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Association of the neuronal cell adhesion molecule (NRCAM) gene variants with autism

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Abstract

Autism is a severe neurodevelopmental disorder of early childhood. Genetic factors play an important role in the aetiology of the disorder. In this study, we considered the *NRCAM* gene as a candidate gene of autism. This gene is expressed in the central nervous system and located in the 7q region, a susceptibility locus of autism. We conducted a case-control study of 18 single nucleotide polymorphisms (SNPs) within the *NRCAM* gene for possible association with autism in 170 autistic patients and 214 normal controls in a Japanese population. Seven SNPs in the *NRCAM* gene were significantly associated with autism, among which rs2300045 indicated the most prominent result ($p=0.0009$ uncorrected, $p=0.017$ corrected). In haplotype analyses, several individual haplotypes, including a common NRCAM haplotype C-T-T-C-T-T-G-C for rs3763463, rs1859767, rs1034825, rs2300045, rs2300043, rs2300039, rs722519, and rs2216259, showed a significant association after Bonferroni correction ($p=0.0035$ uncorrected, $p=0.028$ corrected). These haplotypes were located in the 5′ intron-2 region of the gene. In addition, we also assessed the above mentioned SNPs and haplotypes using the transmission disequilibrium test with 148 trios of autistic families. Haplotype G-T-T-T-T-C-G-C in the same eight SNPs was also associated with autism. In summary, our findings provide evidence for a significant association of NRCAM with autism. Considering the important role of the *NRCAM* gene in brain development, our results therefore indicated that the *NRCAM* gene is one of the strong candidate genes for autism.

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Key words: Association study, autistic disorder, haplotype block, *NRCAM* gene.

Introduction

Autism (MIM 209850) is a severe neurodevelopmental disorder characterized by marked social deficits, delay and deviance in language development and communication skills, and a restricted range of stereotypical, repetitive behaviour and interests. The disorder usually manifests itself during the first 3 years of life.

Twin and family studies have indicated the robust role of genetic factors in the development of autism (Folstein and Rosen-Sheidley, 2001). The respective concordance rates of the disorder in monozygotic and dizygotic twins were reported to be 91% and 0% (Steffenburg et al., 1989) and 60% and 0% (Bailey et al., 1995), respectively. Statistical models suggest that between two and 10 loci (Pickles et al., 1995) or >15

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(Risch et al., 1999) loci are probably implicated. Although the results of linkage studies have not confirmed a specific locus, several studies have provided evidence for the chromosomal 7q region as a susceptibility locus (or loci) of autism (Folstein and Rosen-Sheidley, 2001).

The neuronal cell adhesion molecule (NRCAM: MIM 601581) gene encodes for the NRCAM protein, a member of the immunoglobulin superfamily of cell adhesion molecules (CAMs). CAMs are found in the nervous system (Lane et al., 1996). The *NRCAM* gene is located at 7q31, a candidate region for autism, and contains 34 exons extending over 316 kb (Dry et al., 2001).

The NRCAM protein is expressed at its highest levels in several regions, including the brain and the spinal cord during nervous system development (Lustig et al., 2001). The protein interacts with other molecules and promotes directional signalling during axonal cone growth (Lane et al., 1996; Lustig et al., 2001). NRCAM also serves as a receptor for several different neuronal recognition molecules (Lustig et al., 1999). These facts suggest that NRCAM may be involved in organizing the neural network during brain development. Thus, NRCAM may play a significant role in the development of the autistic brain.

To our knowledge, three studies have investigated the genetic association between NRCAM and autism. While two of them observed the nominal association between the *NRCAM* gene and autism without multiple correction (Bonora et al., 2005; Sakurai et al., 2006), the other reported a negative result (Hutcheson et al., 2004). We, therefore, attempted to confirm whether significant associations exist between this gene and autism by using a case-control design between 170 autistic patients and 214 normal controls and applying Bonferroni correction.

