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Autism is a complex neurodevelopmental disorder with severe cognitive and communication disabilities, that has a strong genetic predisposition.[1] Reelin, a protein involved in neuronal migration during development, is encoded by a gene located on 7q22,[2] within the candidate region on 7q showing increased allele sharing in previous genome scans.[3-8] A case/control and family-based association study recently reported a positive association between a trinucleotide repeat polymorphism (GGC) located in the 5' untranslated region (UTR) of the reelin gene and autism.[9] We performed a transmission disequilibrium test (TDT) analysis of the 5'UTR polymorphism in 167 families including 218 affected subjects (117 trios and 50 affected sib pairs) and found no evidence of linkage/association. Our results do not support previous findings and suggest that the reelin gene is unlikely to play a major role as a susceptibility factor in autism and/or genetic heterogeneity.

There is compelling evidence from twin and family studies that genetic factors contribute substantially to the predisposition to autism (for review, see Lamb *et al*[1]). Indeed, the concordance rate in monozygote twins is approximately 80%-90%,[10,11] the sibling recurrence risk is estimated at 45,[12] and the heritability is >90%.10 Estimates from genetic epidemiology and linkage studies of the number of genes involved vary from 3[13] to more than 15.[14] Although genome-wide screens have identified several susceptibility loci,[3-8,14,15] some of which overlap, no specific genes associated with autism have yet been identified.

The neurodevelopmental hypothesis of autism is supported by the age at onset in early childhood (before three years of age), the presence of motor impairments detected during the first few months of life and some times even at birth in children later developing autism,[16] and more specifically by the observation of post-mortem neuroanatomical anomalies, suggestive of migration alterations that could occur early during development (first trimester).[17,18] Genes coding for proteins involved in neurodevelopment are thus candidate genes in autism.

Increasing evidence suggest that the reelin protein could be involved in the pathophysiology of some major psychiatric disorders (for review, see Fatemi[19]). A 50% decrease in reelin protein was found in the cortex, cerebellum and hippocampus of schizophrenic and bipolar patients.[20,21] A preliminary report also described a decrease in reelin in cerebellar homogenates of autistic patients (cited in Fatemi[19]). In addition, a form of lissencephaly was found linked to a mutation of the reelin gene in one pedigree.[22]

Reelin is the defective protein in the different strains of reeler mice, whose phenotype is characterized by severe ataxia and major anomalies of the lamination in the cortex, hippocampus and cerebellum (for review, see [19,23]). The pattern of these anomalies resembles some of the anomalies seen in autism. Reelin is expressed during development in Cajal-Retzius cells, which play a crucial role in cortex lamination, and is later expressed by interneurons of the cortex and hippocampus and by granular cells in the cerebellum. The gene encoding reelin (RELN) is located on 7q22.[2] This region lies within the chromosome 7q autism susceptibility

locus identified in several genome scans.[3-8] Recently, a polymorphic trinucleotide repeat (GGC)n was described in the 5' untranslated region (UTR) of the gene and was found positively associated to autism in both family-based and population-based association studies.[9]

We performed a family-based linkage/association study in a sample of 167 nuclear families, comprising 50 sib pairs and 117 trios. Preferential allelic transmission from heterozygous parents to affected offspring was analyzed using the extended transmission/disequilibrium test (ETDT) for multiallele marker loci.[24] Genotype distribution and allele frequencies of the patient and parent groups for the 5'UTR polymorphism are shown in Table 1. The genotyping revealed 10 alleles, ranging from 4 to 16 repeats. The global distribution of alleles in our autistic patients differed significantly from that previously reported by Persico *et al*[9] in a sample of autistic patients of Italian descent ($\chi^2 = 27.6$, df = 10, $P = 0.002$). Further analyses revealed that this difference was mainly due to the rare alleles. Indeed, as in the initial report, alleles 8 and 10 were the most frequent and there was no statistically significant difference between the frequency of these two alleles in patients in our sample and those of the previous report: 39.2% and 54.4% in our sample versus 44% and 45% reported by Persico *et al*[9] *(*$P = 0.24$ and $P = 0.036$, respectively, not significant after Bonferroni correction). In contrast, the frequency of allele 12 was significantly lower in our sample (0.46%) compared to that found in the previous study (5.8%) ($\chi^2 = 18.5$, df = 1 $P = 0.0001$ exact P value). Moreover, we did not observe the long allele with 23 repeats previously reported but found a new 16 repeat allele. These differences in allelic distribution most likely reflect the differences in ethnic composition between our sample and that of Persico *et al*.[9] Our family data set is composed mostly of Caucasian families (94%) recruited in seven countries (France, Sweden, Norway, Italy, Austria, Belgium, and the United States), whereas the families studied by Persico *et al* were either Italian or Caucasian-American.

