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ORIGINAL RESEARCH ARTICLE

Association between a *GABRB3* polymorphism and autism

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Autistic disorder (OMIM 209850) is a disease with a significant genetic component of a complex nature.[1] Cytogenetic abnormalities in the Prader-Willi/Angelman syndrome critical region (15q11–13) have been described in several individuals with autism.[1] For this reason, markers across this region have been screened for evidence of linkage and association, and a marker (155CA-2) in the γ-aminobutyric acid type-A receptor β3 subunit gene (*GABRB3*) has been associated in one study[2] but not others.[3–5] We completed an association analysis with 155CA-2 using the transmission disequilibrium test (TDT) in a set of 80 autism families (59 multiplex and 21 trios). We also used four additional markers (69CA, 155CA-1, 85CA, and A55CA-1) localized within 150 kb of 155CA-2. The use of multi-allelic TDT (MTDT) ($P < 0.002$), as well as the TDT ($P < 0.004$), demonstrated an association between autistic disorder and 155CA-2 in these families. Meiotic segregation distortion could be excluded as a possible cause for these results since no disequilibrium was observed in unaffected siblings. These findings support a role for genetic variants within the GABA receptor gene complex in 15q11–13 in autistic disorder.
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Autism is a development disorder characterized by impairments in three domains: communication, reciprocal social interactions, and repetitive or stereotyped behaviors and interests (for review see Tager-Flusberg *et al*[1]). The concordance rate for monozygotic twins is much higher than that of dizygotic twins, which indicates that genetic factors play an important role in the etiology of autism. In addition, family studies indicate that the recurrence risk to siblings, estimated from multiple studies at 1–3%, is profoundly higher than the risk to the general population, which has been estimated at ~0.5–2 per 1000. The mode of inheritance of autism appears to be complex; latent-class analyses suggest that 3–10 genes may underlie the disorder,[6] although an interpretation of at least one genome-wide linkage analysis has argued for >10 genes underlying the disorder.[7]

Cytogenetic studies have demonstrated that duplications within the 15q11–q13 region can be associated with autism (for review see Tager-Flusberg *et al*[1]). Moreover, symptoms of autism can be associated with both Prader–Willi and Angelman syndromes, both of which involve alterations in the 15q11–q13 region. Because of this, the15q11–q13 region has been examined for genetic linkage to autism. A mapping of nine markers spanning a region of ~2 Mb within this region in 132 families demonstrated linkage disequilibrium at a marker within the *GABRB3* gene, 155CA-2.[2] An additional locus within *GABRB3* that is ~150 kb away from 155CA-2 (D15S97) did not demonstrate linkage disequilibrium.

A genome-wide scan involving 51 autistic multiplex families demonstrated a broad peak with a LOD score of about 1 over the *GABRB3* region.[8] Similarly, multipoint analyses over the region in 63 families demonstrated a peak Z-score of 1.78 in the region, near the marker D15S217.[9] The same group did not observe linkage disequilibrium with 155CA-2 in 123 families but found suggestive association with an additional marker (*GABRB3*) in the region.[5] In contrast, two other genome-wide scans did not observe evidence for linkage in this region,[7,10] nor was there evidence for linkage disequilibrium using 155CA-2 or other nearby markers in these samples (comprised of 94[3] and 139[4] families) using the TDT.[3,4]

We have examined the marker 155CA-2 within the *GABRB3* gene for association in a cohort of 59 families with two or more individuals affected with either autism, borderline autism, or Asperger syndrome, and an additional 21 families with one autistic proband (see Methods). In the sample comprised of 59 multiplex autism families, an association was found between autism and the *GABRB3* marker 155CA-2 (MTDT $\chi^2 = 26.36$, 9 df, $P = 0.0018$) (Tables 1 and 2) (we analyzed the multiplex families separately in case this subgroup has increased genetic susceptibility). Similarly, in the sample comprised of the 59 multiplex autism families and 21 singleton autism families, an

Table 1 Transmission data to affecteds for *GABRB3* 155CA-2

Allele (bp)	*Multiplex families only*				*Singleton and multiplex families*			
	Transmitted	*Non-transmitted*	χ^2	*P*	*Transmitted*	*Non-transmitted*	χ^2	*P*
85	0	1			0	1		
87	14	34	8.33	0.0039*	17	41	9.93	0.0016*
89	16	4	7.20	0.0073	17	5	6.55	0.0105
93					0	1		
95	5	5	0.00	1.0000	6	5	0.09	0.7629
97	7	3	1.60	0.2059	7	3	1.60	0.2059
99	16	9	1.96	0.1615	21	12	2.46	0.1172
101	9	12	0.43	0.5125	11	12	0.04	0.8357
103	15	15	0.00	1.0000	20	18	0.11	0.7459
105	3	2	0.20	0.6547	4	2	0.67	0.4141
107	6	1	3.57	0.0588	6	1	3.57	0.0588
109	0	2			0	4		
111	0	3			0	4		

*Significant at $P < 0.05$, after correction for multiple testing. The critical *P* value (defined as 0.05/number of alleles with total counts of five or more) with nine tests was 0.0056.

