Original Article

Mutational Screening of *ARX* Gene in Iranian Families with X-linked Intellectual Disability

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Abstract

Background: Mutations in the human aristaless-related homeobox (*ARX*) gene are amongst the major causes of developmental and neurological disorders. They are responsible for a wide spectrum of phenotypes, including nonsyndromic X-linked intellectual disability (NS-XLID), and syndromic (XLIDS) forms such as X-linked lissencephaly with abnormal genitalia (XLAG), Partington syndrome (PRTS), and X-linked infantile spasm syndrome (ISSX). The recurrent 24 bp duplication mutation, c.428_451dup(24 bp), is the most frequent *ARX* mutation, which accounts for ~40% of all cases reported to date.

Methods: We have screened the entire coding sequences of the *ARX* gene in 65 Iranian families with intellectual disabilities in order to obtain the relative prevalence of *ARX* mutations.

At first these families were screened for the most recurrent mutation, the c.428_451dup(24 bp). For samples with negative results, single strand conformation polymorphism (SSCP) analysis was performed.

Results: We identified one family with the c.428_451dup(24 bp) duplication. Three shifts (one shift in exon 5 and two shifts in exon 4) were also identified among the total families. According to the results of the sequencing analysis, two shifts were not associated with any mutation and the other one was a c.1347C>T (p.G449G) substitution in exon 4.

Conclusion: Hence, we suggest that molecular analysis of ARX mutations as a second cause of XLID should be considered as routine diagnostic procedure in any male who presents with either NS-XLID or XLIDS.

Keywords: X-linked intellectual disability, *ARX* gene, Iran

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Introduction

The aristaless-related homeobox (*ARX*) gene (MIM# 300382) is located in Xp22.13 in proximity to the 3' end of *POLA* (encoding DNA polymerase-alpha). It is composed of five exons and transcribed into a 2.8 kb mRNA, which encodes a protein of 562 amino acids.[1–3] This gene is expressed in the fetal and adult brains and in skeletal muscle, where two shorter mRNA isoforms are found.[2,3]

The ARX protein belongs to a class of homeoproteins, the paired (Prd) class. It is characterized by a paired homeodomain, a C-terminal domain (also known as OAR domain, C-peptide), and a conserved octapeptide motif, which functions as transcriptional repressor.[4,5] The *ARX* gene plays a critical role during vertebrate embryogenesis and is involved in the differentiation and maintenance of specific neuronal cell types, particularly in the central nervous system.[3,6–8]

The phenotypic expression varies, and mutations in the *ARX* gene are responsible for different human phenotypes, including X-linked lissencephaly with abnormal genitalia (XLAG), hydranencephaly with abnormal genitalia (HYD-AG), X-linked infantile spasm (ISSX), X-linked myoclonic seizures, spasticity and intellectual disability (XMESID), Partington syndrome (PRTS), Ohtahara syndrome, Proud syndrome, idiopathic infantile epileptic-dyskinetic encephalopathy (IEDE), and nonsyndromic X-linked intellectual disability (NS-XLID).[1,9–13] *ARX* mutations have been identified in several women with isolated agenesis of the corpus callosum and in a few men with hydranencephaly.[13] This establishes that *ARX* mutations significantly contribute to both syndromic X-linked intellectual disability (XLIDS) and NS-XLID. *ARX* mutations have been identified in 9.5% of X-linked intellectual disability families from the European XLID consortium.[14–16] Also, recent studies have shown *ARX* mutations in 7.5% of families that have multiply-affected males from two or more generations connected by an obligate carrier female.[17]

Two different polyalanine expansion mutations [c.428_451dup(24 bp) and c.333_334ins(GCG)7] represent 46% of all currently known mutations and show considerable pleiotropy.[5,14,15] However, the 24 bp duplication mutation is more frequent (~45% of all *ARX* mutations) and it is the most common mutation in the *ARX* gene.[5,14–16] This mutation can yield different phenotypes: PRTS, X-linked West syndrome with infantile spasms, intellectual disability (ID) with seizures, and NS-XLID.[14–29] To date, various types of seizures, moderate to profound ID, and a combination of minor congenital anomalies have been reported in patients with the c.428_451dup(24 bp) mutation.[16,18,25,28]

Inter- and intrafamilial phenotypic variations have also been observed in the *ARX* gene.[22–24,29,30] Deletions, insertions, missense, and nonsense mutations leading to frame shifts and premature truncation and different duplications have also been reported.[13,22,24,29–33]

