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Mutation screening of the *ARX* gene in patients with autism

Pauline Chaste^{1,2}, Gudrun Nygren³, Henrik Anckarsäter³, Maria Råstam³, Mary Coleman⁴,
Marion Leboyer^{1,5}, Christopher Gillberg^{3,6}, Catalina Betancur^{1*}

¹ INSERM U513, Université Paris XII, Créteil, France

² AP-HP, Hôpital Robert Debré, Service de Psychopathologie de l'Enfant et de l'Adolescent, Paris, France

³ Department of Child and Adolescent Psychiatry, Goteborg University, Goteborg, Sweden

⁴ Department of Pediatrics, Georgetown University School of Medicine, Washington, District of Columbia

⁵ AP-HP, Albert Chenevier and Henri Mondor Hospitals, Department of Psychiatry, Créteil, France

⁶ Saint George's Hospital Medical School, London, United Kingdom

*Correspondence to: Dr Catalina Betancur, INSERM U513, Faculté de Médecine, 8 rue du Général Sarrail, 94010 Créteil Cedex, France. E-mail: Catalina.Betancur@creteil.inserm.fr

Running title: Mutation screening of the *ARX* gene in autism

Abstract

Mutations in the *ARX* gene are associated with a broad spectrum of disorders, including nonsyndromic X-linked mental retardation, sometimes associated with epilepsy, as well as syndromic forms with brain abnormalities and abnormal genitalia. Furthermore, *ARX* mutations have been described in a few patients with autism or autistic features. In this study, we screened the *ARX* gene in 226 male patients with autism spectrum disorders and mental retardation; 42 of the patients had epilepsy. The mutation analysis was performed by direct sequencing of all exons and flanking regions. No *ARX* mutations were identified in any of the patients tested. These findings indicate that mutations in the *ARX* gene are very rare in autism.

Key words: X chromosome, mental retardation, epilepsy

INTRODUCTION

Mutations in the X-linked gene *Aristaless related homeobox (ARX)* have recently been shown to cause mental retardation, either isolated or associated with a broad spectrum of neurological disorders. The first *ARX* mutations were found in families with mental retardation and various forms of epilepsy, including West syndrome (infantile spasms, hypsarrhythmia, and mental retardation), myoclonic epilepsy, or Partington syndrome (dystonic movements of the hands, mental retardation, and seizures) (Stromme et al., 2002b). Subsequently, *ARX* mutations have been reported in familial and sporadic cases of nonsyndromic mental retardation (Bienvenu et al., 2002), X-linked lissencephaly with abnormal genitalia (Kitamura et al., 2002), agenesis of the corpus callosum, hydranencephaly, ataxia, and different types of epilepsy (for review see Suri 2005). Furthermore, the reevaluation of two families with an *ARX* mutation originally diagnosed with X-linked mental retardation (Stromme et al., 2002b) showed that one patient had autism and three had autistic features (Turner et al., 2002). This striking phenotypic variability is partly explained by the type of mutation, with duplications and missense mutations resulting in mental retardation alone or with epilepsy or autism, whereas loss-of-function mutations result in more severe phenotypes with brain malformations and abnormal genitalia (Suri 2005). The most frequently reported mutation is a 24-bp duplication in exon 2, c.428-451dup(24 bp), which results in the expansion of a polyalanine tract and accounts for about 30% of all described mutations. Based on the results of the first mutation screening of *ARX* in individuals with mental retardation (Bienvenu et al., 2002), it was suggested that *ARX* mutations could be as common as fragile X syndrome among the mentally retarded population (Sherr 2003). More recent studies, however, have shown that *ARX* mutations are not as frequent as initially suspected (Gronskov et al., 2004). The human *ARX* gene maps to chromosome Xp22.13 and is composed of five coding exons. It encodes a transcription factor expressed predominantly in the fetal and adult brain (Stromme et al., 2002b), thought to regulate essential processes during brain development, including neuronal proliferation, migration and differentiation (Kitamura et al., 2002; Friocourt et al., 2006).

Autism is a behaviorally defined neurodevelopmental syndrome with a strong heritable component and marked genetic heterogeneity (Veenstra-Vanderweele et al., 2004). Monogenic disorders (e.g., fragile X syndrome, tuberous sclerosis complex, neurofibromatosis, various rare metabolic disorders) and chromosomal abnormalities are identified in about 10% of patients, but the underlying cause remains unknown in the majority of patients (Gillberg and Coleman 2000). The search for genetic variants conferring liability to autism using linkage and association analyses has failed to identify any definite genes (Veenstra-Vanderweele et al., 2004). The prevalence of autism is approximately four times higher in males than in females. The higher prevalence of males together with the description of cases with chromosomal aberrations involving the X chromosome (Vorstman et al., 2006), suggest the implication of X-linked genes in autism. Mutations in several X-linked genes have been identified in individuals with autism spectrum disorders. The most frequently reported genetic disorder in autism is the fragile X syndrome, involving CGG expansions in the *FMR1* gene, found in about 2% of patients

with autism (Wassink et al., 2001). Other mutations of X-linked genes described in a few cases with autism spectrum disorders include *MECP2* (Carney et al., 2003; Zappella et al., 2003), neuroligins *NLGN3* and *NLGN4X* (Jamain et al., 2003), *DMD* (Wu et al., 2005), *CDKL5* (Evans et al., 2005), and the creatine transporter *SLC6A8* (Salomons et al., 2003). Together, these findings strongly suggest that other genes on the X chromosome are also potential candidates for autism. In particular, the *ARX* gene is a compelling candidate. Indeed, approximately 75% of subjects with autism also have mental retardation, and about 30% have epilepsy. The discovery of *ARX* mutations in patients with mental retardation, often associated with epilepsy and sometimes with autism (Nawara et al., 2006; Turner et al., 2002), suggests that *ARX* could be involved in other cases of autism. Thus, the aim of this study was to assess the frequency of *ARX* mutations in autism. We screened the *ARX* gene in 226 males with autism spectrum disorders and mental retardation; 42 of the patients had epilepsy.

