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To cite this version:

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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, hypersarrhythmia, 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), agensis 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.

ACKNOWLEDGMENTS

We are grateful to all the patients and their parents for their cooperation. We thank ML Picoche for technical assistance, the DNA and cell bank of the INSERM U679 (IFR des Neurosciences, Hôpital Pitié-Salpêtrière), and the Centre d'Investigations Cliniques, Hôpital Robert Debré, for obtaining the samples from the French families. This research was supported by Fondation Jérôme Lejeune, INSERM, Assistance Publique-Hôpitaux de Paris, Fondation pour la Recherche Médicale, Fondation France Télécom, Fondation de France, and the Swedish Medical Research Council.


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