Review Article

Role of Abelson Helper Integration Site 1, Nebulin, and Paired Box 3 Genes in the Development of Nonsyndromic Strabismus in a Series of Iranian Families: Sequence Analysis and Systematic Review of the Genetics of Nonsyndromic Strabismus

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Abstract

Purpose: To look for causative genetic mutations in a series of Iranian families with strabismus. In addition, we systematically reviewed all the published articles regarding the role of genetic variations in primary and nonsyndromic comitant strabismus.

Methods: Four families with a history of multiple cases of primary and nonsyndromic comitant strabismus were enrolled in this study. Polymerase chain reaction and Sanger sequencing of exons 23, 11, and 3 of the Abelson helper integration site 1 (*AHI1*), nebulin (*NEB*), and paired box 3 (*PAX3*) genes were performed, respectively. One offspring of a consanguineous marriage underwent whole-exome sequencing (WES) to look for possible causative variants. To conduct a systematic review, we thoroughly searched PubMed, Scopus, and ISI Web of Knowledge extracting relevant publications, released by April 2021.

Results: We examined four Iranian strabismus pedigrees with multiple affected offspring in different generations. Among these 17 participants, 10 family members had strabismus and 7 were healthy. Sanger sequencing did not reveal a causative mutation. Therefore, to further investigate, one affected offspring was chosen for WES. The WES study demonstrated two possible variants in *MYO5B* and *DHODH* genes. These genetic variants showed high allele frequency in our population and are thought to be polymorphisms in our series of Iranian families.

Conclusions: We demonstrated that mutations in *AHI1*, *NEB*, and *PAX3* genes were not common in a series of Iranian patients with familial strabismus. Moreover, by performing WES, we revealed that two variants of uncertain significance as possible causative variants for strabismus are not related to this disease in our population.

Keywords: Abelson helper integration site 1, Genotype, Nebulin, Paired box 3, Strabismus, Whole-exome sequencing

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Introduction

The widespread presence of strabismus thoroughly differs around the world, so that the Caucasian population in Western countries may have a higher incidence and prevalence of strabismus than in other countries.[1] Strabismus is a common

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ocular problem referred to as a condition, in which the eyes are not aligned with each other properly and may be accompanied by abnormal movement of one or both eyes, decreased and double vision, and also abnormal head posture.[2,3] This ocular problem affects at least around 2% of the population regardless of their gender and may end up in amblyopia if not diagnosed and treated in time in most cases.[2] As a result of the disease, overall health-related quality of life including psychosocial factors such as low self-esteem, social anxiety, and problems with interpersonal relationships are reduced. Moreover, eye-related quality of life which is correlated with functional measures such as vision, self-perception, and a visuomotor function is also hindered in these patients.[4] While the exact cause of strabismus may not always be determined, the cause is generally referred to be due to refractive and sensory or motor innervation causes.[2] Some factors including both maternal and paternal age, preeclampsia, maternal cigarette smoking, infant prematurity, neonatal hypoxia, and low birth weight have been considered risk factors for strabismus.[5] Moreover, many population-based studies suggested familial clustering of this phenotype.[6,7] Furthermore, it has been observed that different racial groups may have a distinct and different incidence of specific types of strabismus around the world.[8] Genetic factors may also play a role in the development of this ocular disease. From the genetic point of view, strabismus can be divided into syndromic and nonsyndromic. On one hand, the syndromic forms frequently occur as ocular manifestations in the setting of other known genetic syndromes such as Apert syndrome, incontinentia pigmenti syndrome, Noonan syndrome, trisomy 18, and Prader–Willi syndrome. On the other hand, family studies demonstrated inheritance patterns of dominant, recessive, and sex-linked for nonsyndromic strabismus.[9] Some studies suggested specific candidate genes that are thought to be effective in the development of nonsyndromic strabismus. Paired box 3 (*PAX3*), Abelson helper integration site 1 (*AHI1*), and nebulin (*NEB*) are three of these genes which are reported in different studies. The *AHI1* gene product includes three domains consisting of an SH3 domain, 6 WD40 repeats, and a coiled-coil domain.[8] Recently, mutations in the conserved SH3 domain have been suggested to play a crucial role in strabismus.[8] Similar to *AHI1*, the *NEB* gene product which at least constructs 4% of the total myofibrillar protein, regulates the length of actin filaments and contraction strength, and has been reported to be downregulated in strabismic eye muscles.[10] In addition, *PAX3* gene mutation has also been linked to the strabismic phenotype of Waardenburg syndrome as this gene participates in important pathways during fetal development. Family studies have also recently proposed this gene as a possible susceptibility factor for strabismus.[9]