Methods and materials

Subjects

The patients for the case-control study comprised 170 unrelated Japanese with autism (147 males, 23 females, mean age 20.8 yr, range 3–41 yr). In addition, families chosen for the transmission disequilibrium test (TDT) consisted of 148 Japanese unrelated autistic individuals (134 males, 14 females) and their parents. They were recruited from the outpatient clinics of the Department of Psychiatry, Tokyo University Hospital and Tokai University Hospital, and seven day-care facilities for subjects with developmental disorders. All the hospitals and facilities are located around Tokyo. Ninety-eight cases from Tokai University were assigned from probands in the trios for TDT. The diagnosis of autistic disorder was reached if the subject's condition met all the conditions for autistic disorder according to DSM-IV criteria. The Child Behaviour Questionnaire – Revised (CBQ-R; Izutsu et al., 2001) was used as a supplementary scale for diagnosis. We used the CBQ-R to find out the characteristic behaviour of autism in the patients. Parents were asked to rate their child for each item.

The CBQ-R is a supplementary scale for the diagnosis of pervasive developmental disorders in child and adult patients. The reliability and validity of the questionnaire as a supplementary tool for diagnosis has been confirmed in 269 pervasive developmental disorder (PDD) subjects (age 2–26 yr) and 76 mental retardation (MR) subjects (age 3–26 yr) (Izutsu et al., 2001).

The controls consisted of 214 unrelated Japanese healthy volunteers (145 males, 69 females; mean age 34.6 yr, range 21–65 yr). All controls were briefly interviewed by one of the authors to confirm that they were unrelated. The controls were mainly recruited from the hospital and facility staff. All the controls resided in the same area (Kanto District or around Tokyo) as the patients. All the patients and controls were ethnically Japanese, with no parents or grandparents of ethnicity other than Japanese. In the subjects, no apparent physical anomalies were observed.

At the interview with the parent(s), the CBQ-R was used to assist in the evaluation of autism-specific behaviour and symptoms. After the initial observation and interview, we followed up by examining the patients' behaviour and symptoms for several months (for at least 6 months in most cases), and those who were not considered to meet DSM-IV criteria during the follow-up were excluded from the sample.

The present study was approved by the Ethical Committee, Faculty of Medicine of the University of Tokyo and Tokai University. The objective of the present study was clearly expressed, and written informed consent was obtained from all subjects and healthy volunteers.

Single nucleotide polymorphism (SNP) selection and genotyping

Peripheral blood was obtained and genomic DNA was extracted using the standard phenol-chloroform method. SNPs were analysed by the Taqman method-based assay using the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA).

Table 1. Allele frequencies of 18 SNPs of the *NRCAM* gene in autistic patients and controls

Locus	db SNP ID	Allele A/B[a] (amino acids)[b]	Minor allele frequencies									
			Patients		Controls						HW	
			n	(%)	*n*	(%)	χ^2	*p* value	OR	(95% CI)	Patients	Controls
SNP 1	rs3763463	C/G	44	(13)	58	(14)	0.10	0.7509	1.07	(0.70–0.63)	0.55	0.56
SNP 2	rs1859767	A/T	117	(35)	194	(46)	8.88	**0.0029**	1.56	(1.16–2.10)	0.39	0.20
SNP 3	rs1034825	T/C	195	(58)	200	(47)	7.98	**0.0047**	1.51	(1.13–2.02)	0.13	0.12
SNP 4	rs2300045	T/C	109	(33)	186	(45)	10.96	**0.0009**	0.60	(0.45–0.82)	0.67	0.12
SNP 5	rs2300043	T/C	189	(56)	197	(46)	7.39	**0.0066**	1.49	(1.12–1.98)	0.50	0.20
SNP 6	rs2300039	T/C	92	(27)	99	(23)	1.59	0.2071	1.24	(0.89–1.72)	0.56	0.85
SNP 7	rs722519	G/A	17	(5)	15	(4)	1.04	0.3077	1.44	(0.71–2.94)	0.49	0.14
SNP 8	rs2216259	C/A	109	(32)	132	(31)	0.08	0.7755	1.05	(0.77–1.42)	0.23	0.66
SNP 9	rs2300012	C/A	161	(48)	204	(48)	0.04	0.8460	0.97	(0.73–1.29)	0.61	0.017
SNP 10	rs1269655	G/C	72	(21)	60	(14)	6.87	**0.0088**	1.65	(1.13–2.41)	0.54	0.49
SNP 11	rs1269642	T/G	77	(22)	64	(15)	7.37	**0.0066**	1.66	(1.15–2.40)	0.33	0.93
SNP 12	rs1269627	T/C	44	(13)	54	(13)	0.00	0.9678	1.01	(0.66–1.55)	0.56	0.12
SNP 13	rs1269621	A/G (Asn/Asn)	89	(26)	84	(20)	4.71	**0.0301**	0.69	(0.49–0.97)	0.19	0.46
SNP 14	rs383341	T/A	44	(13)	51	(12)	0.13	0.7176	1.08	(0.70–1.67)	0.56	0.22
SNP 15	rs402029	T/A	42	(13)	48	(11)	0.23	0.6341	1.11	(0.72–1.73)	0.66	0.39
SNP 16	rs6958498	C/G (Pro/Ala)	42	(12)	53	(13)	0.01	0.9364	1.02	(0.66–1.57)	0.67	0.30
SNP 17	rs449514	G/T	75	(23)	97	(23)	0.10	0.7465	1.06	(0.75–1.49)	0.54	0.15
SNP 18	rs409969	G/A	75	(22)	93	(22)	0.01	0.9327	1.01	(0.72–1.43)	0.56	0.13

