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Exome sequencing identifies a novel *UNC5D* mutation in a severe myopic anisometropia family
A case report

Lei Feng, MD[a], Daizhan Zhou, PhD[b,c], Zhou Zhang, PhD[c], Lin He, PhD[c], Yun Liu, PhD[d,e,*], Yabo Yang, PhD[a,*]

Abstract

Introduction: Severe myopic anisometropia has been identified to have heritability, but the pathogenesis of anisometropia still remains obscure.

Case description: Here, we presented a Chinese severe myopic anisometropia family with 5 members affected. Though using the exome sequencing, we identified a novel mutation in the UNC5D gene (c.1297C>T, p.R433C), which was predicted to have a damage effect on the protein function and kept highly conserved throughout evolution across species. As previously described, the UNC5D gene belongs to the UNC5 protein family and may have functions to regulate neuronal migration, axon guidance, and cell survival. The expression of UNC5D was also co-located at the visual areas of the mouse cortical regions at early postnatal ages.

Conclusion: Our data provide the first evidence for involvement of UNC5D gene in the severe myopic anisometropia.

Abbreviations: BCVA = best-corrected visual acuity, SER = spherical equivalent refraction, SNVs = single nucleotide variants, SVZ = sub-ventricular zone.

Keywords: anisometropia, exome sequencing, UNC5D

1. Introduction

Anisometropia is defined as a visual condition in which the 2 eyes have unequal refractive power.[1,2] A difference in spherical equivalent refraction (SER) of 1 diopter or more (SER difference ≥ 1.00 D) is usually used as the definition for anisometropia. In clinical research, anisometropia is showed association with visual impairment, binocular defects, and amblyopia.[3] Previous epidemiologic studies showed that the prevalence of anisometropia is age-dependent, with a relatively low prevalence (1.6–4.3%) among young children and a higher prevalence among adults.[4–6]

There has been some evidence in the genetic basis for myopic anisometropia. The heritability of ocular refraction and its determinants has been reported in several twins' studies.[7–9] Genetic factors have also been conformed to play a significant role in refractive error (myopia and hypermetropia) as well as in ocular biometrics, particularly axial length. Previously, we reported on a Chinese 4-generation family with severe myopic anisometropia, within which 5 individuals were affected with the disease, including a pair of 6-year-old MZ twins.[10] The co-occurrence of severe myopic anisometropia in 5 individuals of the family supports a genetic basis for the disease. However, the genes or mutants responsible for this anisometropia family remained unknown, and the pathogenesis of anisometropia still remains obscure.

Here, by using whole exome sequencing, we presented genetic evidence that a novel mutation in UNC5D (c.1297C>T, p.R433C) cause severe myopic anisometropia in the Chinese family. In addition, a previous functional study also showed that UNC5D is primarily expressed by layer 4 cells in the primary sensory areas of the developing neocortex and may mediate the effect of netrin-4 on cortical cell survival in a lamina-specific manner, and its expression was particularly strong in sensory cortices including somatosensory and visual areas.[11–13] Hence, our study constructs a new relation between UNC5D and anisometropia, and also provides biological relevance for studying the pathogenesis of anisometropia.

a Eye Center, The Second Affiliated Hospital of Zhejiang University School of Medicine, b Department of General Surgery, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou, c Bio-X Center, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders (Ministry of Education), Shanghai Jiao Tong University, d Institutes of Biomedical Sciences, Fudan University, e Key Laboratory of Molecular Medicine, The Ministry of Education, Department of Biochemistry and Molecular Biology, Fudan University Shanghai Medical College, Shanghai, PR China.

* Correspondence: Yabo Yang, Eye Center, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, PR China (e-mail: yabyang@hotmail.com); Yun Liu, Key Laboratory of Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Fudan University Shanghai Medical College, Shanghai, PR China (e-mail: superliuyun@gmail.com).

2. Materials and methods

2.1. Subjects

The Ethics Committee of the Second Hospital Affiliated to Zhejiang University approved this study, and this study was in accordance with the ethical guidelines of the Declaration of Helsinki. The experiments in this study were completely in accordance with the approved guidelines. Written informed consent was obtained from each participating individuals before sample collection.

In this study, 18 family members, including 5 affected individuals, were enrolled (Fig. 1A). Ophthalmologic examinations including uncorrected visual acuity, cycloplegic retinoscopy and manifest refractive power, best-corrected visual acuity (BCVA), ocular movements, slitlamp, and fundus examination were performed. B-scan ultrasonography (IU22, Philips) and A-scan ultrasonography (CineScan, Quantel Medical) were additionally performed for each affected patient for further evaluation.

