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[¶] http://www.well.ox.ac.uk/~maestrin/iat.html

Abstract

Autism spectrum disorders (ASD) are complex genetic disorders more frequently observed in males. Skewed X chromosome inactivation (XCI) is observed in heterozygous females carrying gene mutations involved in several X-linked syndromes. In this study, we aimed to estimate the role of X-linked genes in the susceptibility to ASD by ascertaining the XCI pattern in a sample of 543 informative mothers of children with ASD and in a sample of 163 affected girls. The XCI pattern was also determined in two control groups (144 adult females and 40 young females) with a similar age distribution to the mothers sample and affected girls sample, respectively. We observed no significant excess of skewed XCI in families with ASD. Interestingly, two mothers and one girl carrying known mutations in X-linked genes (*NLGN3, ATRX, MECP2*) showed highly skewed XCI, suggesting that ascertainment of XCI could reveal families with X-linked mutations. Linkage analysis was carried out in the subgroup of multiplex families with skewed XCI (80:20) and a modest increased allele sharing was obtained in the Xq27-Xq28 region, with a peak Z-score of 1.75 close to rs719489. In summary, our results suggest that there is no major X-linked gene subject to XCI and expressed in blood cells conferring susceptibility to ASD. However, the possibility that rare mutations in X-linked genes could contribute to ASD cannot be excluded. We propose that the XCI profile could be a useful criteria to prioritize families for mutation screening of X-linked candidate genes.

Keywords

autistic disorder, skewed X-inactivation, X-linked mutation, linkage study

Introduction

Autism spectrum disorders (ASD) are characterized by impairments in communication, social interaction, and repetitive and stereotyped patterns of behaviors and interests. Although the genes for ASD remain largely unknown, twin and family studies indicate a large genetic contribution to ASD [Freitag, 2007]. ASD is more frequent in males than females, with an approximate ratio of 4:1. Despite this male susceptibility, whole genome scans conducted in families with at least two affected members did not detect major X-linked loci [Freitag, 2007; Autism Genome Project Consortium et al., 2007]. The Y chromosome was also studied using informative single nucleotide polymorphisms (SNP), but no haplotype was found associated with ASD [Jamain et al., 2002]. Although these results suggest a limited role of the sex chromosomes in the susceptibility to ASD, they do not exclude the role of some X or Y-linked genes in a subset of affected individuals. Indeed, mutations in two X-linked genes encoding neuroligins *NLGN3* and *NLGN4X* were identified in males with ASD [Jamain et al., 2003]. In addition, mutations in the X-linked genes for fragile X-related protein 1 (*FMR1*) and methyl-CpG-binding protein 2 (*MECP2*) are associated with ASD in some cases [Carney et al., 2003; Reddy, 2005].

To ensure equal expression of X-linked genes in both sexes, one X chromosome is inactivated at random in every cell in females. X chromosome inactivation (XCI) occurs early in the development of embryos and the process has been divided into three phases: initiation, spreading, and maintenance [Heard et al., 1997]. Non-random XCI or skewing is a rare event in the normal female population [Amos-Landgraf et al., 2006]. In contrast, an extremely skewed XCI can be observed in heterozygous females carrying gene mutations involved in X-linked syndromes such as X-linked mental retardation, Barth syndrome and X-linked sideroblastic anemia [Orstavik et al., 1998; Cazzola et al., 2000; Plenge et al., 2002]. In ASD, no study had been performed to ascertain the XCI pattern in mothers of affected children, but Talebizadeh et al. [2005] reported an excess of XCI skewing in affected girls (33%, 10/30) compared to controls (11%, 4/35). The reason for this non-random X inactivation remains unclear and could be the consequence of a selection against or for cells carrying mutations on the active X chromosome [Muers et al., 2007]. In this study, we aimed to estimate the role of X-linked genes in the susceptibility to ASD by ascertaining the XCI patterns in a large sample of mothers of patients with ASD and affected female patients.

