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The *DLX1* and *DLX2* genes and susceptibility to autism spectrum disorders

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An imbalance between excitation and inhibition in the cerebral cortex has been suggested as a possible etiology of autism. The *DLX* genes encode homeodomain-containing transcription factors controlling the generation of GABAergic cortical interneurons. The *DLX1* and *DLX2* genes lie head-to-head in 2q32, a region associated with autism susceptibility. We investigated 6 Tag SNPs within the *DLX1/2* genes in two cohorts of multiplex (MPX) and one of simplex (SPX) families for association with autism. Family-based association tests showed strong association with five of the SNPs. The common alleles of *rs743605* and *rs4519482* were significantly associated with autism ($P < 0.012$) in the first sample of 138 MPX families, with the latter remaining significant after correction for multiple testing ($P_{\text{cor}} = 0.0046$). Findings in a second sample of 169 MPX families not only confirmed the association at *rs4519482* ($P = 0.034$) but also showed strong allelic association of the common alleles at *rs788172*, *rs788173* and *rs813720* ($P_{\text{cor}} = 0.0003$ – 0.04). In the combined MPX families, the common alleles were all significantly associated with autism ($P_{\text{cor}} = 0.0005$ – 0.016). The GGGTG haplotype was over transmitted in the two MPX cohorts and the combined samples [$P_{\text{cor}} < 0.05$: $P_{\text{cor}} = 0.00007$ for the combined MPX families, Odds Ratio: 1.75 (95% CI: 1.33–2.30)]. Further testing in 306 SPX families replicated the association at *rs4519482* ($P = 0.033$) and the over transmission of the haplotype GGGTG ($P = 0.012$) although P -values were not significant after correction for multiple testing. The findings support the presence of two functional polymorphisms, one in or near each of the *DLX* genes that increase susceptibility to, or cause, autism in MPX families where there is a greater genetic component for these conditions.

European Journal of Human Genetics (2009) 17, 228–235; doi:10.1038/ejhg.2008.148; published online 27 August 2008

Keywords: autism spectrum disorders; epilepsy; genetics; *DLX1* and *DLX2* genes; GABAergic system; gene association

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Received 5 March 2008; revised 15 July 2008; accepted 16 July 2008; published online 27 August 2008

Introduction

Autism Spectrum Disorders (ASDs) are a group of neuro-developmental disorders known as pervasive developmental disorders, characterized by impairments in social interaction and communication, and the presence of restricted activities and interests. ASDs are life-long conditions that are heterogeneous in etiology and highly variable in phenotype.

Genetic epidemiological studies (twin,^{1–3} sibling^{4,5} and family^{6,7} studies) have provided considerable evidence for the presence of genetic factors in autism. ASDs have been proposed to result from the interaction of multiple genes and the interaction of genes and environmental factors.

Based in part on the high prevalence of epilepsy and seizures in individuals with autism,⁸ it has recently been suggested that an imbalance in the ratio of excitatory to inhibitory impulses in the brain may contribute to the development of autism by causing hyperexcitability in the cortex.^{9,10} Rubenstein and Merzenich¹¹ proposed an elegant model for autism in which there is an increased ratio of excitation to inhibition in those neural circuits that are responsible for language development and social behavior. They support their hypothesis with an extensive review of studies focused on understanding neural systems that are involved in memory, affiliative behaviors, and sensory–perceptual processing, and the modulation and integration of these systems, all of which are compromised or abnormal in individuals with autism. In their comprehensive review of the neuroanatomy and neurotransmitter systems in autism, Polleux and Lauder⁹ further suggest that an imbalance between excitation and inhibition in the cortex, resulting from genetic and environmental factors affecting either the pattern of neuronal populations and/or the synaptic assembly of these neuronal networks, provides a compelling model for the underlying neurobiological defects that result in autistic behaviors. Therefore, the investigation of genes that control the growth of populations of inhibitory or excitatory neurons as potential autism candidate genes is warranted. Among these, the *DLX* genes, which encode a family of homeobox transcription factors that are expressed during forebrain development,¹² are attracting growing interest.

Mouse models have revealed the involvement of the *Dlx* genes in the differentiation and migration of GABAergic neurons. Both differentiation and migration of striatal interneurons are blocked in *Dlx1/2*-null mice,^{13,14} and GABAergic neurons fail to migrate to the cerebral cortex.^{15,16} Although *Dlx1/2* and *Dlx2* ablations are fatal, *Dlx1*-null mice are born healthy, but experience a time-dependent reduction in GABAergic interneurons in the hippocampus,¹⁷ a brain region involved in learning and memory. Post-mortem studies have implicated abnormalities in the development of the hippocampus in autism.⁹ In *Dlx1*-null mice, the reduction in GABAergic inhibitory impulses also causes late-onset epilepsy,¹⁷ which is more prevalent in individuals with autism than in the general population.⁸ Both *Dlx2* and *Dlx5* induce expression of glutamic acid decarboxylases,¹⁸ which are involved in the synthesis of GABA. A functional alteration in *DLX* genes may alter the differentiation and migration of GABAergic interneurons, consequently changing the inhibition/excitation ratio in some parts of the brain. Thus we have

hypothesized that the *DLX* genes play a role in ASDs through their proven involvement in the development of the GABAergic system.