DNA from participating family members was extracted from peripheral-blood lymphocytes by standard extraction procedures of the QuickGene DNA whole-blood kit (Fujifilm).

2.2. Exome sequencing and data analysis

We performed exome sequencing on DNA from individuals II:4 and IV:1 using the Agilent SureSelect Human All Exome 38 Mb kit on an Illumina GAIIx instrument with 2×75 bp read length, and we also performed exome sequencing on DNA from individuals II:1, II:2, II:6, III:1, III:2, III:3, and IV:2 using the Illumina Truseq Human All Exome 62 Mb kit on an Hiseq2000 instrument with 2×100 bp read length. The raw paired 75 bp or 100 bp-long reads were mapped to the human reference genome (build hg19) using the Burrows-Wheeler Aligner (BWA, version 0.5.9).[14] The BWA-aligned sequencing reads were processed by

Picard tools (version 1.52) to mark PCR duplicates. The Genome Analysis Toolkit (GATK, version 1.4)[15] was then used to perform local realignment and recalibrate base quality scores, producing a cleaned BAM file for each individual.[16] The raw single nucleotide variants (SNVs) were filtered based on the variant quality score recalibration module in GATK. We set the truth sensitivity level at 99.0, and got a Ti/Tv ratio at 2.35 for the novel single nucleotide polymorphisms (SNPs). The raw INDELs were filtered by the VariantFiltration module in GATK using the recommended argument: (i) QD < 2.0; (ii) ReadPosRankSum ≤20.0; (iii) FS > 200.0. For SNPs and indels, only positions with at least 4-fold coverage were considered.

To narrow down the candidate set, we used snpEff (http://snpeff.sourceforge.net) to predict the possible functional impact of the variants, and excluded the variants with low or modifier impact (e.g., synonymous coding, intron, and intergenic).

According to the pedigree of the family, both dominant and recessive genetic models seemed to be reasonable for the variants filter. Under the dominant model, we identify the candidate mutations as site where the 5 patients and 2 carrier (II:2, II:4, III:1, IV:1, IV:2, II:1, and III:2) were heterozygous for the reference sequence and the other 2 control subjects (II:6 and III:3) were homozygous. Under the recessive model, we identify the candidate mutations as site where the 5 patients (II:2, II:4, III:1, IV:1, IV:2) were homozygous for the mutant sequence and the other 4 subject (II:1, III:2, II:6, and III:3) were heterozygous.

2.3. Sanger sequencing and causal mutation identification

All the candidate mutations were genotyped in all 18 individuals. PCR amplification and bi-directional direct Sanger DNA sequencing performed mutation analysis. The causal mutations were identified according to the genetic relationship between family members.
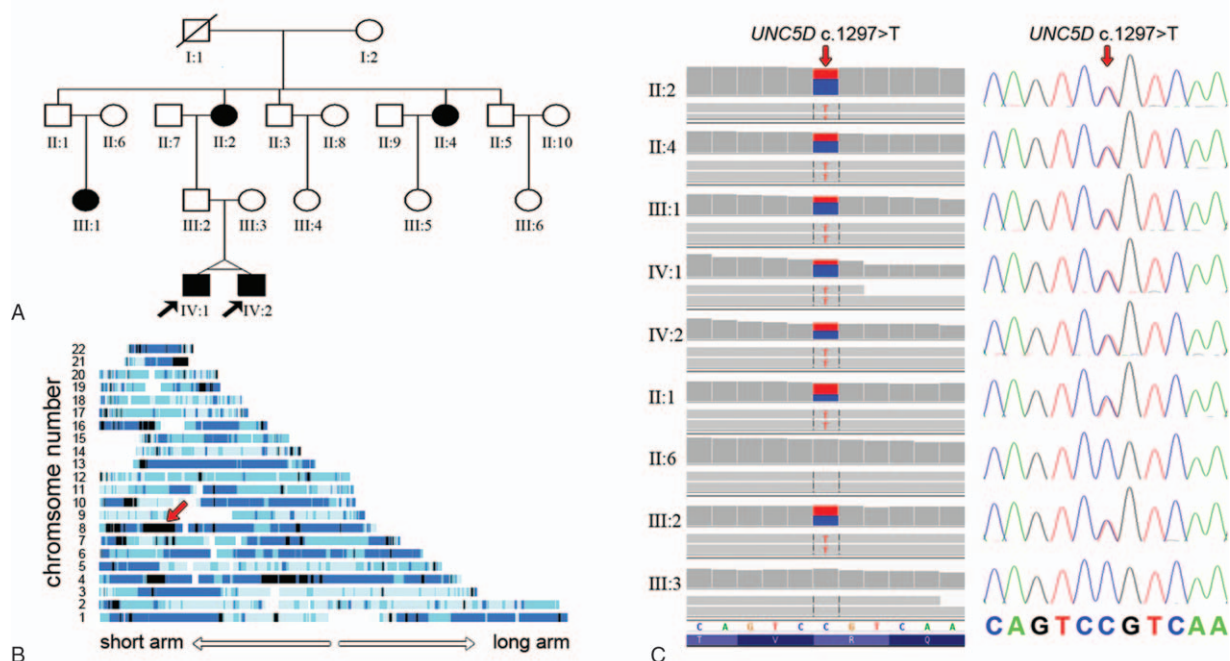
Figure 1. Information about the family with severe myopic anisometropia, (A) pedigree of the severe myopic anisometropia family, (B) the genotype of the UNC5D mutation (c.1297C>T, p.R433C) in all 9 exome-sequencing member. Left: exome sequencing data in IGV, right: validation data in Sanger-Sequencing. Red block indicates locus. (C) The homozygosity haplotype analysis of the 5 patients (II:2, II:4, III:1, IV:1, and IV:2), the largest region of homozygosity located at chrosome 8 (marked with the red arrow). IGV = integrative genomics viewer

