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Association of Reelin gene polymorphisms with autism

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Abstract

Genome scans indicate a linkage of autism to the chromosome 7q21–q36 region. Recent studies suggest that the Reelin gene may be one of the loci contributing to the positive linkage between chromosome 7q and autism. However, these studies were relatively small scale, using a few markers in the gene. We investigated 34 single nucleotide polymorphisms (SNPs) in the Reelin gene with an average spacing between the SNPs of 15 kb for evidence of association with autism. There were significant differences in the transmission of the alleles of exon 22 and intron 59 SNP to autistic subjects. Our findings support a role for the Reelin gene in the susceptibility to autism.

Keywords: Reelin; Autism

Autism is a complex trait with a significant genetic component [1–3]. Recent genome screening studies found that multiple regions distributed over many chromosomes had a multipoint maximum lod score greater than 1 [4–10]. The most consistent results were for the 7q21–q32 region. The Reelin gene (approved gene symbol *RELN*) locus maps to chromosome 7q22 between D7S658 (113.92 cM) and D7S1532 (113.92 cM) on the Marshfield map, in proximity to or within the portion of chromosome 7q yielding positive lod scores in most autism linkage studies published to date. Reelin is a large secreted extracellular matrix protein implicated in modulation of neuronal signaling, synaptic transmission, and plasticity [11]. It plays a pivotal role in the development of laminar structures, including the cerebral cortex, hippocampus, cerebellum, and brain-stem nuclei. Reelin controls cortical layering by signaling through the very low density lipoprotein receptor and apolipoprotein E receptor 2, thereby inducing tyrosine phosphorylation of the adaptor protein Disabled-1 and suppressing tau phosphorylation in vivo [12]. Several recent studies suggest Reelin may be one of the loci contributing to the positive linkage between chromosome 7q and autistic disorder. Biochemical analyses of postmortem autistic brain point to Reelin as being involved in the pathology of autism [13]. Blood levels of unprocessed Reelin (410 kDa) are significantly reduced in autistic twins, their fathers, their mothers, and their phenotypically normal siblings versus controls [14]. Linkage disequilibrium of autism with variants in the Reelin gene has also been reported, but not confirmed in other studies [15–21]. However, these studies were relatively small scale, using a few markers in the gene. Reelin may also influence other neurodevelopmental disorders including schizophrenia, bipolar disorder, major depression, and lissencephaly [22,23].

We investigated 34 single nucleotide polymorphisms (SNPs), including five coding and splice region SNPs in the Reelin gene for association with autism. These SNPs are annotated in the dbSNP database and include a G/C SNP in exon 22 (dbSNP: rs362691, V997L), a C/G SNP in exon 34 (dbSNP: rs2229860, P1703R), an A/G polymorphism in exon 45 (dbSNP: rs362746, V2370V), and a G/A SNP in exon 48 (dbSNP: rs2075038, P2510P). We also investigated an A/G transversion (dbSNP: rs607755) located in the 5′ splice junction of exon 6. This SNP has been previously studied in autistic subjects and is expected to affect splicing of the Reelin pre-mRNA [15]. The polymorphisms in exon 22 (L997V) and exon 34 (P1703R) alter amino acid composition of the Reelin protein. The average spacing between the SNPs was 15 kb.

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Table 1
Summary of pedigrees used in this study

Affected subjects per pedigree	Broad diagnosis	Narrow diagnosis
1	4	67
2	179	124
3	15	4
4	2	1
Total pedigrees	200	196

The narrow category includes individuals with a diagnosis of autism based on the Autism Diagnostic Interview-Revised (ADI-R). Broad diagnosis includes in addition those who narrowly miss meeting the ADI-R criteria and those who are diagnosed with PDD or Asperger syndrome.

Our studies revealed significant differences in the transmission of the alleles of SNPs in exon 22 and intron 59 to autistic subjects.

Results and discussion

We investigated 196 Caucasian families ascertained through the Autism Genetics Resource Exchange (AGRE). The summary of pedigrees used in this study is shown in Table 1. The male–female ratio for autistic subjects was 3.5, consistent with increased prevalence of the disorder in boys. The mean age of the children at the time of testing was 7 years. The probands tested negative for possible secondary autism such as inverted duplication of chromosome 15, abnormal neurological examination or neuroimaging, chromosomal abnormalities, perinatal trauma, or an identified genetic syndrome such as Fragile X. Families with identical twins were also excluded from the study due to concerns of possible environmental factors.