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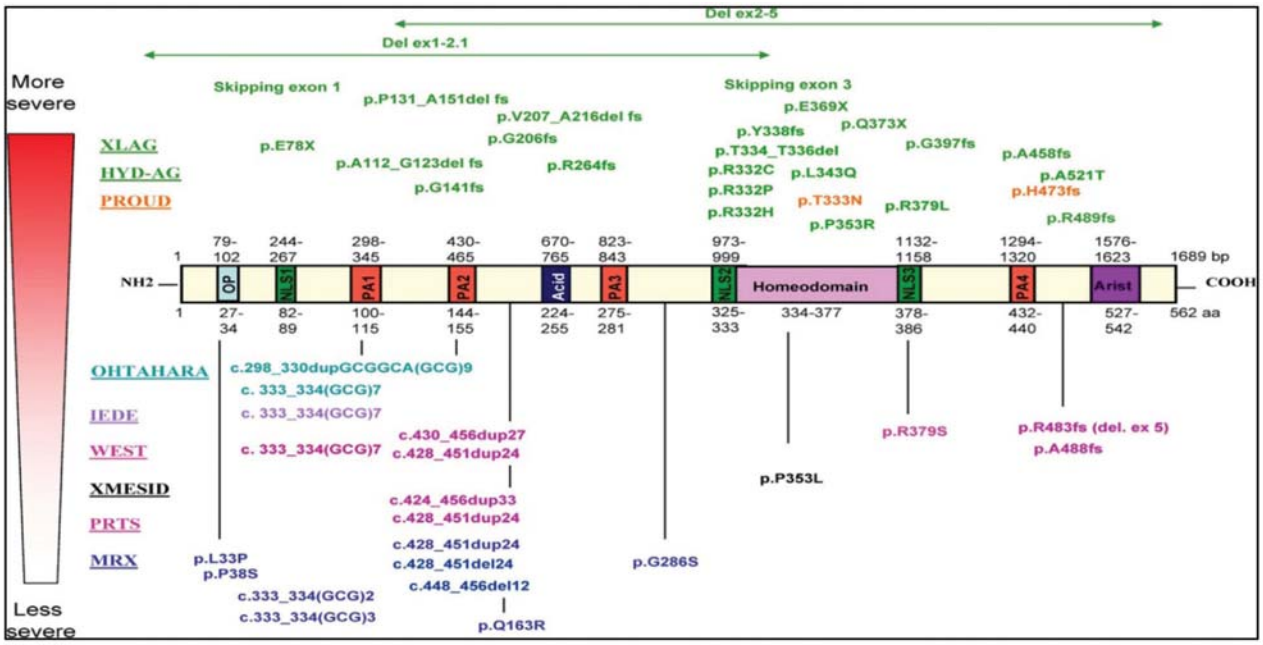

Figure 1. *ARX* gene mutations with related phenotypes.[32]

All mutations and related phenotypes resulting from *ARX* mutations are shown in Figure 1. In order to determine the relative prevalence of *ARX* mutations in the Iranian population, we screened for the entire gene in families with XLID.

Materials and Methods

Patients

A total of 65 families comprising at least two mentally retarded males (aged between 8–45 years) referred from clinician offices in different provinces of Iran to the Genetics Research Center (GRC), Tehran, Iran were selected for this study. These families belonged to various ethnicities from different provinces of Iran.

Physical, cognitive and behavioral data were assessed and collected for each affected individual. A family pedigree was drawn. All studied families had the XLID pattern of inheritance.

DNA extraction and mutation screening

A blood sample (10 mL) was drawn from each patient after obtaining informed consent. Genomic DNA was isolated from blood leucocytes by a standard salting out method.[34] Fragile X testing (CGG expansion of the *FID1* gene) was carried out by polymerase chain reaction (PCR) and Southern blot analysis; all of the results were negative. We also performed standard 450 G-band karyotyping in order to exclude cytogenetically visible chromosomal aberrations.

Mutation screening of all *ARX* coding exons and flanking sequences was performed in two steps. In the first step all families were screened for the most common form of mutation, 24 bp duplication, and if the result of each sample was negative, we then performed single strand conformation polymorphism (SSCP) analysis.