Table 2 MTDT between autism and markers in 15q11–q13

Marker	*Distance to next marker*	*Multiplex families only*			*Singleton and multiplex families*		
		χ^2	*df*	*P*	χ^2	*df*	*P*
69CA	125 kb	0.64	2	0.7248	0.58	2	1.000
155CA-1	3 kb	10.57[a]	6	0.1027	9.46[b]	7	0.2214
155CA-2	60 kb	26.36[c]	9	0.0018*	27.67[d]	9	0.0011*
85CA	45 kb	14.06	9	0.1201	13.39	9	0.1459
A55CA-1		8.52	10	0.5780	8.57	11	0.6616

[a]The 80-, 82-, 92-, and 96-bp alleles were pooled (see Table 3).
[b]The 82- and 92-bp alleles were pooled, as were the 74-, 78-, 80-, 96- and 98-bp alleles (see Table 3).
[c]The 85-, 109-, and 111-bp alleles were pooled (see Table 1).
[d]The 85-, 93- and 111-bp alleles were pooled, as were the 105- and 109-bp alleles (see Table 1).
*Significant at $P < 0.05$, after correction for multiple testing. The critical *P* value with five tests was 0.01.

association was also observed (MTDT $\chi^2 = 27.67$; 9 df, $P = 0.0011$) (Tables 1 and 2). Because siblings not classified as affected were genotyped where available, transmission to these siblings could be determined. In both samples the MTDT results for these siblings were as expected by chance and therefore were not consistent with meiotic segregation distortion (data not shown). As an example, the results for transmission of marker 155CA-2 to all genotyped siblings not classified as affected ($n = 55$) were MTDT $\chi^2 = 10.71$, 9 df, $P = 0.30$. Studies with four additional markers both proximal and distal to 155CA-2 did not demonstrate significant association by the MTDT (Table 2). However, evidence suggestive of association was observed for 155CA-1 using the TDT (Table 3). Markers 155CA-2, 155CA-1 and 69CA lie in a single large intron between exons 3 and 4 of the *GABRB3* gene, with 155CA-1 at a distance of 7 kb from 155CA-2 (markers 85CA and A55CA-1 are 5′ to the *GABRB3* gene near the *GABRA5* gene).[11] Stratification of the data based on maternal *vs* paternal transmission did not provide evidence for a parent-of-origin effect (data not shown). Nonparametric multipoint linkage analyses in multiplex families did not provide evidence for linkage (peak Z-scores of 0.9 over marker 85CA) (data not shown). Furthermore, parametric analysis under dominant and recessive modes of inheritance provided evidence against linkage across this region (LOD scores < –6) (data not shown).

Autism appears to have a significant genetic component involving several or many genes of weak effect. Traditional genome-wide linkage analysis at a density of 5–20 cM may not be the method of choice for identifying such genes. In contrast, family-based association studies such as the TDT—using candidate gene polymorphism or genome-wide, dense polymorphisms—may have greater power to identify such genes. In the current study, we have followed up on previous linkage and association studies to determine whether genes in 15q11–q13 might contribute to autism. In our study

Table 3 Transmission data to affecteds for *GABRB3* 155CA-1

Allele (bp)	*Multiplex families only*				*Singleton and multiplex families*			
	Transmitted	*Non-transmitted*	χ^2	*P*	*Transmitted*	*Non-transmitted*	χ^2	*P*
74					1	0		
76	15	3	8.00	0.0047*	17	4	8.05	0.0046*
78					0	1		
80	1	1			1	1		
82	1	2			1	2		
84	5	6	0.09	0.7629	7	6	0.08	0.7814
86	23	26	0.18	0.6680	25	33	1.10	0.2936
88	17	20	0.24	0.6220	21	22	0.02	0.8795
90	6	7	0.08	0.7814	7	9	0.25	0.6171
92	1	3			1	3		
94	18	20	0.11	0.7459	21	21	0.00	1.0000
96	1	0			1	0		
98					0	1		

*Significant at $P < 0.05$, after correction for multiple testing. The critical *P* value with six tests was 0.0083.

sample, we observed an association for *GABRB3* 155CA-2 with autism.

