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Alleles of a Reelin CGG Repeat do not Convey Liability to Autism in a Sample From the CPEA Network

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A recent study by Persico et al. [2001: Mol Psychiatry 6:150–159] suggests alleles of a CGG polymorphism, just 5′ of the reelin gene (*RELN*) initiator codon, confer liability for autism, especially alleles bearing 11 or more CGG repeats (long alleles). The association is consistent across both a case-control and family-based sample. We attempted to replicate their finding using a larger, independent family-based sample from the NIH Collaborative Programs of Excellence in Autism (CPEA) Network. In our data, allele transmissions to individuals with autism versus unaffected individuals are unbiased, both when alleles are classified by repeat length and when they are classified into long/short categories. Because of the apparent linkage of autism to chromosome 7q, particularly related to the development of language, we also evaluate the relationship between Reelin alleles and the age at which autism subjects use their first word or first phrase. Neither is significantly associated with Reelin alleles. Our results are not consistent with a major role for Reelin alleles in liability to autism.

KEY WORDS: autism; Reelin; language development; genetic association; autistic disorder

INTRODUCTION

Liability to autism, a neurodevelopmental disorder manifesting early in childhood, appears to be largely generated by genetic variation [Bailey et al., 1995]. Because the exact mechanisms of the etiology of autism are obscure, the hunt for genetic variants causing liability is ongoing. Once some of these variants are identified, our hope is that the findings will lead to a deeper understanding of the neurological mechanisms underlying the disorder.

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As so aptly described by Risch [2000], the observation that a disease has a genetic basis, even a substantial one, does not necessarily mean those genetic variants will be easy to find. In fact, the evidence for autism suggests that its genetic basis may be subtle indeed [Risch et al., 1999], and more recent evidence suggests that it may involve epigenetic components [Yu et al., 2002]. If the latter proves to be true, it will change how we search for genetic variants affecting liability to autism.

In the meantime, the search for genetic components proceeds along two fronts, linkage and association analyses. Linkage findings point to areas of chromosomes 2, 7, 13, and 16 [Buxbaum et al., 2001; Collaborative Linkage Study of Autism, 2001; IMGSAC, 2001a; Liu et al., 2001a; Auranen et al., 2002], but the results are not definitive. Linkage only identifies large regions of chromosomes, and in the case of the diffuse findings from autism families, sifting through these regions to find true liability mutations is likely to be onerous.

A shortcut method is association analysis of candidate genes, which are candidates on the basis of biological reasoning. Ideally there would also be evidence for linkage in the vicinity of the biological candidate. The biological and linkage evidence need not coincide, however, if the variants in the gene have relatively small effect on liability, because then linkage analysis will have low power without an exceptionally large number of families with a homogeneous etiology [Risch and Merikangas, 1996; Risch, 2000]. On the other hand, if the variants in the targeted candidate genes have no impact on disease liability, then association analysis has no power except in the rarest of cases: by pure chance, a "liability gene" lies adjacent to a candidate.

Recently Persico et al. [2001] put forth the gene encoding reelin (*RELN*) as a plausible candidate gene. Several lines of evidence support this hypothesis: reelin plays a crucial role in migration and development of various neural connections [D'Arcangelo et al., 1995], and abnormal neural connections are thought to play a role in the manifestation of autistic symptoms [Bauman and Kemper, 1994; Bailey et al., 1998; Minshew et al., 1999; Aylward et al., 2002; Luna et al., 2002]. Some of the anomalies seen in the strains of reelin-deficient mice are also seen in the brains of individuals with autism; RELN is located in 7q22, near some substantial linkage peaks [Ashley-Koch et al., 1999; Barrett et al., 1999; Risch et al., 1999; IMGSAC, 2001b; Alarcon et al., 2002].