The exon 6 splice region SNP was genotyped using restriction enzyme digestion-based assay [15]. The remaining 28 SNPs were genotyped by high-throughput single-base primer extension assay through Orchid BioSciences (exons 34, 45, and 48) and Genaissance. The DNA sequences for designing genotyping assays were obtained from the dbSNP database at http://www.ncbi.nlm.gov/SNP/. The high-throughput genotyping assays were developed, validated, and performed at the genotyping facilities of Orchid BioSciences and Genaissance. The reference ID, location within gene,

Table 2
Reelin gene SNPs investigated in our studies

SNP	dbSNP ID	Location	Allele	Amino acid	Allele frequency	Genotyping rate	Mendelian error rate	HWE p value	Power
1	rs12538307	5′ UTR	C/T	–	0.65/0.35	0.93	0	0.34	0.99
2	rs7800855	Intron 1	A/G	–	0.28/0.72	0.92	0	0.08	0.99
3	rs722277	Intron 1	A/G	–	0.44/0.56	0.99	0.003	0.82	0.99
4	rs3735630	Intron 1	A/G	–	0.51/0.49	0.94	0.003	0.73	0.99
5	rs2299395	Intron 2	A/G	–	0.93/0.07	0.98	0.003	0.19	0.99
6	rs2299388	Intron 2	C/T	–	0.94/0.06	0.98	0.003	0.54	0.98
7	rs39360	Intron 3	A/C	–	0.41/0.59	0.92	0.003	0.46	0.99
8	rs39353	Intron 3	A/C	–	0.41/0.59	0.91	0.003	0.9	0.99
9	rs264373	Intron 3	C/G	–	0.50/0.50	0.92	0.05	0.01	0.97
10	rs2237635	Intron 4	C/G	–	0.24/0.76	0.97	0	0.84	0.99
11	rs607755	Intron 6	A/G	–	0.53/0.47	0.95	0	1	0.98
12	rs661575	Intron 7	C/T	–	0.63/0.37	0.96	0.01	0.83	0.99
13	rs3213611	Intron 7	C/T	–	0.89/0.11	0.97	0.001	0.77	0.99
14	rs2237632	Intron 8	A/G	–	0.11/0.89	0.98	0.001	0.9	0.99
15	rs2283023	Intron 9	A/C	–	0/1	0.97	0.005	N/A	0
16	rs2073559	Intron 11	C/T	–	0.51/0.49	0.92	0.003	0.35	0.99
17	rs2073557	Intron 11	A/G	–	0.43/0.57	0.94	0.01	0.67	0.99
18	rs2240965	Intron 13	C/T	–	0.76/0.24	0.96	0.003	0.03	0.99
19	rs2073551	Intron 16	G/T	–	0.34/0.66	0.97	0.005	0.32	0.99
20	rs977639	Intron 20	C/T	–	0.11/0.89	0.93	0	0.55	0.99
21	rs362691	Exon 22	C/G	V997L	0.89/0.11	0.93	0	1	0.99
22	rs123714	Intron 24	C/T	–	0.29/0.71	0.98	0.001	0.68	0.99
23	rs123713	Intron 27	C/T	–	0.28/0.72	0.99	0	0.43	0.99
24	rs144525	Intron 28	A/G	–	0.69/0.31	0.90	0.008	0.09	0.99
25	rs362726	Intron 31	C/T	–	0.62/0.38	0.97	0.005	0.4	0.99
26	rs2229860	Exon 34	C/G	P1703R	0.995/0.005	0.80	0	1	0.002
27	rs362746	Exon 45	A/G	V2370V	0.97/0.03	0.96	0	0.05	0.68
28	rs362710	Intron 46	C/T	–	0.69/0.31	0.99	0.006	0.29	0.99
29	rs2256658	Intron 46	C/T	–	0.09/0.91	0.96	0.02	0.03	0.99
30	rs2075038	Exon 48	A/G	P2510P	0.02/0.98	0.98	0	1	0.33
31	rs736707	Intron 59	C/T	–	0.23/0.77	0.95	0.003	0.006	0.99
32	rs17150616	Exon 60	C/T	A3358A	0.12/0.88	0.87	0.01	0.4	0.99
33	rs3808045	Intron 61	C/T	–	0.15/0.75	0.90	0.002	0.63	0.99
34	rs7811571	3′ UTR	A/G	–	0.997/0.003	0.94	0	0.98	0.002

These SNPs were genotyped in 196 AGRE families. The reference ID, location within gene, alleles, amino acid change, allele frequencies, genotyping rate, Hardy-Weinberg equilibrium check, and power to detect association at a significance level of 5×10^{-8} are shown. HWE indicates p values for Hardy-Weinberg proportions.

alleles, amino acid change, allele frequencies, genotyping rate, Mendelian error rate, Hardy–Weinberg equilibrium check, and power to detect association at a significance level of 5×10^{-8} are shown in Table 2. Five SNPs in intron 3, intron 13, exon 45, intron 46, and intron 59 demonstrated deviation from Hardy–Weinberg equilibrium (p value not corrected for multiple testing).

We calculated the power of our studies for the different SNPs using the TDT-PC v1.2 program (http://biosun01.biostat.jhsph.edu/~wmchen/pc.html) [24,25]. Assuming a gene effect with genotypic relative risk, $g = 6.0$ for the homozygote, and $g = 3.0$ for the heterozygote, we had sufficient power to detect association at a significance level of $\alpha = 1 \times 10^{-8}$ for all SNPs except SNPs 24, 25, and 28 (dbSNP rs2229860, rs362746, and rs2075038) (Table 2). However, we had less than 50% power to detect an association for a variant with genotypic relative risk $g = 4.0$ for the homozygote. We also had less than 50% power to detect association with rare variants (minor allele frequency less than 0.2) or where linkage disequilibrium between marker and risk allele was less than 0.8. We used a very conservative level of significance of 5×10^{-8} for the power calculation to account for multiple testing of potentially thousands of variants that can be tested in this sample. Recent data suggest that as more SNPs are genotyped, increasing numbers of spurious signals that are stronger than those created by real disease predisposing alleles will be derived [26]. It has been suggested that association studies should be considered as a means to investigate provisionally large sets of genes, to identify polymorphisms that are potentially in linkage disequilibrium with the trait [26]. Replication will then be required to differentiate the real associations from the spurious ones.

