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Analysis of the autism chromosome 2 linkage region: *GAD1* and other candidate genes

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Abstract

Autism has a strong and complex genetic component, involving several genes. Genomic screens, including our own, have shown suggestive evidence for linkage over a 20–30 cM region on chromosome 2q31-q33. Two subsequent reports showed that the linkage evidence increased in the subset of families with phrase speech delay (PSD), defined as onset of phrase speech later than 3 years of age.

To further investigate the linkage in the presumptive candidate region, microsatellite markers in a 2 cM grid covering the interval from 164 to 203 cM were analyzed in 110 multiplex (2 or more sampled autism patients) families. A maximum heterogeneity LOD (HLOD) score of 1.54 was detected at D2S1776 (173 cM) in the overall dataset (dominant model), increasing to 1.71 in the PSD subset. While not conclusive, these data continue to provide suggestive evidence for linkage, particularly considering replication by multiple independent groups. Positive LOD scores extended over the entire region, continuing to define a broad candidate interval.

Association studies were performed on several functional candidates mapping within the region. These included *GAD1*, encoding GAD67, whose levels are reduced in autopsy brain material from autistic subjects, and *STK17B*, *ABI2*, *CTLA4*, *CD28*, *NEUROD1*, *PDE1A*, *HOXD1* and *DLX2*. We found no evidence for significant allelic association between autism and any of these candidates, suggesting that they do not play a major role in the genetics of autism or that substantial allelic heterogeneity at any one of these loci dilutes potential disease–allele association.
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Autism is a neurodevelopmental disorder presenting with severe disturbances in social, behavioral and communicative functioning. It has a substantial but complex genetic etiology. The number of genes estimated to be involved in autism range from a few (2–10) [12,34] to 15 or more [37] to a 100 loci [35]. Although several candidate genes have been suggested as autism risk factors, no major gene effect has been confirmed [5,19,21,36].

At least 10 independent genomic screens [2,4,7,17,18,23,33,37,41,43] have been performed in search of autism risk genes. Several consensus regions of probable linkage have emerged, including the 2q31-33 region. Four different studies [4,18,33,41], including Shao et al. [41] ($N = 99$ families with ≥2 autism patients sampled), showed support for linkage to an interval (170–198 cM) on distal 2q, although no single dataset independently provided conclusive evidence (LOD ≥ 3) for linkage.

The phenotypic and genetic heterogeneity of autism confound genetic analysis and are likely related. That is,

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phenotypic subsetting by clinical symptoms may help reduce genetic heterogeneity, and thus increase power to detect linkage. Subsetting by phrase speech delay (PSD), as defined by onset of phrase speech later than 3 years of age, has been shown to increase linkage scores for 2q in two independent studies [4,40]. Thus, PSD subsetting may be a useful approach in examining linkage in this region.

There are numerous genes located in the broad candidate interval. Several are potentially compelling as functional candidates for autism susceptibility and need to be investigated for major gene effects. *SLC25A12*, encoding a mitochondrial aspartate/glutamate carrier expressed in brain, has been indicated as a possible genetic risk factor for autism [36]. In their analysis, Ramoz et al. found significant association to two SNPs within *SLC25A12*. Recent analysis in our data, using the same two associated SNPs and an additional 7 SNPs located within the gene, failed to replicate these findings (Rabionet, unpublished data), excluding this gene from further analysis for a common allelic effect. *GAD1* has also been specifically related to autism [11]. Other functional candidate genes in this region include neurogenic differentiation 1 (*NEUROD1*), homeobox D1 (*HOXD1*), distal-less 2 (*DLX2*), phosphodiesterase 1A (*PDE1A*), cytotoxic T lymphocyte-associated 4 (*CTLA4*), *CD28*, serine/threonine kinase 17B (*STK17B*), and abl interactor 2 (*ABI2*; Fig. 1).

Three of these genes are transcription factors involved in brain development. NeuroD1 is a basic helix–loop–helix transcription factor expressed in developing brain and pancreas. Absence of the *NEUROD1* gene in mice leads to defects in hippocampal formation, resulting in the absence of the dentate granule cell layer, and epilepsy [24]. *HOXD1* is part of the *HOX*-Homeobox gene cluster located in chromosome 2. It is the only gene of the cluster expressed in brain, where it plays a role in the development of the diencephalon, in combination with PAX6 and HOXA2 [42]. *DLX2* is another homeodomain-encoding gene involved in the development of the forebrain, regulating the migration and differentiation of neurons in the subcortical telencephalon [44]. It is also one of the defining transcription factors expressed in GABAergic (γ-aminobutyric acid) neurons of the neocortex [22], which raises interest due to a proposed role of the GABAergic system in autism [10,16].