The transmission data for the alleles of the reelin gene polymorphism are presented in Table 2, for the total sample and separately for the population of trios and sib pairs. The extended TDT analysis did not reveal a transmission disequilibrium in the total sample, either with the allele-wise TDT ($\chi^2 = 8.99$, df = 9, $P = 0.44$) or the genotype-wise TDT ($\chi^2 = 20.15$, df = 14, $P = 0.13$). Similarly, no preferential transmission of any of the alleles was detected when analyzing the samples of trios and sib pairs separately (trios: allele-wise TDT, $\chi^2 = 10.09$, df = 8, $P = 0.26$; genotype-wise TDT, $\chi^2 = 15.94$, df = 12, $P = 0.19$; sib pairs: allele-wise TDT, $\chi^2 = 5.92$, df = 6, $P = 0.43$; genotype-wise TDT, $\chi^2 = 9.69$, df = 7, $P = 0.21$). Moreover, stratification of the data set based on sex of probands (transmissions to male probands: allele-wise TDT, $\chi^2 = 6.65$, df = 9, $P = 0.67$; genotype-wise TDT, $\chi^2 = 15.36$, df = 14, $P = 0.35$) and maternal versus paternal transmissions did not reveal any significant transmission disequilibrium (data not shown).

Our results do not support the previous report by Persico *et al*,[9] which found excess transmission of the longer alleles of the (GGC)n 5'UTR polymorphism of the reelin gene (≥11 repeats) in patients affected with autism from 172 trios collected in Italy and the USA. There is

no obvious explanation for this discrepancy. Autism is a heterogeneous disorder and clinical heterogeneity could be a source of variation between the two samples, although the same diagnostic criteria were used in both studies. A false negative cannot be totally excluded although our sample is rather large. Interestingly, the sample of sib pairs examined here was the same used in the genome scan in which a region overlapping the location of RELN gene showed a multipoint maximum lod score of 0.83.[4] Ethnic heterogeneity might offer some explanations to the difference with the previous report. Persico *et al* found significant differences in the genotype and allele distributions of GGC triplet repeats between Italian and Caucasian-American patients; moreover, the association was more marked in the American families, whereas the Italian families displayed only a weak association.[9] Separate analysis of the subsample of American families of European descent in our data set (n=16) failed to reveal any transmission distortion (allele-wise TDT, $\chi^2 = 1.97$, df = 3, $P = 0.58$; genotype-wise TDT, $\chi^2 = 1.98$, df =3, $P = 0.58$). Similarly, we found no evidence of linkage disequilibrium when we analyzed separately the subsamples of Caucasian families of French (allele-wise TDT, $\chi^2 = 7.7$, df = 8, $P = 0.46$; genotype-wise TDT, $\chi^2 = 12.34$, df =11, $P = 0.34$) or Scandinavian descent (allele-wise TDT, $\chi^2 = 3.26$, df = 4, $P = 0.51$; genotype-wise TDT, $\chi^2 = 5.06$ df =5, $P = 0.41$). The possibility of a spurious association in the previous report cannot be excluded, particularly since the difference in transmission concerns only rare alleles with 11 repeats or longer, particularly allele 12, which is found in only two patients in the present sample, but represents 5.8 % of the alleles in the study by Persico and coworkers.

Genetic heterogeneity in autism has previously been suggested by linkage studies. In mice, different mutations of genes involved in the reelin pathway lead to phenotypes similar to that of the reeler mice (e.g., scrambler mice, yotari mice or knock-out of mDab1 or cdk5).[23,25,26] Indeed, reelin is known to be part of a complex pathway (for review see [19,23]) leading to phosphorylation of the microtubule-stabilizing protein tau[27] and the expression of reelin is regulated by transcription factors such as homeoprotein[28] and brain-derived neurotrophic factor (BDNF).[29] Therefore, the absence of association seen in this study does not discard the hypothesis that reelin and/or other genes in this pathway are involved in the pathophysiology of autism.

In conclusion, using a large sample of families, we did not confirm the association between the 5'UTR GGC repeat polymorphism and autism initially reported.[9] Thus, our results suggest that the reelin gene does not play a major role in the predisposition to autism. Further studies are warranted to determine the precise role of reelin and/or the reelin pathway in autism.

Materials and methods

Clinical population

Fifty families with at least two affected siblings (including one family with three autistic children) and 117 trios, comprising 218 affected individuals, were included in the study.