Since neighboring SNPs, if sufficiently close, will be inherited with a disease-causing SNP, the inheritance of a haplotype can be used to tag a region of the genome uniquely for closer study. The Reelin gene encompasses 500 kb of genomic DNA with an average spacing between the SNPs of 15 kb. Inspection of the intermarker linkage disequilibrium (LD) (Fig. 1) with the GOLD software package shows that not all marker pairs exhibited a useful level of LD ($D' > 0.7$). However there were useful levels of LD between the SNPs in the regions from intron 11 to intron 28.

Family-based association analyses were performed using the transmission disequilibrium test (TDT), in which prefer-

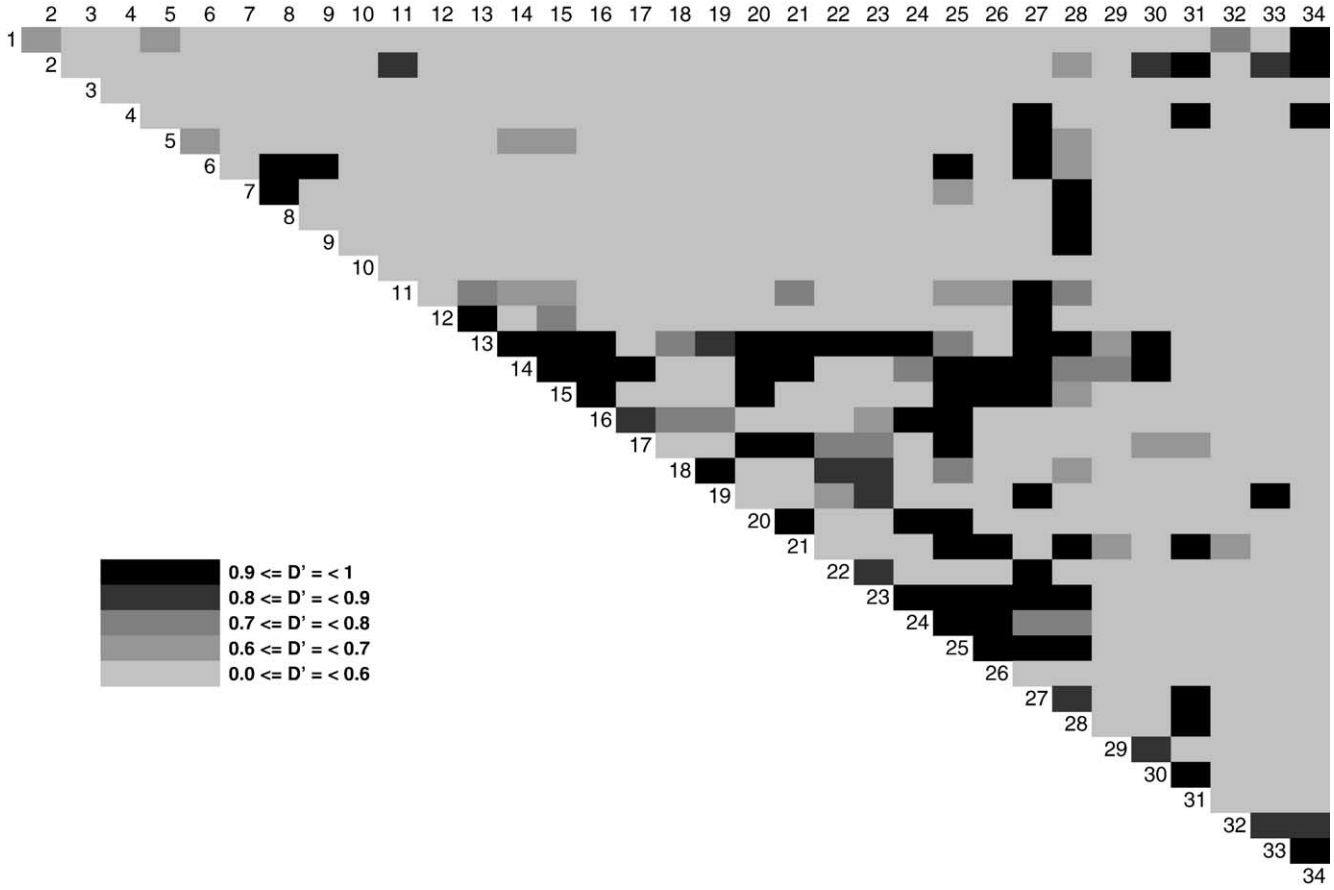

Fig. 1. Linkage disequilibrium among the SNPs in the Reelin gene. The coefficient of disequilibrium, D, is the difference between the observed haplotype frequency and the frequency expected under statistical independence. The D' measure is a proportion of the maximum value of D, whose range extends from −1 to +1, with −1 and +1 representing complete LD and 0 representing free association. SNPs 1 to 34 correspond to the dbSNP IDs shown in Tables 2–5.