The PCR protocol, described by Poirier et al.,[14] was used as a primary screening test to detect the 24 bp duplication. In the case of the 24 bp duplication, familial segregation was directly analyzed on an 8% polyacrylamide gel. Samples in which no mutations could be identified at that stage were further analyzed by SSCP. SSCP analysis was considered as an inexpensive, rapid method for detecting sequence variation. This technique is performed under non-denaturing conditions and at a reduced temperature, single-stranded DNA molecules assume various unique conformations depending on their nucleotide sequences. These conformational changes result in detectable differences in mobility. Following the detection of a mutation by SSCP, it was subsequently confirmed by sequencing.[35]

Oligonucleotide primers were designed and tested for selectivity, specificity, and sensitivity of target detection. As shown in Table

Table 1. Parameters for amplification of *ARX* gene fragments.

Name	PRIMER 5›→3'		PCR product size (bp)
	Forward	Reverse	
ARX-1#	CGGGAGAGGCAGCCGGCGAT	AGTAAGTGCCTGACGGGAGC	277
ARX-2#	CTGATAGCTCTCCCTTGCCC	CCCCTGCGCCGTCCGGCCGTTC	258
ARX-2B*	CCCCTCCGCCGCCACCGCCAAC	TCCTCCTCGTCGTCCTCGGTGCCGGT	313
ARX-2C	TGGCCGGCGGCCCGGGCAGC	CAGCTCCTCCTTGGGTGACA	266
ARX-2X	GCAGTGGCCACAGAGGGCG	CGCGACCACCCTACGCGCAT	255
ARX-3#	TCTGTGTGTTGCTTTCTTAT	CCCGCCACCAACCCATCTCT	147
ARX-4A	GCCGACCCTGGGCTCTCTCTGCC	CAGTCCAAGCGGAGTCGAGCG	204
ARX-4B	ACCCACCCGCTCAGCCCCTA	AGACAGACAGACTTCCGAGGCTGC	261
ARX-5#	CCATGACCGCGCTGTTTGCT	GAGTGGTGCTGAGTGAGGTG	334

* Primers used for the detection of the 24 bp duplicate mutation. #Primer pairs designed by Dr. K. Poirier's group.[14] The remainder of the primers were designed by the primer3 website.

