• Basic Research •

Identification of rare paired box 3 variant in strabismus by whole exome sequencing

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Abstract

• AIM: To identify the potentially pathogenic gene variants that contributes to the etiology of strabismus.

• METHODS: A Chinese pedigree with strabismus was collected and the exomes of two affected individuals were sequenced using the next-generation sequencing technology. The resulting variants from exome sequencing were filtered by subsequent bioinformatics methods and the candidate mutation was verified as heterozygous in the affected proposita and her mother by sanger sequencing.

• RESULTS: Whole exome sequencing and filtering identified a nonsynonymous mutation c.434G-T transition in paired box 3 (PAX3) in the two affected individuals, which were predicted to be deleterious by more than 4 bioinformatics programs. This altered amino acid residue was located in the conserved PAX domain of PAX3. This gene encodes a member of the PAX family of transcription factors, which play critical roles during fetal development. Mutations in PAX3 were associated with Waardenburg syndrome with strabismus.

• CONCLUSION: Our results report that the c.434G-T mutation (p.R145L) in PAX3 may contribute to strabismus, expanding our understanding of the causally relevant genes for this disorder.

• KEYWORDS: strabismus; whole exome sequencing; paired box 3

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INTRODUCTION

Strabismus is a common ocular disorder which is characterized by the misalignment of the eyes[1-4]. Strabismus is often associated with amblyopia of children, which can cause visual disturbance[1]. It is reported that the prevalence of strabismus is 2.4% in Hispanic/Latinos, 2%-4% in Caucasians, 2.5% in African-Americans, and 1% in East-Asians[5-8]. Additionally, the incidence of specific types of strabismus also shows differences in different racial groups, in which Asian strabismus are exotropia, suggesting the relevance of genetic factors[9-11]. Summing data from the medical literature show that the etiology of strabismus has a genetic component because the familial clustering of strabismus has been recognized[12-13].

Three inheritance patterns including dominant, recessive and sex-linked have been associated with nonsyndromic strabismus in family studies[14-15]. Parikh *et al*[16] found that a family of nonsyndromic strabismus conformed to the recessive inheritance model, and they identified susceptibility locus 7p22.1 with a multipoint LOD score of 4.51. However, linkage to 7p in 6 other families was not observed. In addition, 7p22.1 of dominant inheritance model, 16p13.12-p12.3 of recessive inheritance model and 4q28.3 dominant inheritance model has been selected as comitant strabismus associated locus[17-19]. Considering the genetic heterogeneity among families, the identity of the relevant candidate genes remains a challenge. Further work should be conducted to identify more causally relevant genes, improving the understanding of this disorder.

After the exciting finding that exomes sequencing was first developed in 2009[20], exome sequencing was widely used to locate causative genes in rare Mendelian diseases or complex diseases with high sensitivity and specificity[21-25]. Advances in genetic methodology may provide insight into the genetic basis for inherited strabismus. In the current study, we used whole exome sequencing to identify the causative gene for the two affected individuals in a Chinese strabismus family. Several evidences supported the causal role of paired box 3 (PAX3) in strabismus susceptibility.