Materials and Methods

Subjects

The study sample included a total of 621 mothers of children with ASD and 182 affected girls from three research groups: the Paris Autism Research International Sib-pair (PARIS) study, the Finnish study group for ASD and the International Molecular Genetic Study of Autism Consortium (IMGSAC; Table 1). The two control groups used for this study were obtained from anonymous blood donors, and consisted in 162 adult female subjects and 47 young female subjects (Table I). Four ASD families carrying previously identified X-linked mutations were included for comparison purposes: three sib pair families carrying maternally-inherited mutations in the *NLGN3*, *NLGN4X* [Jamain et al., 2003] and *ATRX* genes, respectively, as well as a singleton family with a de novo R294X mutation of MECP2 in a girl with autism and no classical features of Rett syndrome.

The assessment methods and inclusion criteria for the three family collections have been previously described [Philippe et al., 1999; IMGSAC, 2001; Auranen et al., 2002]. Briefly, all patients met ICD-10 and/or DSM-IV diagnostic criteria for autism or pervasive developmental disorder not otherwise specified (PDD-NOS). Diagnosis was based on the Autism Diagnostic Interview-Revised (ADI-R) [Lord et al., 1994], the Autism Diagnostic Observation Schedule (ADOS) [Lord et al., 1989] and clinical evaluation. Cases were excluded if they had known medical disorders or chromosomal abnormalities. Informed, written consent was obtained from all participating subjects or their parents.

X Inactivation Analysis

The X inactivation assay was carried out on genomic DNA extracted from peripheral blood or lymphoblast cell lines. XCI was calculated as the ratio of the peak heights of two alleles of the highly polymorphic CAG repeat of the androgen receptor (AR) after digestion with the methylation sensitive enzyme HpaII, corrected with the ratio of the peak heights of the two alleles before digestion for

preferential amplification of one of the alleles [Amos-Landgraf et al., 2006]. Briefly, 100 ng of genomic DNA was digested with 5 U *Rsa*I plus 15 U *HpaII*, and with 5 U *Rsa*I alone at 37°C overnight. Then the mixture was PCR amplified with forward primer 5'-TCC AGA ATC TGT TCC AGA GCG TGC-3', fluorescently labeled with 6-FAM (6-carboxyfluorescein) and reverse primer 5'-GCT GTG AAG GTT GCT GTT CCT CAT-3'. The resulting PCR products were run on ABI 3700 or ABI 3730 DNA sequencer and the peak height was measured by Genescan software (Applied Biosystems, Foster City, CA).

The range of XCI ratio is from 50:50 (random XCI) to 100:0 (complete non-random XCI). One female sample with known XCI ratio (80:20 or 95:5) and one male sample were used as controls in each batch of samples to verify the complete digestion and replication. For all IMGSAC samples the X inactivation assay was run in duplicate, showing very high reproducibility (mean standard deviation between replicas = 1.21). Skewed XCI was assigned by two thresholds: 80:20 and 90:10. The frequency of XCI skewing was analyzed with Fisher's exact test using SPSS 12.0.

Linkage study

For the linkage analysis of the X chromosome we utilized the genotyping data generated by the Autism Genome Project (AGP) using Affymetrix 10K v2 SNP array [Autism Genome Project Consortium et al., 2007]. SNP data were available for 185 multiplex families from IMGSAC and PARIS, which passed quality check and were informative for XCI analysis. Linkage analysis was also carried out in the subgroup of 28 multiplex families with XCI skewing (80:20). No complete SNP data was available for four IMGSAC and six PARIS families with skewed XCI. Non-parametric linkage (NPL) analysis was performed using the NPL "all" statistic implemented in the Merlin program [Abecasis et al., 2002], that uses the Kong and Cox linear model to evaluate the evidence for linkage. This model is designed to identify small increases in allele sharing spread across a large number of families. To model linkage disequilibrium in Merlin, we used an option that allows to calculate pairwise r^2 between neighboring markers and create a cluster joining markers for which pairwise $r^2 > 0.2$. Empirical significance was estimated by simulating 1,000 replicates of the X chromosome genotypes conditional on the original family structure, marker spacing, allele frequencies and missing data.