ential allelic transmission from heterozygous parents to affected offspring is tested by applying the $(b - c)^2/(b + c)$ statistics and the χ^2 test [27]. The transmission ratio (transmitted/not transmitted) and the 95% confidence interval around the transmission ratio were calculated for each allele of the SNPs. Under a multiplicative model the transmission ratio is an estimator of the genotype relative risk [28]. TDT was performed using UNPHASED, which is a suite of programs for association analysis of multilocus haplotypes from unphased genotype data [29]. Tables 3 and 4 demonstrate results of TDT and TDT-AE studies. There was a significant difference in the transmission of the alleles of a C/T SNP in intron 59 (corrected p value 0.0005) and a C/G SNP in exon 22 (corrected p value 0.003) to autistic subjects, when a broad diagnosis of autism was considered. A synonymous A/G SNP in exon 48 also demonstrated nominally significant difference in the transmission of alleles to the autistic subjects. TDT-AE revealed similar findings. TDT-AE is a likelihood-based transmission disequilibrium test, which maintains a correct type I error rate in the presence of random genotyping errors [32]. Traditional TDT assumes that genotype data are errorless.

Family-based association analyses were also performed using PDT (pedigree disequilibrium test), which is a variation of TDT developed for use in general pedigrees [30,31]. As multiple affected sibs in each family were genotyped for some SNPs, traditional TDT may not be a valid test for association [27]. PDT provides a general and valid test of linkage disequilibrium in such situation [30,31]. The PDT results are shown in Table 5. The most significant result was for a C/T SNP in intron 59 (corrected p value 0.003), when a broad diagnosis of autism was considered. The common C allele was more often transmitted. There was also a nominally significant difference in the transmission of the alleles of C/G SNP in exon 22 ($p = 0.02$) to autistic subjects. The common G allele, which corresponds to valine at amino acid position 997, was more often transmitted. A synonymous A/G SNP in exon 48 also demonstrated a nominally significant ($p = 0.03$)

Table 3
TDT and TDT-AE analyses of the SNPs in the Reelin gene using broad criteria for diagnosis of autism

SNP	dbSNP ID	TDT				Ratio (T/NT)	95% CI for ratio	TDT-AE		
		T/NT	LRS	p value	p value C			LRS	p value	p value C
1	rs12538307	71/82	0.79	0.37	0.34	0.87	0.63–1.19	0.79	0.37	1
2	rs7800855	66/64	0.03	0.86	0.94	1.03	0.73–1.46	0.03	0.86	1
3	rs722277	142/165	1.72	0.18	0.68	0.86	0.69–1.08	1.64	0.20	0.85
4	rs3735630	129/140	0.45	0.5	0.94	0.92	0.72–1.17	0.5	0.48	1
5	rs2299395	44/35	1	0.31	0.81	1.26	0.81–2.00	0.34	0.56	1
6	rs2299388	82/78	0.1	0.75	0.98	1.05	0.77–1.44	0.004	0.95	0.98
7	rs39360	115/115	0	1	1.00	1.00	0.77–1.30	0.015	0.90	1
8	rs39353	123/127	0.06	0.8	1.01	0.97	0.75–1.24	0.03	0.86	1
9	rs264373	159/122	4.64	0.03	0.26	1.30	1.03–1.66	3.5	0.06	0.41
10	rs2237635	48/55	0.48	0.49	0.98	0.87	0.59–1.28	0.49	0.48	1
11	rs607755	138/157	1.2	0.27	0.77	0.88	0.70–1.10	1.18	0.28	0.95
12	rs661575	139/147	0.22	0.64	0.91	0.95	0.75–1.19	0.48	0.49	1
13	rs3213611	61/56	0.21	0.64	0.95	1.09	0.76–1.58	0.09	0.76	1
14	rs2237632	61/62	0.008	0.92	0.98	0.98	0.69–1.41	0.004	0.95	1
15	rs2283023	63/53	0.86	0.35	0.85	1.19	0.83–1.73	0.18	0.67	1
16	rs2073559	123/124	0.34	0.56	0.91	0.99	0.77–1.27	0.33	0.57	0.97
17	rs2073557	139/146	0.17	0.68	0.92	0.95	0.75–1.20	0.13	0.72	1
18	rs2240965	117/99	1.5	0.22	0.75	1.18	0.91–1.55	1.08	0.3	0.93
19	rs2073551	147/139	0.22	0.64	0.99	1.06	0.84–1.34	0.15	0.70	1
20	rs977639	45/65	3.66	0.06	0.41	0.69	0.46–1.00	3.66	0.06	0.5
21	**rs362691**	**83/43**	**9.47**	**0.002**	**0.03**	**1.93**	**1.36–2.87**	**9.47**	**0.002**	**0.05**
22	rs123714	137/127	0.38	0.54	0.92	1.08	0.85–1.38	0.46	0.50	0.94
23	rs123713	138/127	0.48	0.49	1.04	1.09	0.85–1.39	0.46	0.50	1
24	rs144525	116/119	0.04	0.84	0.95	0.97	0.75–1.26	0.61	0.44	1
25	rs362726	160/137	1.78	0.18	0.77	1.17	0.93–1.47	1.58	0.21	0.79
26	rs2229860	1/0	1.39	0.24	0.74			0	1.00	1
27	rs362746	8/9	0	1	1.03	0.89	0.30–2.42	0.06	0.81	1
28	rs362710	158/128	2.58	0.11	0.53	1.23	0.98–1.56	1.93	0.16	0.91
29	rs2256658	38/44	0.44	0.51	0.91	0.86	0.55–1.33	0.007	0.94	1
30	rs2075038	4/14	5.23	0.02	0.23	0.29	0.03–0.71	6.97	0.009	0.1
31	**rs736707**	**106/52**	**18.83**	**1.40×10^{-5}**	**0.0005**	**2.04**	**1.49–2.91**	**17.51**	**0.0001**	**0.003**
32	rs17150616	40/42	0.05	0.83	1.01	0.95	0.61–1.48	0.02	0.89	1
33	rs3808045	43/41	0.048	0.84	0.98	1.05	0.68–1.62	0.16	0.69	1
34	rs7811571	2/0	2.7	0.09	0.51			1.6	0.2	0.97