SNP, Single nucleotide polymorphism; HW, Hardy–Weinberg equilibrium (uncorrected).

[a] Alleles 'B' are minor alleles.

[b] Amino residues in parentheses correspond to allele A/B.

Bold values indicate $p < 0.05$.

The primers and probes of the ABI Assays-on-Demand™ kit (Applied Biosystems) were used for genotyping.

The SNPs of the *NRCAM* gene were selected from the primer-probe list of Assays-on-Demand™ products for the ABI 7900HT to cover the full length of the gene. To increase statistical power, we selected SNPs considering the minor allele frequencies indicated in the ABI primer-probe list and the National Center for Biotechnology Information (NCBI) dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). We chose 18 sets of primers and probes for each SNP. The db SNP IDs of the SNPs are listed in Table 1. The locations of the SNPs are shown in Figure 1a.

Statistical analysis

Statistical analyses were performed using the SAS Genetics 9.1 software (SAS Institute Inc., Cary, NC, USA). The frequencies of the alleles and genotypes of each SNP were compared between patients and controls using the χ^2 test. Subsequently, coefficients D' of the linkage disequilibrium (LD) between SNPs were analysed. The HAPLOVIEW program (Barrett et al., 2005) was used to calculate D' as a measure for LD. D' is a standardized, pairwise disequilibrium value independent of allele frequencies. The frequencies of haplotypes consisting of SNPs, which were at high values of D' (the frequencies of the haplotype blocks), were estimated. To compare the haplotype frequencies between patients and controls, we calculated the exact *p* values based on the likelihood ratio test with 10 000 permutations. We used the Bonferroni method to correct multiple testing.

In the replication analysis using TDT samples, SAS Genetics software was also used for analysis of each SNP. We then assessed haplotype analysis by the TDT using the UNPHASED program (http://www.mrc-bsu.cam.ac.uk/personal/frank/#software) with 10 000 permutations.