***MECP2* and *FMR1* screening**

Mutation screening of *MECP2* and *FMR1* was performed by sequencing all the coding regions and splice junctions. Primers and conditions used for *MECP2* have been previously described [Beyer et al., 2002]. *FMR1* primers and PCR conditions are available on request. Real-time quantitative PCR analysis of the *MECP2* gene was carried out using the comparative ddCt method with SYBR-green as previously described [Van Esch et al., 2005]. Two primer sets of the *MECP2* gene [Van Esch et al., 2005] were validated to have the same efficiency of amplification to that of the primer pair for the *NXF5* gene on Xq22, used as reference sequence for normalization. For relative quantification the reaction mix consisted of Power SYBRGreen PCR Master Mix (Applied Biosystems), with 500 nM of

each primer and 10 ng of DNA in a final volume of 15 µl. Reactions were done in triplicate and run on 7500 Fast Real-Time PCR System (Applied Biosystems). One normal male sample and one male sample carrying a large duplication including the *MECP2* gene were used as controls in each run.

Results

Three independent samples of autism families and two control samples were analyzed (Table II). ASD families were subdivided according to the sex of affected children: families with affected males only (MO) and families containing one or more affected females (female containing, FC), since MO families are more likely to carry X-linked recessive mutations.

The rate of skewed XCI in unaffected females is known to increase with age [Busque et al., 1996]. For this reason, a group of 144 adult females with an age distribution similar to the sample of mothers was used as control population. Similarly, a group of 40 young females was used as control for the sample of affected girls. The pattern of XCI in the adult female control sample (80:20: 12.5%; 90:10: 3.5%) was similar to previously published studies [Amos-Landgraf et al., 2006].

We did not identify a different distribution of XCI patterns between mothers of affected children and the adult controls (P = 1.00, Chi-square test; Fig. 1). Data for the XCI skewing threshold of 80:20 and 90:10 are shown in Table II. No significant differences were present for the subgroups of MO mothers and FC mothers in each independent sample (PARIS, Finland, and IMGSAC) as well as in the combined sample. We also observed no statistical difference between affected females and the sample of young female controls.

Interestingly, three out of the four families carrying known X-linked mutations had skewed XCI. In the first family, the mother had extremely skewed XCI (92:8) and transmitted a R451C mutation of *NLGN3* to her two affected sons [Jamain et al., 2003]. In the second family, the mother showed XCI skewing (95:5) and transmitted an *ATRX* mutation G1676A to her two affected sons. *NLGN3* (56 cM) and ATRX (57 cM) are tightly linked to AR (53 cM) and therefore recombination is an unlikely event among them. n both cases, the mutations were associated with the inactivated allele of AR. In the singleton family, the affected girl had highly skewed XCI (92:8) and a de novo R294X mutation of *MECP2*. As expected, in the family with a frameshift mutation in the *NLGN4X* gene, the carrier mother had random XCI, since *NLGN4X* escapes XCI [Carrel and Willard, 2005].

A total of 86 mothers of affected children without known mutations showed highly skewed XCI (80:20), 45 of which are from multiplex families. These can be classified in three groups: 26 families with two affected boys (brothers or cousins), thus consistent with segregation of a X-linked recessive mutation; 14 families with male-female probands or two female probands; the female probands from these families showed random inactivation except for two girls with skewed XCI ≥ 80:20. Finally, five extended pedigrees contained affected cousin pairs related through their fathers, and are thus incompatible with X-linked segregation. There were 41 mothers of singletons, of which 33 had one affected son and 8 had one affected daughter with random XCI.

Using X-linked markers, we investigated if the subgroup of multiplex families with highly skewed XCI showed evidence of linkage, which could point to a region containing possible X-linked causative gene(s). NPL analysis was performed on multiplex families from the IMGSAC and PARIS collections. Genotypes were available for 185 multiplex families informative for XCI.

