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ARIX and PHOX2B polymorphisms in patients with congenital superior oblique muscle palsy.

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Abstract

To identify ARIX gene and PHOX2B gene polymorphisms in patients with congenital superior oblique muscle palsy, 3 exons of the ARIX gene and PHOX2B gene were sequenced by genomic DNA amplification with polymerase chain reaction (PCR) and direct sequencing in 31 patients with congenital superior oblique muscle palsy and in 54 normal individuals. A family with a father and one daughter each having congenital superior oblique muscle palsy was also included in this study. Eleven patients with congenital superior oblique muscle palsy had heterozygous nucleotide changes in the ARIX gene, including 4 patients reported on previously. One patient with atrophy of the superior oblique muscle had a new change of T-4G in the promoter region of the ARIX gene. The other 6 patients had a heterozygous nucleotide change of G153A in the 5'-untranslated region (UTR) of the exon 1 of the ARIX gene. These nucleotide changes of the ARIX gene, taken together, had a significant association with congenital superior oblique muscle palsy($P = 0.0022$). One patient and 5 patients had heterozygous nucleotide changes of A1106 C and A1121 C in exon 3 of the PHOX2B gene, respectively, while these changes were absent in the normal individuals. Two patients had both the G153A change in the 5'-UTR of exon 1 of the ARIX gene and the A1121 C change in exon 3 of the PHOX2B gene. In conclusion, the polymorphisms of the ARIX gene and PHOX2B gene may be genetic risk factors for the development of congenital superior oblique muscle palsy.

KEYWORDS: congenital superior oblique muscle palsy, congenital fibrosis of the extraocular muscles (CFEOM), ARIX, PHOX2B, polymorphism

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Original Article

ARIX and PHOX2B Polymorphisms in Patients with Congenital Superior Oblique Muscle Palsy

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To identify ARIX gene and PHOX2B gene polymorphisms in patients with congenital superior oblique muscle palsy, 3 exons of the ARIX gene and PHOX2B gene were sequenced by genomic DNA amplification with polymerase chain reaction (PCR) and direct sequencing in 31 patients with congenital superior oblique muscle palsy and in 54 normal individuals. A family with a father and one daughter each having congenital superior oblique muscle palsy was also included in this study. Eleven patients with congenital superior oblique muscle palsy had heterozygous nucleotide changes in the ARIX gene, including 4 patients reported on previously. One patient with atrophy of the superior oblique muscle had a new change of T-4G in the promoter region of the ARIX gene. The other 6 patients had a heterozygous nucleotide change of G153A in the 5'-untranslated region (UTR) of the exon 1 of the ARIX gene. These nucleotide changes of the ARIX gene, taken together, had a significant association with congenital superior oblique muscle palsy ($P = 0.0022$). One patient and 5 patients had heterozygous nucleotide changes of A1106 C and A1121 C in exon 3 of the PHOX2B gene, respectively, while these changes were absent in the normal individuals. Two patients had both the G153A change in the 5'-UTR of exon 1 of the ARIX gene and the A1121 C change in exon 3 of the PHOX2B gene. In conclusion, the polymorphisms of the ARIX gene and PHOX2B gene may be genetic risk factors for the development of congenital superior oblique muscle palsy.

Key words: congenital superior oblique muscle palsy, congenital fibrosis of the extraocular muscles (CFEOM), ARIX, PHOX2B, polymorphism

Congenital superior oblique muscle palsy as an isolated event is a common cause of vertical deviation of the eyes. The etiologies of congenital superior oblique muscle palsy suggested so far include aplasia of the trochlear nucleus, nerve injury and anatomical abnormalities of the superior oblique tendon and/or the muscle [1-4]. Recent studies of the inheritance trait indicated that congenital superior oblique muscle palsy could be attributed to a genetic abnormality, which may either occur sporadically or be inherited [5-8].

The ARIX gene is involved in the development of the oculomotor and trochlear nerve in mice and zebrafish [9-11]. Furthermore, ARIX gene mutations have been discovered in families with congenital fibrosis of the extraocular muscles type 2 (CFEOM2), in which the superior oblique muscle is involved [12]. More recently, we identified ARIX gene polymorphisms in several patients with congenital superior oblique muscle palsy as

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one of the genetic risk factors for its development [13]. An ARIX paralogue, PHOX2B, is 100% identical to the ARIX in the homeodomain region and 71% identical over the whole gene [12]. These 2 genes are coexpressed in the oculomotor and trochlear nuclei which control eye movement [14].