Based on available evidence regarding the possible role of these genes in strabismus, we aimed to conduct a study on specific families with offspring with nonsyndromic strabismus to confirm the possible role of these three genes in the development of strabismus. We also used whole-exome sequencing (WES) to identify other possible causative genes in the pathogenesis of this ocular disorder. We have also comprehensively reviewed the current literature in a systemic manner to further elucidate the genetic background of this disorder.

Identification and classification of risk factors for strabismus are indeed advantageous in the identification of high-risk individuals.[11]

According to environmental risk factors, pregnancy and perinatal risk factors including retinopathy of prematurity, birth weight, and gestational age; maternal and paternal influence including smoking and age of parents; demographic and social factors including ethnicity, socioeconomic status, and housing; refraction including anisometropia and hyperopia; and hereditary factor are comprehensively described in a systematic review of the literature.[11]

Regarding hereditary factors, there are some families and twin studies indicating strabismus possesses a polygenic inheritance that several determinants including genetic and environmental are involved, and simple Mendelian models cannot be fully distinguished.[11]

Genetic studies using linkage analysis and genome-wide searches revealed susceptible loci including 4q28.3 in the dominant model (heterogeneity logarithm of the odds [LOD] = 3.32) and 7q31.2 in the recessive model (heterogeneity LOD = 3.33 and 3.80 at 125.2 cM and 107.8 cM), 12q24.32 with almost complete maternal imprinting, and the most significant one 7p22.1, now called the recessive STBMS1 locus.[12]

Although our understanding of the genetics of strabismus has increased substantially, there are still many gaps in our knowledge regarding nonsyndromic forms of strabismus and amblyopia.[13] One reason would be the variable expression pattern of affected individuals with known mutations. This fact indicated that there may be additional genetic and environmental factors affecting related phenotypes. Strabismus in some patients with no identified gene mutation could also have resulted from somatic mutations or environmental factors that lead to similar effects of germline mutations. Moreover, in many pedigrees, there is no or little genetic information to help to identify the related mutation. For these subjects, there still need much more comprehensive data concerning underlying environmental and genetic mechanisms.[14]

Most of the evidence concerning the genetics of nonsyndromic strabismus comes from different studies.

Parikh *et al*. conducted a study to clarify a locus for comitant strabismus using the linkage analysis method.[13] These studies proposed that according to various ethnic backgrounds, there would be a unique genetic locus demonstrating genotype–phenotype correlation for strabismus.[15]

Although the sequence analysis is highly important for finding the possible causative variants, the level of the gene's expression

might be also interfering with the normal condition despite the normal sequence of gene status.[15] In one example, Altick *et al*. evaluated the gene expression profile difference between strabismic extraocular muscles (EOMs) and normal EOMs.[1]

WES is considered a powerful and cost-effective tool for exploring pathogenic variants of complex diseases such as strabismus.

Genome-wide association studies (GWAS) have also been applied for nonsyndromic strabismus. Commonly occurring polymorphisms within the general population may increase the risk of the disease.[16]

Methods

To explore the possible causative mutations in a family with a primary and nonsyndromic comitant strabismus including esotropia or exotropia or both, four Iranian strabismus pedigrees with multiple affected offspring in different generations who were referred to Khatam Eye Hospital, Mashhad, Iran, were enrolled in the present study. Every participant with strabismus had primary and nonsyndromic comitant strabismus including esotropia or exotropia. Written informed consent was obtained from all individuals who participated in this study. Then, 5 ml of venous blood was taken from 17 subjects of these four families. Among these 17 participants, 10 family members had strabismus and 7 were healthy. The family pedigrees are summarized in Table 1. The present research was approved by the Mashhad University of Medical Sciences Ethics Committee. Variants involving a mean allele frequency of <1% in the EXAC and dbSNP databases which were located in exons or splice sites and had a high impact on protein function were chosen. Furthermore, variants with reading depth lower than seven and those variants which did not pass the quality filter were excluded.