DLX genes are arranged in bi-gene clusters, two of which, the *DLX1/DLX2* and *DLX5/DLX6* clusters, are located in chromosomal regions 2q32 and 7q22, respectively. These regions have shown association with autism susceptibility in genome scan studies,¹⁹ making these *DLX* genes good positional candidates for ASDs.

Although the two previous association studies examining single-nucleotide polymorphisms (SNPs) in the *DLX2* and *DLX6* genes did not find association with autism,^{20,21} these studies were limited to the analysis of the coding regions of the genes. The study by Hamilton *et al*²² of rare variants in the *DLX1/DLX2* and *DLX5-DLX6* regions in families with autistic children also did not establish an association of these variants with autism susceptibility; however, only those variants leading to non-synonymous amino acid substitutions were investigated. Therefore, we investigated 6 Tag SNPs in the region covering the *DLX1* and *DLX2* genes in two independently recruited cohorts of multiplex (MPX) families, as well as a simplex (SPX) cohort, for association with susceptibility to autism. Our findings on the two MPX cohorts strongly support a role for these genes in the etiology of ASDs.

Materials and methods

We performed the study in a two-stage design. Sample 1 consisted of DNA samples from 138 MPX families purchased from the Autism Genetics Resource Exchange (AGRE: www.agre.org), including 288 affected children (219 males and 69 females). A second group of families, Sample 2, consisted of 169 MPX families (208 affected males and 145 affected females) recruited from across North America by the Autism Spectrum Disorders – Canadian–American Research Consortium (ASD-CARC; www.AutismResearch.ca). In addition, we examined 306 SPX families recruited by ASD-CARC, including 256 families with affected males and 50 with affected females. All MPX families had at least two affected siblings. Family selection criteria for the Sample 1 families from AGRE have been described previously.^{23,24} Diagnoses of the affected individuals varied in the AGRE families, with 220 meeting criteria for autism, 20 for ‘not quite autism’ and 45 for ‘broad spectrum’. The designations ‘not quite autism’ and ‘broad spectrum’ are not DSM-IV diagnoses but are defined by AGRE (<http://www.agre.org/agrecatalog/algorithm.cfm>). The diagnoses of the affected individuals from the Sample 2 and SPX families were based on multiple approaches including comprehensive assessments by collaborating ASD-CARC clinical geneticist-led interdisciplinary teams and Autism Diagnostic Interview-Revised (ADI-R)²⁵ and/or ADOS²⁶ or PDD Behavior Inventory

(PDDBI)^{27,28} data. All of the affected individuals in the MPX families had a clinical standard diagnosis of ASD by no less than the ADI-R and ADOS and standardization according to DSM-IV criteria. Among the 306 affected children in the SPX families, 262 have ADI-R data available, all meeting diagnostic criteria for autism or not quite autism. PDDBI information was available for the remaining and all met the cutoff for autism or PDD.

This study was approved by the Research Ethics Board of all participating ASD-CARC institutions including Queen's University, New York State Institute for Basic Research in Developmental Disabilities, University of Manitoba, and University of British Columbia. Written informed consent was obtained from parents of all participating family members from Canada and the United States (and through AGRE for the AGRE families).

SNPs were selected based on haplotype structure information available from the HAPMAP project (<http://www.hapmap.org/>). Tag SNPs were identified with the assistance of the Haploview program (v3.32) (<http://www.broad.mit.edu/mpg/haploview/>)²⁹ and the Applied Biosystems SNPbrowser™ software (v3.5) (<http://www.appliedbiosystems.com>), using the following criteria: (1) SNPs with minor allele frequencies of >0.05 and (2) CEPH samples as a reference group because our ASD families are mostly of European descent. Six Tag SNPs in a 30-kb flanking region of the *DLX1* and *DLX2* genes were selected for study (see Figure 1).