Based on the facts that both the ARIX and PHOX2B gene are expressed in the trochlear nucleus and that the cross-regulation and interaction of these 2 closely related genes occur during neurogenesis, we hypothesized that congenital superior oblique muscle palsy may be associated with both the ARIX and PHOX2B genes. In this study, we analyzed polymorphisms of the ARIX and PHOX2B genes in patients with congenital superior oblique muscle palsy and in normal individuals.

Materials and Methods

Thirty one patients diagnosed with congenital superior oblique muscle palsy were included in the study (Table 1). Of these 31 patients, 13 had been reported on in our previous paper [13]. All patients were questioned about their age at onset, any history of previous head trauma, and any family history of strabismus. Clinical examinations were performed as described previously [13]. Twenty two patients underwent orbital magnetic resonance imaging to evaluate the status of the superior oblique muscle. Fifty four normal individuals who were confirmed to have no ophthalmologic diseases also participated in this study. All patients and normal individuals were ethnic Japanese. In one family with a father and one daughter who each had congenital superior oblique muscle palsy, the unaffected members of this family also participated (as was also reported in our previous paper [13]). The study was approved by the Institutional Review Board at Okayama University Medical School, and written consent was obtained from each patient or a parent when the patient was below age 15. All of the procedures conformed to the Declaration of Helsinki.

Genomic DNA from the 31 patients and 54 normal individuals was used for the study. Briefly, peripheral leukocytes were isolated from 10 ml blood by gradient centrifugation, and the genomic DNA was purified by phenol/chloroform extraction and ethanol precipitation. Polymerase chain reaction (PCR) amplification for the ARIX gene was performed as described previously [13]. For the PHOX2B gene, 4 sets of primers were used to amplify exons 1, 2 and 3 from 100 ng of genomic DNA. PCR was carried out with AmpliTaq Gold DNA Polymerase (Roche, Branchburg, NJ, USA) for exons 1 and 2: initial denaturation at 94 °C for 12 min, followed by 40 cycles at 94 °C for 30 sec, at 57 °C for 30 sec, and at 72 °C for 1 min, and final extension at 72 °C for 7 min. For exon 3 (3a and 3b fragments), we carried out PCR amplification with HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany): initial denaturation at 95 °C for 15 min, followed by 35 cycles at 94 °C for 40 sec, at 52–57 °C for 1 min, and at 72 °C for 1 min, and final extension at 72 °C for 10 min. The sequences of the primers for ARIX and PHOX2B are shown in Table 2. The PCR products were purified with ExoSAP-IT (USB, Cleveland, OH, USA) and used as a template for direct sequencing with the ABI 310 Genetic Analyzer (Perkin-Elmer, Foster, CA, USA) using the BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer). Both strands were sequenced for each DNA fragment. The DNA sequences were aligned with the published human ARIX sequences (GenBank Accession Numbers: AF022722, AF022723, and AF022724) and PHOX2B sequence (GenBank Accession Number: AF117979).

Results

The characteristics of 31 patients with congenital superior oblique muscle palsy are summarized in Table 1. The first 13 patients were reported on in our previous paper [13]. Of the 31 patients with congenital superior oblique muscle palsy, 11 had heterozygous nucleotide changes in the ARIX gene, including 4 patients reported on previously [13] (Table 1, Fig. 1A). In total, 6 kinds of nucleotide changes in the exon 1 or the promoter region of the ARIX gene were found in 11 patients with congenital superior oblique muscle palsy (Table 1, Fig. 1A). These nucleotide changes were absent in the 54 normal individuals, except for one nucleotide change (G153A), which was also found in 4 normal individuals. The presence of any kind of ARIX gene polymorphism was significantly associated with congenital superior oblique muscle palsy ($P = 0.0022$, Fisher exact probability test, Table 3). When the 22 patients who underwent orbital magnetic resonance imaging were divided into 2 groups, 12 patients with a normal superior oblique muscle and 10 patients with muscle atrophy, the presence of nucleotide changes did not show a significant association with the presence of the muscle atrophy ($P = 0.6749$, Fisher exact probability test, Table 3).

