EXTENDED REPORT

ARIX gene polymorphisms in patients with congenital superior oblique muscle palsy

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Br J Ophthalmol 2004;88:263–267. doi: 10.1136/bjo.2003.021527

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Accepted for publication
12 May 2003

Aim: To identify *ARIX* gene polymorphisms in patients with congenital superior oblique muscle palsy and to find the relation between the *ARIX* gene and congenital superior oblique muscle palsy.

Methods: The three exons of the *ARIX* gene were sequenced by genomic DNA amplification with polymerase chain reaction (PCR) and direct sequencing in 15 patients with superior oblique muscle palsy (13 with congenital and two with acquired palsy) and 54 normal individuals. PCR products cloned into plasmids were also sequenced. A family with father and a daughter each having congenital superior oblique muscle palsy was also involved in this study.

Results: Four patients with congenital superior oblique muscle palsy carried heterozygous nucleotide changes in the *ARIX* gene. One patient with the absence of the superior oblique muscle had T7C in the 5'-UTR of the exon 1 and C-44A in the promoter region, both of which were located on the same strand. Another unrelated patient with congenital superior oblique muscle palsy had C76G in the 5'-UTR of the exon 1 and C-9A in the promoter region on the same strand. G153A in the 5'-UTR of exon 1 was found in common in two affected members of a family with congenital superior oblique muscle palsy. This G153A in the 5'-UTR of exon 1 was also present in four unrelated normal individuals. No other heterozygous nucleotide changes were found in normal individuals.

Conclusions: The nucleotide change (G153A) in the 5'-UTR of exon 1 co-segregated with congenital superior oblique muscle palsy in one family. Four other nucleotide changes in the exon 1 or the promoter region were found only in patients with congenital superior oblique muscle palsy. These nucleotide polymorphisms may be one of the risk factors for the development of congenital superior oblique muscle palsy.

Superior oblique muscle palsy is the most frequent isolated cranial nerve palsy encountered in strabismology.¹ In addition, it is probably the most common cause of vertical deviation in the primary gaze. Congenital superior oblique muscle palsy has a high incidence, accounting for 25% to 44% of cases.^{1–3} Although the true aetiology of congenital superior oblique muscle palsy remains speculative, possible causes include hypoplasia of the trochlear nucleus or nerve, perinatal nerve injury, and anatomical defects of the superior oblique tendon or the trochlea.^{3–7} Until now, familial aggregation of congenital superior oblique muscle palsy has been occasionally reported, but its genetic cause has not yet been studied.^{8–11}

Congenital fibrosis of the extraocular muscles (CFEOM), characterised by congenital ptosis and restrictive external ophthalmoplegia, is a clinical entity of the congenital ocular motility disorder that arises probably from dysfunction of the oculomotor, trochlear, and abducens nerves and atrophy of the extraocular muscles which these nerves innervate.^{12–13} CFEOM2 is a subtype in which the patients have bilateral ptosis and ophthalmoplegia with their eyes partially or completely fixed in an exotropic position, or mildly in a hypertropic or hypotropic position.¹⁴ Recently, mutations of *ARIX*, a homeobox-containing gene, have been found in families with CFEOM2.¹⁵ *ARIX* is indeed expressed in the brainstem nuclei for oculomotor and trochlear nerves.^{16–17} Based on the facts that the superior oblique muscle is involved in CFEOM and that *ARIX* is expressed in the trochlear nucleus, we hypothesise that congenital superior oblique muscle palsy may be a milder variant of CFEOM. In the present study, we analysed *ARIX* gene polymorphisms in patients with superior oblique muscle palsy and normal individuals to find whether the *ARIX* gene might be related to

congenital superior oblique muscle palsy. We also showed an *ARIX* gene polymorphism which co-segregated with congenital superior oblique muscle palsy in one family.

