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Linkage-Disequilibrium Mapping of Autistic Disorder, with 15q11-13 Markers

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Summary

Autistic disorder is a complex genetic disease. Because of previous reports of individuals with autistic disorder with duplications of the Prader-Willi/Angelman syndrome critical region, we screened several markers across the 15q11-13 region, for linkage disequilibrium. One hundred forty families, consisting predominantly of a child with autistic disorder and both parents, were studied. Genotyping was performed by use of multiplex PCR and capillary electrophoresis. Two children were identified who had interstitial chromosome 15 duplications and were excluded from further linkage-disequilibrium analysis. Use of the multiallelic transmission-disequilibrium test (MTDT), for nine loci on 15q11-13, revealed linkage disequilibrium between autistic disorder and a marker in the γ-aminobutyric acid$_A$ receptor subunit gene, *GABRB3* 155CA-2 (MTDT 28.63, 10 df, P = .0014). No evidence was found for parent-of-origin effects on allelic transmission. The convergence of *GABRB3* as a positional and functional candidate along with the linkage-disequilibrium data suggests the need for further investigation of the role of *GABRB3* or adjacent genes in autistic disorder.

Introduction

Autistic disorder is a complex genetic disease with evidence of high MZ twin concordance, relative to DZ twin concordance (Folstein and Rutter 1977; Ritvo et al. 1985; Steffenburg et al. 1989; Bailey et al. 1995), and a high relative sibling recurrence risk (reviewed in Smalley 1997). Several genomewide screens are in progress. Although several candidate-gene associations have been reported (Comings et al. 1991; Hérault et al. 1993; Warren et al. 1996), only one has reported the results of a family-based control design (Cook et al. 1997*a*). In this latter study, a haplotype consisting of the short form of a promoter variant and an intron 2 polymorphism in the serotonin transporter gene (*HTT* = *SLC 6A4*) was preferentially transmitted in a U.S. sample. However, this finding would not be significant if type I error was Bonferroni corrected for all loci tested. In addition, a different haplotype at *HTT* recently was found to be preferentially transmitted in a sample of German subjects with autistic disorder (Klauck et al. 1997). It is anticipated that several loci will contribute to autistic disorder susceptibility (Pickles et al. 1995).

Historically, cytogenetic abnormalities have sometimes provided information helpful in the localization of disease-susceptibility loci. In recent years, duplication of the Prader-Willi/Angelman syndrome critical region (15q11-13) has been described in several individuals with autistic disorder (Gillberg et al. 1991; Robinson et al. 1993; Baker et al. 1994; Bundey et al. 1994; Leana-Cox et al. 1994; Schinzel et al. 1994; Crolla et al. 1995; Hotof and Bolton 1995; Flejter et al. 1996; Cook et al. 1997*b*). Because patients seen in our clinic who had 15q11-13 duplications had classic symptoms of autistic disorder, we initiated linkage-disequilibrium studies of this region, in early 1996.

To determine whether the patients with 15q11-13 duplications indicated an autistic disorder–susceptibility locus in the region, linkage-disequilibrium studies of 15q11-13 loci between D15S128 and D15S156 were conducted. Previously reported duplications of 15q11-13 in autistic disorder have been exclusively of maternal origin, and two reports have suggested that duplications of maternal, but not paternal, origin increase the risk for developmental disorders (Browne et al. 1997; Cook et al. 1997*b*). *UBE3A* is expressed exclusively from the

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Table 1

MTDT between Autistic Disorder and 15q11-q13 Markers

Marker	Distance to Next Marker (kb)	Observed Heterozygosity	MTDT χ^2	df	*P* Value
D15S128	300[a,b]	.80	6.72	8	.5667
D15S1506	100[b]	.70	4.52	8	.8074
UBE3A Intron 1	50[b]	.21	.16	1	.6892
D15S122	20[b]	.75	12.10	8	.1466
D15S10	.2	.51	3.31	3	.3458
UBE3A OP2 3A/3B	1,000[b]	.32	.05	1	.8231
D15S97	150[c]	.83	8.59	9	.4763
GABRB3 155CA-2	800[c,d]	.79	28.63	10	.0014
D15S156		.54	3.23	4	.5193

NOTE.—The relationship between genetic and physical distance varies in this region, but this area of ~2.4 Mb has a sex-averaged genetic distance of ~8.1 cM (Robinson and Lalande 1995). Distances are physical estimates based on available physical or genetic mapping data.

