ARIX gene polymorphisms in patients with congenital superior oblique muscle palsy

Y Jiang, T Matsuo, H Fujiwara, S Hasebe, H Ohtsuki, T Yasuda

EXTENDED REPORT

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 17C 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 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. 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. Although the true aetiology of congenital superior oblique muscle palsy remains speculative, possible causes include hypoplasia of the trochlear nucleus or nerve, perinaital nerve injury, and anatomical defects of the superior oblique tendon or the trochlea. Until now, familial aggregation of congenital superior oblique muscle palsy has been occasionally reported, but its genetic cause has not yet been studied.

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. 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. Recently, mutations of ARIX, a homeobox-containing gene, have been found in families with CFEOM2. ARIX is indeed expressed in the brainstem nuclei for oculomotor and trochlear nerves.

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

See end of article for authors’ affiliations

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amplify exons 1, 2, and 3 of the ARIX 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). Both strands were sequenced for each DNA nucleotide changes were located on the same strand or not, AF022722, AF022723, and AF022724). To examine whether common homozygous changes, compared with the GenBank sequence, were found in the ARIX gene of all 15 patients with superior oblique muscle palsy patients and 54 normal individuals as follows (not shown in fig 2): A393C and T7C, C-44A, C-9A, C76G, C87, G-16A, G-13C, T-50C, G-40C, A-16 C, and G-13C (not shown in fig 2), amino acid substitutions except for T399G producing a single amino acid substitution (Asn76Lys). In addition, common amino acid substitutions in intron 1 of the ARIX gene in all patients and normal individuals were T-50C, G-40C, A-16C, G-13C, T-50C, G-40C, A-16C, and G-13C (not shown in fig 2).

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 ARIX 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 ARIX gene in all patients and normal individuals were T-63C, T-60C, T-57C, G-54C, A-52C, T-50C, G-40C, A-16C, and G-13C (not shown in fig 2). 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 ARIX 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.

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

<table>
<thead>
<tr>
<th>Case No</th>
<th>Sex</th>
<th>Age at onset</th>
<th>Eye</th>
<th>History</th>
<th>Past</th>
<th>Family history</th>
<th>Deviation at far (prism dioptres)</th>
<th>Magnetic resonance imaging</th>
<th>Surgical procedure</th>
<th>Nucleotide changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>12 years</td>
<td>L</td>
<td>No</td>
<td>No</td>
<td></td>
<td>25LHT/16XT</td>
<td>LSO atrophy</td>
<td>LIO recess 10 mm</td>
<td>G153A</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>3 months</td>
<td>L</td>
<td>No</td>
<td>No</td>
<td></td>
<td>14LHT/4XT</td>
<td>Normal</td>
<td>LIO recess 10 mm</td>
<td></td>
</tr>
<tr>
<td>3†</td>
<td>F</td>
<td>Childhood</td>
<td>R</td>
<td>No</td>
<td>Father: RE, SOP</td>
<td>25RHT/10XT</td>
<td>Normal</td>
<td>RIO recess 10 mm</td>
<td>G153A</td>
<td></td>
</tr>
<tr>
<td>4†</td>
<td>M</td>
<td>40s</td>
<td>R</td>
<td>No</td>
<td>Daughter: RE, SOP</td>
<td>30RHT/6XT</td>
<td>Normal</td>
<td>LIR recess 4 mm</td>
<td>G153A</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>3 months</td>
<td>R</td>
<td>No</td>
<td>No</td>
<td>hypertropic</td>
<td>25RHT/14XT</td>
<td>RSO atrophy</td>
<td>RIO recess 10 mm</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>8 years</td>
<td>L</td>
<td>No</td>
<td>No</td>
<td></td>
<td>10LHT/4XT</td>
<td>Normal</td>
<td>LIO recess 10 mm</td>
<td></td>
</tr>
<tr>
<td>7*</td>
<td>M</td>
<td>Childhood</td>
<td>R</td>
<td>No</td>
<td>No</td>
<td></td>
<td>35RHT/6ET</td>
<td>RSO absence</td>
<td>RIO recess 14 mm</td>
<td>C-44A†</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>4 months</td>
<td>R</td>
<td>No</td>
<td>No</td>
<td></td>
<td>10RHT</td>
<td>Normal</td>
<td>RIO recess 5 mm</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>20s</td>
<td>R</td>
<td>No</td>
<td>No</td>
<td></td>
<td>16RHT/14XT</td>
<td>Normal</td>
<td>RIO recess 10 mm</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>3 years</td>
<td>L</td>
<td>No</td>
<td>No</td>
<td></td>
<td>8LHT/2XT</td>
<td>LSO atrophy</td>
<td>LIO recess 10 mm</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>3 months</td>
<td>L</td>
<td>No</td>
<td>No</td>
<td></td>
<td>10LHT/6XT</td>
<td>LSO atrophy</td>
<td>LIO recess 10 mm</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>Birth</td>
<td>R</td>
<td>No</td>
<td>No</td>
<td></td>
<td>10RHT/6XT</td>
<td>Normal</td>
<td>RIO recess 4 mm</td>
<td>C-9A†</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>2 years</td>
<td>R</td>
<td>No</td>
<td>No</td>
<td></td>
<td>8RHT/30XT</td>
<td>NA</td>
<td>RSR recess 3 mm</td>
<td>C76G‡</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>60 years</td>
<td>L</td>
<td>No</td>
<td>SAH</td>
<td>operation</td>
<td>16LHT/6XT</td>
<td>LSO atrophy</td>
<td>LIO recess 10 mm</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>38 years</td>
<td>L</td>
<td>No</td>
<td>Traffic accident</td>
<td>25LHT/8XT</td>
<td>LSO atrophy</td>
<td>LIO recess 10 mm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA = not available; SAH = subarachnoid haemorrhage; LH = left hypertropia; RH = right hypertropia; XT = exotropia; ET = esotropia; LSO = left superior oblique; LIO = left inferior oblique; LIR = left inferior rectus; LSR = left superior rectus; RIO = right inferior oblique; RSO = right superior oblique; 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.

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:3) also harbour this nucleotide change without any symptoms at this time.
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 transla-
tion.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
teach eye movement, and encodes a homeodomain trans-
scription 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, conclud-
ing 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 develop-
mental handicap of the trochlear nerve and nucleus could
covably 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
nerves...
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 ARIX 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. 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 ARIX gene. Since we sequenced mainly the three exons and the splicing sites of the ARIX 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 ARIX gene is considered as only one of genetic risk factors for the disease. For example, PHOX2B, a close relative of ARIX (also known as PHOX2A) with an identical homeodomain, is co-expressed with ARIX at most sites, such as in the brainstem 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. Based on the facts that the oculomotor and trochlear nuclei are present in PHOX2B−/− mice, but absent in ARIX−/− mice, both oculomotor and trochlear nuclei would consist mainly of ARIX dependent neurons and would not necessarily require PHOX2B for the normal development. In contrast, cross regulation and interaction do occur between ARIX and PHOX2B, 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 ARIX expression. One explanation would be that ARIX 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. 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. Further studies of ARIX and PHOX2B genes are needed to explain this discrepancy.

In conclusion, the ARIX 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 ARIX 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.

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