2.4. Function prediction, evolutionary conservation, and structure analysis

The pathogenicity of the identified missense mutations was tested using PolyPhen-2,[17] PROVEAN,[18] SIFT,[19] and Mutation Taster[20] online software (see Web Resources).

Data on UNC5D orthologous genes were downloaded from the homologene database (http://www.ncbi.nlm.nih.gov/homologene). Multiple-sequence alignment was carried out using the ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

The tertiary structure modeling of UNC5D protein was performed by I-TASSER[21] and visualized using RasMol 2.7.5[22] to construct and compare the 3-dimensional structure of the wild UNC5D protein and mutated UNC5D protein.

2.5. Genome-wide SNP genotyping and homozygosity haplotype analysis

DNA samples from all 5 affected individuals were genotyped with Affymetrix 6.0 SNP microarrays (Affymetrix). Genotyping was performed according to the manufacturer's instructions. The call rate of the 5 samples was greater than 98%.

Miyazawa et al[23] reported that the HH analysis could detect both dominant and recessive genes because the HH analysis looks for the ancestral segments and does not require the information from the pedigree. Therefore, the HH analysis was compatible with the pedigree of the family under ether dominant or recessive genetic models. So the common regions of homozygosity of the 5 affected individuals were identified using the Homozygosity Haplotype software according to the standard tutorial.[23]

3. Results

The detail information and clinical characters of eyes have been showed in our previous report[24] (Supplemental Table 1, http://links.lww.com/MD/B737). The family comprised 20 individuals, of whom 18 participated in this study. Difference in spherical equivalent of cycloplegic refractions between the right and left eyes in all 5 effected individuals were >3.00 D. The pedigree of the family is shown in Fig. 1A.

An average of 8.1G bases were obtained for each sequencing sample, with a mean depth of >40-fold (Table 1). A total of 151161 SNVs (including 18,558 indels and 132,603 SNPs) were identified in all sequenced individuals after quality filter. 61426 SNVs showed no less than 4-fold in all 9 individuals (Table 2). After filtering, 4 SNVs (FAM 59A, c.1791G>A, p.V579I; C18orf34, c.1258G>A, p.D420N; IGSF5, c.905G>A, p. R302H; and UNC5D, c.1297C>T, p.R433C) remained under

the dominant model and 3 SNVs (ABCB1, c.2677T>G, p.S893A; PON2, c.443C>G, p.A148G; and IL34, c.367G>C, p.E123Q) remained under the recessive model (Fig. 1B and Table 3). All 3 candidate SNVs under the recessive model were further excluded because they were all common SNPs with the minor allele frequency over 10% in different population (Table 4). The other 4 candidate SNVs under the dominant model were genotyped in all 18 members by Sanger sequencing, only the novel mutation in the UNC5D gene (c.1297C>T, p.R433C) were confirmed to be consistent with the genetic relationship between family members (Supplemental Table 2, http://links.lww.com/MD/B737).