[a] From Christian et al. (in press).
[b] From Sutcliffe et al. (1997).
[c] From Glatt et al. (1997).
[d] From Robinson and Lalande (1995).

maternal chromosome (Albrecht et al. 1997), at some neuroanatomical sites reported to be abnormal in autistic disorder (e.g., the hippocampus and cerebellar Purkinje cells) (reviewed in Courchesne 1997). Therefore, several *UBE3A* markers were included. Results are reported for the first 138 consecutive families meeting inclusion criteria of a planned sample of 350 families.

Subjects and Methods

Subjects

Consecutive subjects consenting to participate in a family-based linkage-disequilibrium study of candidate loci for autistic disorder were studied between June 1994 and October 1997 at the University of Chicago Developmental Disorders Clinic and between July 1990 and October 1997 at the Laboratory for Research on the Neuroscience of Autism, Children's Hospital Research Center, La Jolla. The study was approved by both institutional review boards. Inclusion criteria included the following: (1) diagnosis of autistic disorder, by use of the Autism Diagnostic Interview–Revised (ADI-R) (Lord et al. 1994) and age-appropriate versions of the Autism Diagnostic Observation Schedule (Lord et al. 1989) or the Pre-Linguistic Autism Diagnostic Observation Schedule (DiLavore et al. 1995); (2) mental age >18 mo,

Table 2

Transmission Data for Each Allele of *GABRB3* 155CA-2

			Paternal			Maternal			Combined		
Allele	*n*	Percentage	TR	NT	χ^2	TR	NT	χ^2	TR	NT	χ^2
85	1	.2	0	0	0	1	0	1	1	0	1
87	167	32	19	32	3.31	22	30	1.23	44	65	4.05
89	53	10.2	11	16	.93	7	15	2.91	18	31	3.45
91	1	.2	0	0	0	0	1	1	0	1	1
93	1	.2	0	1	1	0	0	0	0	1	1
95	16	3.1	2	5	1.29	5	4	.11	7	9	.25
97	11	2.1	3	2	.2	6	0	6	9	2	4.45
99	84	16.1	15	12	.33	18	17	.03	34	30	.25
101	68	13	10	11	.05	11	16	.93	21	27	.75
103	87	16.7	29	11	8.1	19	8	4.48	50	21	11.85
105	12	2.3	3	3	0	3	3	0	6	6	0
107	4	.8	0	3	3	1	0	1	1	3	1
109	9	1.7	3	0	3	3	3	0	6	3	1
111	6	1.1	2	0	2	3	1	1	5	1	2.67
113	2	.4	0	1	1	0	1	1	0	2	2

NOTE.—TR = transmitted; NT = not transmitted. The genotype of CEPH individual 884-15 was 99/103. Only transmissions from heterozygous parents were included. For parent-child pairs, only transmissions from a heterozygous parent to a heterozygous child with a different genotype were included.

as assessed by the Vineland Adaptive Behavior Scales (Sparrow et al. 1984); (3) nonverbal IQ >35; (4) confirmation of the diagnosis of autistic disorder, by a child psychologist (C.L.) and a child psychiatrist (E.H.C. or B.L.L.) or by a child psychologist (A.L.) and a child neurologist (R.H.); and (5) exclusion of known etiologies of autistic disorder, by physical examination, including neurological examination and Wood's lamp examination to exclude tuberous sclerosis (Smalley et al. 1992). *FRAXA* DNA testing was performed and was found to be negative for 136 of the 140 probands. Owing to insufficient DNA and the absence of clinical suspicion of fragile X syndrome, *FRAXA* DNA testing was not performed in four of the subjects. For three of these four subjects, *FRAXA* DNA testing was negative for an affected sibling but was not performed for the proband. In the process of genotyping, two individuals with autistic disorder, of the 140 consecutive cases meeting criteria for inclusion in this study, were identified to have interstitial 15q11-13 duplications. The first case has been reported elsewhere (Cook et al. 1997*b*), because the affected sibling shared the interstitial duplication of maternal origin and because the unaffected mother had a duplication of paternal origin. The second case has a de novo interstitial duplication of maternal origin.

The sample of families meeting full inclusion criteria includes six affected-sibling pairs in which each child meeting the criteria was considered as a separate proband, for generation of parent-child trios (no parent-child pairs in the families with two affected children). There were 13 parent-child pairs and 125 trios, consisting of a child and both parents.