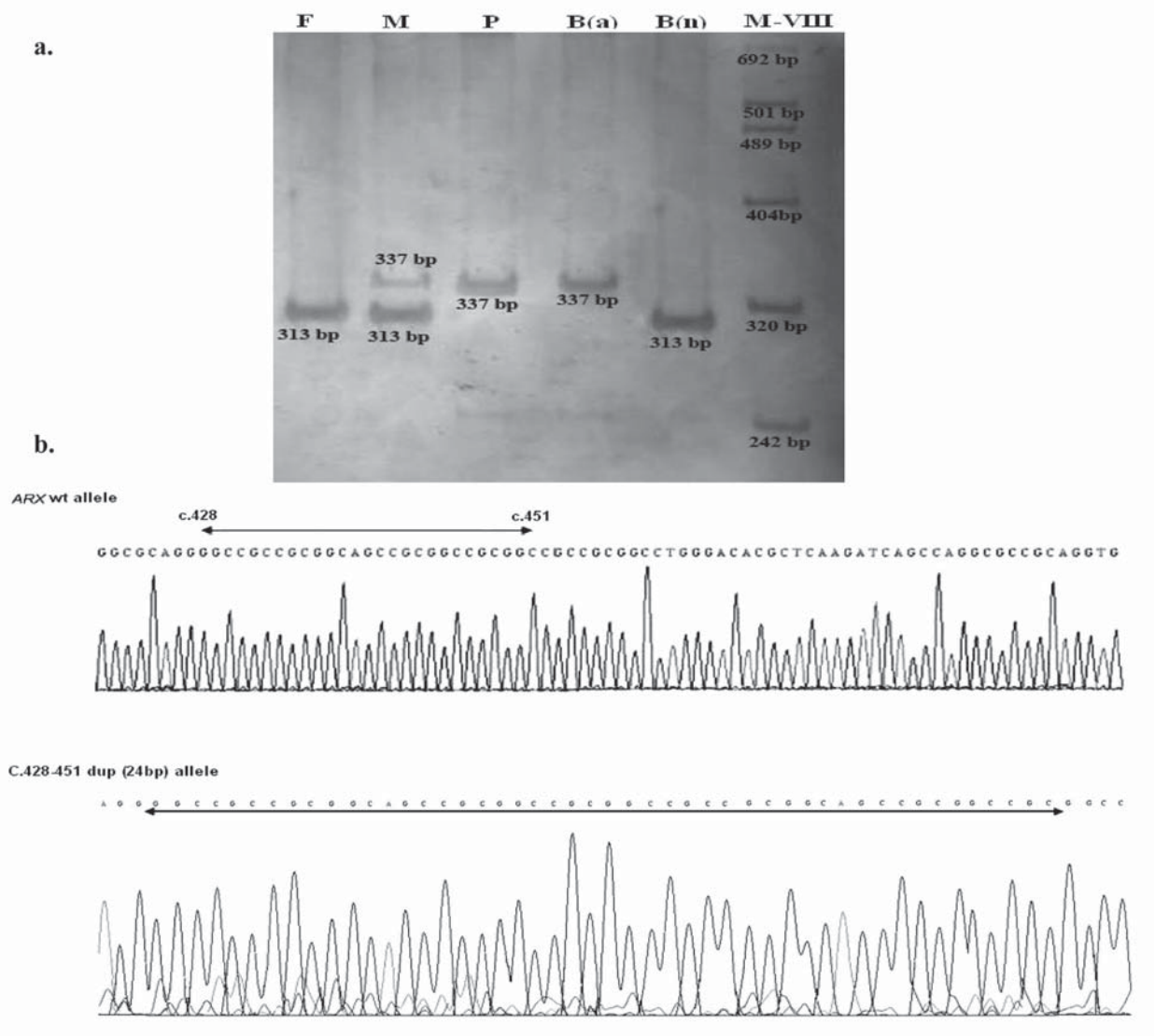

Figure 2. The family with c.428_451dup(24 bp) mutation |; **a)** Polyacrylamide gel electrophoresis [M-VIII = marker VIII, F = father, M = mother, P = proband; B(a): Proband's brother (affected), B(n): Proband's brother (normal)], **b)** DNA sequence chromatograms for c.428_451dup(24 bp) mutation in the affected male compared with the control.

1, some of the primers used for PCR analysis were previously designed by Poirier et al.[14] and the remainders were selected from the primer3 website (http://www.fokker.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi).

Results

In total, we selected 65 families of whom 16 had at least two sons in different sibships that were related through their mothers (established XLID); 49 families had a minimum of two affected sons in one generation (putative XLID).

Among the 21 families with syndromic ID there were three families with an established X-linked pattern of inheritance, whereas in 44 families with nonsyndromic ID, 13 cases had an established X-linked pattern of inheritance. Severity of ID among these families varied from mild to profound.

After obtaining clinical information, all families were screened for the most common mutation in exon 2, c.428_451dup(24 bp), of the *ARX* gene by PCR. Thus far, we have detected the mutation in a family with the XLID pattern of inheritance (Figure 2). The patient with 24 bp duplication, a 24-year-old male was the third of four children of an unrelated marriage. There was no history of prenatal, perinatal, or postnatal complications with normal measurements and severe ID. The proband's oldest brother who had no clinical abnormalities, had a similar genotype and phenotype as the proband. The mother was the carrier for this mutation and was not affected with ID. There were no other clinical symptoms except for ID in the two affected siblings.

Among the total families, three shifts (one shift in exon 5 and two shifts in exon 4) were identified in three families by using SSCP. The sequencing analysis showed that two shifts were not associated with any mutation and the other one was a silent mutation in exon 4 (c.1347C>T, G449G) (Figure 3). However, this substitution has been reported previously.[10–13] We did not detect any variant in exons 1, 3, and 5. The patient with a silent mutation, an 18-year-old male, was the first child of an unrelated marriage. He has a severe intellectual disability without any other clinical symptoms.