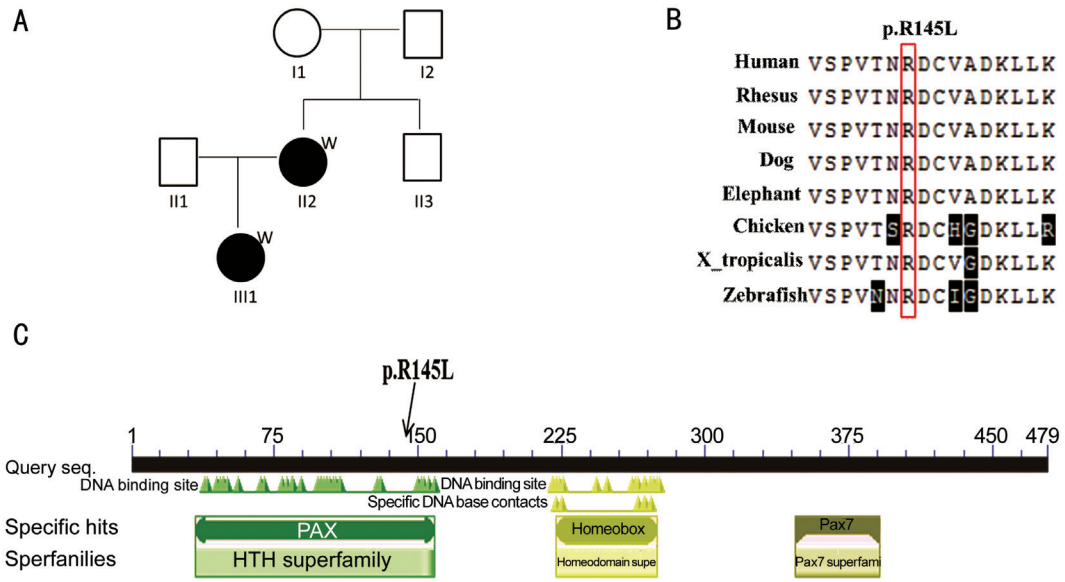

Figure 1 The analysis of PAX3 in strabismus A: Pedigree for the Chinese family with strabismus, individuals II:2 and III:1 underwent exome sequencing; B: Affected amino acid residue was highly conserved across species; C: Conserved domains in PAX3. The mutation c.434G-T (p.145R-L) was located in the conserved PAX domain of PAX3.

SUBJECTS AND METHODS

Subjects For the purpose of this study, a three-generation Chinese strabismus family with two affected individuals was recruited (Sample II:2 and III:1) (Figure 1A). The proposita was a 7-year-old girl from Shandong Province who presented with intermittent exotropia of unknown etiology, leading to amblyopia (uniocular visual neglect). Once she was tired, one of the eyes will turn outwards intermittently when looking into the distance. The amount of tropia was -50△ (near) and -50△ (far) and the unaided visual acuity was 1.0 in the right eye and 1.0 in the left eye. Moreover, her mother was 35-year-old and also presented with intermittent exotropia with similar phenotypes. The amount of tropia was -40△ (near) and -40△ (far). Her best-corrected visual acuity was OD 1.0 and OS 1.0. Given the high suspicion for a congenital strabismus family, the two affected individuals were enrolled for the exome sequencing screen. Peripheral blood samples were collected in EDTA tubes from the participants for DNA extraction. The written informed consent was then obtained from study subjects or guardian before the study. Our study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (2015-012).

Exome Capture Analysis Genomic DNA was extracted from the blood samples obtained from the available patients according to the standard procedures. The 2 μg of genomic DNA was fragmented with about 200 bp, then ligated with adapters and amplified by ligation-mediated polymerase chain reaction (PCR). The qualified genomic DNA was used for exome capture and high-throughput sequencing. Agilent SureSelect Human All Exon 50 Mb Exon Kit was used to perform exome target enrichment. The captured library was sequenced on the Illumina HiSeq 2500 Sequencer with paired-end 125 bp and mean coverage of 100X.

Variant Calling and Filtering Raw data of exome sequencing was filtered by removing adapter, contaminating reads and low quality reads, and remains were the clean ones. The exome sequencing clean reads were mapped to the reference human genome sequence (hg19) (http://genome.ucsc.edu/) using the Burrows-Wheeler Alignment (BWA) tool, which can do short reads alignment to a reference genome and support paired-end mapping[26]. The sequence alignment/map (SAM) file was then generated. Picard was used to mark and exclude the duplicate reads. Variants [single nucleotide variants (SNVs), insertions and deletions] calling was performed using the Genome Analysis Toolkit (GATK)[27] and MuTect software[28].