GAD1 is also related to the γ-aminobutyric acid (GABA) pathway. This gene maps within 2 cM of our peak LOD score at D2S1776, and it encodes GAD67, a protein responsible for the conversion of glutamic acid into GABA. Fatemi et al. [11] showed a 61% reduction ($p < 0.03$) in the levels of GAD67 in the parietal cortex of autistic patients when compared to controls. The involvement of GAD67 in the GABAergic system together with its level reduction in autistic patients makes *GAD1* an excellent candidate gene for autism susceptibility.

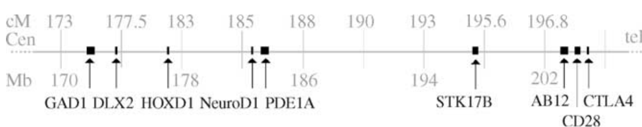

Fig. 1. Map of the region of interest with the location of the genes analyzed.

PDE1A is a calmodulin-dependent cyclic nucleotide phosphodiesterase. This family of proteins is involved in signal transduction by regulating the concentrations of cyclic nucleotides. In particular, certain splice variants of *PDE1A* have been shown to be specifically expressed in brain [30]. *CTLA4* and *CD28* are also located in the region of interest. These genes are putatively involved in autoimmune disorders, and an autoimmune component has been proposed for autism [8]. Both genes code for proteins from the immunoglobulin family, involved in T-cell regulation. CD28 is an antigen binding protein that recognizes the B7 antigen in the antigen-presenting cell, and delivers antigen independent co-stimulating signals to the T-cell. These signals are necessary for normal activation of the T-cell in addition to the antigen-dependent stimulation. CTLA4 is involved in the same pathway as CD28, but it delivers an inhibitory signal by binding B7 molecules and thereby blocking CD28 binding, and the subsequent stimulatory signal. The region encompassing *CTLA4* and *CD28* has been implicated in several autoimmune disorders, such as psoriasis, diabetes, hypothyroidism, and coeliac disease [6,20,31]. STK17B, also called DRAK2, is a serine/threonine kinase involved in the regulation of apoptosis [38]. Apoptosis is an important process in the development of the nervous system. During nervous system development, excess neurons and synapses are generated, and later eliminated via apoptosis or programmed cell death [26]. Therefore, a protein involved in the regulation of this process is potentially a good functional candidate gene. ABI2 is a substrate for Abl tyrosine kinase that is involved in cytoskeletal reorganization after growth factor stimulation. In the mouse, *Abi1* and *Abi2* are expressed in the developing nervous system and undergo changes in expression and phosphorylation during development [9], thus making *ABI2* potentially relevant for neurodevelopmental disorders such as autism.

To investigate the possible involvement of these selected functional candidate genes in autism, we performed association analysis using multiple SNPs located within these genes. Additionally, in an effort to fine map this region we examined 110 multiplex families for linkage using 19 microsatellites in a 2 cM grid.

Linkage analysis was performed in 110 multiplex families (those for which we obtained DNA from at least two affected individuals) from the Collaborative Autism Team (CAT) collected by the Duke Center for Human Genetics and the WS Hall Psychiatric Institute. A subset of these multiplex families (99) had been genotyped previously for a limited set of markers in this region [40].

Association analyses were performed on a total of 406 families (209 multiplex and 197 parent–child trios (families for which DNA from only one affected individual was obtained)). Ninety-nine multiplex families from the Autism Genetic Resource Exchange (AGRE) were also included in the association analysis of candidate genes to increase power.

Linkage to this region was previously reported in the AGRE families [4].

All participants were ascertained on the basis of an autism diagnosis. Both multiplex and trio families were included in this study. Detailed diagnostic evaluations of the family data have been previously described [39]. Briefly, the Autism Diagnostic Interview-Revised (ADI-R) [25] was used to confirm the clinical diagnosis of autism. The classification of an individual with autism required that an individual exceed cutoff scores in each of the three areas: social behavior, communication (nonverbal or verbal), and restricted, repetitive behaviors. PSD was defined as the failure to acquire phrase speech before 36 months, and a multiplex family was classified as PSD when at least two autistic patients met criteria for PSD. This resulted in the PSD classification of 64 families from the AGRE multiplex subset, and 56 multiplex and 50 trio families from the CAT subset.