TDT was performed by TDTPHASE software. LRS indicates value of the likelihood ratio statistic in TDTPHASE and TDT-AE analyses. T, transmitted; NT, not transmitted; ratio, transmission ratio (T/NT). Under a multiplicative model the transmission ratio is an estimator of the genotype relative risk. TDT-AE allows genotyping errors in the analysis of SNP data. p value C indicates after p value Benjamini and Hochberg false recovery rate correction for multiple testing.

Table 4
TDT and TDT-AE analyses of the SNPs in the Reelin gene using strict criteria for diagnosis of autism

SNP	dbSNP ID	TDT				Ratio (T/NT)	95% CI for ratio	TDT-AE		
		T/NT	LRS	*p* value	*p* value C			LRS	*p* value	*p* value C
1	rs12538307	57/64	0.41	0.52	1	0.89	0.62–1.27	0.41	0.52	1
2	rs7800855	49/53	0.16	0.69	0.98	0.92	0.62–1.37	0.16	0.69	1
3	rs722277	98/125	3.3	0.07	0.79	0.78	0.6–1.02	2.9	0.09	1
4	rs3735630	99/94	0.13	0.72	0.91	1.05	0.79–1.4	0.13	0.72	0.94
5	rs2299395	33/26	0.83	0.36	1	1.27	0.76–2.18	0.86	0.35	1
6	rs2299388	63/55	0.54	0.46	1	1.15	0.8–1.66	0.17	0.68	1
7	rs39360	80/85	0.15	0.7	0.95	0.94	0.69–1.28	0.04	0.83	0.94
8	rs39353	89/95	0.2	0.66	0.97	0.94	0.7–1.25	0.12	0.73	0.92
9	rs264373	108/91	1.5	0.23	1	1.19	0.9–1.58	0.33	0.57	1
10	rs2237635	32/36	0.24	0.63	0.97	0.89	0.54–1.43	0.24	0.63	1
11	rs607755	102/97	0.13	0.72	0.94	1.05	0.8–1.39	0.001	0.97	0.97
12	rs661575	95/107	0.71	0.4	1	0.89	0.67–1.17	0.52	0.47	1
13	rs3213611	49/43	0.39	0.53	1	1.14	0.76–1.74	0.39	0.53	1
14	rs2237632	48/47	0.01	0.92	0.98	1.02	0.68–1.54	0.01	0.92	1
15	rs2283023	46/39	0.58	0.45	1	1.18	0.77–1.83	0.14	0.71	0.96
16	rs2073559	87/73	0.09	0.76	0.89	1.19	0.87–1.64	0.07	0.8	0.94
17	rs2073557	91/100	0.42	0.51	1	0.91	0.68–1.21	0.14	0.71	1
18	rs2240965	78/68	0.69	0.41	1	1.15	0.83–1.6	0.52	0.47	1
19	rs2073551	99/98	0.01	0.94	0.97	1.01	0.76–1.34	0.003	0.96	1
20	rs977639	34/49	2.7	0.09	0.8	0.69	0.44–1.06	2.73	0.1	0.85
21	rs362691	51/38	1.9	0.17	1	1.34	0.89–2.08	1.91	0.17	1
22	rs123714	87/84	0.05	0.82	0.93	1.04	0.77–1.4	0.16	0.69	1
23	rs123713	89/84	0.09	0.76	0.92	1.06	0.79–1.43	0.16	0.69	1
24	rs144525	86/84	0.02	0.88	0.97	1.02	0.76–1.39	0.001	0.97	1
25	rs362726	106/99	0.24	0.62	1	1.07	0.81–1.41	0.09	0.76	0.92
26	rs2229860	1/0	1.4	0.24	1		–	0.81	0.37	1
27	rs362746	8/6	0.29	0.59	1	1.33	0.45–4.91	0.97	0.32	1
28	rs362710	104/87	1.5	0.22	1	1.20	0.9–1.6	0.58	0.45	1
29	rs2256658	26/30	0.29	0.59	1	0.87	0.5–1.47	0.21	0.66	1
30	rs2075038	3/10	3.98	0.05	0.85	0.30	0–0.85	3.14	0.09	1
31	**rs736707**	**74/37**	**12.57**	**0.0004**	**0.01**	**2.00**	**1.38–3.07**	**10.12**	**0.002**	**0.07**
32	rs17150616	33/28	0.41	0.52	1	1.18	0.71–1.99	1.03	0.31	1
33	rs3808045	36/30	0.55	0.46	1	1.20	0.74–1.99	0.86	0.36	1
34	rs7811571	1/0	1.4	0.24	1		–	0.81	0.37	1