Table 1 Clinical characteristics of 31 patients with congenital superior oblique muscle palsy and nucleotide changes in the ARIX gene and the PHOX2B gene

Case No.	Sex	Age at onset	Laterality	Past history	Family history	Deviation at far (prism diopters)	Magnetic resonance imaging	Surgical procedure		Nucleotide changes		
								First	Second	Promoter of ARIX	5′-UTR of ARIX-exon1	PHOX2B-exon3
1	F	12 yrs	L	No	No	25LHT/16XT	LSO Atrophy	LIO recess LLR recess 6 mm				
2	M	3 mos	L	No	No	14LHT/4XT	Normal	LIO recess				A112C
3#	F	Childhood	R	No	Father: R) SOP	25RHT/10XT	Normal	RIO recess			G153A	
4#	M	Forties	R	No	Daughter: R) SOP	30RHT/6XT	RSO Atrophy	LIR recess 4 mm			G153A	A112C
5	F	3 mos	R	No	Aunt: Hypertropia	25RHT/14XT	RSO Atrophy	RIO recess RSR recess 3 mm	LIR recess 3 mm LLR recess 6 mm			
6	M	8 yrs	L	No	No	10LHT/4XT	Normal	LIO recess				A106C
7*	M	Childhood	R	No	No	35RHT/6ET	RSO Absence	RIO recess	LIR recess 3.5 mm LSR resect 3.5 mm	C-44A**	T7C**	
8	M	4 mos	R	No	No	10RHT	Normal	RIO recess				
9	F	Twenties	R	No	No	16RHT/14XT	Normal	RIO recess				
10	M	3 yrs	L	No	No	8LHT/2XT	LSO Atrophy	LIO recess				
11	F	3 mos	L	No	No	10LHT/6XT	LSO Atrophy	LIO recess				
12	F	Birth	R	No	No	10RHT/6XT	Normal	RIO recess RSR recess 3 mm		C-9A**	C76G**	
13	M	2 yrs	R	No	No	8RHT/30XT	NA	RLR recess 5 mm RMR resect 5 mm				
14	M	5 mos	R	No	No	20RHT/16XT	Normal	LIR recess 4.5 mm			G153A	A112C
15	F	Birth	R	No	Grandfather strabismus	20RHT/12XT	Normal	RIO recess				
16	F	Birth	R	No	No	18RHT	NA	RIO recess				

ET, esotropia; LHT, left hypertropia; LIO, left inferior oblique; LIR, left inferior rectus; LLR, left lateral rectus; LSO, left superior oblique; LSR, left superior rectus; NA, not available; RHT, right hypertropia; RIO, right inferior oblique; RIR, right inferior rectus; RLR, right lateral rectus; RMR, right medial rectus; RSO, right superior oblique; RSR, right superior rectus; SOP, superior oblique muscle palsy; XT, exotropia; 5′-UTR, 5′-untranslated region.

*Patient with superior oblique muscle and trochlea absence. #Father and daughter with superior oblique muscle palsy. **These 2 changes are located on the same strand (reported previously [13]).

Table 1 Continued

Case No.	Sex	Age at onset	Laterality	Past history	Family history	Deviation at far (prism diopters)	Magnetic resonance imaging	Surgical procedure		Nucleotide changes		
								First	Second	Promoter of ARIX	5'-UTR of ARIX-exon1	PHOX2B-exon3
17	M	1.5 yrs	R	No	No	30RHT	Normal	RIO recession			G153A	
18	F	10 yrs	R	No	No	14RHT/8XT	Normal	RIO recession				
19	F	4 yrs	R	No	No	16RHT/16XT	RSO Atrophy	RIO recession				
20	F	Birth	R	No	No	14RHT	NA	RIO recession				
21	M	5 yrs	L	No	No	20LHT/20ET	NA	LIO recession				
22	F	9 yrs	R	No	No	10RHT/18XT	RSO Atrophy	LIR recession 4 mm LLR recession 6 mm				A112IC
23	M	5 yrs	L	No	No	8LHT	NA	LIO recession				A112IC
24	M	Birth	R	No	No	16RHT	NA	RIO recession			G153A	
25	M	2 yrs	R	No	No	6RHT/6XT	Normal	LR recession 6 mm	RLO recession			
26	F	2 yrs	R	No	Mother: L) SOP	14RHT	Normal	RIO recession			G153A	
27	F	38 yrs	L	No	No	4LHT/8XT	LSO Atrophy	LIO recession		T-4G		
28	F	2 yrs	L	No	No	14LHT	LSO Atrophy	LIO recession			G153A	
29	F	Childhood	R	No	No	20RHT/4XT	RSO Atrophy	RIO recession				
30	F	Birth	R	No	No	30RHT/14ET	NA	RIO recession			G153A	
31	F	Birth	L	No	No	20LHT/14XT	NA	LIO recession				