PATIENTS AND METHODS

The study included 15 patients in whom a diagnosis of superior oblique muscle palsy was made: 13 with congenital palsy and two with acquired palsy. All patients were questioned about the age at onset, a history of previous head trauma, and family history of strabismus. Clinical examinations included visual acuity, inspection of head posture, deviation measurement at 5 metres and 0.3 metre by alternate prism and cover test in nine diagnostic positions of the gaze, version, Bielschowsky head tilt test, vertical fusional amplitude, and TNO stereotest. Orbital magnetic resonance imaging was performed in all patients to evaluate the status of the superior oblique muscle except one patient (case 13 in table 1). Fifty four normal individuals who were confirmed as having no ophthalmological diseases also participated in this study. All patients and normal individuals were ethnic Japanese. In one family with father and a daughter, each having congenital superior oblique muscle palsy, unaffected members also participated (fig 1). The study was approved by the institutional review board at Okayama University Hospital, and written consent was obtained from each patient or parent when the patient was below the age of 15. All the procedures conformed to the Declaration of Helsinki.

Genomic DNA of 15 patients and 54 normal individuals was used for the study. Briefly, peripheral leucocytes were isolated from 10 ml blood by gradient centrifugation and genomic DNA was purified by phenol/chloroform extraction and methanol precipitation. Five sets of primers were used to

Table 1 Clinical characteristics of patients with congenital and acquired superior oblique muscle palsy and nucleotide changes in the *AR1X* gene

Case No	Sex	Age at onset	Eye	Past history	Family history	Deviation at far (prism dioptres)	Magnetic resonance imaging	Surgical procedure		Nucleotide changes	
								First	Second	Promoter	5'-UTR of exon1
1	F	12 years	L	No	No	25LHT/16XT	LSO atrophy	LIO recess 10 mm			
2	M	3 months	L	No	No	14LHT/4XT	Normal	LLR recess 6 mm			
3†	F	Childhood	R	No	Father: RE, SOP	25RHT/10XT	Normal	LIO recess 10 mm			G153A
4†	M	40s	R	No	Daughter: RE, SOP	30RHT/6XT	RSO atrophy	LIR recess 4 mm			G153A
5	F	3 months	R	No	Aunt: hypertropia	25RHT/14XT	RSO atrophy	RIO recess 10 mm	LIR recess 3 mm		
6	M	8 years	L	No	No	10LHT/4XT	Normal	RSR recess 3 mm	LLR recess 6 mm		
								LIO recess 10 mm			
7*	M	Childhood	R	No	No	35RHT/6ET	RSO absence	RIO recess 14 mm	LIR recess 3.5 mm	C-44A‡	T7C‡
									LSR resect 3,5 mm		
8	M	4 months	R	No	No	10RHT	Normal	RIO recess 5 mm			
9	F	20s	R	No	No	16RHT/14XT	Normal	RIO recess 10 mm			
10	M	3 years	L	No	No	8LHT/2XT	LSO atrophy	LIO recess 10 mm			
11	F	3 months	L	No	No	10LHT/6XT	LSO atrophy	LIO recess 10 mm			
12	F	Birth	R	No	No	10RHT/6XT	Normal	RIO recess 4 mm		C-9A‡	C76G‡
								RSR recess 3 mm			
13	M	2 years	R	No	No	8RHT/30XT	NA	RIR recess 5 mm			
								RMR resect 5 mm			
14	F	60 years	L	SAH operation	No	16LHT/6XT	LSO atrophy	LIO recess 10 mm	RIR recess 3 mm		
15	F	38 years	L	Traffic accident	No	25LHT/8XT	LSO atrophy	LIO recess 10 mm			

NA = not available; SAH = subarachnoid haemorrhage; LHT = left hypertropia; RHT = right hypertropia; XT = exotropia; ET = esotropia; LSO = left superior oblique; LIO = left inferior oblique; LLR = left lateral rectus; LIR = left inferior rectus; LSR = left superior rectus; RIO = right inferior oblique; RSO = right superior oblique; RSR = right superior rectus; RLR = right lateral rectus; RIR = right inferior rectus; RMR = right medial rectus; SOP = superior oblique muscle palsy. *Patient with superior oblique muscle and trochlea absence. †Father and daughter with superior oblique muscle palsy. ‡These two changes are located on the same strand. Cases 1–13 represent patients with congenital superior oblique muscle palsy and cases 14 and 15 represent the patients with acquired superior oblique muscle palsy.