SNP rs788172 was genotyped using traditional RFLPs. A 217-bp fragment covering this SNP was amplified by PCR using the following primers 5'-CTCTGTCTGTGCGCTGGTAA-3' and 3'-AGGCGAAGTCCATTCTCAA-5'. PCR was performed on a Peltier Thermal Cycler (MJ Technologies) using 8 ng DNA in a reaction volume of 4 μ l, containing 1 $\times$ PCR buffer (Invitrogen), 4 $\times$ 0.2 mM dNTP, 24 ng each of the forward and reverse primers, 2.0 mM MgCl₂, and 0.2 U Taq DNA Polymerase (Invitrogen). PCR consisted of 30 cycles of 45 s at 95°C, 30 s at 61°C, and 30 s at 72°C. Cycling was preceded by a 5 min denaturation step at 95°C and followed by a 6 min incubation at 72°C. PCR products were digested with *Pst*I restriction endonuclease (New England Biolabs). Digestions were carried out overnight at 37°C in a reaction volume of 7 μ l, containing 15 mM MgCl₂ (Invitrogen), 1 $\times$ PCR buffer, 2 $\times$ BSA (New England Biolabs), 0.67 mM DTT, 0.6 U of *Pst*I restriction enzyme, and 4 μ l of PCR product. Electrophoresis of digested samples was carried out in a 2% agarose gel and the DNA fragments were stained with ethidium bromide. Samples were scored based on the presence of the expected bands for each allele and each result was read independently by two persons.

Genotyping of the other five SNPs was carried out using validated custom TaqMan SNP Genotyping Assays (<http://www.appliedbiosystems.com>) on an ABI Prism 7900HT, using 384-well plates. Duplicate samples and negative controls were included in each plate to check the accuracy

Figure 1 Linkage disequilibrium (LD) map of the *DLX1/DLX2* genes. Thirteen SNP markers within the 30 kb of genomic sequence spanning the *DLX1/DLX2* genes. Pairwise LD among SNPs was estimated using D' with Haploview. The number inside each rhombus is a D' value*100. The structure and position of the *DLX1/DLX2* genes, the positions of the 6 Tag SNPs for genotyping are indicated (SNP markers in bold).

of genotyping. Genotypes were automatically scored with the SDS 2.2.2 software using standard parameters.

Before data analysis, each polymorphism was assessed in parents and affected cases using χ^2 for deviations from Hardy–Weinberg equilibrium with the HWE program (Ott J, 1999).³⁰ This step was performed to determine whether there was any departure from HWE because such a finding can suggest that the marker is linked to a susceptibility or protective allele.

Genotypes were checked for Mendelian inconsistencies using the FBAT program (v1.7.3)³¹ and all identified Mendelian errors were either corrected by regenotyping individual samples or omitted from data analysis. Linkage disequilibrium (LD) of each pair of SNPs was assessed using the 2LD program.³² To perform family-based single-marker and multi-locus tests of association, the FBAT program (v1.7.3) was used.³¹ FBAT is an extension of the original transmission disequilibrium test.³³ It uses a generalized score statistic to perform a variety of transmission disequilibrium tests for single marker alleles, haplotypes and genotypes in the presence or absence of linkage. Because FBAT uses conditional distributions in deriving the

Table 1 Single SNP FBAT for association of 6 Tag SNPs in the *DLX1/DLX2* genes in MPX families with ASD

SNP	Allele	Sample 1 MPX (N = 138)					Sample 2 MPX (N = 169)					Combined MPX (N = 307)				
		S	E(S)	Z	P	P _{cor}	S	E(S)	Z	P	P _{cor}	S	E(S)	Z	P	P _{cor}
rs788172	A	139	152	-1.617	0.11		101	121	-2.814	0.0049	0.039	240	273	-3.095	0.002	0.016
	G	247	235	1.617			223	203	2.814			470	438	3.095		
rs788173	A	135	145	-1.256	0.21		89	118	-4.108	0.00004	0.0003	224	263	-3.72	0.0002	0.0016
	G	239	230	1.256			229	200	4.108			468	430	3.72		
rs813720	C	141	155	-1.725	0.085		107	133	-3.4	0.0007	0.0054	249	288	-3.548	0.0004	0.0031
	G	245	232	1.725			249	224	3.4			495	457	3.548		
rs13390848	T	106	103	0.494	0.62		116	100	2.388	0.017	0.14	222	202	2.02	0.043	
	G	218	222	-0.494			190	207	-2.388			408	428	-2.02		
rs4519482	A	97	123	-3.441	0.0006	0.0046	84	98	-2.123	0.034		181	221	-3.982	0.00007	0.0005
	T	217	192	3.441			166	152	2.123			383	344	3.982		
rs743605	A	139	159	-2.5	0.012	0.096	171	181	-1.24	0.21		310	340	-2.641	0.0083	0.066
	G	213	193	2.5			231	221	1.24			444	414	2.641		

P_{cor}: Corrected *P*-values. See Methods for a description of the correction applied for multiple testing.

distribution for the test statistic under the null hypothesis, biases owing to population admixture, mis-specification of the trait distribution, and/or selection based on a trait can be avoided. FBAT uses data from related triads and therefore can handle MPX families when testing for transmission disequilibrium. All family-based association tests in this study were performed using an additive model under the null hypothesis of no association in the presence of linkage. The individual haplotype tests were conducted under the 'biallelic' mode in haplotype FBAT.

