WES analyzes the exons and coding regions of thousands of genes simultaneously using next-generation sequencing technique [20]. By sequencing the exome of a patient and comparing it with a normal reference sequence, variations in an individual's DNA sequence can be identified and related to the individual's medical concerns in an effort to discover the cause of the disorder [20]. The overall molecular diagnostic rate is higher than several comparable genetic tests, including chromosome studies (5%-10%) and chromosomal microarray analysis (15%-20%)[26-28]. Recent increases in accuracy have enabled the development of clinical exome sequencing for mutation identification in patients with suspected genetic diseases [29-31]. We anticipate applications for WES that include the discovery of genes and alleles contributing to Mendelian and complex traits, especially for disease like CORDs, which are exceptionally heterogeneous in genotype and phenotype[5,19,29,30].

The *CRX* gene located on 19q13 contains 5 exons and encodes a protein with 299 amino acid residues; it is expressed in the inner nuclear layer, where it plays a significant role in the differentiation and maintenance of photoreceptor cells by synergistic interaction with other transcription factors such as NRL and RX [7,31,32]. CRX molecule possesses a paired-like homeodomain followed by a basic region, a WSP domain, and a C-terminal OTX tail. The OTX tail is important for its transcriptional activation [32,33]. CRX exerts its activity on dimerization such as with the neural retina leucine zipper *via* the OTX tail[34,35]. Therefore, mutations in CRX abolishing the OTX tail (*e.g.* c.504delA, c.587delCCCC, c.816delCACinsAA, and c.C766T in this study) affect photoreceptor-specific gene expression with a

Table 1 Overview of whole-exome sequencing data production

Exome capture statistics	III-12 (Proband)	III-10 (Unaffected)	III-4 (Affected)	IV-7 (Affected)
[1]Target region (bp)	51339787	51391525	51339787	51391525
Raw reads	133565546	147779980	136823886	140113624
Raw data yield (Mb)	12021	13300	12314	12610
Reads mapped to genome	118841720	129869013	119724061	122370094
[2]Reads mapped to target region	57175597	66226757	63991407	62336567
Data mapped to target region (Mb)	4440.09	5159.21	4981.38	4850.57
Mean depth of target region (×)	86.48	100.39	97.03	94.38
Coverage of target region (%)	99.73	99.72	99.72	99.69
Average read length (bp)	89.95	89.97	89.94	89.93
Rate of nucleotide mismatch (%)	0.22	0.18	0.22	0.23
Fraction of target covered ⩾4× (%)	99.30	99.35	99.32	99.26
Fraction of target covered ⩾10× (%)	98.27	98.50	98.45	98.31
Fraction of target covered ⩾20× (%)	95.45	96.24	96.10	95.75
[3]Capture specificity (%)	48.93	52.08	54.32	51.92
[4]Reads mapped to flanking region	9072073	9020405	9210197	8861715
Mean depth of flanking region (×)	20.97	22.39	22.24	21.54
Coverage of flanking region (%)	98.92	98.67	98.75	98.59
Fraction of flanking covered ⩾4× (%)	91.19	89.02	90.10	89.20
Fraction of flanking covered ⩾10× (%)	66.13	63.98	65.45	64.00
Fraction of flanking covered ⩾20× (%)	39.15	40.16	40.49	39.22
Fraction of unique mapped bases on or near target (%)	56.14	58.73	61.64	58.83
[5]Duplication rate (%)	6.80	9.41	8.49	8.39
Mean depth of chrX (×)	96.40	58.22	107.72	54.62
Mean depth of chrY (×)	-	55.05	-	53.52
GC rate (%)	45.46	45.92	46.14	45.50
Gender test result	F	M	F	M

[1]Target regions refer to the regions that are actually covered by the designed probes; [2]Reads mapped to target regions are reads that are within or overlap with the target region; [3]Capture specificity is defined as the percentage of uniquely mapped reads aligning to the target region; [4]Flanking region refers to regions ±200 bp on both sides of each target region; [5]PCR duplicates would have the same start and end for both mates, which rarely occurs by chance. Duplication rate is the fraction of duplicated reads in the raw data.

Table 2 Summary of SNPs for exome capture sample

Categories	III-12 (proband)	III-10 (unaffected)	III-4 (affected)	IV-7 (affected)
[1]Number of genomic positions for calling SNPs	134975362	135126064	134975362	135126064
[2]Number of high-confidence genotypes	128995083	128400486	128538834	128338404
Number of high-confidence genotypes in TR	50782259	50828750	50782665	50812238
Total number of SNPs	110292	108907	109560	108350
Nonsense	114	115	122	120
Readthrough	57	50	53	55
Missense	11030	11033	11010	10849
[3]Splice site	2704	2767	2729	2673
5-UTR	3813	3777	3836	3767
3-UTR	7426	7276	7327	7187
NR_exon	10219	10113	10086	9933
Synonymous-coding	5856	5939	5929	5908
Intron	65755	64506	65128	64450
Intergenic	3318	3331	3340	3408
Homozygous	45692	45421	45114	45639
Heterozygous	64600	63486	64446	62711

[1]Genomic positions for calling SNPs include capture target regions and their 200-bp flanking regions; [2]Consensus genotype with quality score of at least 20; [3]Intronic SNPs within 10 bp of an exon/intron boundary.

dominant-negative effect because the OTX tail establishes interactions with other transcription factors [3,7,36,37]. Upon loss of the OTX tail, the mutant protein occupies the CRX-binding site in target promoters without establishing an interaction and, consequently, may block transactivation.

To date, more than 31 *CRX* mutations have been reported

[http://www.retina-international.org/files/sci-news/crxmut.htm (update from June 16, 2005)] to underlie 3 different phenotypes: CORD, autosomal dominant retinitis pigmentosa (RP), and autosomal dominant Leber congenital amaurosis (LCA)[3,5]. The first mutation in *CRX* was detected in a Greek family with adCORD[7]. Thereafter, a number of mutations in *CRX* have been identified as being responsible for CORD as well as LCA or RP [24]. However, the severity and progression vary considerably both within and across families [38]. All *CRX* mutations appear to be completely penetrant and cause disease in heterozygotes. Missense mutations preferentially affect the conserved homeobox (codons 39-98), and all frameshift mutations leave the homeodomain intact but alter the OTX motif encoded by codons 284-295 at the carboxy terminus[3,5]. So far, there does not appear to be an obvious relationship between the type of dominant mutation (missense *vs* frameshift) in *CRX* and the severity of the resulting retinal disease[3,5].

In conclusion, we have used WES to identify a novel nonsense mutation c.C766T(p.Q256X) of *CRX* in a Chinese pedigree with adCORD. Although the assessment of disease-associated variation in CORD is still a difficult task for ophthalmologists and geneticists, WES offers a direct and robust diagnostic tool to substantially advance our knowledge, specifically in CORD-associated diseases and in ophthalmic genetics in general.

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