amplify exons 1, 2, and 3 of the *AR1X* gene from 100 ng of genomic DNA. Polymerase chain reaction (PCR) was carried out with HotStarTaq DNA polymerase (Qiagen, Hilden, Germany): initial denaturation at 95°C for 15 minutes, followed by 35 cycles at 94°C for 40 seconds, at 56–60°C for 1 minute, and at 72°C for 1 minute, and final extension at 72°C for 10 minutes. PCR products were purified with GeneClean DNA Purification Kit (Qbiogene, Montreal, Canada) and used as a template for direct sequencing with ABI 310 Genetic Analyser (PE-Applied Biosystems, CA, USA)

using BigDye Terminator Cycle Sequencing Kit (PE-Applied Biosystems). Both strands were sequenced for each DNA fragment. DNA sequences were aligned with the published human *AR1X* sequences (GenBank Accession Numbers: AF022722, AF022723, and AF022724). To examine whether nucleotide changes were located on the same strand or not, PCR products were cloned into plasmids using TOPO TA Cloning Kit (Invitrogen, CA, USA) and sequenced.

RESULTS

The characteristics of 15 patients with superior oblique muscle palsy are summarised in table 1. Of the 15 patients with superior oblique muscle palsy, 13 were diagnosed with congenital superior oblique muscle palsy, while two were diagnosed with acquired superior oblique muscle palsy, which was preceded by traffic accident and subarachnoid haemorrhage surgery, respectively.

Several common homozygous changes, compared with the GenBank sequence, were found in the *AR1X* gene of all 15 superior oblique muscle palsy patients and 54 normal individuals as follows (not shown in fig 2): A393C and T399G in exon 2, A636G, A666G, A672G, A690G, T960C, A978C, and T1020C in exon 3, which were not responsible for amino acid substitutions except for T399G producing a single amino acid substitution (Asn76Lys). In addition, common changes found in intron 1 of the *AR1X* gene in all patients and normal individuals were T-63C, T-60C, T-57C, G-54C, A-52C, T-50C, G-40C, A-16 C, and G-13C (not shown in fig 2, nucleotide numbers in the intron 1 assigned relative to the beginning of the exon 2).

Among the 13 patients with congenital superior oblique muscle palsy, four had heterozygous nucleotide changes in

Figure 1 Pedigree of a family with congenital superior oblique muscle palsy (SOP) in this study. Squares and circles refer to males and females, respectively. Solid symbols = affected individuals; open symbols = normal individuals. Hatched circle represents an asymptomatic individual who happens to have a change. The arrow points to the proband. Roman and Arabic numerals indicate generations and position of individuals within generations, respectively. Nucleotide change is shown below the symbol. In this family, both father (I:1) and his eldest daughter (II:1) harbour G153A in the 5'-UTR of the exon 1 of *AR1X* gene, co-segregating with their phenotypes. His youngest daughter (II:3) also harbours this nucleotide change without any symptoms at this time.

Figure 2 Nucleotide changes of *ARIX* gene in patients with congenital superior oblique muscle palsy (above the line) in this study and CFEOM2 patients (below the line) reported previously.¹⁵ The numbering system indicates the position of the sequence of the *ARIX* mRNA (GenBank accession number: NM-005169) and all nucleotide numbers are assigned relative to the transcription start site. The positions of nucleotide changes are pointed out by arrows and changed nucleotides are shown. T7C in the 5'-UTR of exon 1 and C-44A in the promoter region occur in one patient with congenital superior oblique muscle absence. C76G in the 5'-UTR of exon 1 and C-9A in the promoter region occur in another unrelated patient with congenital superior oblique muscle palsy. G153A in the 5'-UTR of exon 1 was found in common in two members with congenital superior oblique muscle palsy in a family. In contrast with *ARIX* nucleotide changes in patients with congenital superior oblique muscle palsy, mutations in CFEOM2 patients are shown at the bottom of the figure: C386T with an amino acid substitution of Ala72Val (shown in parentheses), G to A change at position +1 of the GT splice donor sequence of exon 1, and G to A change at position -1 of the AG splice acceptor site of exon 3.

the *ARIX* gene (table 1, fig 2). One patient (case 7) with the absence of the superior oblique muscle had T7C in the 5'-UTR of exon 1 and C-44A in the promoter region, both of which were located on the same strand of genomic DNA. Another unrelated patient (case 12) with congenital superior oblique muscle palsy who showed the normal muscle on magnetic resonance imaging had C76G in the 5'-UTR of exon 1 and C-9A in the promoter region on the same strand. These changes were absent in the 54 normal individuals. The combination of such nucleotide changes in the 5'-UTR and the promoter region reached statistical significance ($p = 0.0353$, Fisher's exact probability test) between two of the 13 patients with congenital superior oblique muscle palsy and none of the 54 normal individuals).