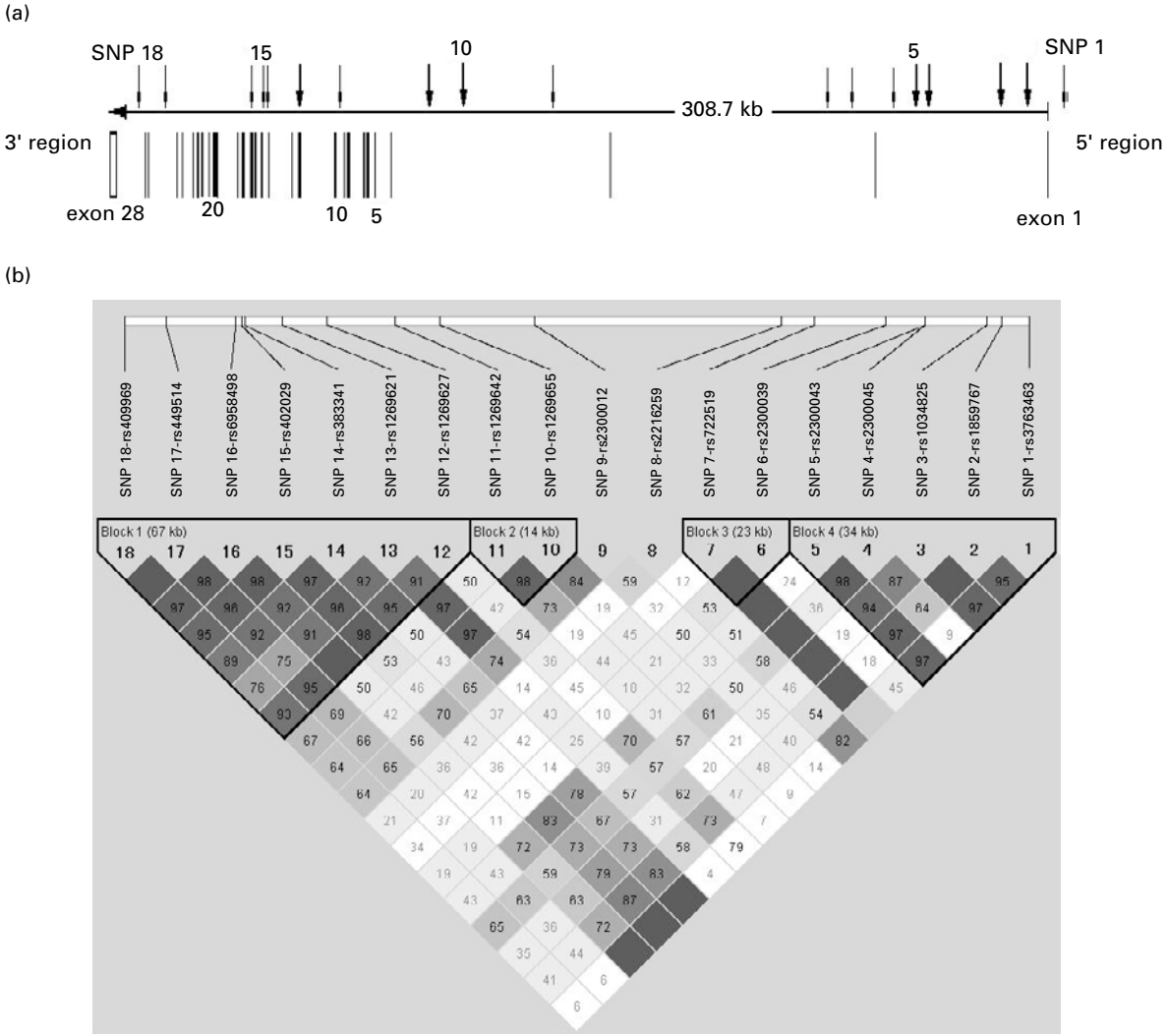

Figure 1. (a) The structure of the *NRCAM* gene, and positions of the single nucleotide polymorphisms (SNPs) (presented from the 5′ to the 3′ region of the gene). All SNPs are denoted numerically with reference to Table 1. The vertical black bars indicate exons. Open boxes indicate untranslated regions. The SNPs associated with autism in allele distributions are indicated in bold style. (b) Haplotype heat-map of the *NRCAM* gene region. Pairwise linkage disequilibrium (LD) among SNPs was investigated using HAPLOVIEW software (Barrett et al., 2005). Regions of high LD and high LOD scores ($D' = 1$ and LOD > 2) are shown in black without values of '100'. Markers with lower LD ($D' < 1$) are in light grey with the intensity decreased with decreasing D' value. White regions represent low LD and low LOD scores (LOD < 2). Haplotype blocks were determined by identifying the first and last markers in a block, which are in strong LD with all intermediate markers.

Results

The allele frequencies of the SNPs of the *NRCAM* gene are summarized in Table 1. We found a significant association with autism for 7/18 SNPs (rs1859767, rs1034825, rs2300045, rs2300043, rs1269655, rs1269642, and rs1269621). Association was identified for the SNPs in intron 1 (rs1859767, rs1034825, rs2300045, rs2300043), intron 3 (rs1269655, rs1269642), and exon 11 (rs1269621) of the *NRCAM* gene (Table 1). The association for rs2300045 remained significant after Bonferroni correction for all 18 SNPs.