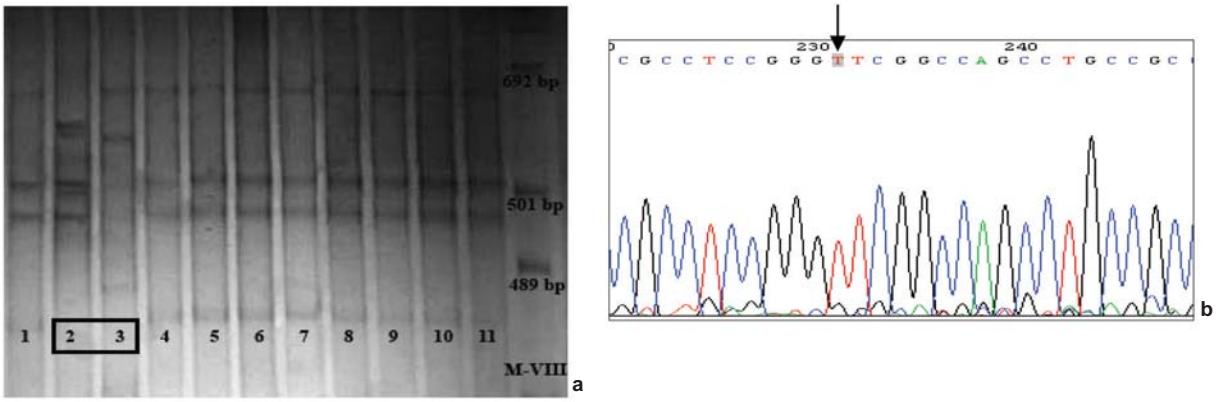

Figure 3. PCR-SSCP and sequence analysis of a c.1347C>T substitution |; **a)** PCR-SSCP analysis. Two samples located in the box show a shift that the c.1347C>T substitution is identified in sample 3 [M-VIII: Marker VIII], **b)** Chromatogram for DNA sequencing showing c.1347C<T substitution. The substitution is located in the gray box and is shown by an arrow.

Discussion

In 2002, the *ARX* gene was independently identified by two groups. Stromme et al. mapped a XLIDS trait ISSX syndrome to a seven-megabase region in Xq22, and they tested *ARX* as a positional candidate gene expressed in the brain.[6,7,15,16,33]

Mutations in *ARX* have recently been reported to give rise to a broad spectrum of phenotypes, from nonspecific intellectual disability to syndromic forms such as PRTS and XLAG.[24,33,36] *ARX* mutations have been suggested to be more frequent in XLID families than mutations in other known XLID genes, apart from *FMR1*.[15,16,22,32,33,36] The pleiotropic effects of mutations in the *ARX* gene recognized to date are all neuropsychological.[24,32,33]

The c.428_451dup(24 bp) which leads to an expansion of the second poly A tract in the ARX protein is the highly recurrent mutation identified in the *ARX* gene.[7,12,16,22,33] This mutation accounts for about 70% of NS-XLID families linked to Xp22.1.[14,15,33] This mutation is associated with both the inter- and intrafamilial variabilities of expression and has been found in families with PRTS, ISSX, and XLIDS.[5,13,16,17,24,25,32,33]

More recently, expansions of alanine tracts in at least seven genes, particularly transcription factor genes, have been reported to cause several human diseases.[37] They are characterized by intellectual disability and developmental malformations that range from defects in the formation of digits to the central nervous system.[12,16,33,36] Recent data has shown a high frequency (6.1%) of the c.428_451dup(24 bp) in XLID families, a low rate in brother pair (BP) families (1.5%), and 0.1% in sporadic cases.[10,14–17]

In this study, screening for the c.428_451dup(24 bp) mutation in 65 male samples showed one family with this mutation among 16 established XLID (6.25%) cases. We did not find this duplication in 49 BP families. The frequency of this mutation among all selected families (XLID and BP) was 1.5%. The results obtained here were similar to other published studies in different countries.[10,11,14–17]

With regards to our data concerning the c.1347C>T substitution, other studies have already identified this su sibstitution as a polymorphism.[10,11,13] In silico analysis of the altered sequence performed by Gronskov et al. has shown that a strong binding site is created by c.1347C>T substitution for SRp55.SR proteins which are splicing factors that bind to exonic splice enhancers (ESE).[11] However when the RESCUE-ESE program by Gestinari-Duarte et al. was used, the achieved results were not same with Gronskove et al. So, they did not confirm any possible EST sites for this substitution.[10]

Considering that two shifts have been identified without any mutations emphasizes the fact that SSCP is not efficient enough for mutational screening. Therefore, the mutations should be confirmed by DNA sequencing.

In conclusion, our data show that the 24 bp duplication in exon 2 of the *ARX* gene is considerably frequent among XLID families. We therefore recommend that molecular analysis of the 24 bp duplication in the *ARX* gene should be considered as a routine test in any male who presents with NS-XLID and/or XLIDS.

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