Both father (case 4) and his eldest daughter (case 3) having congenital superior oblique muscle palsy as a common manifestation in one family were found to harbour a heterozygous nucleotide change of G153A in the 5'-UTR of exon 1. This change was also found in his unaffected youngest daughter as well as in four of the 54 normal individuals (table 1, fig 1). The daughter showed the normal superior oblique muscle on magnetic resonance imaging while the father showed atrophy of the muscle. Two patients diagnosed with acquired superior oblique muscle palsy in this study did not carry any nucleotide changes. The 54 normal individuals did not have the other heterozygous nucleotide changes except for four with G153A in the 5'-UTR of exon 1 as mentioned above.

DISCUSSION

In the present study, several homozygous cosense changes in exons 2 and 3 of the *ARIX* gene in all patients and normal individuals were found in comparison with the GenBank sequence. These changes would be common traits in the Japanese population or errors in the initial sequence. In addition, one homozygous missense change of T399G (Asn76Lys) was detected as a common trait in exon 2 of all patients and normal individuals. This change of Asn76Lys is located beyond the brachyury-like domain which is a short peptide sequence (amino acids 61–75) of *ARIX* with high conservation.^{18–19} Nakano and coworkers¹⁵ also proved that the sequence of *ARIX* analysed in their patients and normal individuals differed from the published sequence at the same T399G which altered an amino acid from Asn to Lys and brought the human sequence in agreement with that of the mouse and rat, providing additional evidence that this

Asn76Lys is a polymorphism. Similarly, several changes in intron 1 of *ARIX* gene, which exist in all participants, are considered as *ARIX* gene polymorphisms.

Two kinds of the unique combinations of heterozygous nucleotide changes in the promoter region and the 5'-UTR of the same genomic DNA strand were found separately in two patients with congenital superior oblique muscle palsy. These changes were absent in the 54 normal individuals. Furthermore, it should be noted that one (case 7) of the two patients showed congenital absence of the superior oblique muscle. The 5'-UTR is involved in the binding of the 40S ribosomal subunit to the mRNA at the internal ribosomal entry site and subsequent scanning along the 5'-UTR region until the initiation codon is reached. Mutations in the 5'-UTR sequence indeed inhibit the initiation of translation.^{20–22} On the other hand, mutations in the promoter region reduce promoter activity and lead to downregulation of the transcription.^{23–24} The combinations of nucleotide changes in the promoter region and the 5'-UTR of exon 1 found in the present study are presumed to reduce *ARIX* transcription and translation, giving rise to low levels of the normal protein.

ARIX, a homeobox-containing gene, is expressed in the brainstem nuclei for oculomotor and trochlear nerve which control eye movement, and encodes a homeodomain transcription factor protein previously shown to be required for oculomotor and trochlear nerve development in mouse and zebrafish.^{16–17–25–26} Nakano and coworkers¹⁵ reported *ARIX* mutations in the exon 1 as well as in two separate splice sites in CFEOM2 patients, which were predicted to disrupt a highly conserved subdomain of *ARIX* and the transcript upstream of its homeodomain region, respectively, concluding *ARIX* as the gene causing CFEOM2 (fig 2).