Regarding genotype distribution, the whole of the 95% confidence interval of odds ratios of minor/minor genotypes remained < 1.00, or > 1.00 in six of the seven SNPs described above (rs1859767,

Table 2. Genotype distributions of the *NRCAM* gene in autism patients and controls

SNP …		Major/major		Major/minor		Minor/minor		Odds ratios (vs. major/major)	
Allele A/B[a]		*n*	(%)	*n*	(%)	*n*	(%)	Minor/minor (95% CI)	Major/minor (95% CI)
SNP 2 rs1859767	Case	73	(44)	71	(43)	23	(14)	**0.43**	0.68
A/T	Control	67	(32)	96	(45)	49	(23)	(**0.24–0.78**)	(0.43–1.07)
SNP 3 rs1034825	Case	35	(21)	73	(43)	61	(36)	**2.10**	1.42
T/C	Control	64	(30)	94	(45)	53	(25)	(**1.21–3.66**)	(0.85–2.37)
SNP 4 rs2300045	Case	77	(46)	71	(43)	19	(11)	**0.37**	0.70
T/C	Control	70	(33)	92	(44)	47	(22)	(**0.20–0.69**)	(0.45–1.10)
SNP 5 rs2300043	Case	35	(21)	79	(47)	55	(33)	**2.11**	1.56
G/C	Control	67	(31)	97	(45)	50	(23)	(**1.20–3.69**)	(0.94–2.58)
SNP 10 rs1269655	Case	106	(63)	54	(32)	9	(5)	**4.42**	1.47
G/C	Control	156	(73)	54	(25)	3	(1)	(**1.17–16.69**)	(0.94–2.31)
SNP 11 rs1269642	Case	103	(61)	55	(33)	11	(7)	**3.27**	1.51
T/G	Control	153	(72)	54	(25)	5	(2)	(**1.10–9.68**)	(0.96–2.38)
SNP 13 rs1269621	Case	95	(56)	59	(35)	15	(9)	2.19	1.35
A/G	Control	139	(65)	64	(30)	10	(5)	(0.95–5.09)	(0.87–2.09)

SNP, Single nucleotide polymorphism.
p values are based on 10 000 permutation test with 2 d.f.
[a] Alleles 'B' are minor alleles.
Bold face indicates significant SNPs and odds ratios.

rs1034825, rs2300045, rs2300043, rs1269655, rs1269642), which were significant in allele distributions (see Table 2).

Deviation from Hardy–Weinberg equilibrium was observed in the distribution of SNP rs2300012 ($p = 0.017$) in the controls. The disequilibrium for rs2300012 in the *NRCAM* gene was not statistically significant after correction for the 36 tests (cases and controls of the 18 SNPs). Thus, this may have been a chance observation.

Figure 1b shows LD within the *NRCAM* gene in the form of a heat-map. According to the heat-map, the D' values of SNPs 1–5, 6–7, 10–11, and 12–18 in the *NRCAM* gene were forming haplotype blocks respectively. The first block, containing SNPs 1–5 (rs3763463, rs1859767, rs1034825, rs2300045, rs2300043), covered the 34-kb 5′ intron-1 region. The second block, covered the 23-kb region of intron 1–intron 2, and included SNPs 6–7 (rs2300039, rs722519). The third block, located within a 14-kb region of intron 3, included SNPs 10–11 (rs1269655, rs1269642). The fourth block extended over a 73-kb region of intron 10–intron 28 and included SNPs 12–18 (rs1269627, rs1269621, rs383341, rs402029, rs6958498, rs449514, rs409969). Regarding the pairwise D' values, we therefore tested the five-marker haplotype of SNPs 1–5, the two-marker haplotype of SNPs 6–7, the two-marker haplotype of SNPs 10–11, and the seven-marker haplotype of SNPs 12–18. In addition, considering the LD map (Figure 1), we also tested the eight-marker haplotype of SNPs 1–8 and the 10-marker haplotype SNPs 9–18 which were respectively determined by the first and last markers that are in strong LD.

The estimated haplotype frequencies are shown in Table 3. We found two major haplotypes, C-A-C-T-C and C-T-T-C-G (with frequencies of 45% and 35%, respectively), for SNPs 1–5, which showed significant associations with autism. The appearance frequency of the C-A-C-T-C haplotype significantly increased in the autism patients ($p = 0.0071$). Similarly, the frequency of the C-T-T-C-G haplotype significantly decreased in these patients ($p = 0.00080$). Both of these associations remained significant after Bonferroni correction for testing five major haplotypes ($p = 0.0355$, $p = 0.0040$, corrected respectively; haplotype frequencies $>3\%$). In addition, a major extended haplotype, C-T-T-C-T-T-G-C for SNPs 1–8 [frequencies: 21.9% (decreased), $p = 0.0035$ uncorrected, $p = 0.028$ corrected; haplotype frequencies $>3\%$] showed significant associations with autism even after Bonferroni correction. Furthermore, we also found two major haplotypes, G-T and C-G [frequencies: 81% (decreased) and 17% (increased), $p = 0.014$ and $p = 0.011$ uncorrected, respectively], for SNPs 10–11, which showed significant associations with autism. Both of these