Also, as reported by Fatemi et al. [2002], plasma reelin levels are reduced in autism subjects, their parents, and normal sibs. The same group reported reduced reelin levels in cerebellar tissue from five individuals with autism relative to levels in controls [Fatemi et al., 2001b]. This latter finding may not be specific to autism as reduced reelin protein levels have also been reported in cerebellar and other brain regions in schizophrenia, bipolar disorder, and major depression [Impagnatiello et al., 1998; Fatemi et al., 2000, 2001a; Guidotti et al., 2000]. Finally, in humans, null mutations in RELN cause an autosomal recessive form of lissencephaly, a severe brain developmental disorder. The phenotype includes severe developmental delay, an abnormal cerebral cortex resulting from impaired neuronal migration and cerebellar hypoplasia [Hong et al., 2000]. The pattern of brain development in these subjects mirrors that seen in reeler mice.

Persico et al. [2001] molecular results suggest that a CGG repeat polymorphism, just 5′ of the RELN initiator codon, does confer liability for autism. They find an association, especially for a long/short dichotomy of repeat length, in both their family-based and case-control samples. Their findings from the family-based sample are important because they cannot be due to the effects of population substructure [Spielman et al., 1993], a potential confounder for population-based studies [Devlin et al., 2001a,b]. In addition, it is plausible that the CGG repeat polymorphism could have an impact on RELN expression, potentially accounting for the reduced levels of reelin in plasma and brain from autism subjects. For these reasons, we evaluate the hypothesis that RELN CGG alleles affect liability to autism using data from the Collaborative Programs of Excellence in Autism (CPEA) Network. We also evaluate whether RELN alleles are associated with rudimentary language phenotypes (age at first word and age at first phrase) on the basis of recent findings that language phenotypes yield better linkage signals to chromosome 7 in autism families [Bradford et al., 2001; Alarcon et al., 2002].

MATERIALS AND METHODS

Simplex and multiplex autism families were recruited from ongoing research projects at six NIH CPEA Network sites: University of California Irvine, University of Pittsburgh, University of Rochester, University of Utah, University of Washington, and Yale University. Three hundred ninety-five families were genotyped. Of these, approximately 52.5% were simplex families and the rest were multiplex families. Of the multiplex families, most contained an affected sibling pair (88%), and a few were multigenerational (3.6%); nonetheless, linkage/association information was almost always confined to nuclear families. Diagnoses were based on the Autism Diagnostic Interview-Revised (ADI-R) [Lord et al., 1994], the Autism Diagnostic Observation Schedule-Generic [Lord et al., 2000], DSM-IV, and clinical evaluation to rule out known medical causes of autism. For most families, DNA was available from both parents, and 87.8% of all parents were genotyped. The ancestry of most of the families was European (88%).

Data for age at first word and phrase were also obtained through the ADI-R interview. Ages were recorded in months. The ADI-R also has several special codes for these variables. For children reported to have language regression (993), we recorded the data as missing (also 997 [not known but delayed] and 999 [not known]). For the few 996, "not known but apparently normal," we took the value to be 18 months. For milestone not reached, we set the value to 60 months, recognizing that this value was likely to be an underestimate. Larger values had no impact on the conclusions.

RELN CGG genotypes were determined essentially as described by Persico et al. [2001]. Because genotyping was performed at four different sites (Rochester, Utah, Washington, Yale), a panel of 20 control samples

obtained from the Coriell Cell Repository was genotyped at each site and the results compared to insure that genotyping methods across sites produced identical results.

For our analyzes, two groups of affected individuals were considered. The first group included all individuals diagnosed with autism, pervasive developmental disorder—not otherwise specified (PDD-NOS), and Asperger's syndrome (broad diagnosis). The second group included only individuals with autism (narrow diagnosis). Following Persico et al. [2001], in some situations we dichotomize alleles into short/long on the basis of whether the allele was less than 11 repeats or not (biallelic analysis).

Markers and pedigrees were evaluated for Mendelian errors using the PedCheck program [O'Connell and Weeks, 1998]. Only 3 genotyping errors were noted from over 1600 genotypes, and these were set to missing. To test for differential transmission of alleles, a generalization [Rabinowitz and Laird, 2000] of the TDT test [Spielman et al., 1993], as implemented in the program FBAT (http://www.biostat.harvard.edu/~fbat/) was used. To analyze linkage/association, we chose an additive model, which is often powerful even when the true model deviates from additivity. Finally, the data were analyzed for both broad and narrow diagnosis. All analyzes ignored the possibility of linkage in the region surrounding RELN; the tests would be slightly anticonservative if this assumption were false.