Blood was obtained from patients and other family members under IRB-approved procedures. Genomic DNA was isolated from whole blood samples using Puregene (Gentra Systems, Minneapolis, MN).

SNPs within candidate genes were identified among Applied Biosystems (ABI, Foster City, CA) Assay on Demand (AoD) products. SNPs rs3791344 (*DLX2*) and rs6710142 (*HOXD1*) were identified in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP/), and ordered as custom ABI Assays-by-Design. A list of markers is provided in Table 2. All SNPs were genotyped using the TaqMan® allelic discrimination method, according to manufacturer's recommendations. From analysis of all SNPs, rs1470789 and cv1354384 (*ABI2*) showed perfect linkage disequilibrium (LD) ($r^2 = 1$) in a test on 70 unrelated individuals. On the same 70 individuals, testing for LD between rs1519602 and rs1054537 (*STK17B*) also showed $r^2 = 1$. Therefore, further analysis was conducted only for one SNP of each pair, rs1470789 and rs1519602. Validation of rs3791344 showed a very low minor allele frequency, and further studies on this SNP were not performed.

Nineteen markers in the chromosome 2 candidate region were selected, creating a 2 cM grid covering the region between the peaks obtained by the different genomic screens, from 170 to 198 cM [4,33,18,40]. All markers were amplified by 40 cycles (94–55–72 °C) PCR using 30 ng DNA in 10 μl reactions containing 0.05 u/μl Platinum Taq (Invitrogen), 3 mM $MgCl_2$, 0.6 mM dNTPs and 4 ng/μl each primer, and then run on acrylamide gels and scanned. Gel analysis was performed using Bioimage.

For quality control procedures, two CEPH standards were included on each 96-well plate, and samples from six individuals were duplicated across all plates as quality controls (QCs), with the laboratory technicians blinded to their identities. Analysis required that identical QC samples within and across plates had matching genotypes, in order to identify errors in loading and reading, and thus minimize the error rate in genotypes assignment. After QC verification, genotypes of the samples were uploaded into the PEDIGENE® database and merged into the Lapis management system for creating analysis input files [13].

We performed parametric two-point linkage analysis using Fastlink, and the HOMOG program [32] to consider the

Table 1
Parametric and non-parametric two-point linkage analysis results for our complete dataset and PSD subset

Position (cM)	Marker	All Duke ($N = 110$)			PSD Duke ($N = 56$)			Marker information content
		2pt LOD		ASPEX MLS	2pt LOD		ASPEX MLS	
		dom	rec		dom	rec		
169.41	**D2S2330**	0.00	0.11	0.22	0.02	0.17	0.38	0.413
173.00	**D2S1776**	**1.54**	**1.16**	0.75	**1.62**	1.19	1.14	0.404
175.91	**D2S2194**	0.02	0.23	0.01	0.02	0.07	0.25	0.421
175.91	**D2S2302**	0.14	0.22	0.32	0.13	0.21	0.21	0.600
175.91	**D2S335**	0.10	0.13	0.09	0.11	0.28	0.14	0.448
177.53	**D2S326**	−0.04	0.03	0.01	−0.04	−0.01	0.01	0.451
180.79	**D2S2188**	0.07	0.05	0.00	0.17	0.19	0.13	0.420
182.96	**D2S138**	0.23	0.22	0.04	0.00	0.05	0.19	0.413
186.2	**D2S1391**	1.12	1.04	0.56	1.30	1.25	0.75	0.420
186.2	**D2S2273**	0.09	0.03	0.00	−0.02	−0.01	0.01	0.668
186.21	**D2S364**	0.13	0.09	0.00	0.01	0.04	0.02	0.449
188.11	**D2S1361**	0.24	0.23	0.01	0.01	0.11	0.16	0.570
190.00	**D2S426**	0.60	0.67	0.31	0.48	0.56	0.61	0.430
193.26	**D2S425**	0.19	0.13	0.17	0.32	0.48	0.37	0.324
194.45	**D2S117**	1.28	**1.16**	0.19	1.19	1.02	0.58	0.513
196.85	**D2S311**	0.25	0.19	0.03	0.17	0.14	0.13	0.650
198.65	**D2S116**	0.38	0.64	**1.06**	1.22	**1.66**	**2.09**	0.576
198.65	**D2S309**	0.86	1.01	0.74	1.07	0.95	0.68	0.666
198.65	**D2S2309**	0.39	0.46	0.61	1.38	1.47	1.39	0.506
200.43	**D2S1384**	0.11	0.10	0.06	0.76	0.55	0.29	0.438
202.9	**D2s1782**	0.03	−0.02	0.00	0.18	0.25	0.23	0.405

Marker cM position is derived from the Marshfield map. 2pt LOD: two-point parametric LOD scores; dom: dominant model; rec: recessive model; MLS: maximum non-parametric (sibpair) LOD score.

presence of genetic heterogeneity. Dominant and recessive affected only low-penetrance models were considered. Non-parametric affected sib-pair analysis of the data was performed using ASPEX software [14].