Then, these results were further confirmed by the Homozygosity Haplotype analysis. The largest region of homozygosity, shared by all 5 effected individuals, were located at chrosome 8 (chr8: 23,237,687–40,108,728), spanning approximately 16Mbp (Fig. 1C). The UNC5D gene, located at chr8: 35,092,975–35,652,181, was just right in the homozygosity Haplotype region. The c.1297C>T, p.R433C mutation was a novel mutation compare to the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org/), which spanned 60,706 unrelated individuals sequenced as part of various disease-specific and population genetic studies. In addition, the mutation was predicted to be pathogenic by the algorithms of PROVEAN, SIFT, Polyphen2 and Mutation Taster (categorized as "deleterious," "damaging," "probably damaging," and "disease causing," respectively). The identified mutation (p.R433C) was also highly conserved throughout evolution across species (Fig. 2)

Table 2

The exome sequencing filtering process.

Filter process		Number of SNVs
Total SNVs after quality filter		151,161
	SNPs	132,603
	Indels	18,558
The depth filter (≥4 in all individuals)		61,426
SnpEff filter (*moderate* and *high* impact)		12,159

Genetic model	Dominant	Recessive
In-house controls filter*	1814	Not done
Filter with all 5 patients (II:2, II:4, III:1, IV:1, and IV:2)	12	1592
Filter with II:1 and III:2	7	7
Filter with II:6 and III:3	4	3

SNVs = single nucleotide variants.
* In-house controls included 23 normal Chinese subjects.

Table 1

Detailed statistic of the exome sequencing.

Sample	Total_Reads	Read_ Length (bp)	Reads_ mapped (%)	Exome_ Capture Kits	Reads_ on_Target	Mean_ Depth	≥1 Coverage (%)	≥8 Coverage (%)
III:2	79971986	100	99.55%	TruSeq (62M)	46726605	55.56	96.2	92
II:1	82895203	100	99.55%	TruSeq (62M)	48686623	57.9	96	92.2
II:6	91766964	100	99.55%	TruSeq (62M)	53127006	63.21	96.5	92.8
III:3	97545985	100	99.60%	TruSeq (62M)	57271985	68.29	96.4	93
II:2	96026975	100	99.38%	TruSeq (62M)	56458387	65.43	95.8	92.1
III:1	85230021	100	99.41%	TruSeq (62M)	45478951	54.41	96.4	91.9
IV:2	87208893	100	99.36%	TruSeq (62M)	47797315	58.94	96.7	92.2
II:4	56632712	75	99.62%	Sure_Select (38M)	33117657	40.1	96.3	86.8
IV:1	74670554	75	99.03%	Sure_Select (38M)	45238486	56.82	96.2	87.7

The genotype and depth for the candidate variants with exome sequencing.

Model	Gene	Chr:Position	Ref>Alt	ID	Function	II:4 G*	II:4 D†	IV:1 G*	IV:1 D†	II:2 G*	II:2 D†	III:1 G*	III:1 D†	IV:2 G*	IV:2 D†	II:1 G*	II:1 D†	III:2 G*	III:2 D†	II:6 G*	II:6 D†	III:3 G*	III:3 D†
RECESSIVE	ABCB1	7:87160618	A>C	rs2032582	MISSENSE	1*1	0:58	1*1	0:61	1*1	0:56	1*1	0:67	1*1	0:67	0*1	13:8	0*1	9:14	0*1	24:10	0*1	31:27
	PON2	7:95041016	G>C	rs12026	MISSENSE	1*1	0:34	1*1	0:59	1*1	0:153	1*1	3:125	1*1	1:62	0*1	27:19	0*1	18:31	0*1	16:24	0*1	28:31
	IL34	16:70690989	G>C	rs8046424	MISSENSE	1*1	0:6	1*1	0:8	1*1	0:35	1*1	0:33	1*1	0:15	0*1	34:76	0*1	31:43	0*1	39:64	0*1	50:58
DOMINANT	UNC5D	8:35579907	C>T	.	MISSENSE	0*1	43:28	0*1	43:30	0*1	34:24	0*1	17:14	0*1	9:8	0*1	14:18	0*1	14:14	0*0	44:0	0*0	33:0
	FAM59A	18:29848727	C>T	rs3891458	MISSENSE	0*1	28:39	0*1	46:34	0*1	13:10	0*1	4:4	0*1	6:3	0*1	12:11	0*1	10:10	0*0	19:0	0*0	17:0
	C18orf34	18:30847180	C>T	rs58448816	MISSENSE	0*1	31:32	0*1	53:48	0*1	58:56	0*1	73:36	0*1	25:26	0*1	23:29	0*1	24:25	0*0	65:0	0*0	70:0
	IGSF5	21:41151203	G>A	rs78244022	MISSENSE	0*1	6:12	0*1	12:14	0*1	9:17	0*1	17:16	0*1	8:9	0*1	29:15	0*1	20:11	0*0	42:0	0*0	54:0

* Genotype: 0*0 = homozygous for reference sequence, 0*1 = heterozygous for reference sequence, 1*1 = homozygous for alternative sequence.