The genomic DNA was extracted using the salting-out method from the participant's whole blood. The quality and concentration of DNA samples were checked using NanoDrop2000 (Thermo Scientific, USA).

Amplification of the *AHI1*-, *NEB*-, and *PAX3*-specific exons[9,10] which had the highest mutation frequency in previous studies was carried out by polymerase chain reaction (PCR) using the forward and reverse primers as listed with their product sizes in Table 2. Amplification was performed in Applied Biosystems Veriti Thermal Cycler (one cycle of 95°C for 7 min, 42 cycles including the 30s of 95°C, 30s for annealing, 30s of 72°C, and one cycle of 7 min for final extension).

The PCR products of 17 participants (10 affected and 7 unaffected) were used for direct DNA sequence analysis by an automated sequencer (Genetic Analyzer 3130XL, Kowsar Biotech). The sequence alignment against the NCBI database (Build = 151) was done using Chromas® and CLC sequence viewer® software.

Among 17 participants, one individual was chosen for WES with ×200 coverage. In this regard, 2 ml of the whole blood sample from the selected patient was used for WES with a reading depth of ×200 (Illumina Sequencer). Bioinformatics analysis of the sequencing results was performed using international databases and standard bioinformatics software. The entire *AHI1*, *NEB*, and *PAX3* exons were then checked for any pathogenic or likely pathogenic as well as variants of unknown significance (VUS) according to the American College of Medical Genetics (ACMG 2015). As there were not any such variants reported in the patients, other possible genes previously linked to strabismus were also checked, and 2 VUS in *MYO5B* and *DHODH* genes were chosen for further population analysis.

Table 1: Summary of the participant's demographic data including gender, family number, and the status on the relevant pedigree

Family number	Gender	Patient on supplementary pedigree	Age	Onset of disease	Phenotype
1	Male	4–16	33	Before age of 6 months	Esotropia
	Male	5–25	5	Before age of 6 months	Exotropia
	Male	5–26	1	Before age of 6 months	Exotropia
	Female	4–24	27	-	Healthy
2	Male	2–10	45	After age of 6 months	Exotropia
	Female	2–19	19	Before age of 6 months	Esotropia
	Male	3–16	17	-	Healthy
	Male	3–17	13	-	Healthy
	Female	3–18	10	-	Healthy
3	Female	3–19	33	Before age of 6 months	Esotropia
	Male	4–26	4.5	Before age of 6 months	Esotropia
	Male	4–27	4.5	Before age of 6 months	Esotropia
	Male	3–22	33	-	Healthy
4	Female	2–3	32	-	Healthy
	Male	2–10	32	-	Healthy
	Female	3–15	3	Before age of 6 months	Exotropia
	Male	3–16	8	Before age of 6 months	Esotropia

The 2 VUS in *MYO5B* and *DHODH* genes were chosen for further analysis by amplification-refractory mutation system (ARMS) PCR and sequencing techniques to investigate other affected and unaffected family members and 100 unrelated healthy controls by ARMS PCR method. The desired primers and their products' sizes are provided in Table 3, and the PCR condition was as follows: one cycle of 95°C for 5 min, 35 cycles of 95°C, annealing temperature as mentioned in and 72°C, all steps for 30 s each, and one cycle of final extension for 7 min.

A comprehensive literature search was performed using keywords ("strabismus" OR "squint" OR "eye misalignment" OR "esotropia" OR "esophoria" OR "exotropia" OR "exophoria") AND ("genetics" OR "mutation" OR "variations" OR "familial"). PubMed, Scopus, and Web of Science up to April 2021 (including all available years) were searched. Languages other than English were excluded. Reference lists of included studies were also reviewed for additional articles. The abstract and title of the articles were reviewed by an author to check the quality, reliability, and relevance of the studies, and all possible case–control, linkage analysis, proteomics, and gene expression studies, as well as sequencing analysis, were included for a secondary review. Then, two authors carefully checked the specified articles, and the final papers were selected under the supervision of the third author. The study selection and exclusion process are illustrated in Figure 1. Exclusion criteria were defined as not relating to the subject of interest and reported syndromic strabismus.