TDT was performed by TDTPHASE software. LRS indicates value of the likelihood ratio statistic in TDTPHASE and TDT-AE analyses. T, transmitted; NT, not transmitted; ratio, transmission ratio (T/NT). Under a multiplicative model the transmission ratio is an estimator of the genotype relative risk. TDT-AE allows genotyping errors in the analysis of SNP data. *p* value C indicates after *p* value Benjamini and Hochberg false recovery rate correction for multiple testing.

difference in the transmission of alleles to the autistic subjects. There was no sex difference in the transmission of variants.

Although we investigated a total of 34 SNPs, due to the large size of Reelin genes, a sufficient degree of LD (>0.7) was not present between the SNPs to be useful for haplotype studies (Fig. 1). Linkage disequilibrium study with a denser map of SNPs and improved understanding of the haplotype structure in the AGRE sample will be required to tag the region of the Reelin gene containing any putative etiologic variant.

Our results suggest an association of autism with a SNP in intron 59 of the Reelin gene. Although TDT studies revealed significant results for exon 22 SNP, only intron 59 SNP remains significant in PDT studies after Benjamini and Hochberg false recovery rate correction for multiple testing [35]. At present, there is no general consensus as to how to correct for multiple testing in genetic association studies [36]. As the SNPs are related, Bonferroni correction is too conservative and may not be appropriate [37]. Although Benjamini and Hochberg false recovery rate correction is less stringent than Bonferroni correction, it provides a good balance between discovery of statistically significant associations and limitations of false positive occurrences [35].

The analyses in this study were conducted using a broad diagnostic definition. When strict criteria for diagnosis of autism are used, similar but less significant results were obtained (Tables 3 and 4). The more significant results with a broad diagnosis definition may be due to additional information that is gained by inclusion of more affected individuals who do not meet the strict criteria for diagnosis of autism. Alternatively, this may be an indication of biased results.

In our study the common allele of intron 59 SNP was more often transmitted to autistic subjects, suggesting that the rare allele is associated with a protective effect, although one must consider chance nontransmission of the rare allele.

Table 5
PDT analyses of the SNPs in the Reelin gene

SNP	dbSNP ID	Strict diagnosis PDT			Broad diagnosis PDT		
		χ^2	*p* value	*p* value C	χ^2	*p* value	*p* value C
1	rs12538307	0.2	0.9	1	0.045	0.83	1
2	rs7800855	1.1	0.28	1	0.16	0.68	1
3	rs722277	2.9	0.09	0.77	1.07	0.30	0.78
4	rs3735630	0.13	0.71	1	1.20	0.27	0.83
5	rs2299395	0.58	0.45	1	1.43	0.23	0.78
6	rs2299388	0.14	0.71	1	0.04	0.85	0.99
7	rs39360	0.02	0.89	1	0.009	0.93	0.99
8	rs39353	0.18	0.67	1	0.01	0.92	1
9	rs264373	0	1	1	1.67	0.20	0.85
10	rs2237635	0.21	0.65	1	0.11	0.74	1
11	rs607755	0.08	0.78	1	1.97	0.16	0.91
12	rs661575	0.3	0.58	1	2.41	0.12	0.82
13	rs3213611	0.28	0.6	1	0.25	0.62	0.96
14	rs2237632	0.04	0.84	1	0.27	0.61	0.99
15	rs2283023	0.04	0.84	1	1.72	0.19	0.92
16	rs2073559	0.004	0.95	1	0.38	0.54	1
17	rs2073557	0.005	0.95	1	0.05	0.83	1
18	rs2240965	0.49	0.48	1.3	1.14	0.29	0.82
19	rs2073551	0.02	0.9	1	0.03	0.87	0.99
20	rs977639	3.7	0.06	1	3.99	0.05	0.42
21	rs362691	1.8	0.18	1	5.98	0.02	0.34
22	rs123714	0.23	0.53	1	0.31	0.58	1
23	rs123713	0.17	0.68	1	0.09	0.77	1
24	rs144525	0	1	1	0.96	0.33	0.70
25	rs362726	0.005	0.95	1	1.54	0.21	0.79
26	rs2229860	1	0.32	1	1	0.32	0.72
27	rs362746	0.33	0.57	1	0	1	1
28	rs362710	0.1	0.74	1	1.04	0.31	0.75
29	rs2256658	0.49	0.48	1	0.72	0.4	0.8
30	rs2075038	3	0.08	0.9	5	0.03	0.34
31	**rs736707**	**7.1**	**0.007**	**0.24**	**15.15**	**0.0001**	**0.003**
32	rs17150616	1.1	0.3	1	0.10	0.75	1
33	rs3808045	0.7	0.4	1	0.29	0.59	1
34	rs7811571	0	1	1	0	1	1