† Depth = the depth of reference sequence + the depth of alternative sequence.

The miner allele frequency of the candidate variants in the exome aggregation consortium (ExAC) database.

Gene	Chr:Posi	Ref>Alt	ID	Allele frequency							
				African	European (Finnish)	European (non-Finnish)	Latino	Other	South Asian	East Asian	Total
ABCB1	7:87160618	A>C	rs2032582	0.9101	0.47	0.5523	0.5395	0.5187	0.3496	0.4653	0.5432
PON2	7:95041016	G>C	rs12026	0.2883	0.4429	0.2484	0.2111	0.3156	0.3634	0.192	0.271
IL34	16:70690989	G>C	rs8046424	0.9143	0.399	0.4827	0.6714	0.5553	0.7016	0.6644	0.5764
FAM59A	18:29848727	C>T	rs3891458	0.0009042	0.006053	0.001598	0.05586	0.008028	0.001839	0.04929	0.01055
C18orf34	18:30847180	C>T	rs58448816	0.134	0.02708	0.03463	0.03157	0.03812	0.04851	0.1282	0.05114
IGSF5	21:41151203	G>A	rs78244022	9.66E−05	0	0.0003024	0.0004362	0.003311	0.01341	0.02738	0.004013
UNC5D	8:35579907	C>A	rs199800648	0	0	4.54E−05	0	0	0	0	2.50E−05
UNC5D	8:35579908	G/A	rs144081400	0.0008672	0.0127	0.00289	0.0003457	0.001116	0	0.0002312	0.002431
UNC5D	8:35579907	C>T		NA	NA	NA	NA	NA	NA	NA	NA

ExAC database = exome aggregation consortium database.

4. Discussion

Severe myopic anisometropia has been identified to have heritability in the twin studies.[7–9] However, the pathogenesis of anisometropia still remains obscure, and there was no report on the genes associated with anisometropia. The purpose of present study was to identify the causal genes and mutations responsible for this anisometropia family.

In this study, both exome sequencing study and the homozygosity haplotype analysis found a same region, where the UNC5D gene located in. Meanwhile, a novel mutation (c.1297C>T, p.R433C) in the UNC5D gene was further

identified, and it was predicted to have a damage effect on the protein function. The p. R433 site was also highly conserved throughout evolution across species. In the Exome Aggregation Consortium (ExAC) database, there were 2 mutations (c.1297C>A, p.R433S; and c.1298G/A, p.R433 H) at the code of p. R433 site of the UNC5D gene, and the minor allele frequency of both mutations were less than 0.5% (Table 4), and all 3 mutations were predicted to be deleterious to the protein function by SIFT software. However, the c.1297C>T, p.R433C mutation was not detected in a 400 samples in-house exome-sequencing database and the Exome Aggregation Consortium (ExAC) database. The c.1297C>T, p.R433C mutation seemed to
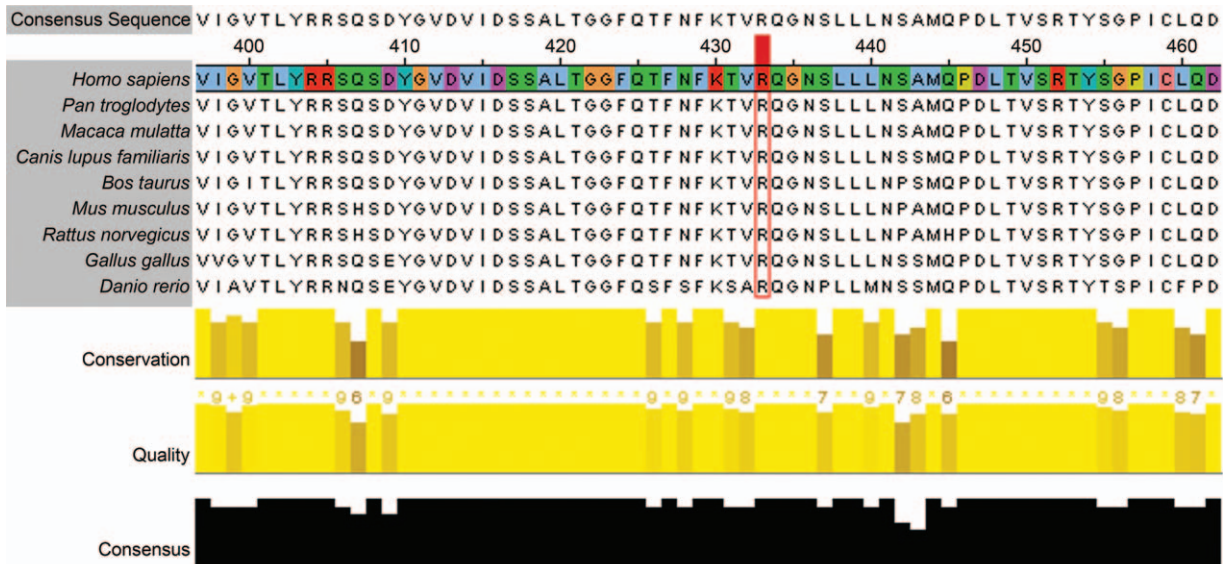

