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, and STK17B, ABI2, CTLA4, CD28, NEUROD1, PDE1A, HOXD11 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,
controls. The involvement of GAD67 in the GABAergic system in the parietal cortex of autistic patients when compared to 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.

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 trios 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® ABI Assays-by-Design. A list of markers is provided in Table 2. All SNPs were genotyped using the TaqMan® al-

### Table 2. All SNPs were genotyped using the TaqMan® ABI Assays-by-Design. A list of markers is provided in Table 2. All SNPs were genotyped using the TaqMan® al-

<table>
<thead>
<tr>
<th>Position (cM)</th>
<th>Marker</th>
<th>All Dake (N = 110)</th>
<th>PSD Duke (N = 56)</th>
<th>Marker information content</th>
</tr>
</thead>
<tbody>
<tr>
<td>169.41</td>
<td>D2S2330</td>
<td>0.00</td>
<td>0.11</td>
<td>0.22</td>
</tr>
<tr>
<td>173.00</td>
<td>D2S1776</td>
<td>1.54</td>
<td>1.16</td>
<td>0.75</td>
</tr>
<tr>
<td>175.91</td>
<td>D2S2894</td>
<td>0.02</td>
<td>0.25</td>
<td>0.01</td>
</tr>
<tr>
<td>175.91</td>
<td>D2S2882</td>
<td>0.14</td>
<td>0.22</td>
<td>0.32</td>
</tr>
<tr>
<td>175.91</td>
<td>D2S35</td>
<td>0.10</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>177.53</td>
<td>D2S326</td>
<td>−0.04</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>180.79</td>
<td>D2S2308</td>
<td>0.07</td>
<td>0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>182.96</td>
<td>D2S138</td>
<td>0.23</td>
<td>0.22</td>
<td>0.04</td>
</tr>
<tr>
<td>186.62</td>
<td>D2S1381</td>
<td>1.12</td>
<td>1.04</td>
<td>0.56</td>
</tr>
<tr>
<td>186.2</td>
<td>D2S2273</td>
<td>0.00</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td>186.21</td>
<td>D2S364</td>
<td>0.13</td>
<td>0.09</td>
<td>0.00</td>
</tr>
<tr>
<td>188.11</td>
<td>D2S1381</td>
<td>0.24</td>
<td>0.23</td>
<td>0.01</td>
</tr>
<tr>
<td>190.00</td>
<td>D2S426</td>
<td>0.60</td>
<td>0.67</td>
<td>0.31</td>
</tr>
<tr>
<td>193.26</td>
<td>D2S425</td>
<td>0.19</td>
<td>0.13</td>
<td>0.17</td>
</tr>
<tr>
<td>194.45</td>
<td>D2S147</td>
<td>1.28</td>
<td>1.16</td>
<td>0.19</td>
</tr>
<tr>
<td>196.85</td>
<td>D2S314</td>
<td>0.25</td>
<td>0.19</td>
<td>0.03</td>
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<tr>
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<tr>
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<td>D2S309</td>
<td>0.86</td>
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<td>0.74</td>
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<tr>
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<td>D2S309</td>
<td>0.39</td>
<td>0.46</td>
<td>0.61</td>
</tr>
<tr>
<td>200.43</td>
<td>D2S1384</td>
<td>0.11</td>
<td>0.10</td>
<td>0.06</td>
</tr>
<tr>
<td>202.9</td>
<td>D2S782</td>
<td>0.03</td>
<td>−0.02</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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.
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 haploFBA T [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 $r^2 > 0.9$) between markers in each gene was calculated and is presented in Table 3. We found high levels of LD $(D' > 0.91)$ between markers in ABI2 (100 kb), CTLA4 (10 kb) and STK17B (30 kb).

Only three SNPs were nonnullly significant below the $p < 0.05$ level in the overall dataset: cv/158448 in the ABI2 gene (PDT $p = 0.034$, geno-PDT $p = 0.069$), rs6170142 in HOXD1 (PDT $p = 0.01$, geno-PDT $p = 0.04$), and rs2228184 in DLX2 (PDT $p = 0.032$, geno-PDT $p = 0.015$).

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 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>r^2</th>
<th>D'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD1</td>
<td>0.896</td>
<td>0.081</td>
</tr>
<tr>
<td>HOXD1</td>
<td>0.991</td>
<td>0.166</td>
</tr>
<tr>
<td>PDE1A</td>
<td>0.798</td>
<td>0.156</td>
</tr>
<tr>
<td>STK7B</td>
<td>0.734</td>
<td>0.082</td>
</tr>
<tr>
<td>ARI2</td>
<td>0.983</td>
<td>0.96</td>
</tr>
<tr>
<td>CE28</td>
<td>0.932</td>
<td>0.976</td>
</tr>
</tbody>
</table>

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.

Acknowledgements

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The research conducted in this study complies with current U.S. laws.

References