Results

Among the study participants, exons 23, 11, and 3 of *AHI1*, *NEB*, and *PAX3* genes, respectively, were sequenced, and no disease-causing mutation was found. Therefore, to further investigate, one affected offspring was chosen for WES. This individual was a 5-year-old boy whose brother, mother, and uncle had the same ophthalmic manifestation without any other abnormal finding in physical examinations. The WES result revealed 300,564 variants. According to this variant filtering protocol from a total number of 300,564 variants, 585 variants remained. Thereafter, those with mean allele frequency <1% in Ensemble and dbSNP databases were filtered. The mutant protein structure of the final 45 variants was predicted in SIFT, PROVEAN, dbSNP, and Polyphen2, and only the pathogenic, likely pathogenic, or variants with unknown significance were included.

Among remained variants, the only genetic variants which were located on genes that were considered possible causes of strabismus were evaluated, and 2 VUS in *MYO5B* (*rs78626055*., A > G or Leu59Pro) and *DHODH* (*rs61733129,* C > T or Ala341Val) genes were chosen and sequenced in the study population as well as 100 healthy control subjects. The zygosity of each studied individual is demonstrated in their family pedigree and presented in Figure 2.

Among the healthy population, 66, 26, and 8 individuals were heterozygote (GT), healthy homozygote (GG), and mutant heterozygotes (TT), respectively, for the *MYO5B* variant. The results of tetra ARMS PCR for the proband are presented in Figure 3. The result is confirmed by Sanger sequencing

Table 2: Primer pairs were used for amplification of Abelson helper integration site 1, nebulin, and paired box 3 genes

Gene	Exon number	Strand	Product size	Primer sequences
AHI1	23	Forward	488	GCTGCTCTTCTGCAAGAGGAAGT
		Reverse	488	GGTCCCCGAGGATAAGAAGTCCAT
PAX3	3	Forward	284	ATTCAGCGAGGAGCATCCC
		Reverse	284	GGGGTAATAGCGACTGACTGTC
NEB	11	Forward	287	GGACCTAAGAATGGCTTGCTGAAG
		Reverse	287	GAAGTCACTAAGGAAAGGGGTCTC

AHI1: Abelson helper integration site 1, *PAX3*: Paired box 3, *NEB*: Nebulin

Table 3: Primers for *MYO5B* and *DHODH* genes

Sequencing name	Strand	Sequencing length	Primer
MYO5B			
Mutant	Forward inner	150	GATATCTGGATTCCGTAAGAAGGTCG
	Reverse outer		GTGATTCCAGCTAAGAAGACAGGACA
Native	Forward outer	116	TCAAATTATGCAAAACTGCAGGCTC
	Reverse inner		AATTGATGTACAACGCAACCATCT
Con	Forward outer	217	TCAAATTATGCAAAACTGCAGGCTC
	Reverse outer		GTGATTCCAGCTAAGAAGACAGGACA
DHODH			
Mutant	Forward inner	388	TGAGCAGCGGGCAGGAAGC
	Reverse outer		CAAGGGAGGGGAGCCTGAGG
Native	Forward inner	388	TGAGCAGCGGGCAGGAAGT
	Reverse outer		CAAGGGAGGGGAGCCTGAGG

and illustrated in Figure 4. Among the healthy population, 43 and 57 individuals were heterozygote (CT) and healthy homozygote (CC), respectively, for the *DHODH* variant. The results of allele-specific PCR for the proband are presented in Figure 5. The result is confirmed by Sanger sequencing and illustrated in Figure 6.

Three hundred and seventy-three abstracts and full texts were established in the first phase. Three hundred and twelve articles were removed after review due to their lack of relevance. Only 20 of the 61 studies that were reminded met our criteria and could be included in the systematic review [Figure 1].

The results of the analysis showed that three linkage analysis, GWAS, and WES were the most frequent techniques, and the Caucasian race was investigated in nine studies. The comprehensive results of a systematic review of the previous studies are shown in Table 4.