No significant linkage was present in the whole sample. When the subgroup of 28 multiplex families with skewed XCI (≥80:20) and consistent with X-linked segregation (18 MO families and 10 FC families) was analyzed, only a modest increased allele sharing was detected in the Xq27-Xq28 region, with a peak Z-score of 1.75 close to rs719489 (Fig. 2). The empirical P-value of this result, calculated through simulation, was not significant (P = 0.39). A very similar NPL linkage profile was obtained for the smaller subgroup of 18 MO families with skewed XCI (≥80:20) (data not shown). Although the sample size of the skewed XCI group is too small to provide conclusive results, the lack of significant linkage suggests that no major X-linked locus is likely to be involved in these ASD families with skewed XCI. We note, however, that within the Xq27-q28 region, two genes (FMR1 and MECP2) are associated with ASD in some cases and therefore represent candidates for the disorder. FMR1 is responsible for the fragile X syndrome. The presence of $(CCG)_n$ trinucleotide repeat expansions has been previously excluded in at least one affected male from each family of our sample [Philippe et al., 1999; IMGSAC, 2001; Auranen et al., 2002]. Moreover, mutation screening of the coding region of FMR1 in 22 IMGSAC multiplex families with skewed XCI and allele sharing at Xq27-28 failed to identify any causative mutation. The MECP2 gene is responsible for up to 80% of the cases of Rett syndrome in females and for a proportion of syndromic and non-syndromic forms of mental retardation (MR) in males. Recently, Xq28 duplications including *MECP2* have been identified in families with MR, with asymptomatic carrier females showing extreme (>85%) skewing of XCI [Beyer et al., 2002; Del Gaudio et al., 2006]. For these reasons, we performed a mutation screen and quantitative real time PCR analysis (qPCR) of *MECP2* in 22 unrelated probands from all IMGSAC multiplex families showing XCI skewing and compatible with linkage at Xq27-28, and in 11 probands from IMGSAC singletons families with skewed XCI. We did not find any point mutations, deletions or duplications in *MECP2*.

Discussion

Our study showed no significant excess of XCI skewing in a large sample of mothers of children with ASD and no evidence for a large excess of XCI skewing in females with ASD, in contrast to the findings obtained in a previous study [Talebizadeh et al., 2005]. Using XCI approach, our results are consistent with previously published linkage studies [Freitag, 2007], suggesting that there is no major X-linked gene subject to XCI conferring susceptibility to ASD. However, the possibility that X-linked genes escaping XCI could contribute to the excess of males with ASD cannot be excluded. Indeed, not all X-linked genes are subject to XCI and not all X-linked mutations lead to XCI skewing. In addition, no conclusions can be drawn for genes that are not expressed in blood or blood stem cells. Despite these limitations, our results demonstrate that the XCI profile is useful for detecting families carrying

X-linked mutations since families with known mutations in NLGN3, ATRX, and MECP2 were detected by this approach. Therefore, we propose that the XCI profile could be used to reduce heterogeneity by selecting families more likely to carry X-linked mutations and to improve the identification of new genes associated with ASD. According to this hypothesis, we stratified the linkage analysis by selecting families with high XCI skewing. Using the subgroup with XCI skewing (80:20), we observed a modest increase of allele sharing in the region Xq27-Xq28, which did not reach empirical significance. The non-significant result can be due to the small sample size of families showing XCI skewing; therefore we cannot exclude that one or more genes in this region could play a role in the susceptibility to ASD in a minority of cases. Previous studies have also provided modest support for an autism susceptibility locus at the same X chromosomal region. Vincent et al. [2005] achieved a maximum MLOD of 1.7 at the Xq27-q28 locus in 22 multiplex autism families. In addition, Gauthier et al. [2006] reported an association with ASD at DXS8043 located on Xq27.3 ($P = 0.01$ for allele analysis and $P = 0.00001$ for haplotype analysis) in French-Canadian males. Within this broad region, FMR1 and MECP2 are strong candidate genes, but mutation screening of MECP2 and FMR1 failed to identify any causative mutations in IMGSAC families with skewed XCI. Among the other interesting candidate genes within this region is the ribosomal protein gene RPL10, which has been previously shown to harbor missense mutations in two independent autism families [Klauck et al., 2006].