To pinpoint the functionally important variants, the resulting SNVs were annotated with ANNOVAR tool (http://www.openbioinformatics.org/annovar/)[29], and the information for variant frequencies and location within genes were obtained. Moreover, the SNVs were sequentially filtered and given higher priority with the following criteria: 1) minor allele frequency (MAF) <0.01 in 1000 genomes project; 2) nonsynonymous SNVs; 3) damaging as predicted by more than 4 bioinformatics programs (*e.g.* SIFT, Polyphen2, LRT, MutationTaster, MutationAssessor, FATHMM, RadialSVM, LR); 4) consistent with model of dominant disease transmission. Besides, more than 5X coverage of the given positions were required for genotype calling.

Variant Validation To validate the variants identified through exome sequencing, candidate SNVs were selected and sanger sequencing was performed at Majorbio (Shanghai, China). Peripheral blood samples were obtained from additional 7 affected individuals and 3 unaffected individuals. Genomic DNA was extracted and SNVs were tested in the original two individuals who underwent exome sequencing and ten

Table 1 Summary of exome sequencing data

Sample	Raw data (Gb)	Clean data (Gb)	Map bases rate (%)	Target region map bases (Gb)	Target region map bases rate (%)	Coverage (%)	Mean depth
II:2	10.58	10.42	99.04	5.89	56.53	81.37	104.23
III:1	20.18	19.85	98.27	11	55.42	84.94	186.56
Average	15.38	15.135	98.655	8.445	55.975	83.155	145.395

additional individuals. Oligonucleotide primers for PCR were designed by well-known program Primer 3[30].

Silico Analysis Protein conservation was analyzed using the multiple alignment tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The affected residue was visualized using MEGA7.0. The conserved domains present in the protein sequence were identified using the Conserved Domain Search Service (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

RESULTS

Exome Sequencing Identifies a Candidate Gene The whole exomes of II:2 and III:1 were sequenced, followed by variant detection and filtering. Totally, we generated 10.58 Gb and 20.18 Gb raw sequences as paired-end 125 bp reads for II:2 and III:1, respectively. After removing adapter, contaminating reads and low quality reads, 10.42 Gb and 19.85 Gb clean data were retained. Above 98% clean reads can be aligned to the human reference sequence (Table 1). The exome sequencing led to the detection of 525787 SNVs.

Considering that a causal mutation is usually a rare variant or novel in the known database, the SNVs with global MAF>0.01 in 1000 genomes project were excluded and 111 738 SNVs were retained. Among the variants identified through exome sequencing, we focused on the 1340 nonsynonymous SNVs in exonic region, which can alter the coding sequence and were more likely associated with the disease. With the assumption of dominant-inherited mode of the strabismus pedigree, 193 SNVs were retained which were shared by the two affected individuals. It is well known that most pathogenic variants are predicted to be deleterious. Total of eight bioinformatics programs were used to assess the likely functional impact of nonsynonymous SNVs. Further filtering resulted in a list of 27 SNVs that were damaging as predicted more than 4 bioinformatics programs (Table 2). Given that strabismus is an eye development disease, we surveyed the literature and narrowed down the gene list to two genes of PAX3 and MYO10 that may be associated with strabismus.

MYO10 encodes a member of the myosin superfamily. Myosins are actin-dependent molecular motors that play important roles in muscle contraction. The head domain is a molecular motor, which utilizes ATP hydrolysis to generate directed movement toward the plus end along actin filaments. A cyclical interaction between myosin and actin provided the driving force for movement of the extraocular muscles[31-32]. The mutation of c.493G>A in MYO10 (p.E165K) was highly conserved and the altered amino acid residue (p.E165K) was located in the conserved motor domain. Even so, the association of MYO10 and strabismus has not been reported. Therefore, the candidate mutant in MYO10 was further excluded.