Corrections for multiple comparisons

As the SNPs that we examined show a high degree of intermarker LD (ie, non-independence of the tests) and there are two haplotype sub-blocks, we corrected for multiple comparisons by applying a correction factor of 8 (2 (samples) × 2 (test-retest) × 2 (haplotype sub-blocks)) for all the tests. The corrected *P*-values (*P*_{cor}) were calculated by multiplying the *P*-values by eight. Results were considered significant at *P*_{cor} < 0.05.

Estimating odds ratios

Odds Ratios were calculated according to the suggestions of Ackerman *et al*, 2005,³⁴ by comparing the observed number of transmitted alleles or haplotypes and the number of non-transmitted alleles or haplotypes. The number of non-transmitted alleles or haplotypes were calculated by multiplying the number of expected transmissions by two and subtracting the number of observed transmissions.

Results

Figure 1 shows the locations of the six SNPs tested. The alleles in the cases and parents from both sets of MPX

families and the SPX families were in Hardy-Weinberg Equilibrium (HWE).

The results of the single marker FBAT analyses are summarized in Table 1. In the single marker FBAT tests in Sample 1 families, we found highly significant preferential transmission of the common T allele of rs4519482 [*P* = 0.0006, *P*_{cor} = 0.0046, *OR* = 1.96 (95% CI: 1.44–2.76)] and significant preferential transmission of the common G allele of rs743605 [*P* = 0.012, *OR* = 1.74 (95% CI: 1.26–2.41)] although this was marginal after correction (*P*_{cor} = 0.096). Both SNPs are located in *DLX2*. The remaining four SNPs (rs788172, rs788173, rs813720 and rs13390848), which are in or near the *DLX1* gene, did not show significant association although there was a tendency towards over transmission of the common alleles for three of the SNPs: rs788172-G, rs788173-G and rs813720-G.

The T allele of rs4519482 was significantly over transmitted in the Sample 2 families, although the significance was not as strong as in the Sample 1 families. The significant association of rs743605 in the Sample 1 families was not evident in the Sample 2 families. Interestingly, three common alleles at rs788172-G, rs788173-G and rs813720-G, which showed only a tendency towards over transmission in the Sample 1 families, were very significantly over transmitted from parents to affected offspring [*P*_{cor} = 0.039, 0.0003 and 0.0054, respectively; *OR* = 1.70 (95% CI: 1.23–2.35), 2.21 (95% CI: 1.59–3.08) and 1.86 (95% CI: 1.53–2.37), respectively]. Unlike in the Sample 1 families, the rs13390848-G allele was also over transmitted in the Sample 2 families [*P* = 0.017, *OR* = 1.63 (95% CI: 1.16–2.29)], although this was not significant after correction.

All of the common alleles of the markers were significantly over transmitted in the combined MPX families (Table 1).

We also tested the 6 Tag SNPs in 306 SPX families. Only the common T allele at rs4519482 showed an association with ASD [$P = 0.033$, $OR = 1.59$ (95% CI: 1.11–2.27)] before correction (Table 2).

As the common alleles at rs788172, rs788173, rs813720, rs4519482, and rs743605 were individually found to be associated with ASD, we next tested whether a haplotype consisting of these alleles was over transmitted in the different family groups. Strikingly, a haplotype composed of rs788172-G, rs788173-G, rs813720-G, rs4519482-T, and rs743605-G was significantly over transmitted in both sets of MPX families, as well as in the combined MPX families [$P_{cor} = 0.036$, 0.0022 and 0.00007, respectively; $OR = 1.89$ (95% CI: 1.25–2.86), 1.96 (95% CI: 1.33–2.9) and 1.75

(95% CI: 1.33–2.30) respectively] (Table 3). There was also a tendency towards over transmission of this haplotype in the SPX families ($P_{cor} = 0.096$).