Hardy-Weinberg equilibrium was assessed using exact tests implemented in the Genetic Data Analysis program [45]. Pairwise linkage disequilibrium (D' and r^2) between markers within each gene was calculated using the software package GOLD [1].

The pedigree disequilibrium test (PDT) [28,29] and the geno-PDT [27] were used to examine single-locus disease–allele or disease–genotype association to autism. PDT examines transmissions from parents to affected offspring, and also compares genotypes of affected individuals with their unaffected siblings. The geno-PDT test, while not as powerful as the PDT under an additive model, is more powerful under a recessive or dominant model, and is able to test association of specific genotypes. To consider multiple loci simultaneously, we used the haploFBAT [15], a test of association with haplotypes. We obtained global scores for pairwise haplotype analysis considering all SNPs within each gene. For each SNP pair, observed haplotypes with less than 10 families were not considered.

Results of linkage analysis for our independent dataset of 110 multiplex autism families gave a peak LOD (dominant model) of 1.54 for marker D2S1776 (173 cM). PSD subsetting of the families resulted in an increased LOD (dominant model) of 1.62, further supporting the finding that the PSD subset contributes significantly to the overall linkage seen in the region (Table 1). Heterogeneity LOD scores (HLOD) obtained did not differ substantially from the overall LOD scores presented in Table 1. Peak HLODs (dominant affected only model) for the overall and PSD subsets were also at marker D2S1776, with scores of 1.54 ($\alpha = 1$) and 1.72 ($\alpha = 0.6$), respectively. D2S116 gave the peak score for the non-parametric sibpair MLS results for both subsets (1.06 and 2.09 for the entire dataset and the PSD subset, respectively). Multipoint analysis (data not shown) did not increase the LOD score or further narrow the candidate region.

Association analysis for *ABI2*, *GAD1*, *CTLA4*, *CD28*, *DLX2*, *HOXD1*, *NEUROD1*, *PDE1A* and *STK17B* was performed on our complete dataset of 407 families (multiplex and trio families, including 99 AGRE multiplex families). Association results from PDT and geno-PDT analysis on all candidate genes are shown in Table 2. Intermarker LD within each gene was calculated and is presented in Table 3. We found high levels of LD ($D' > 0.9$) between markers in *ABI2* (100 kb), *CTLA4* (10 kb) and *STK17B* (30 kb).

Only three SNPs were nominally significant below the $p \leq 0.05$ level in the overall dataset: cv158148 in the *ABI2* gene (PDT $p = 0.034$, geno-PDT = 0.069), rs6710142 in *HOXD1* (PDT $p = 0.02$, geno-PDT = 0.04), and rs2228184 in *DLX2* (PDT $p = 0.053$, geno-PDT $p = 0.032$).

No evidence was found for association between the SNPs located in *GAD1*, *STK17B*, *CD28*, *CTLA4*, *PDE1A* and *NEUROD1* and autism. We also analyzed a microsatellite marker

Table 2
Association analysis (complete dataset and PSD dataset) by PDT, geno-PDT and FBAT