Table 3. Estimated frequencies and permutation p values for association of major NRCAM haplotypes

Haplotype	Frequencies			χ^2	p value
	Case	Control	Combined		
(SNPs 1–5) rs3763463, rs1859767, rs1034825, rs2300045, rs2300043					
				global $p=0.2021$	
C-A-C-T-C	**0.544**	**0.440**	**0.486**	**8.1894**	**0.0071**
C-T-T-C-G	**0.194**	**0.305**	**0.255**	**12.3188**	**0.0008**
G-T-T-T-G	0.083	0.072	0.077	0.2907	0.5843
C-A-T-C-G	0.061	0.051	0.056	0.3639	0.5795
G-T-T-C-G	0.047	0.055	0.053	0.2193	0.6657
(SNPs 1–8) rs3763463, rs1859767, rs1034825, rs2300045, rs2300043, rs2300039, rs722519, rs2216259					
				global $p=0.2794$	
C-T-T-C-T-T-G-C	**0.163**	**0.266**	**0.219**	**11.7703**	**0.0035**
C-A-C-T-C-T-G-A	0.225	0.192	0.205	1.2269	0.3114
C-A-C-T-C-T-G-C	0.175	0.132	0.151	2.7739	0.1168
C-A-C-T-C-C-G-C	0.096	0.075	0.086	1.0827	0.3498
G-T-T-T-T-C-G-C	0.074	0.065	0.068	0.2365	0.7009
G-T-T-C-T-T-G-C	0.047	0.057	0.053	0.3754	0.6039
C-A-T-C-T-C-G-C	0.041	0.042	0.041	0.0323	0.8882
C-T-T-C-T-T-G-A	0.033	0.040	0.037	0.2331	0.7717
(SNPs 10–11) rs126965, rs1269642					
				global $p=0.0598$	
G-T	**0.769**	**0.847**	**0.813**	**7.5619**	**0.0142**
C-G	**0.210**	**0.138**	**0.170**	**6.8322**	**0.0108**
(SNPs 9–18) rs2300012, rs1269655, rs1269642, rs1269627, rs1269621, rs383341, rs402029, rs6958498, rs449514, rs409969					
				global $p=0.2021$	
A-G-T-T-A-T-T-C-G-G	0.416	0.438	0.428	0.3967	0.575
C-G-T-T-A-T-T-C-G-G	0.283	0.297	0.291	0.188	0.6853
C-C-G-T-G-T-T-C-T-A	0.078	0.058	0.066	1.2474	0.297
C-C-G-C-G-A-A-G-T-A	0.064	0.051	0.056	0.6871	0.433
C-G-T-C-G-A-A-G-T-A	0.028	0.043	0.037	1.1757	0.3026
C-C-G-T-G-T-T-C-G-G	**0.050**	**0.019**	**0.033**	**5.3982**	**0.0316**

SNP, Single nucleotide polymorphism.
Haplotype frequencies were estimated to be $>3\%$.
Bold face indicates significant haplotypes.

associations remained significant after Bonferroni correction for testing two major haplotypes ($p=0.028$, $p=0.022$, corrected respectively; haplotype frequencies $>3\%$).

Regarding SNPs 6–7, haplotype frequencies were not associated with autism (data not shown). For SNPs 12–18, the presence of haplotype T-G-T-T-C-G-G significantly increased in these patients (3.8%, $p=0.0135$, data not shown). Furthermore, regarding extended haplotype SNPs 9–18, the presence of haplotype C-C-G-T-G-T-T-C-G-G significantly increased in these patients (3.3%, $p=0.0316$). However, these associations of SNPs 12–18 and 9–18 were not significant after Bonferroni correction in terms of the number of major haplotypes (frequency $>3\%$).