The positive results of the current study and those of Cook *et al*,[2] contrast with three other studies[3–5] and might be false positive results. We would like to note, however, that the report of Cook *et al* made use of the MTDT, while the three follow-up studies used somewhat different methodologies, relying either on the TDT,[3,4] or on the T_{sp}.[5] The MTDT with highly polymorphic markers such as 155CA-2 can have increased power as compared to allele-wise TDT if multiple alleles are in transmission disequilibrium because the χ^2 values are summed. It also must be considered that in a complex disorder involving multiple genes of weak effect and potential locus heterogeneity, association with a particular polymorphism may be true in one sample while not found in another. With linkage analysis in autism there is little concordance of results, which may be in part due to the same causes.

When one considers transmissions and analyzes the previously published data using the same MTDT method as used in the original report,[2] one obtains individual study MTDT χ^2, df, and *P* values as summarized in Table 4. As can be seen, the MTDT results for the three prior studies where it can be determined are either consistent with a role for a gene in 15q in autism,[2] or do not represent compelling evidence against such a role.[3,4] For the most recent study,[5] only a *P*-value ($P = 0.71$) using a relatively novel analytical method was reported, so the MTDT could not be calculated.

Note that in the current study, we made use of one affected per family, which allows for the evaluation of 'linkage with linkage disequilibrium' and is a valid test for association[12,13] (analyzing all affecteds in all families, we observed an MTDT χ^2 of 25.0; 10 df, $P = 0.0053$). In prior studies, 155CA-2 was examined using more than one affected per family in all studies except one (where transmission to one and to all affecteds was reported[3]). Although a valid test of 'linkage' (in the sense of co-segregation of alleles), the inclusion of multiple affected individuals per family yields results which are not a rigorous test of association.[12,13] For a meta-analysis of the evidence for linkage in all previous studies, one can make use of the T_{sp}[5] or derived or reported MTDT (Table 4) *P*-values (using the *P*-value derived for all affecteds from the Maestrini *et al* report). This yields a meta-analysis *P* value of 0.0024 when examining all prior studies. It would be interesting to examine transmission of individual alleles in the study of Martin *et al* and to determine the MTDT in that sample. However, the meta-analyses of all studies (which included the *P* value of Martin *et al*) support linkage of 155CA-2 with autism. Only the current study and the study of Maestrini *et al* report transmission data to one affected and can therefore be considered rigorous tests for association. The MTDT results for these two studies (*P* of 0.0011 and 0.13, respectively) would together suggest that association of 155CA-2 with autism warrants study in further samples. To convincingly demonstrate that a polymorphism in the *GABRB3* region increases risk for autism, the variant(s) that confers risk will need to be identified (ideally with a demonstrated functional change) and further association studies carried out.

In the current study, the 87-bp allele of 155CA-2 showed significant transmission disequilibrium after correction for multiple testing. This allele was preferentially not transmitted to affected individuals. This same allele shows reduced transmission in the previous positive study,[2] and it also shows reduced transmission in childhood absence epilepsy (referred to as allele 95 in that study).[14] The apparent decrease in transmission of the 87-bp allele could reflect one of two things. First, the allele might actually be protective, as has been suggested for HLA-DQ or HLA-DR in IDDM[15] and Apo E2 in Alzheimer's disease.[16,17] In both these examples, molecular models based on the changes in

Table 4 Re-analysis with MTDT of published results for *GABRB3* 155CA-2

Study	*Number of families (Multiplex/singleton)*	*MTDT*	*df*	*P*
Cook *et al*, 1998[2] (Transmissions to all affecteds)	132 (6/126)	28.63	10	0.0014
Maestrini *et al*, 1999[3] (Transmissions to all affecteds)	94 (86/8)	17.21[a]	10[a]	0.070[a]
Maestrini *et al*, 1999[3] (Transmissions to a single affected)	94 (86/8)	13.79[b]	9[b]	0.13[b]
Salmon *et al*, 1999[4] (Transmissions to all affecteds)	139 (139/0)	17.57[c]	11[c]	0.092[c]
Current study (all families) (Transmissions to a single affected)	80	27.67	9	0.0011