Based on haplotype structure information from HAPMAP (Figure 1) and the data from this study (not shown), rs788172, rs788173, rs813720, rs4519482 and rs743605 are in the same major haplotype block. However, it is clear that there are two sub-blocks, with rs788172, rs788173 and rs813720 being in the first sub-block covering the *DLX1* gene and rs4519482 and rs743605 in the sub-block covering the *DLX2* gene. We therefore performed haplotype transmission tests on the two sub-blocks of haplotypes and, similar to the single marker analysis, we found: (1) the rs788172-G rs788173-G rs813720-G haplotype in the first sub-block was very significantly over transmitted in the Sample 2 and combined MPX families [$P_{cor} = 0.00058$ and 0.0005, respectively; $OR = 1.88$ (95% CI: 1.32–2.69) and 1.57 (95% CI: 1.25–1.97) respectively] (Table 3), with marginal and no significance in Sample 1 MPX families and SPX families, respectively; (2) in contrast, the rs4519482-T rs743605-G haplotype in the second sub-block showed highly significant preferential transmission in Sample 1 and combined MPX families [$P_{cor} = 0.014$ and 0.0034; $OR = 1.85$ (95% CI: 1.28–2.67) and 1.53 (95% CI: 1.2–1.96) respectively], with marginal and no significance in the Sample 2 MPX and SPX families, respectively (Table 3).

Table 2 Single SNP FBAT for association of 6 Tag SNPs in *DLX1/DLX2* in SPX families with ASD

SNP	Allele	S	E(S)	Z	P	P_{cor}
rs788172	A	137	143	−0.715	0.47	
	G	233	228	0.715		
rs788173	A	154	159	−0.553	0.58	
	G	256	252	0.553		
rs813720	C	162	169	−0.784	0.43	
	G	262	256	0.784		
rs13390848	T	109	104	0.712	0.48	
	G	213	218	−0.712		
rs4519482	A	85	99	−2.16	0.033	0.26
	T	173	159	2.16		
rs743605	A	186	187	−0.122	0.9	
	G	222	221	0.122		

P_{cor} , Corrected P -values. See Materials and methods for a description of the correction applied for multiple testing.

Discussion

In this study, we provide support for a role of the *DLX1/DLX2* genes in the susceptibility or cause of ASDs. Evidence for association of 5 Tag SNPs in the *DLX1/DLX2* genes, namely rs788172, rs788173, rs813720, rs4519482 and rs743605, was presented. Family-based association analysis revealed that the rs4519482-T allele consistently showed

Table 3 Family-based haplotype association for the rs788172, rs788173, rs813720, rs4519482, and rs743605 SNPs in the *DLX1/DLX2* genes in families with ASD

Haplotypes	Family set	Frequency	S	E(S)	Z	P	P_{cor}
GGGTG	Sample 1 MPX	0.35	90	76	2.84	0.0045	0.036
	Sample 2 MPX	0.34	103	85	3.63	0.00028	0.0022
	Combined MPX	0.34	193	163	4.44	0.00001	0.00007
	SPX	0.37	140	124	2.51	0.012	0.096
GGG--	Sample 1 MPX	0.637	229	214	1.93	0.054	
	Sample 2 MPX	0.65	222	195	3.97	0.00007	0.00058
	Combined MPX	0.644	451	410	4.00	0.00006	0.0005
	SPX	0.64	214	208	0.73	0.47	
---TG	Sample 1 MPX	0.384	116	98	3.14	0.0017	0.014
	Sample 2 MPX	0.42	130	120	1.83	0.068	
	Combined MPX	0.405	246	218	3.54	0.00042	0.0034
	SPX	0.433	149	139	1.47	0.14	

P_{cor} , Corrected P -values. See Materials and methods for a description of the correction applied for multiple testing.

significant association in the 138 Sample 1 MPX families, 169 Sample 2 MPX and the combined group of 307 MPX families, as well as in 306 SPX families. We also showed that the rs788172-G, rs788173-G and rs813720-G alleles were associated with ASD in the Sample 2 MPX families, whereas the rs743605-G allele was associated with ASD in the Sample 1 MPX families, with these four SNPs all showing significant association in the combined MPX family cohort. Interestingly, Hamilton *et al*²² reported an increase in the frequency of the rs743605-G allele in the sample of AGRE families they examined when compared with a control group of samples ($P=0.04$). We also identified a risk allele-containing haplotype, rs788172-G rs788173-G rs813720-G rs4519482-T rs743605-G, that was highly significantly over transmitted from parents to autistic children in all the families studied, with the significance in MPX families ($P_{\text{cor}}=0.00007$) being much greater than that in SPX families. We suggest that the two sub-blocks in this major haplotype block may be linked to different functional polymorphisms in the different cohorts of families.

To the best of our knowledge, this is the first comprehensive Tag SNP-based study covering the *DLX1/DLX2* region using two different sets of MPX family samples. The Sample 1 MPX families were obtained from AGRE and the majority is from the United States. The Sample 2 MPX and all SPX families were recruited by ASD-CARC through an on-line Research Registry and by collaborating clinical geneticists whose clinics include a large number of individuals with definitively diagnosed ASDs. These families are predominantly from Canada, although some are also from the United States.