Of the probands, 119 were male and 19 were female. One hundred fourteen were Caucasian, 6 were African American, 13 were Asian American, and 5 were Hispanic. The mean age was 7.6 ± 6.2 years. The mean nonverbal mental age was 6.0 ± 5.8 years, and the mean nonverbal IQ was 79.2 ± 25.3. The mean ADI-R scores were as follows: social, 21.7 ± 4.7; communication, 14.6 ± 3.9; and restricted and repetitive behaviors, 6.3 ± 2.2.

For the first 86 families of this sample, preliminary findings for the *HTT* gene have been published previously (Cook et al. 1997*a*). For the larger sample, genotyping at *HTT* is ongoing. Other genes that have been screened, in part of this sample, by use of the transmission-disequilibrium test (TDT), include *HTR1A, HTR2A, HTR2C, HTR6, HTR7, TPH, PRKCQ, HRAS, DRD2,* and *TNFA,* a gene in the major histocompatibility complex region.

Genotyping

Blood was collected, by venipuncture, into lavender top Vacutainer tubes. Blood was extracted by use of the PureGene DNA Isolation Kit (Gentra Systems). Genotyping was performed blind to the inclusion status of the probands and to family information.

Multiplex PCR reactions were optimized by starting with equal concentrations of all primers and then were adjusted to yield peak heights of one-third the maximum fluorescence-detection limit. PCR amplification for the first set of four microsatellite markers was carried out in a final volume of 10 μl consisting of 50 ng genomic DNA, 10 mM Tris HCl, 50 mM KCl, 200 μM dNTPs, 2.5 mM $MgCl_2$, 0.3 units *Taq* Gold polymerase, 0.001% gelatin, and sense and antisense primers of the following concentrations: 100 nM sense and 313 nM antisense D15S128, 1.25 μM D15S122, 232 nM D15S97, and 48 nM D15S156. The initial activation step for heat-activated DNA polymerase was carried out at 95°C for 12 min, followed by 10 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s and by 28 subsequent cycles in which the denaturation temperature was reduced to 89°C. Final extension was at 72°C for 30 min. D15S1506 and D15S10 were amplified under identical multiplex conditions, except for the concentration of primers (200 nM sense and 160 nM antisense D15S1506 and 500 nM D15S10), the annealing temperature (55°C), and the use of 30 cycles with a denaturation temperature of 95°C and no cycles at 89°C denaturation. A third PCR was run for *GABRB3* 155CA-2 (Glatt et al. 1994). The final volume of 10 μl consisted of 50 ng genomic DNA, 10 mM Tris HCl, 50 mM KCl, 0.001% gelatin, 1.5 mM $MgCl_2$, 0.6 units *Taq* polymerase, 200 μM dNTPs, 5% dimethyl sulfoxide, by volume, and a concentration of sense and antisense primers of 500 nM *GABRB3* 155CA-2. Initial activation for DNA polymerase was carried out at 94°C for 2 min, followed by 29 cycles at 95°C for 15 s, 61°C for 30 s, and 72°C for 60 s. Final extension was at 72°C for 10 min. A 7-bp insertion/deletion polymorphism was identified in which there was a common deletion, at 805–811 bp, of the OP2 genomic sequence (Entrez database [http://www.ncbi.nlm.nih.gov] accession L23501) that overlaps with the transcription-initiation site of at least one *UBE3A* transcript (Kishino et al. 1997). Primers OP2 3A (5′-HEX-TGG TTA TAG TTG TGA GGC GGA TAC-3′) (1 μM) and OP2 3B (5′-AAC TTG CAA CTT TGT TGA TAA GCC-3′) (1 μM) were used in a PCR with the same conditions as the first and second PCRs described above, except that the annealing temperature was 53.3°C and the final extension was 2 min. A 3-bp insertion/deletion polymorphism after the first coding exon was amplified by use of 1 μM *UBE3A* e1A (5′-HEX-CAC AGG TTA ATC ACT TCA GTG C-3′) and 1 μM *UBE3A* e1B (5′-TAA GCA CAG TGA TTA GTA CA-3′). Conditions were the same as those used for the first and second PCRs described above, except that the annealing temperature was 58°C.