[a]Derived from Maestrini *et al*, 1999,[3] Table II, pooling alleles 3 and 11.
[b]Derived from Maestrini *et al*, 1999,[3] Table II, pooling alleles 3, 10 and 11.
[c]From Salmon *et al*, 1999,[4] Table II. Note that there is an error in the reported transmissions for the 114-bp allele due to a typographical error; the reported χ^2 value for this allele is correct (J Hallmayer, personal communication).

protein have been proposed to account for protective effects. Alternatively, if there are minor alleles that confer risk, there will be decreased transmission of other alleles to affected individuals, a phenomenon that will be easiest to detect in common alleles. In this case, there would be no protective function for the 87-bp allele. Note that in contrast to the previous positive study[2] the 103-bp allele did not show excess transmission in the current study.

Rates of anxiety disorder and social phobia have been reported to be higher in first-degree relatives of autistic probands.[18,19] Therefore, given its role in anxiety, the GABAA receptor might be considered a candidate gene for autism. The GABAA receptor also appears to play a role in several behaviors associated with autism; mice with a targeted deletion of the murine *garb3* gene are hyper-responsive to human contact, fail to nurture offspring, are hyperactive, often run in tight circles, and have occasional epilepsy.[20–22] It should be noted, however, that several recent studies have demonstrated that linkage disequilibrium varies dramatically between pairs of markers, with less than 50% of markers at distances <5 kb displaying significant disequilibrium, but disequilibrium occasionally detectable at 500 kb.[23,24] For this reason, the current data are consistent with the presence of a susceptibility allele in the 15q11–q13 region, but not specifically for an allele within the *GABRB3* gene.

The lack of evidence for linkage using a nonparametric LOD score based approach and the significant evidence against linkage using parametric analysis contrasts with the evidence for association with the TDT. This situation is similar to that of the HLA locus and autoimmune thyroid disease.[25] Such results imply that while a genetic alteration in 15q11–13 may increase susceptibility to autism (as shown by the existence of the 155CA-2 association), the major genetic influence on the inheritance of autism must be at another locus.[25,26]

In conclusion, we have observed association with a marker in *GABRB3* in our families. Further sequencing within the region around 155CA-2 may identify genetic variants associated with autism in certain individuals.

Methods

Subjects

Opportunistic recruitment of multiple incidence families for genetic studies of autism was initiated in 1994 at the Seaver Autism Research Center (SARC) at the Mount Sinai School of Medicine. Many of the families were ascertained in collaboration with the Autism Research Genetic Exchange (AGRE) and reviewed by Mount Sinai School of Medicine (see Geschwind *et al*[27] for a description of the AGRE sample and the AGRE consortium). With parents providing written informed consent, families were assessed if they had at least two members affected with a clinical diagnosis of autism, pervasive development disorder, or Asperger syndrome. All potentially affected individuals were assessed using the Autism Diagnostic Interview-Revised (ADI-R)[28] to determine a research diagnosis of autism or borderline autism. The ADI-R is a standardized, investigator-based, semi-structured instrument that employs a diagnostic algorithm based on ICD-10 criteria. To be considered affected with autism for this study, an individual had to satisfy the pre-specified cut-off scores in all three symptom areas of the ADI-R (social impairment, language and communication impairment, unusual and restricted interests) and present with evidence for an onset prior to 36 months of age. A research diagnosis of Asperger syndrome was given to those without autism, but who met criteria for Asperger syndrome according to DSM-IV. Individuals who failed to meet the ADI-R algorithm criteria for autism by no more than one point in the social domain and either the communication or repetitive behavior domain (but not both) that did not meet cri-

teria for Asperger syndrome were given a research diagnosis of borderline autism. A diagnosis of borderline autism was also given to individuals in which all three domains were above threshold but the onset criterion for autism was not met. Dr Catherine Lord has previously called this definition of borderline autism, 'not-quite-autism' (personal communication). Families were excluded from the study if there was not at least one affected individual with a research diagnosis of autism.