PAX3 is a member of the PAX family of transcription factors, which play critical roles during fetal development. Mutations in PAX3 were associated with Waardenburg syndrome with strabismus, and associated with craniofacial-deafness-hand syndrome with short palpebral fissures and hypertelorism. Considering that, we speculated that the mutant c.G434T (p.R145L) in PAX3 was the most likely causative gene mutant in this Chinese strabismus pedigree. The mutation of c.G434T in PAX3 was highly conserved (Figure 1B) and the altered amino acid residue (p.R145L) was located in the conserved PAX domain (Figure 1C).

Sanger Sequencing of the Candidate Causative Variants To further confirm the variant of c.434G>T in PAX3 in strabismus, Sanger sequencing was performed in additional ten individuals. The results showed that the variants were not observed in additional ten individuals with strabismus, strongly supporting the genetic heterogeneity of strabismus.

DISCUSSION

Strabismus was a large group of ophthalmic diseases with genetic heterogeneity among families. Accumulating evidences have suggested that the etiology of strabismus has important genetic factors[12-13,33]. While only the susceptibility locus 7p22.1 was reported[16], leaving the genetic basis of this disorder remains unclear and challenging. In the present study, we enrolled two individuals with strabismus in a Chinese strabismus pedigree. In this pedigree, the proposita and her mother were diagnosed as intermittent exotropia. We suggested this was a congenital strabismus family and it was consistent with the model of dominant disease transmission. Therefore, exome sequencing was ideally suited to screen for the causal genes of the strabismus pedigree. Our result identified a novel heterozygous mutation in PAX3 (c.G434T; p.R145L), which was not reported in dbSNP 138, 1000 genome project or ESP6500. This change may be associated with strabismus.

The pathology of strabismus inheritance was complex[15,34-35]. In the current study, genetic analysis was conducted on a Chinese strabismus pedigree, and a mutation in PAX3 was identified that may be responsible for hereditary susceptibility of strabismus. PAX3 encoded a member of PAX family of

Table 2 Deleterious rare variants (MAF<0.01) identified in the family with strabismus

Chr	Position	Ref	Var	Gene	Variant type	Amino acid change	1000 genome frequency	EA-ESP frequency	rs
1	40422828	C	T	MFSD2A	Nonsynonymous	p.P55S	0.000399	-	rs181094032
1	45797401	G	A	MUTYH	Nonsynonymous	p.A345V	0.001398	-	rs35352891
2	74474313	C	T	SLC4A5	Nonsynonymous	p.E637K	-	-	-
2	223160264	C	A	PAX3	Nonsynonymous	p.R145L	-	-	-
3	156979081	G	A	VEPH1	Nonsynonymous	p.R782C	0.000399	-	rs199678437
4	6302757	T	C	WFS1	Nonsynonymous	p.V412A	0.001398	-	rs144951440
4	57340223	T	C	SRP72	Nonsynonymous	p.Y120H	0.0002	-	-
4	74442424	T	A	RASSF6	Nonsynonymous	p.D215V	0.000399	-	rs200656717
4	103647776	C	T	MANBA	Nonsynonymous	p.S81N	-	-	rs372866446
5	896841	C	A	TRIP13	Nonsynonymous	p.P107H	-	-	-
5	16783553	C	T	MYO10	Nonsynonymous	p.E165K	-	-	-
5	96329584	G	T	LNPEP	Nonsynonymous	p.R439L	0.0002	-	-
6	75804894	C	G	COL12A1	Nonsynonymous	p.G1696A	-	-	-
8	33449641	C	T	DUSP26	Nonsynonymous	p.V176M	0.0002	-	-
11	73717970	G	A	UCP3	Nonsynonymous	p.R40C	0.0002	0.000077	rs199727434
11	129795006	C	T	PRDM10	Nonsynonymous	p.R464Q	0.0002	0.0002	rs201242124
12	2224509	G	A	CACNA1C	Nonsynonymous	p.D57N	-	-	-
14	88946042	G	A	PTPN21	Nonsynonymous	p.T578M	-	-	-
15	43132561	C	A	TTBK2	Nonsynonymous	p.L96F	-	-	-
16	87885411	G	A	SLC7A5	Nonsynonymous	p.R195W	-	-	-
17	3957414	G	A	ZZEF1	Nonsynonymous	p.P1791S	-	-	-
17	63156387	G	T	RGS9	Nonsynonymous	p.G81V	-	-	-
17	66890377	A	T	ABCA8	Nonsynonymous	p.N991K	0.0002	-	-
19	38103754	T	C	ZNF540	Nonsynonymous	p.C525R	0.000599	0.0005	rs138665562
19	50796922	G	A	MYH14	Nonsynonymous	p.R1775H	-	0.000077	rs201923258
22	40801217	C	T	SGSM3	Nonsynonymous	p.R120C	-	-	-
X	43652695	T	A	MAOB	Nonsynonymous	p.Y300F	-	-	-