ET, esotropia; LHT, left hypertropia; LIO, left inferior oblique; LIR, left inferior rectus; LLR, left lateral rectus; LSO, left superior oblique; LSR, left superior rectus; NA, not available; RHT, right hypertropia; RIO, right inferior oblique; RIR, right inferior rectus; RLR, right lateral rectus; RMR, right medial rectus; RSO, right superior oblique; RSR, right superior rectus; SOP, superior oblique muscle palsy; XT, exotropia; 5'-UTR, 5'-untranslated region.

Table 2 Sequences of primers used in polymerase chain reaction for ARIX and PHOX2B amplification

	Forward 5'-3'	Reverse 5'-3'
ARIX exon 1a	TCCACACCTCTGAGCCCTAAGACGG	GCCGCAGGGGGCTGTATTGGAAGC
ARIX exon 1b	CCCCGGGCCGATGGACTACT	AGCGGGCCCAGGGATTC
ARIX exon 2	CCCCGGAGCTGGACACAAC	GCTCCCACACCTCCTTCCA
ARIX exon 3a	GATCTCACTCGAGCCTTGC	CTGCACGTGGACTCCTTGGA
ARIX exon 3b	CGGGCCAAGTTCCGCAAACAGGAG	GGAGTTTCTGGGGGCAGGCTCGGA
PHOX2B exon 1	GCGTTGAGCTGTGCACATCTC	GCTTCCTATATACGGGCGG
PHOX2B exon 2	GAGTCCTCACATTCTAGCTCC	CACTCGAGGCTCCAGGACTTCG
PHOX2B exon 3a	GGCCACCCTAACCGGTGC	CTGCTGCGCCGCCCTTG
PHOX2B exon 3b	CCAGCTGCGGGGCGAATG	CTGGCTCGCCCGCTGTC

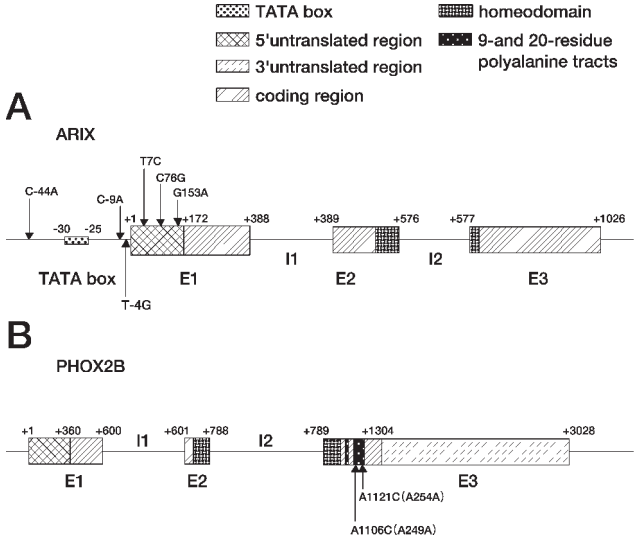

Fig. 1 Nucleotide changes of the ARIX gene and PHOX2B gene in patients with congenital superior oblique muscle palsy in this study. **A**, Nucleotide changes of the ARIX gene. Above the line are previously reported changes [13]. Below the line, a new change, T-4G, in the promoter region in 1 patient with congenital superior oblique muscle palsy. The G153A nucleotide change in the 5'-untranslated region (UTR) of exon 1 was found in the other 6 patients with congenital superior oblique muscle palsy in addition to the 2 family members reported previously [13]. **B**, Nucleotide changes of the PHOX2B gene. A1106C in exon 3 in one patient with congenital superior oblique muscle palsy, resulting in no amino acid substitution (Ala249Ala) (shown in the bracket). A1121C in exon 3 in the other 5 patients with congenital oblique muscle palsy, also resulting in no amino acid substitution (Ala254Ala) (shown in the bracket). Both are located in the 20-residue polyalanine tract. The numbering system indicates the position of the sequence of the ARIX mRNA (GenBank accession number: NM-005169) and the PHOX2B mRNA (GenBank accession number: NM-003924), and all nucleotide numbers were assigned relative to the transcription start site.