D15S10, D15S128, D15S122, D15S97, and D15S156 were obtained from Research Genetics, and *GABRB3* 155CA-2, D15S1506, OP2 3A, and *UBE3A* e1A were synthesized by Applied Biosystems. Unlabeled primers were synthesized at the Cancer Research Center at the University of Chicago. All reactions were performed on a GeneAmp PCR system 9600 thermocycler (Perkin-Elmer).

PCR products were combined for injection, on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems), in a final volume of 13.5 μl consisting of 12 μl deionized formamide, 0.5 μl N,N,N′,N′-tetramethyl-6-carboxyrhodamine GS500 size standard, and 1.0 μl pooled PCR products, consisting of pool 1 (D15S128, D15S122, D15S97, *GABRB3* 155CA-2, and D15S156), pool 2 (D15S1506 and D15S10), and pool 3 (*UBE3A* OP2 and *UBE3A* intron 1). Products were separated by use of Performance Optimized Polymer-4 denaturing polymer (Wenz et al., in press) and were sized with Genescan and Genotyper, version 2.0, software (Applied Biosystems).

Data Analysis

Transmissions were determined by the extended TDT program, version 1.6 (ftp://ftp.gene.ucl.ac.uk/pub/packages/dcurtis). When one parent was missing, families were dropped for diallelic markers and for pairs in which the child was homozygous or shared the same genotype as the parent (Curtis and Sham 1995). At each locus, alleles with parental counts less than five were combined with other loci with counts less than five. If this sum was less than five, these alleles were combined with the least common allele until each allele (or group of alleles) had a minimum count of five. Data were analyzed by the multiallelic TDT (MTDT) (Spielman et al. 1993; Spielman and Ewens 1996). Since 20 loci, including the 9 loci described in this report, have been analyzed in this sample, Bonferroni correction leads to a corrected significance threshold of $P < .05/20 = .0025$.

Results

Linkage disequilibrium was found between autistic disorder and *GABRB3* 155CA-2 (MTDT 28.63, 10 df, $P = .0014$) (table 1). There was no evidence of parent-of-origin effects on *GABRB3* 155CA-2 transmission-disequilibrium (table 2). None of the other markers in the region showed statistically significant evidence of linkage disequilibrium, by use of the MTDT (table 1).

Discussion

Linkage disequilibrium was found between *GABRB3* 155CA-2 and autistic disorder. *GABRB3* 155CA-2 has been physically localized to the third intron, within 10-kb of exons 1a, 1, 2, and 3 (Kirkness and Fraser 1993; Glatt et al. 1994). D15S97 has been localized to the fourth intron of *GABRB3* (Glatt et al. 1997). Owing to a 170-kb third intron, the *GABRB3* 155CA-2 and D15S97 microsatellite markers are at least 150 kb apart (Glatt et al. 1997). Linkage disequilibrium between loci in a heterogeneous population would not be expected to be strong for >50 kb, but linkage disequilibrium between loci within a gene may be larger than that between other loci (Jorde et al. 1994; Jorde 1995). In addition, linkage disequilibrium has extended over much larger distances in single-gene disorders, such as cystic fibrosis. Although the current finding is Bonferroni corrected for the number of loci tested in this sample, the number of loci that may be tested in autistic disorder and the absence of linkage disequilibrium with D15S97 provide caution that the current finding may be a false positive, owing to type I error. Genotyping of several markers in *GABRB3* and adjacent *GABRA5* (Glatt et al. 1994) may be useful in confirming and clarifying the presence and extent of linkage disequilibrium between autistic disorder and markers in this region. A preliminary report of weak linkage in an overlapping region of 15q11-13 (Pericak-Vance et al. 1997) provides some support for the current finding, but a recent 10-cM genome screen of affected-relative pairs did not find significant evidence of linkage in this region (International Molecular Genetic Study of Autism Consortium, 1998).

In addition to its location within 15q11-13, the *GABRB3* gene, which codes for the β3 subunit of the γ-aminobutyric acid $(GABA)_A$ receptor, is a candidate gene for autistic disorder because of the role of the $GABA_A$ receptor agonist benzodiazepine in the treatment of seizures and anxiety disorders. Rates of anxiety disorder previously had been found to be higher in the first-degree relatives of probands with autistic disorder, compared with the first-degree relatives of probands with Down syndrome (Piven et al. 1991). In another study, first-degree relatives of probands with autistic disorder had a 10-fold elevation in social phobia, a specific anxiety disorder, relative to first-degree relatives of probands with tuberous sclerosis complex or a seizure disorder without autistic disorder (Smalley et al. 1995). Testing of linkage between *GABRB3* and panic disorder, an anxiety disorder, was negative (Crowe et al. 1997), but social phobia may be more related to autistic disorder susceptibility than is panic disorder. No significant evidence for linkage was found between *GABRB3* markers and schizophrenia (Byerley et al. 1995) or bipolar mood disorder (Coon et al. 1994).