Many factors may contribute to a failure of replication in association studies of complex genetic disorders,^{35,36} including study design, sample size, recruiting strategies, power issues, and sources of true variability among populations. Consequently, our study illustrating the association of ASD to Tag SNPs in the *DLX1/DLX2* genes in two cohorts of MPX families, as well as SPX families, recruited using different strategies and different subject sources across North America, supports the *DLX1/DLX2* genes as contributing factors to ASD etiology. We suggest that there are two functional polymorphisms in the *DLX1/2* genes, with one present in each of the haplotype sub-blocks, and that the different family cohorts have different proportions of the functional polymorphisms. The Sample 2 families appear to have a higher proportion of a functional allele in the *DLX1* gene and the Sample 1 families have a higher proportion of a functional allele in the *DLX2* gene, reflecting the sub-blocks that are most strongly associated with these respective families. On the basis of this hypothesis, the SPX families would have a higher proportion of families with a functional polymorphism in the *DLX2* gene.

The Homeobox-containing *DLX* gene complexes are of interest because they regulate the development of a subset of cortical and striatal neurons. The *Dlx2* gene has been shown to be involved in forebrain development in the mouse, regulating the migration and differentiation of neurons in the subcortical telencephalon.³⁷ *DLX2* is one of the major transcription factors expressed in GABAergic neurons of the neocortex.³⁸ Among neurotransmitter systems, some of the strongest evidence supports defects in the GABAergic inhibitory system in autism.³⁹ Unlike pyramidal neurons, GABAergic interneurons migrate tangentially for considerable distances, and thus may be susceptible to genetic or environmental factors acting during early development.⁴⁰ Thus the *DLX* gene complexes, which are involved in the differentiation and migration of GABAergic interneurons, can be added to the GABA receptor genes as contributing to the autism phenotype. Furthermore, the *Dlx* genes regulate the expression of the X-linked *Arx* gene in basal ganglia progenitor cell populations,⁴¹ and a subset of patients with a mutation of *ARX* gene have autistic features.^{42,43} Both the neuropathological findings, which indicate altered organization of specific regions of the brain^{44–46} and the co-occurrence of autism with a number of neurological and cognitive disorders, including epilepsy,⁸ support a role for the *DLX* gene complexes in interneuron development and GABAergic-mediated pathophysiological changes that are central components of autism.⁴⁷ We had only a small group of individuals ($n=32$) with confirmed seizures. There was increased transmission of the risk alleles and risk haplotype to affected children in this group of families, although it was much less significant than the overall group of families (data not presented).

Testing for association in SPX families revealed increased transmission of only the rs4519482-T allele ($P=0.033$) in single SNP analyses and over transmission of the GGGTG haplotype ($P=0.012$), for which the significance was lower than that in the MPX families. As most SPX families have relatively small sib-ships, it is possible that this increased transmission is restricted to those families that are 'misclassified' as SPX secondary to reduced penetrance (may only be 10–20%) and small family size (many families had only one or two children). Furthermore, a finding of significant association or a trend towards association with autism in the SPX families strengthens the relevance of our findings, because families with other sporadic genetic causes, as well as those where environmental factors predominate, will reduce the significance substantially.

There are some important limitations to this study. First, we did not have samples from many of the unaffected siblings in the MPX cohorts. For the AGRE families, when we purchased the samples, we chose families in which there were few or no unaffected males. As a result, there could be a bias in the types of families that were included.

This may account for the differences in the *DLX1/2* SNPs that were found to be associated with autism in the two MPX cohorts. If the alternative alleles and haplotypes are significantly transferred to the unaffected siblings, this would strengthen our interpretation. Second, it will be important to obtain more complete information about epilepsy and seizures from each participating family so that we can determine whether there is, indeed, an association of *DLX1/DLX2* haplotypes with this quantitative trait or other traits, such as head circumference or hearing loss, which can be used to stratify the families further. Finally, rare variants in the *DLX1/2* genes will be important to examine as they may lead to the identification of the functional polymorphisms that are responsible for the associations with autism seen in the two MPX family cohorts. Despite these limitations, we believe that the findings do support a role for the *DLX1/DLX2* genes in autism.

To conclude, we hypothesize that functional variants in the *DLX1/DLX2* genes linked with the common risk haplotype found in this study, together with other vulnerability genes and epigenetic and environmental factors, contribute to the development of autism. A search for such functional variants in the *DLX1/DLX2* genes is warranted.