MATERIALS AND METHODS

Patients

A total of 226 males with an autism spectrum disorder and mental retardation were included in the study. They were recruited by the Paris Autism Research International Sibpair (PARIS) study at specialized clinical centers in seven countries (France, Sweden, Norway, Italy, Belgium, Austria, and the United States). There were 77 subjects from families with two or more affected siblings (45 with males only and 32 brother/sister pairs) and 149 sporadic cases. None of the familial cases was ascertained from known X-linked pedigrees with mental retardation or neurological disorders. Diagnoses were based on clinical evaluation by experienced clinicians, DSM-IV criteria, and the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994). Among the patients studied, 218 had autism and 8 had pervasive developmental disorder not otherwise specified. Laboratory tests to rule-out medical causes of autism included standard karyotyping, fragile X testing, and metabolic screening; brain imaging and EEG were performed when possible. Patients diagnosed with medical disorders such as fragile X syndrome or chromosomal abnormalities were excluded from the study. Forty-two patients had epilepsy and 6 had an abnormal EEG but no seizures. There were 198 individuals of Caucasian origin, 12 Black, 2 Asian and 14 of mixed ethnicity. The local Research Ethics Boards reviewed and approved the study. Informed consent was obtained from all families participating in the study.

ARX mutation analyses

Genomic DNA was prepared from blood or lymphoblastoid cell lines. The complete *ARX* coding sequence including the exon-intron boundaries was amplified by PCR. Exon 2 was amplified in two overlapping fragments. Primer sequences were kindly provided by Prof Jozef Gécz (Stromme et al., 2002b) or reported previously (Kato et al., 2004). The amplicons were screened for mutations by direct sequencing using the ABI BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 sequencer (Applied Biosystems).

RESULTS

Genomic DNA from 226 patients with autism spectrum disorders was screened for intragenic *ARX* mutations by sequencing analysis. No pathogenic mutations were identified in any sample. We identified one synonymous variant in exon 4, c.1347C>T (p.G449G), in 10 individuals. This variant has been described previously as a polymorphism (Gronskov et al., 2004; Kato et al., 2004).

DISCUSSION

Our results indicate that coding mutations in the *ARX* gene are not associated with autism in our family sample. Since our analysis was limited to the coding sequence of *ARX*, we cannot exclude that mutations in the promoter and other regulatory sequences may disrupt the activity of the *ARX* gene in some individuals with autism. Similarly, we cannot exclude the presence of duplications or deletions of *ARX* in our patients, as we did not perform quantitative analyses. Furthermore, the absence of coding changes does not preclude epigenetic abnormalities that might affect gene expression.

Autism was described in 4 subjects among 50 mentally retarded males in 9 X-linked families with *ARX* mutations (Stromme et al., 2002a; Turner et al., 2002). They belonged to two families carrying the duplication c.428-451 dup(24 bp), which represents the most frequent mutation of the *ARX* gene found in mental retardation and epilepsy. Interestingly, the same mutation in other family members manifested as isolated mental retardation or was associated with dystonia, infantile spasms, generalized tonic-clonic seizures or ataxia, underlining the intrafamilial phenotypic variability associated with *ARX* mutations (Stromme et al., 2002a). Similarly, Nawara et al. (Nawara et al., 2006) reported 5 families with 19 affected patients with X-linked mental retardation carrying the 24-bp duplication; in one of the families, 2 of 4 affected males had autism.

The precise prevalence of *ARX* mutations in mental retardation is at present unknown but is likely to be low. The first study to explore *ARX* in mental retardation found mutations in 7 of 9 extended families with mental retardation linked to Xp22.1, 2 of 148 families with two affected brothers and 1 of 40 sporadic cases, suggesting that *ARX* mutations were a common cause of mental retardation (Bienvenu et al., 2002). The most recent estimates indicate that mutations in the *ARX* gene account for 9.5% of families with X-linked mental retardation but are less frequent in families with two affected brothers (2.2%) (Poirier et al., 2006). By contrast, the yield of *ARX* mutation analysis in sporadic males with mental retardation is extremely low. A recent Danish study screened exon 2 for two recurrent expansions of polyalanine tracts in 682 males with mental retardation, revealing only one putative mutation (Gronskov et al., 2004). Our negative findings after systematic sequence analysis of *ARX* in a large set of patients with autism and mental retardation, including 45 families with 2 or more affected brothers, 32 brother-sister pairs and 149 sporadic cases, are compatible with those reported for mental retardation.

In conclusion, the results of the present study indicate mutations of the *ARX* gene are a very rare cause of autism, as previously shown for other X-linked genes, such as *MECP2* (Carney et al., 2003; Zappella et al., 2003), *NLGN3*, and *NLGN4X* (Jamain et al., 2003), which have been screened in large

cohorts of patients with autism. Thus, with the exception of *FMR1*, the number of patients found to be mutated for each X-linked gene appears to be very low. In addition, our findings suggest that routine molecular screening of *ARX* is not indicated in families with two siblings with autism or in sporadic patients.

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