Figure 2. Evolutionary conservation analysis of the novel UNC5D gene mutation (c.1297C>T, p.R433C).

be unique in this severe myopic anisometropia family. From the structure model of UNC5D, p.R433 is in a hydrophilic environment, and the mutation resulting in a larger hydrophobic residue might subtly affect the interactions with other amino acid sites or ligands (Supplemental Figure 1, http://links.lww.com/MD/B737); thus, these genetic results indicated the UNC5D gene might be involved in the pathogenesis of anisometropia.

Previous studies have investigated the UNC5D expression during the mammalian neocortex development. First, the expression of UNC5D was observed uniformly distributed along the anterior–posterior axis in the sub-ventricular zone (SVZ) and intermediate zones at E16–18, and UNC5D protein was localized in the multipolar cells of the SVZ.[24] Takemoto et al[24] found the expression of UNC5D showed area-specific in the sensory cortices at early postnatal ages (P0-P5). Zhong et al[25] reported unc5d was specifically expressed in layer 4 developing rat neocortex at postnatal day 7(P7), Layer 4 cells were just born at E16–17 in the ventricular zone and migrated to the sub-ventricular and intermediate zones at E18, and UNC5D expression was strong in the somatosensory and visual areas. Though whole-mount in situ hybridization of P7 mouse brain, Takemoto et al also found UNC5D was strongly expressed in 3 cortical regions, and the regions were found to correspond to the primary somatosensory (S1), visual (V1) and auditory (A1) areas. Thus, these studies provided some support on the connection between UNC5D and visual system development.

UNC5D belongs to the UNC5 protein family UNC5A, UNC5B, UNC5C, or UNC5D), which were the transmembrane receptors for netrins.[26] Both UNC-5 family members and netrins have been demonstrated to regulate neuronal migration, axon guidance and cell survival, and Unc5 proteins could induce apoptosis through their Death domain in the absence of Netrin binding.[27] Moreover, it have been confirmed that the development of the optic nerve head is mediated by netrin-1.[28] This information further supported the UNC5D gene might be involved in the optic nerve development and further suggested UNC5D was involved in the pathogenesis of anisometropia.

In the severe myopic anisometropia family, 10 members carried the novel mutation (c.1297C>T, p.R433C) in the UNC5D gene, but only 5 members had the anisometropia phnotype. We thought there were 2 hypothesizes could be reasonable to explain this situation. First, the prevalence of myopic anisometropia is 1.6–4.3% among young children, and there might be 2 or more genes responsible for the anisometropia, and we just found the one of the causal mutations and genes in this family. Second, Takemoto et al[24] suggested the layer 4 cells was susceptible to environmental influences, so the anisometropia patients might be influenced by some environment factors at their early postnatal ages, but the carriers without anisometropia phenotype might not be induced by these factors. We could not exclude the possibility of both explanations, and this need to be further studied.

In summary, we identified a novel mutation (c.1297C>T, p.R433C) in the UNC5D gene associated with the severe myopic anisometropia in a Chinese family, and the previous studies also provided us credible data to support that the UNC5D gene was involved in the pathogenesis of anisometropia.

Acknowledgments

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