Gene	Marker	PDT		Geno-PDT	
		All (407)	PSD (170)	All (407)	PSD (170)
GAD1	rs2241165	0.899	0.682	0.608	0.890
	cv2177420	0.218	0.362	0.270	0.239
DLX2	rs2228184	0.053	0.806	**0.032**	0.764
	rs3791344	–	–	–	–
HOXD1	rs6710142	**0.02**	0.058	**0.04**	0.139
	rs1374326	0.084	0.077	0.135	0.090
NEUROD1	rs1801262	0.146	0.193	0.224	0.307
PDE1A	cv2074792	0.472	0.187	0.676	0.409
	rs2128043	0.175	0.431	0.230	0.738
	cv2048114	1.000	0.844	0.902	0.570
STK17B	rs1568368	0.703	0.746	0.488	0.445
	rs1519602	0.579	0.203	0.815	0.430
	cv11511607	0.670	0.068	0.202	0.068
	rs1054537	–	–	–	–
ABI2	cv158148	**0.034**	0.153	0.069	0.351
	cv11511088	0.813	0.789	0.515	0.953
	rs1470789	0.203	0.435	0.397	0.707
	cv1354384	–	–	–	–
CD28	cv11459560	0.638	0.144	0.886	0.229
	cv2821002	0.628	0.368	0.836	0.457
	cv2959702	0.232	0.139	0.075	0.045
CTLA4	rs926169	0.699	0.494	0.520	0.812
	rs231723	0.467	0.680	0.557	0.802
	rs231729	0.958	0.720	0.699	0.788

Number of families included in each analysis is indicated in parenthesis. "–" indicates the SNP was run but not analyzed, either because it was in perfect LD with another SNP or because of low minor allele frequency.

located within *GAD1* (D2S2194) and found no evidence for linkage or association. Pairwise association results from haploFBAT also failed to provide evidence for association. Finally, examination of the PSD subsets failed to reveal significant association for any of the markers tested.

We have presented data for 19 microsatellite markers in 110 multiplex autism families covering the chromosome 2 autism linked region using a 2 cM grid interval. We have obtained positive linkage scores (LOD score > 1) for several markers across the region, with a peak two-point heterogeneity LOD score of 1.54 at D2S1776, located at 173 cM (Table 1). While these results continue to support linkage to chromosome 2, the overall candidate region remains broad.

We also performed association analysis on nine functional candidate genes located within this linked region. For this analysis, we also included 197 trio families and an additional 99 multiplex families from AGRE. Many of these multiplex families were included in a previous screen by Buxbaum et al. [4], where they showed linkage to marker D2S364, at 186 cM.

Association studies failed to provide any highly significant results. In fact, only three of the genes analyzed (*ABI2*, *HOXD1* and *DLX2*) showed nominal effects in the complete

Table 3
LD distribution within each gene

GAD1	rs2241165	cv2177420	
rs2241165		0.141	
cv2177420	0.896		
HOXD1	RS6710142	RS1374326	
rs6710142		0.667	
rs1374326	**0.991**		
PDE1A	cv2074792	rs2128043	cv2048114
cv2074792		0.4096	0.0001
rs2128043	0.798		0.1156
cv2048114	0.047	0.428	
STK17B	rs1568368	rs1519602	cv11511607
rs1568368		0.7344	0.8372
rs1519602	**1**		0.8082
cv11511607	**1**	**0.993**	
ABI2	cv158148	cv11511088	rs1470789
cv158148		0.299	0.06
cv11511088	**0.983**		0.365
rs1470789	**1**	**1**	
CD28	cv1145960	cv2821002	cv2959702
cv1145960		0.0448	0.1665
cv2821002	0.227		0.1399
cv2959702	0.425	0.391	
CTLA4	rs926169	rs231723	rs231729
rs926169		0.7379	0.6740
rs231723	**0.958**		0.7174
rs231729	**0.92**	**0.988**	

Shadowed cells indicate r^2, while plain cells show D'.

dataset. Bonferroni correction for multiple testing of 29 markers, would require p values ≤ 0.0017 to achieve significance. Thus, none of these results are considered significant based on that conservative basis. Our findings are consistent with those of Bacchelli et al. [3], who screened nine candidate genes, including *DLX2*, *HOXD1* and *NEUROD1*, in 164 families, and found no evidence of association at any of these loci.

We are particularly intrigued by the lack of association obtained for *GAD1*, which was thought to be a promising candidate gene, both positionally and functionally. These results indicate that the differences in GAD67 levels in autistic brain samples compared to controls observed by Fatemi et al. [11] are probably not due to common mutations or variants in *GAD1*. The lack of evidence for linkage and association for the markers located within this gene suggests that, while it and the other genes analyzed cannot be excluded as susceptibility genes, given the possibility of allelic heterogeneity, they do not possess *common* alleles with major involvement in autism risk.

In summary, we have continued to find evidence in support of linkage of autism to 2q, but the region of interest remains broad. Using allelic association tests to examine functional candidates mapping to the region failed to detect involvement of common alleles in autism. While the presence of rare disease alleles (e.g. mutations) at these genes cannot be excluded as a possibility, our data do not support them as major susceptibility genes in autism.

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