Acknowledgements

This work was supported by a CIHR-Interdisciplinary Health Research Team grant (no. 43820, PI: JJAH) (www.AutismResearch.com), and an OMHF grant (PI: JJAH). NN and AW were trainees with the CIHR/NAAR STIHR ASPIRE (Autism Spectrum Interdisciplinary REsearch)/Training Program (PI: JJAH) (www.AutismTraining.ca). We thank all members of the ASD-CARC team for their support. We are very grateful to the families who participated in this research through our on-line Research Registry (www.AutismResearch.com). We acknowledge the resources provided by the AGRE (Autism Genetics Resource Exchange) consortium and the participating AGRE families. The AGRE is a program of Cure Autism Now and is supported, in part, by grant MH64547 from the National Institute of Mental Health to Daniel H Geschwind (PI).

References

- Bailey A, Le CA, Gottesman I *et al*: Autism as a strongly genetic disorder: evidence from a British twin study. *Psychol Med* 1995; **25**: 63–77.
- Folstein S, Rutter M: Infantile autism: a genetic study of 21 twin pairs. *J Child Psychol Psychiatry* 1977; **18**: 297–321.
- Ritvo ER, Freeman BJ, Mason-Brothers A, Mo A, Ritvo AM: Concordance for the syndrome of autism in 40 pairs of afflicted twins. *Am J Psychiatry* 1985; **142**: 74–77.
- Bolton P, Macdonald H, Pickles A *et al*: A case-control family history study of autism. *J Child Psychol Psychiatry* 1994; **35**: 877–900.
- Hughes C, Plumet MH, Leboyer M: Towards a cognitive phenotype for autism: increased prevalence of executive dysfunction and superior spatial span amongst siblings of children with autism. *J Child Psychol Psychiatry* 1999; **40**: 705–718.
- Abramson RK, Ravan SA, Wright HH *et al*: The relationship between restrictive and repetitive behaviors in individuals with autism and obsessive compulsive symptoms in parents. *Child Psychiatry Hum Dev* 2005; **36**: 155–165.
- Piven J, Palmer P, Jacobi D, Childress D, Arndt S: Broader autism phenotype: evidence from a family history study of multiple-incidence autism families. *Am J Psychiatry* 1997; **154**: 185–190.
- Tuchman R, Rapin I: Epilepsy in autism. *Lancet Neurol* 2002; **1**: 352–358.
- Polleux F, Lauder JM: Toward a developmental neurobiology of autism. *Ment Retard Dev Disabil Res Rev* 2004; **10**: 303–317.
- Cline H: Synaptogenesis: a balancing act between excitation and inhibition. *Curr Biol* 2005; **15**: R203–R205.
- Rubenstein JL, Merzenich MM: Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav* 2003; **2**: 255–267.
- Panganiban G, Rubenstein JL: Developmental functions of the Distal-less/Dlx homeobox genes. *Development* 2002; **129**: 4371–4386.
- Anderson SA, Qiu M, Bulfone A *et al*: Mutations of the homeobox genes *Dlx-1* and *Dlx-2* disrupt the striatal subventricular zone and differentiation of late born striatal neurons. *Neuron* 1997; **19**: 27–37.
- Marin O, Anderson SA, Rubenstein JL: Origin and molecular specification of striatal interneurons. *J Neurosci* 2000; **20**: 6063–6076.
- Anderson SA, Eisenstat DD, Shi L, Rubenstein JL: Interneuron migration from basal forebrain to neocortex: dependence on *Dlx* genes. *Science* 1997; **278**: 474–476.
- Pleasure SJ, Anderson S, Hevner R *et al*: Cell migration from the ganglionic eminences is required for the development of hippocampal GABAergic interneurons. *Neuron* 2000; **28**: 727–740.
- Cobos I, Calcagnotto ME, Vilaythong AJ *et al*: Mice lacking *Dlx1* show subtype-specific loss of interneurons, reduced inhibition and epilepsy. *Nat Neurosci* 2005; **8**: 1059–1068.
- Stuhmer T, Anderson SA, Ekker M, Rubenstein JL: Ectopic expression of the *Dlx* genes induces glutamic acid decarboxylase and *Dlx* expression. *Development* 2002; **129**: 245–252.
- International Molecular Genetic Study of Autism Consortium (IMGSAC): A genomewide screen for autism: strong evidence for linkage to chromosomes 2q, 7q, and 16p. *Am J Hum Genet* 2001; **69**: 570–581.
- Rabionet R, Jaworski JM, Ashley-Koch AE *et al*: Analysis of the autism chromosome 2 linkage region: *GAD1* and other candidate genes. *Neurosci Lett* 2004; **372**: 209–214.
- Nabi R, Zhong H, Serajee FJ, Huq AH: No association between single nucleotide polymorphisms in *DLX6* and *Piccolo* genes at 7q21–q22 and autism. *Am J Med Genet B Neuropsychiatr Genet* 2003; **119**: 98–101.
- Hamilton SP, Woo JM, Carlson EJ, Ghanem N, Ekker M, Rubenstein JL: Analysis of four *DLX* homeobox genes in autistic probands. *BMC Genet* 2005; **6**: 52.
- Geschwind DH, Sowsinski J, Lord C *et al*: The autism genetic resource exchange: a resource for the study of autism and related neuropsychiatric conditions. *Am J Hum Genet* 2001; **69**: 463–466.
- Liu J, Nyholt DR, Magnussen P *et al*: A genomewide screen for autism susceptibility loci. *Am J Hum Genet* 2001; **69**: 327–340.
- Lord C, Rutter M, Le Couteur A: Autism diagnostic interview-revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. *J Autism Dev Disord* 1994; **24**: 659–685.
- Lord C, Rutter M, Goode S *et al*: Autism diagnostic observation schedule: a standardized observation of communicative and social behavior. *J Autism Dev Disord* 1989; **19**: 185–212.
- Cohen IL: Criterion-related validity of the PDD Behavior Inventory. *J Autism Dev Disord* 2003; **33**: 47–53.
- Cohen IL, Schmidt-Lackner S, Romanczyk R, Sudhalter V: The PDD behavior inventory: a rating scale for assessing response to intervention in children with pervasive developmental disorder. *J Autism Dev Disord* 2003; **33**: 31–45.