In summary, our results suggest that there is no major X-linked gene subject to XCI and expressed in blood cells conferring susceptibility to ASD. However, the XCI profile could be a useful criteria to prioritize families for mutation screening of X-linked candidate genes in ASD.

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Table I. Subjects Included in the Study

Samples	N	Mean age±SD
PARIS		
Mothers (multiplex fam)	72	43±8.9
Mothers (singleton fam)	217	41±8.0
Mothers (all fam)	289	42±8.4
Affected girls	85	12±8.3
Finland		
Mothers (multiplex fam)	13	41±7.7
Mothers (singleton fam)	44	43±6.7
Mothers (all fam)	57	43±6.9
Affected girls	22	15±6.2
IMGSAC		
Mothers (multiplex fam)	201	39±6.6
Mothers (singleton fam)	74	40±4.9
Mothers (all fam)	275	39±6.2
Affected girls	75	9±5.6
All samples		
Mothers (multiplex fam)	286	40±7.1
Mothers (singleton fam)	335	41±7.5
Mothers (all fam)	621	41±7.4
Affected girls	182	11±7.6
Controls		
Adult females	162	39±8.0
Young girls	47	8±4.8

Table II. Skewed X-inactivation in ASD Families and Controls

Samples	N	Informative[a]	XCI≥80:20	*P*-value[b]	XCI≥90:10	*P*-value[b]
PARIS						
Mothers of MO	200	168	29(17.3%)	0.155	7 (4.2%)	0.494
Mothers of FC	89	78	9(11.5%)	0.509	3 (3.8%)	0.578
Affected girls	85	75	7(9.3%)	0.164	1 (1.3%)	0.652
Finland						
Mothers of MO	37	31	5 (16.1%)	0.385	2 (6.5%)	0.36
Mothers of FC	20	17	4 (23.5%)	0.184	2 (11.2%)	0.161
Affected girls	22	19	3 (15.8%)	0.593	1 (5.3%)	0.322
IMGSAC						
Mother of MO	197	181	28 (15.5%)	0.274	9 (5.0%)	0.353
Mother of FC	78	68	11 (16.2%)	0.299	3 (4.4%)	0.502
Affected girls	75	69	5 (7.2%)	0.093	1 (1.4%)	0.633
All samples						
Mother of MO	434	380	62 (16.3%)	0.172	18 (4.7%)	0.359
Mother of FC	187	163	24 (14.7%)	0.346	8 (4.9%)	0.37
All mothers	621	543	86 (15.8%)	0.195	26 (4.8%)	0.339
Affected girls	182	163	15 (9.2%)	0.112	3 (1.8%)	0.516
Controls						
Adult controls	162	144	18 (12.5%)		5 (3.5%)	
Girls controls	47	40	7 (17.5%)		0	

[a] Number of families without known mutations informative for XCI analysis.
[b] P-value for one-sided Fisher's exact test.