Families were recruited by the PARIS study at specialized clinical centers in seven countries (France, Sweden, Norway, Italy, Austria, Belgium, and the United States). All patients fulfilled the DSM-IV criteria for autistic disorder and the Autism Diagnostic Interview-Revised (ADI-R) algorithm for ICD-10 childhood autism.[30] Subjects were included after a complete clinical and neuropsychological evaluation described previously.[4] Patients diagnosed with associated organic conditions or other established chromosomal disorders were excluded. Among the 50 sib-pairs, 19 were from Sweden, 15 from France, 6 from Norway, 4 from the United States, 3 from Italy, 2 from Austria, and 1 from Belgium. Among the trios, there were 85 from France, 14 from the United States, 10 from Norway, and 8 from Sweden. All the sib-pairs were Caucasian; among the trios, 107 were Caucasian, 4 Caucasian/African Caribbean, 2 Caucasian/African, 2 Asian, 1 African, and 1 Caucasian/Asian. There were 161 males and 57 females (male:female ratio of 2.8:1). Written informed consent was obtained from all families participating in the study. The study was approved by the ethical committees of the collaborating institutions.

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes or lymphoblastoid cell lines. The 5'UTR polymorphism of the reelin gene (GenBank Accession N° U79716) was amplified by PCR using primers flanking exon 1 (forward: 5'-CGCCTTCTTCTCGCCTTCTC-3' and reverse: 5'-CGAAAAGCGGGGGTAATAGC-3') in a 20 µL volume containing approximately 50 ng of genomic DNA and thermostable Taq Polymerase (AmpliTaq, Perkin Elmer Cetus, Rockville, MD, USA). The forward primer was labeled with IRD800 (MWG Biotech, Germany). PCR products were visualized by 8% polyacrylamide gel electrophoresis, using a LICOR 4000L sequencer. Genotyping was carried out blind to the clinical status, was performed in duplicate and read by two independent investigators.

Statistical analysis

Results were analyzed using the ETDT program,[24] which calculates an allele-wise statistic, which assumes an allele-specific effect on transmission distortion, and a genotype-wise statistic which allows for an independent effect of each parental genotype. TDT analyses were performed only in families were both parents were genotyped. In addition, we compared the allelic frequencies between our sample and that reported previously[9] using a Pearson chi-squared test. When the expected values were less than 5, exact P values were computed with the StatXact software.

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Table 1 Distribution of genotype and allele frequencies of the RELN gene 5'UTR triplet repeat polymorphism in autistic patients and their parents

	Patients (n=218)		*Parents (n=436)*	
Genotypes				
4-8	1	(0.5%)	1	(0.2%)
4-10	1	(0.5%)	1	(0.2%)
5-8	1	(0.5%)	0	
5-10	0		1	(0.2%)
7-8	0		1	(0.2%)
7-10	1	(0.5%)	0	
8-8	36	(16.5%)	68	(15.6%)
8-10	84	(38.5%)	192	(44.0%)
8-11	2	(0.9%)	3	(0.7%)
8-12	1	(0.5%)	3	(0.7%)
8-13	8	(3.7%)	15	(3.4%)
8-14	1	(0.5%)	2	(0.5%)
8-16	1	(0.5%)	2	(0.5%)
10-10	70	(32.1%)	127	(29.1%)
10-11	2	(0.9%)	2	(0.5%)
10-12	1	(0.5%)	3	(0.7%)
10-13	8	(3.7%)	13	(3%)
10-14	0	–	1	(0.2%)
13-13	0	–	1	(0.2%)
Alleles				
4	2	(0.5%)	3	(0.3%)
5	1	(0.2%)	1	(0.1%)
7	1	(0.2%)	1	(0.1%)
8	171	(39.2%)	355	(40.7%)
10	237	(54.4%)	467	(53.6%)
11	4	(0.9%)	5	(0.6%)
12	2	(0.5%)	6	(0.7%)
13	16	(3.7%)	29	(3.3%)
14	1	(0.2%)	3	(0.3%)
16	1	(0.2%)	2	(0.23%)

Table 2 TDT of the RELN gene 5'UTR (GGC)n variant in autistic disorder

Alleles	*Trios*		*Sib pairs*		*Total sample*		χ^2	*P*
	T	*NT*	*T*	*NT*	*T*	*NT*		
4	2	0	0	0	2	0		
5	1	0	0	0	1	0		
7	1	0	0	0	1	0		
8	54	67	49	49	103	116	0.77	0.38
10	63	58	47	45	110	103	0.23	0.63
11	2	1	2	0	4	1		
12	1	3	1	1	2	4		
13	11	7	4	6	15	13	0.14	0.71
14	1	0	0	2	1	2		
16	0	0	1	1	1	1		

T = transmitted, NT = not transmitted. Total sample; allele-wise TDT, $c^2 = 8.99$, df =9, p= 0.44; genotype-wise TDT, $c^2 = 20.15$, df = 14, p = 0.13. In the last two columns are indicated the c^2 and p values for the transmission of individual alleles in the total sample.