As reported previously [13], one patient (Case 7) with the absence of the superior oblique muscle had a combination of C-44A in the promoter region and T7C in the 5'-untranslated region (UTR) of exon 1, while another patient (Case 12) with the normal superior oblique muscle on magnetic resonance imaging had a combination of C-9A in the promoter region and C76G in the 5'-UTR of exon 1. In addition to these 2 patients, this study revealed that a third patient (Case 27) with superior oblique muscle atrophy on magnetic resonance imaging had a new change, T-4G, in the promoter region of the ARIX gene. This new change was not found in the 54 normal individuals. The other 8 patients, including the 2 members of the family in our previous report [13] (Cases 3, 4, 14, 17, 24, 26, 28, and 30) had a heterozygous nucleotide change of G153A in the 5'-UTR of the exon 1. This G153A nucleotide change of the ARIX gene alone had a significant association with congenital superior oblique muscle palsy ($P = 0.0259$, Fisher exact probability test, Table 4). When the 22 patients who underwent magnetic resonance imaging were divided into 2 groups, 12 patients with a normal superior oblique muscle and 10 patients with muscle atrophy, there was no significant association of the G153A nucleotide change with the muscle atrophy ($P = 0.6462$, Fisher exact probability test, Table 4).

As for the PHOX2B gene, one homozygous change of G1234C in exon 3, compared with the GenBank sequence, was found in the PHOX2B gene of all 31 congenital superior oblique muscle palsy patients and the 54 normal individuals, producing an amino acid substitution of Gly292Ala (not shown in Fig. 1). Six patients

Table 3 Any types of polymorphisms of the ARIX gene and congenital superior oblique muscle palsy

Subjects	The total number	The number with ARIX polymorphisms	Fisher exact probability test *P* value
All CSOP patients	31	11	0.0022 (all CSOP patients *vs.* controls)
With SO atrophy	10	3	0.6749 (SO atrophy *vs.* no SO atrophy)
Without SO atrophy	12	5	
With SO absence	1	1	
SO status unknown	8	2	
Controls	54	4	

CSOP, congenital superior oblique muscle palsy; SO, superior oblique muscle.

Table 4 G153A nucleotide change of the ARIX gene and congenital superior oblique muscle palsy

Subjects	The total number	The number with G153A	Fisher exact probability test *P* value
All CSOP patients	31	8	0.0259 (all CSOP patients *vs.* controls)
With SO atrophy	10	2	0.6462 (SO atrophy *vs.* no SO atrophy)
Without SO atrophy	12	4	
With SO absence	1	0	
SO status unknown	8	2	
Controls	54	4	

CSOP, congenital superior oblique muscle palsy; SO, superior oblique muscle.

with congenital superior oblique muscle palsy were found to carry heterozygous nucleotide changes in the PHOX2B gene (Table 1, Fig. 1B): 1 patient (Case 6) with the normal superior oblique muscle had A1106 C in exon 3, resulting in no amino acid substitution (Ala249Ala), while 5 patients (Cases 2, 4, 14, 22, and 23) had A1121 C in exon 3, also resulting in no amino acid substitution (Ala254Ala). These 2 kinds of heterozygous nucleotide changes, located in "the 20-residue polyalanine tract" (Fig. 1B), were absent in the 54 normal individuals.

Among the 31 patients with congenital superior oblique muscle palsy, 2 patients (Cases 4 and 14) had both the G153A change in the 5'-UTR of exon 1 of the ARIX gene and the A1121 C change in exon 3 of the PHOX2B gene. One of these 2 patients (Case 4) is the father of the family with congenital superior oblique muscle palsy, while his affected eldest daughter (Case 3) was found to carry only the G153A change in the ARIX gene, but not the A1121 C change in the PHOX2B gene. However, his unaffected youngest daughter was found to carry both changes in the ARIX gene and the PHOX2B gene.