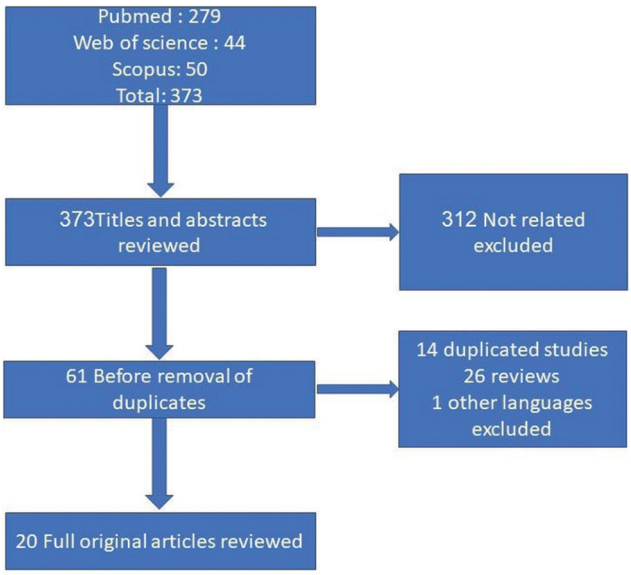

Figure 1: Flowchart showing study selection method

Figure 3: The allele-specific polymerase chain reaction result of the MYO5 gene in the proband

Discussion

Despite recent advances in the identification of the strabismus susceptibility genes, this field has been remained enigmatic, and many biological mechanisms regarding the role of genetics in strabismus have been poorly diagnosed.[9] The present study evaluated the genetic causes of strabismus in a series of Iranian families with multiple affected children. Our result demonstrated that these families did not have the most common mutations in *AHI1*, *NEB*, and *PAX3* genes. Similarly, 2 VUS in *MYO5B* and *DHODH* genes were not related to the development of strabismus as they proved to be polymorphic in our population.

The observation that strabismus can be inherited from parents has been known since the time of Hippocrates.[32] Previous twin studies revealed that in comparison to dizygotic twins, there is a higher concordance rate in monozygotic twins, which suggests the role of genetic factors for strabismus.[33] Some studies confirmed associations between genetic factors and nonsyndromic strabismus. A large linkage analysis study conducted by Parikh *et al*. demonstrated a susceptibility locus of nonsyndromic strabismus to 7p22.1 under a model of recessive inheritance.[13] Today, strabismus is considered a large group of eye diseases with considerable genetic heterogeneity among families in different populations.[10] Genetic forms of strabismus will also provide insights into its possible mechanisms, as possible causal genes will end up in pathophysiological processes disrupting eye misalignment.[34]

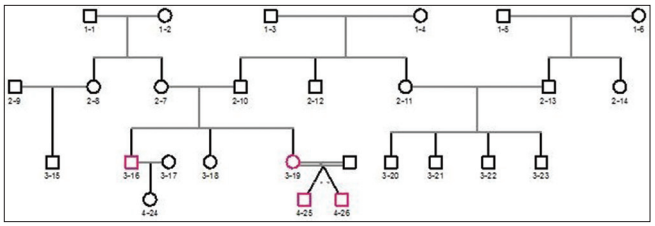

Figure 2: The pedigree of the proband underwent whole-exome sequencing

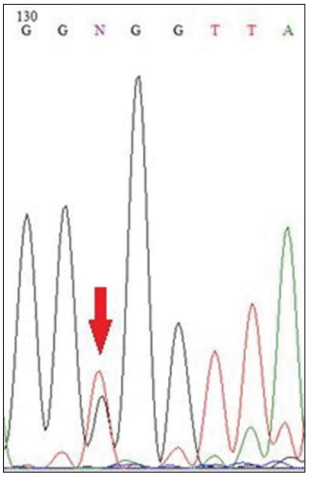

Figure 4: Result of Sanger sequencing for rs78626055 in the proband

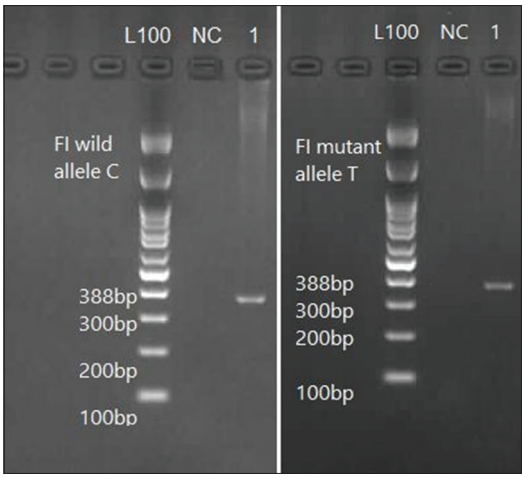

Figure 5: The allele-specific polymerase chain reaction result of the DHODH gene in the proband