- 29 Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263–265.
- 30 Ott J: Methods of analysis and resources available for genetic trait mapping. *J Hered* 1999; **90**: 68–70.
- 31 Laird NM, Horvath S, Xu X: Implementing a unified approach to family-based tests of association. *Genet Epidemiol* 2000; **19** (Suppl 1): S36–S42.
- 32 Zhao JH: 2LD, GENECOUNTING and HAP: computer programs for linkage disequilibrium analysis. *Bioinformatics* 2004; **20**: 1325–1326.
- 33 Spielman RS, McGinnis RE, Ewens WJ: Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993; **52**: 506–516.
- 34 Riley B: Linkage studies of schizophrenia. *Neurotox Res* 2004; **6**: 17–34.
- 35 Ackerman H, Usen S, Jallow M, Sisay-Joof F, Pinder M, Kwiatkowski DP: A comparison of case-control and family-based association methods: the example of sickle-cell and malaria. *Ann Hum Genet* 2005; **69**: 559–565.
- 36 Bartlett CW, Gharani N, Millonig JH, Brzustowicz LM: Three autism candidate genes: a synthesis of human genetic analysis with other disciplines. *Int J Dev Neurosci* 2005; **23**: 221–234.
- 37 Yun K, Fischman S, Johnson J, Hrabec de AM, Weinmaster G, Rubenstein JL: Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. *Development* 2002; **129**: 5029–5040.
- 38 Letinic K, Zoncu R, Rakic P: Origin of GABAergic neurons in the human neocortex. *Nature* 2002; **417**: 645–649.
- 39 Dhossche D, Applegate H, Abraham A *et al*: Elevated plasma gamma-aminobutyric acid (GABA) levels in autistic youngsters: stimulus for a GABA hypothesis of autism. *Med Sci Monit* 2002; **8**: R1–R6.
- 40 Marin O, Rubenstein JL: A long, remarkable journey: tangential migration in the telencephalon. *Nat Rev Neurosci* 2001; **2**: 780–790.
- 41 Cobos I, Broccoli V, Rubenstein JL: The vertebrate ortholog of *Aristaless* is regulated by *Dlx* genes in the developing forebrain. *J Comp Neurol* 2005; **483**: 292–303.
- 42 Turner G, Partington M, Kerr B, Mangelsdorf M, Gecz J: Variable expression of mental retardation, autism, seizures, and dystonic hand movements in two families with an identical ARX gene mutation. *Am J Med Genet* 2002; **112**: 405–411.
- 43 Stromme P, Mangelsdorf ME, Scheffer IE, Gecz J: Infantile spasms, dystonia, and other X-linked phenotypes caused by mutations in *Aristaless* related homeobox gene, ARX. *Brain Dev* 2002; **24**: 266–268.
- 44 Steffenburg S: Neuropsychiatric assessment of children with autism: a population-based study. *Dev Med Child Neurol* 1991; **33**: 495–511.
- 45 Ritvo ER, Creel D, Realmuto G *et al*: Electroretinograms in autism: a pilot study of b-wave amplitudes. *Am J Psychiatry* 1988; **145**: 229–232.
- 46 Gillberg C, Steffenburg S, Jakobsson G: Neurobiological findings in 20 relatively gifted children with Kanner-type autism or Asperger syndrome. *Dev Med Child Neurol* 1987; **29**: 641–649.
- 47 Levitt P, Eagleson KL, Powell EM: Regulation of neocortical interneuron development and the implications for neurodevelopmental disorders. *Trends Neurosci* 2004; **27**: 400–406.