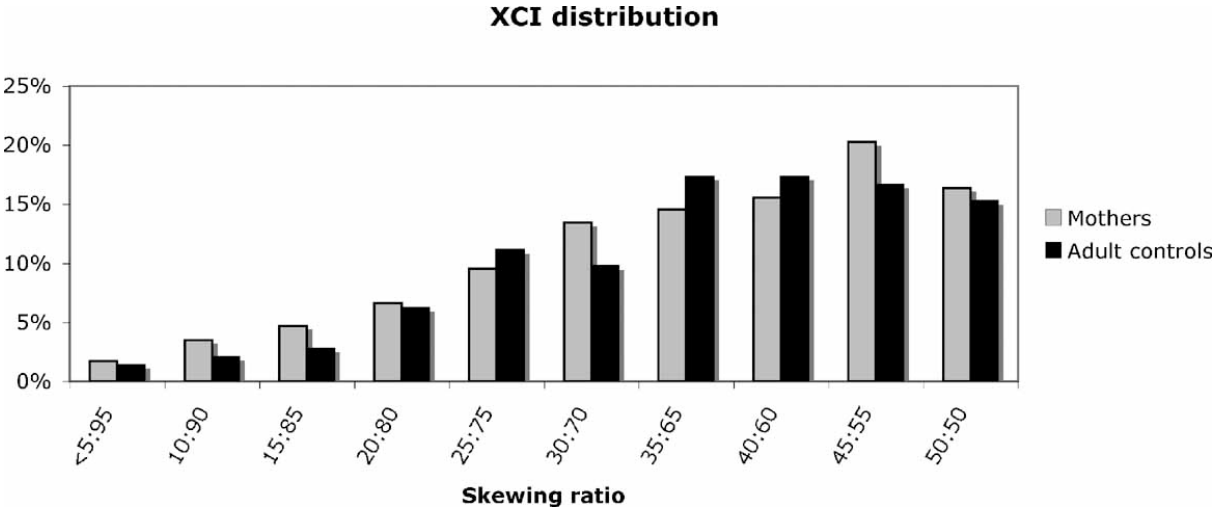

Figure 1. Distribution of X-inactivation ratios in 543 informative mothers and 144 adult controls.

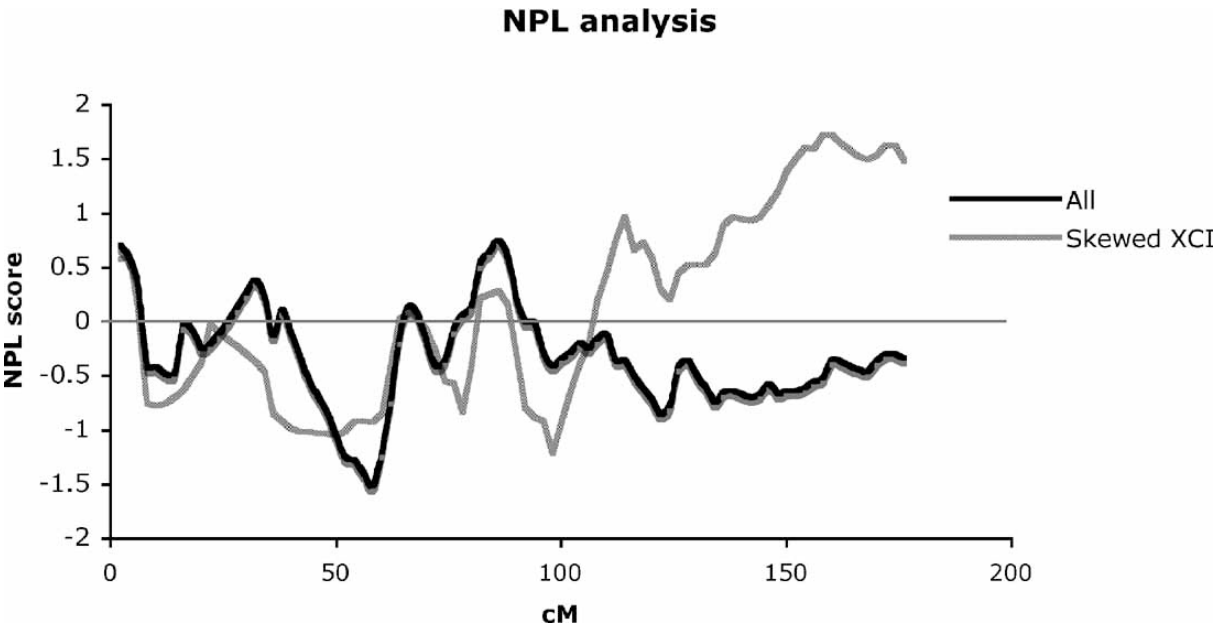

Figure 2. Non-parametric linkage analysis in the 185 multiplex families and in the subgroup of 28 families with skewed XCI 80:20. The peak for the skewed subgroup is Z = 1.75 (LOD = 0.85) at Xq27-Xq28, close to rs719489.