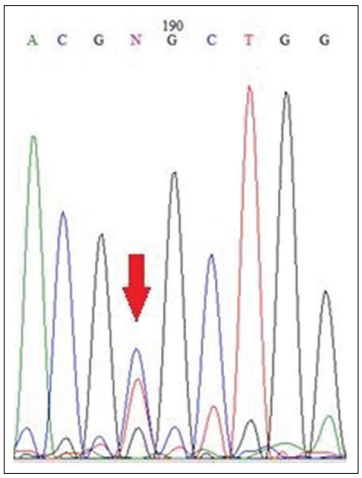

Figure 6: Result of Sanger sequencing for rs61733129 in the proband

AHI1, *NEB*, and *PAX3* are three of these genes which are thought to be effective in the development of strabismus. Mutations in these genes disrupt actin filaments and reduce contraction strength as well as the alteration in myofibrillar length.[1,9,10] Some mutations within the *AHI1* gene are related to Joubert syndrome-related disorders along with retinal dystrophy.[35] A variant affecting the conserved SH3 domain of *AHI1,* named c. A3257G (p.E1086G), can cause strabismus in a homozygous manner.[9] Another finding indicated that in the esotropia form of strabismus, the control group demonstrated amplification of *AHI1*, whereas patients did not show any amplification in this gene.[35] Regarding *NEB* gene, c.A914G mutation has been detected in a Chinese strabismus pedigree using WES.[9] Concerning the *PAX3* gene, c. 434G-T (p.R145 L) mutation affecting a conserved *PAX* domain of this gene is believed to be one of the causing genetic factors in strabismus.[10] However, in the present study, we could not find the prevalent mutations in these three genes. *MYO5B* gene had a VUS in one of our study participants. This gene encodes a protein with 1848 amino acids and is located on chr18:49, 8.[36] The encoded protein is a component of the CART complex which is mostly found in the cellular cytoskeleton.[36] GWAS suggested that *MYO5B* may be associated with myopia and refractive errors.[37] However, in our study, we demonstrated that a VUS variant is also frequent in the healthy population and is not likely to be a causative mutation of strabismus in our population. The other VUS was reported in the *DHODH* gene. *DHODH* gene codes a 395 amino acid protein and is located on 16q22.2. Mutations within this gene are considered a cause of postaxial acrofacial dysostosis which is an autosomal recessive genetic disorder. Patients who develop this syndrome show various phenotypes including ocular problems such as coloboma of eyelids.[38] The subcellular location of the product of this gene is mostly the mitochondrion which provides the required energy for cellular function. Dysfunction of the product of this gene may be correlated with disruption of muscle functions which require appropriate energy for their functions. As same to the *MYO5B* gene, this gene has also been related to eye disorders related to abnormal muscle functions.[37] However, similar to *MYO5B* gene, the *DHODH* gene is not also a causative mutation of strabismus in our Iranian population.

The result of the present study can be discussed in different ways. The first issue affecting the result of the present study is related to the allelic frequencies of genetic variants in different populations. The present study evaluated the three most common genes which have been thought to be related to strabismus and demonstrated that a series of Iranian families with congenital strabismus do not have variants in these genes. These common genes have been studied in other ethnic groups around the world, and therefore the allelic frequencies of these reported variants are different among various populations in other studies. Hence, researchers should focus their effort on studying genetic variants in other possible genes that are reported in our population study. The big challenge ahead of studying multifactorial diseases including strabismus is choosing the appropriate sample size with the most similar phenotypes. While the strabismus patients tend to present with various phenotypes, choosing the patients with similar phenotypes in desirable sample sizes is challenging. Besides, studies on congenital strabismus are mostly pointed toward next-generation sequencing (NGS) techniques. However, the present study did not find any specific genetic variation in suggested genes for strabismus. The next step could be performing other NGS-based techniques including whole-genome sequencing (WGS). As such technique covers almost the entire genomic content, other possible genetic changes in intragenic or intronic places are covered in the analysis and possible causative mutations can be further confirmed by functional studies.