PDT is a form of TDT suitable for analysis of general pedigrees such that all family data may be used without nullifying the validity of the association test, even when there is more than one affected in a family. *p* value C indicates after *p* value Benjamini and Hochberg false recovery rate correction for multiple testing.

The most significant result was obtained with the SNP in intron 59. In TDT studies, there were significant differences in the transmission of alleles of exon 22 and tendencies toward significance for exon 48 SNPs, but after correction for multiple testing only the result for intron 59 was significant in PDT studies. The C/G SNP in exon 22 corresponds to a conservative amino acid change and the G/A SNP in exon 48 (P2501P) does not cause any alteration in amino acid composition. It is likely that intron 59 SNP is not an etiologic variant, but may be in linkage disequilibrium with an etiologic variant. However, there is a steadily accumulating body of evidence to support a role for intronic variants in complex diseases [38]. As the linkage disequilibrium pattern in the Reelin gene is incompletely understood, it is difficult to assess the source of the association signal. Investigation of three additional markers distal to intron 59 SNP did not reveal any association, suggesting that the association signal is unlikely to be due to a mutation in a gene 3′ of Reelin.

It is to be noted that intron 3, intron 13, exon 45, and intron 59 SNPs demonstrated deviations from Hardy–Weinberg proportions (HWE). The *p* values for the deviation from HWE for these SNPs were 0.01, 0.03, 0.05, and 0.006; these *p* values were not corrected for multiple testing. If one routinely performs testing for Hardy–Weinberg proportions for a large number of loci, deviations from HWE for some loci are to be expected. The deviation from expected proportions for intron 59 SNP raises the possibility that the association signal may be due to nonrandom genotyping errors. However, in a recently published association study, the intron 59 SNP was nominally significant in 85 families from AGRE, suggesting that the association signal from intron 59 in this study is real [21].

Linkage disequilibrium of autism with variants in the Reelin gene has been investigated before [15–17]. The Reelin gene 5′ untranslated region contains a low and variable number of GCG repeats, and this has been correlated with genetic susceptibility to autism [15]. Other studies, however, demonstrated no association [16] or equivocal results [17] for the 5′ GCG repeats. AGRE families have previously been used in genetic studies of Reelin [17,21]. The most significant association identified in two previous studies with AGRE samples was for the 5′ UTR GGC repeat; however, in one study a nominally significant difference in the transmission of intron 59 alleles was found [21]. In this study, the 5′ UTR repeat was not studied, but the intron 59 SNP demonstrated significant difference in transmission of alleles to autistic subjects, while polymorphisms in exon 22 and exon 48 demonstrated a trend toward significant difference in the transmission to autistic subjects. Increased transmission was obtained for the frequent allele of intron 59. Although the association could be real, and the common allele could be the disease-causing mutation, from a population genetics perspective, it is unlikely that a disease-causing mutation will become the common allele in the population unless the allele confers some selective advantage. The common allele may, however, be in linkage disequilibrium with another disease-predisposing allele. Alternatively, undetected genotyping errors may cause apparent overtransmission of the common allele in a transmission disequilibrium test [39]. The sequence of Reelin protein begins with a signal peptide followed by a region with similarity to F-spondin, a unique region, and then eight 300-amino-acid-long repeats. Exon 22 corresponds to the second repeat of the Reelin protein, whereas exon 48 corresponds to the sixth repeat. The exon 22 G/C SNP causes a valine to leucine change in amino acid position 997, which is a conservative change in the Reelin protein in the region of the second repeat. Review of the databases suggests that leucine is the common allele in rat and chick, while valine is the common allele in human and mouse. The exon 22 SNP has been studied previously. Bonora et al. did not find any evidence of association of the exon 22 SNP, but they reported a trend toward transmission disequilibrium of maternal alleles of an intron 31 SNP, which was in linkage disequilibrium with the exon 22 SNP.

In a recent study, a trend toward significance was observed for SNPs in exons 6, 45, and 50 [21] in a subset of autism samples.

PI3-kinase (PIK3) interacts with the adaptor protein Dab1 in response to Reelin signaling and is required for normal cortical lamination, suggesting that PI3-kinase signaling is involved in Reelin's regulation of neuronal position during brain development [33,34]. This is of interest as PIK3 signaling is disrupted in tuberous sclerosis, a condition with a very high risk for autism. We have previously reported a nominally positive association of autistic spectrum disorder with variants in PIK3CG and INPP1, two genes that map to areas of linkage to autism and act in the PIK3 pathway [40]. We performed TDT studies of Reelin while conditioning at the loci of the PIK3CG and INPP1 genes. There was no evidence of interaction between the two loci and Reelin variants (data not shown).