MAF: Minor allele frequency; Chr: Chromosome; Ref: Reference allele; Var: Variant allele; EA-ESP: European American Exome Sequencing Project; rs: Accession number in dbSNP138.

transcription factors, which played critical roles during fetal development. PAX3 gene contained 10 exons[36-37] and was mapped to chromosome 2q35[38]. The human PAX3 gene contained a PAX and a paired-type homeobox. Molecular genetic studies were conducted and a series of variations in the PAX3 gene were gradually identified in unrelated patients or family patients with Waardenburg syndrome type 1[39-44]. *In vitro* functional expression studies showed that the mutant proteins of PAX3 had decreased or abolished ability to transactivate the MITF promoter[45]. Watanabe *et al*[46] found that its paired domain or the homeodomain failed to transactivate the MITF promoter, causing Waardenburg syndrome in some individuals. Experiments on the mouse mutant splotch of Waardenburg syndrome showed that mutations in PAX3 were associated with Waardenburg syndrome that was related to human strabismus phenotypes[39,47-48]. Here, we identified the conserved domains present in the protein sequence of PAX3 and found that the candidate causal mutation of PAX3 (c.G434T; p.R145L) was located in the conserved PAX domain. These findings supported our PAX3 variant as the likely causative mutation, which may play roles in the pathological mechanism of strabismus.

Ridgeway and Skerjanc[49] suggested that strabismus was associated with an imbalance between convergence and divergence. More evidences indicated that ocular alignment depended on complex sensory, motor pathways, and the development and function of the extraocular muscles. The expression of PAX3 can control a cascade of transcriptional events, which are necessary for myogenesis[49]. The absence of PAX3 can arrest the muscle development[50]. PAX3/FKHR fusion protein activated a myogenic transcription program involved in several aspects of muscle function[51-53]. The chimeric protein PAX3-FOXO1 was the most common genetic aberration in rhabdomyosarcoma. Roeb *et al*[54] found that myoblasts from transgenic mice expressing PAX3/

FOXO1 were unable to complete myogenic differentiation. A recent study reported that oculo-auriculo-vertebral spectrum (OAVS) presented a generalized myopathy and PAX3 may be responsible for non-branchiomeric myopathy with strabismus and limb hypotrophy[55]. These findings suggested that genetic aberrant of PAX3 may be involved in development and function of the extraocular muscles, which affected the ocular alignment and contributed to strabismus.

Taken together, strabismus was a complex disease with significant genetic heterogeneity, leading to the genetic findings hampered. We demonstrated the presence of a novel causative mutation, c.434G-T (p.145R-L), in PAX3 in the affected individuals, which may potentially contribute to strabismus susceptibility. Further functional studies are needed to gain the pathogenic mechanism and the role of PAX3 in strabismus.

There was a limitation in this study. Herein, we only tested two subjects including the proposita and her mother in the whole exomes sequencing. Other relatives such as, father, uncle and the maternal grandparents of the proband should also be studied. Additionally, larger numbers of sporadic individuals with strabismus are needed to investigate the value of the identified variant. Anyhow, we found the mutation gene of PAX3 in the strabismus family, which provided a new field in understanding the genetic pathology of strabismus.

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