The trochlear nerve was the last of the ocular motility nerves to reach its innervated muscle, and such developmental handicap of the trochlear nerve and nucleus could conceivably result in isolated trochlear nerve palsy. Based on this fact, congenital superior oblique muscle palsy, not only as a milder variant of CFEOM but also as congenital isolated trochlear nerve palsy, is suspected to be related to the *ARIX* gene which regulates the development of the trochlear nerve and nucleus. Compared with the *ARIX* mutations in CFEOM2 patients, one with an amino acid substitution and two in the separate splice sites (fig 2), which resulted in abnormal or truncated proteins, the *ARIX* gene polymorphisms found in the two patients with congenital superior oblique muscle

palsy would only give rise to reduced levels of the normal protein. Such differences in putative proteins would explain why the nucleotide changes in the same *ARLX* gene lead to severe clinical manifestations as CFEOM2 on one occasion and to such milder clinical features as isolated superior oblique muscle palsy on the other occasions.

The nucleotide change, G153A in the 5'-UTR of exon 1, was found in common in a father and his eldest daughter of a family with congenital superior oblique muscle palsy in this study. Despite such co-segregation of superior oblique muscle palsy with the nucleotide change, the youngest daughter in this family and four normal individuals also harboured the change. So it is possible that this G153A is a non-pathogenic change co-segregating with congenital superior oblique muscle palsy by chance as a polymorphism. The youngest daughter was asymptomatic and clinical examinations revealed no sign of superior oblique muscle palsy at the age of 20 when her family members were analysed in this study. The previous reports indicate that congenital superior oblique muscle palsy is an autosomal dominant disorder but that some patients might be asymptomatic and underdiagnosed.⁸⁻¹⁰ We cannot, therefore, exclude a possibility that the youngest daughter might have a subtle change of the superior oblique muscle and tendon as an asymptomatic carrier, which would, later in her life, produce superior oblique muscle palsy.

In the present study, only four patients with congenital superior oblique muscle palsy were found to carry nucleotide changes in the *ARLX* gene. Since we sequenced mainly the three exons and the splicing sites of the *ARLX* gene, we might miss other changes in the introns (about two thirds of the intron 1 and 2 were not sequenced) or 3'-UTR which would be responsible for congenital superior oblique muscle palsy in other patients. Furthermore, the other genes may be responsible for congenital superior oblique muscle palsy in the case that the *ARLX* gene is considered as only one of genetic risk factors for the disease. For example, *PHOX2B*, a close relative of *ARLX* (also known as *PHOX2A*) with an identical homeodomain, is co-expressed with *ARLX* at most sites, such as in the branchiomotor and visceral motor neurons, in the oculomotor and trochlear nuclei, and in the adrenergic and noradrenergic centres such as the locus coeruleus, visceral sensory and parasympathetic ganglia.²⁷⁻²⁸ Based on the facts that the oculomotor and trochlear nuclei are present in *PHOX2B*^{-/-} mice,²⁹ but absent in *ARLX*^{-/-} mice,^{17,25} both oculomotor and trochlear nuclei would consist mainly of *ARLX* dependent neurons and would not necessarily require *PHOX2B* for the normal development.¹⁵ In contrast, cross regulation and interaction do occur between *ARLX* and *PHOX2B*,²⁵⁻³⁰⁻³¹ suggesting that *PHOX2B* also has a role in the normal development of these nuclei.

The patients with congenital superior oblique muscle palsy do not show obvious signs and symptoms of dysfunction in the other areas of the central nervous system, including the locus coeruleus and parasympathetic ganglia. Such clinical facts are inconsistent with the broader areas of *ARLX* expression. One explanation would be that *ARLX* may not be essential for the development of the locus coeruleus and parasympathetic ganglia, possibly because *PHOX2B* or the other analogues specify the development of these neurons.¹⁵⁻³² Alternatively, dysfunction in the other areas would be clinically insignificant or silent even if these neurons are abnormal in individuals with congenital superior oblique muscle palsy.¹⁵ Further studies of *ARLX* and *PHOX2B* genes are needed to explain this discrepancy.

In conclusion, the *ARLX* gene polymorphisms may be one of genetic risk factors for the development of congenital

superior oblique muscle palsy. Since congenital superior oblique muscle palsy may comprise multiple pathogenic diseases, other contributing factors, either genetic or environmental, may come into play in addition to the *ARLX* gene. Further analysis of a large number of patients with congenital superior oblique muscle palsy is necessary to reach the final conclusion because of a small sample size in this study.

ACKNOWLEDGEMENTS

The authors thank Dr Elizabeth C Engle for providing information of the primers for *ARLX* gene.

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