Discussion

In the present study, we found a new nucleotide change, T-4G, in the promoter region of the ARIX gene in one patient who showed superior oblique muscle atrophy on magnetic resonance imaging. This change was not found in the 54 normal individuals. Furthermore, 6 additional patients had the same change of G153A in the 5'-UTR of exon 1 in the present study, as did the 2 family members who both had congenital superior oblique muscle palsy [13]. The G153A nucleotide change alone had a significant association with congenital superior oblique muscle palsy. The presence of any nucleotide changes, including the G153A change, was also significantly associated with congenital superior oblique muscle palsy. The combinations of nucleotide changes in the promoter region and the 5'-UTR of exon 1 were found in Cases 7 and 12. In general, mutations in the 5'-UTR and in the promoter region have been proven to inhibit the initiation of translation and to reduce promoter activity, respectively. These 2 types of combinations of nucleotide changes are therefore presumed to reduce ARIX transcription and translation, giving rise to low levels of the normal protein, as discussed in our previous report [13].

Taken together, the results in the present study support our conclusion, as mentioned in our previous article, that ARIX gene polymorphisms may be one genetic risk factor for the development of congenital superior oblique muscle palsy [13].

The etiology of congenital superior oblique muscle palsy remains the subject of controversy. Earlier works suggested the aplasia of the trochlear nucleus [8, 15], and later studies indicated anatomic abnormalities of the superior oblique tendon and/or the muscle [3, 16]. In this study, we analyzed the association of the ARIX gene polymorphisms and superior oblique muscle palsy with or without the muscle atrophy revealed by magnetic resonance imaging. However, no significant association with the muscle atrophy was found. One explanation might be that the statistics in this study was unreliable because magnetic resonance imaging was not performed in all patients.

In this study, one homozygous missense change of G1234 C in exon 3 of the PHOX2B gene was found in all patients and normal individuals, in comparison with the GenBank sequence, producing an amino acid substitution of Gly292Ala. This change of Gly292Ala is located outside the brachyury-like domain and homeodomain regions, which are highly conserved among species. Therefore, this Gly292Ala change is likely due to either a polymorphism of the PHOX2B gene, a common trait in the Japanese population, or an error in the GenBank Sequence. Two heterozygous changes of A1106 C and A1121 C in exon 3 of PHOX2B, which are located in the 20-residue polyalanine tract, were found in 6 patients with congenital superior oblique muscle palsy, but were absent in normal individuals. These changes might be another genetic risk factor for the development of congenital superior oblique muscle palsy.

The PHOX2B gene maps to chromosome 4p12, and encodes a highly conserved homeobox transcription factor of 314 amino acids with 2 short and stable polyalanine repeats of 9 and 20 residues, respectively [17]. It is expressed in the branchiomotor and visceral motor neurons, the oculomotor and trochlear nuclei, and the sympathetic, parasympathetic and enteric ganglia [18, 19]. Both Arix and Phox2b are expressed in the oculomotor and trochlear nuclei in mice, and the expression pattern of Phox2b in Arix-null mice indicates that Arix regulates Phox2b directly or indirectly in cranial ganglia [14]. Furthermore, cross-regulation and interaction occur between Arix and Phox2b [14, 20, 21]. It has been reported that PHOX2B is the major disease-causing gene in congenital central hypoventilation syndrome (CCHS) and Hirschsprung's disease, indicating the essential role of PHOX2B in the normal patterning of the autonomic nervous system [22–26]. From a clinical point of view, overlapping features are observed between congenital central hypoventilation syndrome (CCHS) and congenital fibrosis of the extraocular muscles type 2 (CFEOM2) [22, 27]. In addition, patients with CCHS sometimes show hypertropia, which might be a manifestation of the congenital superior oblique muscle palsy [27]. These animal experiments and clinical observations in humans, taken together, support the possible role of PHOX2B in the development of congenital superior oblique muscle palsy.

The combination of heterozygous nucleotide changes in the ARIX gene and PHOX2B gene was found in one unrelated patient, and also in the affected father and unaffected youngest daughter of a family with congenital superior oblique muscle palsy, while the affected eldest daughter only carried the G153A change in the ARIX gene [13]. Such inconsistency in the combination of 2 gene polymorphisms suggests that the nucleotide change of G153A in the ARIX gene may be the major polymorphism, which co-segregates with the congenital superior oblique muscle palsy. Furthermore, in this family with superior oblique muscle palsy, there is at least no additive effect of the polymorphisms in the ARIX gene and the PHOX2B gene.

In conclusion, both the ARIX gene and PHOX2B gene polymorphisms may be genetic risk factors for the development of congenital superior oblique muscle palsy, but the ARIX gene may play a more important role.

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