Regarding our systematic review analysis, we gathered all molecular studies conducted on genetic aspects of nonsyndromic strabismus using linkage analysis, gene and protein expression analysis, WES, and GWAS techniques [Table 4]. Most of the studies (10 studies) utilized

Table 4: Genetic variants/loci associated with nonsyndromic strabismus

Method	Population	Variant	Gene/chromosome	Gene function	Clinical significance	Reference
Whole-genome linkage analysis	Caucasian	-	7p22.1	-	Associated with esotropia under a recessive model of inheritance	15
Preliminary linkage screen	Caucasian	-	7p22.1	-	Existence of a susceptibility locus for esotropia in association with hyperopia on chromosome 7p	17
Whole-genome linkage analysis	Caucasian	-	7p22.1	-	Associated with esotropia under a dominant model of inheritance	18
Linkage analysis and mutation screening	Caucasian	c.443A>T	*CHN1*	GTPase-activating protein	A heterozygous missense mutation in a dominant pattern results in an α2-chimaerin Y148F amino acid substitution	19
Linkage analysis WES, WGS	Caucasian	-	14q12	-	4 bp noncoding the deletion was prioritized as the top candidate for the observed strabismus phenotype. The deletion is predicted to disrupt the regulation of *FOXG1*	20
Microarrays and quantitative PCR	Caucasian	-	1. EOM-specific myosin (*MYH13*) and *MYH1*, and related contractile genes 2. Collagen and collagen-related genes 3. Downregulation of *PPARGC1A, PPARGC1B, PRKAB2, PRKAG3*	-	1. Decreases in the expression of contractility genes 2. Increases of extracellular matrix-associated genes involved in energy metabolism	1
GWAS	Caucasian	rs2244352 [T]	*WRB* 21q22.2	Transmembrane receptor complex on the surface of the ER	Associated with nonaccommodative esotropia increased WRB expression to susceptibility to nonaccommodative ET	16
		rs912759	1p31.1 intergenic SNP	-	Accommodative ET	
GWAS	Caucasian	rs6420484	17q25.3 *TSPAN10*	C177Y substitution in the *TSPAN10*	Reduced *TSPAN10* gene expression in brain tissues	21
GWAS	Caucasian	rs397693108	17q25.3 TSPAN10	*TSPAN10*-frameshift-inducing 4-bp indel	Reduced *TSPAN10* gene expression in brain tissues	21
Linkage analysis	Middle East	-	16p13.12-p12.3	-	Variety phenotypes of childhood strabismus are related to this recessive susceptibility locus	22
Linkage analysis	Middle East	-	3p26.3–26.2 and 6q24.2–25.1	-	Oligogenic inheritance for a consanguineous nuclear family infantile esotropia	23
Whole genome linkage analysis	Asian	-	-	-	Insignificant linkage peaks with no definite linkage	24
Genome-wide linkage analyses	Asian	-	MGST2: 4q28.3 WNT2: 7q31.2	-	Associated with comitant strabismus under a dominant (4q28.3)/ recessive (7q31.2) model of inheritance	25,26
Genome-wide linkage analyses	Asian		6q26, 12q24.32, 19q13.11		Genomic imprinting as a possible mode of inheritance in comitant strabismus	27

Contd...

Table 4: Contd...