To evaluate the power of the sample under both the broad and narrow diagnostic schemes, we use the program PBAT [Lange and Laird, 2002a,b]. For input, PBAT requires the family structures present in the sample, in terms of the number of genotyped affected and unaffected offspring and the number of genotyped parents. It also requires specification of the genetic model. For these calculations, we evaluate pure additive, dominant, and recessive models; assume autism prevalence is $K = 0.005$ and the frequency of the disease allele equals the observed frequency of long alleles; and perform the calculations for a range of liability attributable to the RELN CGG repeat (Lange and Laird's attributable fraction AF, namely $AF = 1 - f_0/K$, in which f_0 is the probability of the disease when the individual carries no risk alleles at the locus of interest).

RESULTS

To test the hypothesis that alleles of the RELN CGG repeat are involved in liability to autism [Persico et al., 2001], we genotyped a sample of 202 simplex and 183 multiplex families. In all 852 progeny were genotyped: 35% diagnosed with autism, 12% with PDD-NOS, 4% with Asperger's syndrome, 35% were unaffected, and the remainder did not receive a diagnosis.

The allele distribution from our sample of pedigree founders, mostly parents of children with autism, showed a range of repeat sizes from 4 to 15, with two common alleles of 8 and 10 repeats (Table I). Founder genotypes conformed to Hardy–Weinberg expectations (Table II).

For the broad diagnostic category and 395 families, no significant deviation from the null hypothesis of Mendelian transmissions was seen in our data (multiallelic: $\chi^2 = 1.35$, DF = 4, $P = 0.85$; biallelic: $z = 0.29$, $P = 0.78$). Similar results were obtained for the narrow diagnosis of autism and 341 pedigrees (multiallelic: $\chi^2 = 3.89$, DF = 4, $P = 0.42$; biallelic: $z = -0.62$, $P = 0.54$). Likewise, the conclusions were unchanged if the data are restricted to families of known European ancestry, which limits the sample to 296 families under the broad diagnosis (multiallelic: $\chi^2 = 2.99$, DF = 4, $P = 0.56$; biallelic: $z = -0.35$, $P = 0.73$).

TABLE I. Allele Distribution From the Sample of Pedigree Founders

Repeat number	Relative frequency
4	0.00064
8	0.42850
9	0.00322
10	0.50559
11	0.00516
12	0.01375
13	0.03674
14	0.00382
15	0.00257

The CGG alleles in the 5′ region of RELN also were not associated with two measures of language development, namely age at first word ($z = 0.833$, $P = 0.40$) and age at first phrase ($z = 0.067$, $P = 0.94$). For this analysis, we used the biallelic categorization of the CGG alleles and allowed FBAT to choose an offset.

We also assess whether our sample is large enough to detect subtle effects of long alleles on liability to autism. To evaluate power, we assume the long alleles occur with probability 0.062 in the general population (Table I), and use the family structure for the sample (see "Materials and Methods"). By using PBAT to analyze the power for the sample under the broad or narrow diagnostic categorizations, and by examining a range of liability attributable to the RELN CGG repeat (using the short/long dichotomy), we find greater than 80% power to detect an association of long alleles for additive, dominant, and recessive models and attributable fraction AR as small as 0.075. Power is largest for the recessive model (essentially 100%) and smallest for the dominant model (close to 80%). To put $AF = 0.075$ in perhaps more familiar terms, the genotype relative risk GRR attributable to the RELN CGG repeat is 1.656, for $GRR = f_1/f_0$ and f_1 equal the penetrance of the genotype with one long allele. These calculations assume the sample is representative of the population and that genotyping and diagnostic errors are absent.