The families include 62 families recruited with AGRE and 18 recruited directly by SARC. Families recruited directly by SARC were assessed by CJS or by a rater trained by CJS. CJS has had extensive training with Dr Lord at the University of Chicago, and has been credentialed as an off-site trainer and as a 'reliable rater' against whom another's reliability can be assessed. Raters trained by either Dr Lord or CJS conducted assessments of families recruited by AGRE. All raters met reliability requirements established by Drs Lord and Rutter. Of the families included in this study, 53 had two or more individuals with a research diagnosis of autism, four had one with autism and one with borderline autism, and two had one with autism and one with Asperger syndrome. Of the remaining 21 families, 15 were singleton autism families (although eight out of these families had an additional individual with a clinical diagnosis of a pervasive developmental disorder), and six had identical twins with a research diagnosis of autism. The ratio of male to female affecteds in the study was 111:30 for autism and borderline autism. The ethnicity of the families was 91% Caucasian, 1.25% Black, 1.25% Hispanic, and 6.5% other or unknown, and the age ± SD for the affected individuals was 7.45 ± 3.49. These individuals had ADI scores (mean ± SD) as follows: social: 23.02 ± 5.11; communication, verbal: 16.6 ± 3.76; communication, non-verbal, 12.63 ± 1.57; and, restricted and repetitive behaviors, 6.15 ± 2.28.

Genotype analysis
Blood was collected from affected individuals and their parents; when available, siblings' blood was also collected. Blood was collected by venipuncture into green-top Vacutainer tubes. DNA was extracted using the PureGene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). Genotyping was performed blind to the inclusion status of the probands and to family information.

Microsatellite markers (155CA-1, 69CA, A55CA-1, 155CA-2, 85CA)[11] were amplified by PCR, using an ABI PRISM 877 Integrated Thermal Cycler (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Amplification was carried out using the Perkin Elmer AmpliTaq Gold Polymerase Kit in a final volume of 10 μl, consisting of 50 ng of genomic DNA, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl, 200 μM dNTPs, 0.5 units taq polymerase, and 10 μM sense and antisense primers. Two separate PCR amplification programs were used: C2 and C3. For both programs, the initial activation step for heat activated DNA polymerase was carried out at 95°C for 12 min. For C3, this was followed by 12 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 60 s, then by 23 subsequent cycles in which the denaturation temperature was reduced to 89°C. The final extension was at 72°C for 30 min. For C2, the initial activation step was followed by 12 cycles of 95°C for 15 s, 59°C for 30 s, and 72°C for 60 s, then by 18 subsequent cycles in which the denaturation temperature was reduced to 89°C. The final extension was at 72°C for 30 min. Microsatellite markers 69CA and 155CA-1 were amplified with the C3 program. The remaining three markers were amplified with the C2 program.

The resulting PCR products were then diluted 1–20 (except for 85CA, which was 1–10) with deionized water. An aliquot was injected into an ABI PRISM 310 Genetic Analyzer in a final volume of 17 μl, consisting of 11.5 μl of deionized formamide, 0.5 μl GS500 TAMRA size standard, and 5 μl diluted PCR product. Products were separated by use of Performance Optimized Polymer-4 denaturing polymer and were sized with Genescan 2.1.1 and Genotyper 2.0 software (Perkin-Elmer Applied Biosystems).

Statistical analyses
For TDT analyses, families with a missing parent were included if and only if the child was heterozygous and did not share the same genotype as the genotyped parent.[12] In order to have a rigorous test for association, only one affected individual per family was used in TDT analyses. In the families with two or more individuals with a research diagnosis of autism, a single, randomly selected affected individual was included in TDT analyses. In families where there was only one individual with a research diagnosis of autism (either because the family was singleton, or because the family had autism/borderline autism or autism/Asperger diagnoses), the individual with the definite diagnosis of autism was included in the analyses. Transmissions were determined by the TDT/S-TDT program (http://spielman07.med.upenn.edu/TDT.htm). At each locus, alleles with counts less than five were combined with the least common allele until each group of alleles had a minimum count of five. Data were then analyzed by the MTDT as well as the TDT.[12,13] The primary hypothesis tested was that the marker 155CA-2 was in linkage disequilibrium with autism as determined by the MTDT using the criteria and methods of the original report.[2] Follow-up studies were then carried out for additional markers.

Nonparametric and parametric linkage analysis was carried out using Genehunter.[29] For linkage analysis, all available unaffected and affected (including those with a research diagnosis of borderline autism or Asperger syndrome) individuals were genotyped and included in the analyses. For parametric analyses, penetrance was arbitrarily set to 0.5, and data were analyzed under dominant and recessive models.[30]

Meta-analyses of published results were carried out with a method involving summing $-2 \times \mathrm{LN}(P)$ across n independent studies This sum has a chi-square distri-

bution with $2n$ df. The PVALUES program from Dr Jurg Ott of the Rockefeller University (http://linkage.rockefeller.edu/ott/linkutil.htm) was used to calculate meta-analysis P values.

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