Method	Population	Variant	Gene/chromosome	Gene function	Clinical significance	Reference
SELDI-TOF-MS[a]	Asian	-	Glucagon precursor, pituitary adenylate cyclase-activating polypeptide, camp-dependent protein kinase inhibitor α, and antimetastasis gene (antigen)	-	Detected four differentially expressed proteins in monozygotic twins with discordance of congenital esotropic phenotypes	28
Microarray	Asian	-	Differently expressed genes between strabismic cases and normal controls: *TNMD, HBB, FNDC1, PTHLH, CRISPLD1, NPTX2, COL1A2, CHRDL1, CYS1, SFRP2, LINC01279*, LOC643733	-	Both coding and lncRNA produced certain effects in the development of strabismus	29
WES	Asian	*AHI1* gene: c.A3257G *NEB* gene: c.A914G	*AHI1 NEB*	*AHI1*: Involved in vesicle trafficking and required for ciliogenesis *NEB*: Involved in maintaining the structural integrity of sarcomeres	The c. 3257A-G mutation in *AHI1* resulted in p. 1086E-G change, which was predicted to be damaging by Polyphen2 expression of *NEB* is decreased in strabismic extraocular muscles compared to normal muscles	9
WES	Asian	c.434G-T	*PAX3*	A member of the PAX family of transcription factors, which play roles during fetal development	p.R145L was located in the conserved PAX domain	10
WES	Asian	KCNH2: c.526C>T (p.R176W) CELSR1: c.7312C>T (p.R2438W) TTYH1: c. 1145G>C (p.R382P)	*FAT3, KCNH2, CELSR1, TTYH1*	*FAT3*: Neuronal morphogenesis and retina development *KCNH2*: Cortical physiology, cognitive function, and neuronal repolarization *CELSR1*: Early neurodevelopment *TTYH1*: Notch signaling pathway in neural stem cells	Causative associations with strabismus	30
Mass spectometry, microarrays		-	Increased in strabismic versus normal human eye: *CTGF, IL7, SLIT2, CXCR4, DDR2, IL10RA, NPY1R, NTRK1, NTRK2, PTGER2, TNFRSF11B, MMP2, TIMP1, TIMP2* Decreased in strabismic versus normal human eye: *GDNF, NRG1, PAX7*	-	Quantification of proteins and gene expression showed significant differences in the composition of extraocular muscles of strabismic patients concerning important motor proteins, elements of the ECM, and connective tissue	31

[a]Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. WRB: Tryptophan-rich basic protein, WES: Whole-exome sequencing, WGS: Whole-genome sequencing, PCR: Polymerase chain reaction, EOM: Extraocular muscle, AHI1: Abelson helper integration site 1, NEB: Nebulin ET: Esotropia, GWAS: Genome-Wide Association Studies, PAX: Paired box, ECM: Extracellular matrix, MYH1: Myosin heavy chain-1, ER: Endoplasmic reticulum

linkage analysis to perform their studies. In the first attempt at linkage analysis for nonsyndromic strabismus, no definite association was found in an Asian population.[24] However, Parikh *et al*. for the first time revealed an association between a susceptibility locus (7p22.1) and nonsyndromic strabismus in a Caucasian population. They also demonstrated a phenotype–genotype correlation (esotropia under a recessive model of inheritance was associated with this locus). Furthermore, two other independent pieces of research in the Caucasian population also indicated that 7p22.1 locus is associated with esotropia under a dominant model of inheritance[18] and esotropia in association with hyperopia.[17] Other linkage analysis findings conducted in Asian, Middle Eastern, and Caucasian populations demonstrated various

loci and genes indicating the heterogeneous nature of the disease [Table 4]. For the gene and protein expression analysis, four studies showed interesting results using microarray and mass spectrometry techniques [Table 4]. The studies showed that signaling molecules known to control the EOM plasticity were predominantly expressed in muscle tendon rather than muscle belly, especially in medial and lateral rectus which are not influenced by age. It should be noted that using this technique, more practical results concerning the difference of molecular patterns between healthy and case individuals and/or tissues would be achieved. One study even indicated that both coding and lncRNA produced certain effects in the development of strabismus.[29] Concerning more advanced molecular approaches such as WGS and GWAS methods, there were much less data. Using WGS, only two studies explored the role of *AHI1, NEB,*[9] and *PAX3,*[10] genes in nonsyndromic strabismus and identified related variants in their Asian populations. The roles of *TSPAN1020* and *WRB*15 SNPs in strabismus were also demonstrated in two GWAS. Replication of these findings would help obtain more reliable results.

The present study demonstrated that in a series of Iranian families who had members with strabismus, common mutations in *AHI1*, *NEB*, and *PAX3* genes are not present. Furthermore, using the WES technique, we demonstrated that 2 VUS that belong to possible causative genes for strabismus are not related to the disease in our population. Evaluation of other causative genes in further studies may reveal other causative mutations for strabismus in the Iranian families.

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Conflicts of interest

There are no conflicts of interest.

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