DISCUSSION

Recent evidence, reported in Persico et al. [2001], suggests certain alleles of the *RELN* gene on 7q22 confer liability to autism. The findings from the Persico study involve analyzes of both family- and population-based samples. Using a larger, CPEA family-based sample, we attempt to replicate these findings. Unfortunately, there is no significant association/linkage between the RELN alleles and autism. In the original Persico study,

TABLE II. Observed (Expected) Genotype Distribution in the Top Triangular Portion (Column Label ≥ Row Label) of the Table, and Allele Transmission in the Bottom Portion of the Table (Below the Diagonal)

	4	8	9	10	11	12	13	14	15
4	0 (0)	1 (0.4)	0 (0)	0 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
8	1:0	138 (134.8)	4 (2.0)	317 (318.0)	3 (3.2)	5 (8.6)	25 (23.1)	0 (2.4)	2 (1.6)
9	0:0	2:2	0 (0)	1 (2.4)	0 (0)	0 (0.1)	0 (0.2)	0 (0)	0 (0)
10	0:0	182:197	0:0	187 (187.6)	3 (3.8)	13 (10.2)	27 (27.3)	3 (2.8)	1 (1.9)
11	0:0	0:2	0:0	1:2	0 (0)	0 (0.1)	0 (0.3)	0 (0)	0 (0)
12	0:0	2:1	0:0	8:10	0:0	1 (0.1)	0 (0.7)	0 (0)	0 (0)
13	0:0	13:18	0:0	21:14	0:0	0:0	1 (1.0)	0 (0.2)	1 (0.1)
14	0:0	0:0	0:0	0:3	0:0	0:0	0:0	0 (0)	0 (0)
15	0:0	1:2	0:0	1:1	0:0	0:0	0:0	0:0	0 (0)

For the allele transmissions, read the cell X:Y as X transmissions of the allele on the row label versus Y transmissions of the allele on the column label. We use the broad diagnosis when counting allele transmissions to affected individuals.

long alleles, defined to be alleles of at least 11 repeat units, appear to confer greater liability. Not only is there no significant linkage/association in our sample, in general, we observe under-transmission of long alleles (Table II). In fact, in the competition between short and long alleles, 53 short and 47 long are transmitted. In addition, we could find no association between allele transmission and two rudimentary measures of language development, namely age at first word and age at first phrase.

The family-based sample from the Persico study is largely of European ancestry, consisting of 176 families; the majority of those families are simplex, consisting of parents and a single affected offspring. Our sample of 296 families is notably larger, and about half of those families are multiplex (two or more affected siblings).

Our findings agree with those recently published by Krebs et al. [2002], who analyzed a sample of 117 simplex and 50 multiplex families. The estimated allele distribution from our families is quite similar to that estimated by both the Persico and Krebs studies, showing two common alleles with 8 and 10 CGG repeats, a few uncommon alleles (12 and 13 CGG repeats) and a smattering of rare alleles (4, 9, 11, 14, and 15 repeats). The samples differ slightly in the realization of rare alleles, but that is to be expected. In contrast, Zhang et al. [2002], in a family-based association study of 126 multiplex families, did find that larger RELN alleles ($\geq$11 repeats) were preferentially transmitted to affected children. This study also reported that subjects with delayed speech tended to have one or more of the larger alleles ($\leq$11 repeats). In this work, the allele distribution was similar to that reported here and by the Persico and Kreb studies.

Because our sample is substantially larger than the Persico's study, even when the sample is restricted to families of known European ancestry, we would expect our power to larger as well, at least on the basis of sample size alone. Of course there could be subtle differences between the Persico's study and our own, owing to the complexity of autism etiology, and to different inclusionary and exclusionary criteria of other conditions and features by each of the sites. If that were the case, RELN might play a small role in autism liability but its effects could be absent from our sample. Nonetheless, ours results and that of Krebs et al. [2002] suggest that the CGG alleles in the 5′ region of RELN cannot play a major role in autism liability. While our analysis does not support RELN as a susceptibility locus for autism, this gene may still be important in autism etiology. For example, epigenetic regulation of RELN may be important [Chen et al., 2002]. The hypothesis that down-regulation of RELN could contribute to developmental problems is supported by the fact that mice hemizygous-null for the mouse reelin gene have a phenotype [Liu et al., 2001b].

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