Seizures and electroencephalographic abnormalities have been reported in >25% of subjects with autistic disorder (reviewed in Bailey et al. 1996). Testing of linkage between the *GABRB3/GABRA5/GABRG3* gene cluster and idiopathic generalized epilepsy revealed ev-

idence against linkage, by use of the entire family set and subsets selected from either juvenile absence epilepsy or childhood absence epilepsy. In 61 families of patients with juvenile myoclonic epilepsy, a weakly positive LOD score was found between a broad phenotype of idiopathic generalized epilepsy and this gene cluster (Sander et al. 1997). However, haplotype–relative-risk testing of *GABRB3* 155CA-2 was negative (Sander et al. 1997). Another study, using the *GABRB3* CA repeat 3′ of the gene, found no association in a case-control study of juvenile myoclonic epilepsy (Guipponi et al. 1997). Once a sufficiently large number of families with probands with autistic disorder and epilepsy are identified, testing of linkage disequilibrium in this subgroup would be of interest.

In the mature brain, GABA functions as an inhibitory neurotransmitter, but changes, in the developing brain, in the distribution of expression of $GABA_A$ receptor subunits indicate that it may function as a neurotrophic factor affecting neural differentiation, growth, and circuit organization. The $\beta 3$ subunit of the $GABA_A$ receptor reaches peak expression at different times in different brain regions, during pre- and postnatal murine development. For example, peaks are reached prenatally in the cerebral cortex, the hippocampus, and the thalamus and postnatally in the cerebellar cortex (Laurie et al. 1992; Nadler et al. 1994). After peak expression, rapid down-regulation occurs in the murine thalamus (Laurie et al. 1992) and the inferior olive (Chang et al. 1995), in which climbing fibers constitute a major functional input to Purkinje neurons. Although, by adulthood, the cerebral cortex and, to a lesser extent, the hippocampus have lower levels of expression of the $\beta 3$ subunit, expression in the cerebellum does not change during postnatal maturation (Laurie et al. 1992; Nadler et al. 1994). In the mature murine brain, the $\beta 3$ subunit is most intensely expressed in the cerebellum (Purkinje and granule cells), the hippocampus, and the pyriform cortex (Wisden et al. 1992): all three of these regions consistently have been reported to be anatomically abnormal in autistic patients, in either quantitative magnetic-resonance imaging or autopsy studies (reviewed in Bauman and Kemper 1994; Courchesne 1997).

Mice with homozygous deletions of *gabrb3* and a surrounding region have been shown to have cleft palate (95%), tremor, and jerky gait (Culiat et al. 1994). Mice with a targeted disruption of *gabrb3* have cleft palate (57%) and occasional epilepsy, are hyperresponsive to human contact and other sensory stimuli behavior, fail to nurture offspring, often run in tight circles, and are very hyperactive. Although *gabrb3* knockout mice have difficulty swimming, walking on grids, and remaining on platforms and rotarods, they do not have jerky gait (Homanics et al. 1997).

Although the cytogenetic evidence suggests a role of imprinted-gene expression in 15q11-13 duplications (Martinsson et al. 1996; Browne et al. 1997; Cook et al. 1997*b*), the parent of origin did not have an effect on preferential transmission of *GABRB3* 155CA-2 alleles. It is possible that a mutation in a regulatory region disrupts the usual temporal and/or spatial expression patterns of a gene or genes in the region. Moreover, it is premature to speculate on mechanisms, until a functional variant has been identified.

Until replicated and until functional variants are demonstrated to have biological effects, these results must be considered preliminary. Furthermore, apparent meiotic segregation distortion may contribute to the finding. In addition to possible involvement of *GABRB3,* adjacent loci—including *GABRA5* and expressed genes that may not have been identified previously in the region—may be implicated by linkage disequilibrium. Even if replicated, it is likely that a mutation in the region would be one of several susceptibility loci in autistic disorder and would pertain to a subset of families, owing to the likely heterogeneity of this complex genetic disorder.

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