There is a debate as to whether de novo sporadic mutations in a single gene or a multilocus model involving two or more interacting loci underlie the autistic phenotype [41,42]. A recent study reported novel missense variants in autistic subjects that were absent in a large control group; however, the low frequency of these mutations did not explain the relatively strong linkage results on 7q [18]. It is to be noted that our study relies on the hypothesis that common variants underlie susceptibility to common diseases. Our findings provide additional evidence of a role for Reelin gene polymorphisms in the susceptibility to autism. Investigation of independent samples will help to determine whether our results represent true associations or spurious chance findings.

Materials and methods

Subjects

The study protocol was approved by the Institutional Review Board at Wayne State University. DNA samples from 196 autistic disorder Caucasian families were obtained from AGRE. AGRE, developed and maintained by the Cure Autism Now Foundation, is a central repository of family DNA samples for genetic studies of autism [43]. Medical histories, physical neurological exam data, Peabody scores, Autism Diagnostic Interview—Revised (ADI-R), Autism Diagnostic Observation Scale, Vineland Adaptive Behavior Scales, Raven Progressive Matrices, and handedness testing results with all interview data points and computer-scored algorithm results are available at the AGRE Web site (http://www.agre.org). Diagnoses were confirmed using the ADI-R protocol [44]. Criteria for different diagnostic categories within the spectrum of autism disorders are available at the AGRE Web site (http://www.agre.org). The narrow category includes individuals with a diagnosis of autism based on the ADI-R. For this study, we also used a broad diagnostic definition, and all subjects with a diagnosis of autism, Asperger syndrome, and "not quite autism" (NQA) were considered affected. The broad diagnostic category encompasses "broad spectrum" subjects ranging from mildly to severely impaired and includes individuals who were categorized as having a disorder that was NQA, as well as subjects with pervasive developmental disorder and Asperger syndrome. NQA represents individuals who are no more than 1 point away from meeting criteria for autism in any or all of the three content domains or individuals who meet criteria in all domains but do not meet age-at-onset criteria. Thus, they are individuals who narrowly miss meeting the ADI-R criteria. Broad spectrum defines individuals ranging from mildly to severely impaired.

Genotyping

SNPs were selected from databases such as the dbSNP, human genome variation database, and human gene mutation database, which are representative of the Caucasian population and are thus relevant to the Caucasian families from AGRE that were investigated in this study. The exon 6 SNP was genotyped using a restriction enzyme digestion-based assay. The primers and the PCR conditions for assays for SNPs were as described before [15]. The remaining SNPs were genotyped by either a high-throughput single-base primer extension assay through Orchid BioSciences' SNPstream UHT services (http://www.orchid.com), which uses fluorescence detection (exons 34, 45, and 48) or through Genaissance (http://www.genaissance.com), which employs Sequenom's MassARRAY platform and mass spectrometry to detect the primer extension products (remaining 30 SNPs). Single-base primer extension involves the annealing of an oligonucleotide primer to a single-stranded PCR amplicon at a location that lies immediately adjacent to, but not including, the polymorphic SNP site, followed by the addition of a DNA polymerase and subsequent enzymatic extension of the primer in the presence only of chain-terminating dideoxynucleotides, which are labeled to facilitate subsequent detection of the identity of the single incorporated nucleotide. Each of the primer extension products has also a unique molecular weight that allows the associated genotype to be precisely identified using mass spectrometry.

Statistical and genetic analysis

The PedCheck and Merlin programs were used to detect genotyping errors [45,46]. Mistyping analyses were also performed using Sim Walk2 [47]. Marker allele frequencies were obtained by counting parental genotypes. Linkage disequilibrium between the markers was analyzed by the Sim Walk2 and GOLD software packages using parental gametic haplotypes [47,48]. Family based association analyses were performed using the TDT and PDT [31]. The PDT analysis program allows the analysis of all family data, even when there is more than one affected in a family and thus results in substantial gains in power compared to traditional TDT. Association analyses were also performed with TDT-AE, which is a transmission disequilibrium test that allows for random genotyping errors [32].

For TDT analyses, we used UNPHASED, which is a suite of programs for association analysis of multilocus haplotypes from unphased genotype data [49]. This program provides both global p values, which assess the significance of transmission distortion for all the test haplotypes, and p values that assess the significance of transmission distortion for specific haplotypes. Permutation or bootstrap test procedure for testing significance is available in UNPHASED, when the χ^2 approximations are not appropriate. UNPHASED provides association tests conditioning on additional loci that may already be associated and in linkage disequilibrium with the test loci. For phase-certain haplotype data, this is the Conditional ETDT [50,51]; when haplotypes are uncertain, the test is of equality of odds ratios for haplotypes identical at conditioning loci. There is no specific test for gene–gene interaction, but the likelihoods under various options can be compared to test certain hypotheses. For example, comparing the alternative likelihoods for the conditional test with and without the −maineffects option gives a test for interactions between test and conditioning loci.

The program TDT Power Calculator (TDT-PC v1.2), based on Knapp's first approximation, was used to estimate power for the association analyses [24,25].

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