We then conducted TDT analysis to assess the transmissions of each SNP and the haplotypes that indicated significant association with autism in the case-control study. The TDT results for each SNP are presented in Table 4. No evidence for association was observed in these SNPs. In the regions for SNPs 1–8 and 9–18 where significant association was observed in the case-control study, individual haplotypes G-T-T-T-T-C-G-C for SNPs 1–8 and A-G-G-C-G-A-A-G-T-A for SNPs 9–18 were significantly

Table 4. Transmission disequilibrium test results for each allele

Name		Major allele	Trans.	Untrans.	χ^2	*p* values
SNP 1	rs3763463	C	162	158	0.42	0.52
SNP 2	rs1859767	A	120	108	1.64	0.20
SNP 3	rs1034825	T	79	88	0.87	0.35
SNP 4	rs2300045	T	127	127	0.00	1.00
SNP 5	rs2300043	T	82	87	0.26	0.61
SNP 6	rs2300039	T	136	133	0.13	0.72
SNP 7	rs722519	G	176	177	0.07	0.80
SNP 8	rs2216259	C	126	121	0.32	0.57
SNP 9	rs2300012	C	98	98	0.00	1.00
SNP 10	rs1269655	G	148	144	0.26	0.61
SNP 11	rs1269642	T	142	140	0.07	0.80
SNP 12	rs1269627	T	161	153	1.68	0.19
SNP 13	rs1269621	A	136	127	1.03	0.31
SNP 14	rs383341	T	160	157	0.26	0.61
SNP 15	rs402029	T	163	156	1.32	0.25
SNP 16	rs6958498	C	164	155	2.19	0.14
SNP 17	rs449514	G	144	128	3.66	0.06
SNP 18	rs409969	G	144	126	4.63	0.03

SNP, Single nucleotide polymorphism.

under-transmitted and over-transmitted to affected individuals, respectively ($p=0.028$, $p=0.019$) in the TDT analysis (Table 5). However, haplotype A-G-G-C-G-A-A-G-T-A for SNPs 9–18 was excluded from consideration due to a deficiency in estimated frequency (frequency: 1.5%, global $p=0.0697$, data not shown). The significant individual haplotype for SNPs 1–8 had different combinations of alleles from the individual haplotypes indicating significant association in the case-control study, and this association did not remain after Bonferroni correction for testing six major haplotypes.

We additionally tested some haplotypes, including rs2300045 and adjacent markers in the case-control study. The lowest *p* value was obtained for haplotypes consisting of SNPs 4–5 (global $p=0.0069$, individual $p=0.0099$ and 0.0035 for two major individual haplotypes, total frequency 89%) and SNPs 4–6 (global $p=0.0208$, individual $p=0.0241$ and 0.0006 for two major individual haplotypes, total frequency 76%) (data not shown).

Discussion

Thus far, three association studies of NRCAM SNPs with autism have been conducted. One study (Hutcheson et al., 2004) examined the *NRCAM* gene for association with autism susceptibility in 30 families using the pedigree disequilibrium test. They found no significant association of NRCAM with autism. However, the sample size in their study might be too small to be considered as reliable negative data. The other two studies (Bonora et al., 2005; Sakurai et al., 2006) conducted an association analysis with haplotypes. These studies showed that the haplotypes comprising these SNPs were associated with autism. However, the associated haplotypes in these studies were incompatible, and correction for multiple testing was not applied to their results. It might be possible that the Caucasian and African American subject used in these studies had different allele or haplotype distributions from Japanese subjects used in our study, and that some disagreements existed between the results of these former studies and our results. However, at least their results certainly indicated the trends towards the *NRCAM* gene being nominally associated with autism. Thus, their reports are still very informative.

Our study is the first to conduct an association study with autism using multiple corrections and a replication study, and indicated significant results. We tested for association with autism among 18 NRCAM SNPs and their haplotypes in a Japanese population. Regarding rs2300045, the association with the disease

Table 5. Estimated Transmission disequilibrium test haplotyes in autism parent–offspring triads for SNPs 1–8 (rs3763463, rs1859767, rs1034825, rs2300045, rs2300043, rs2300039, rs722519, rs2216259)

Haplotype	Translated[a] (frequency)	Untranslated[a] (frequency)	χ^2	p value
C-T-T-C-T-T-G-C	61.91 (0.2211)	60.81 (0.2172)	0.01032	0.92
C-A-C-T-C-T-G-A	63.92 (0.2283)	58.8 (0.21)	0.2799	0.60
C-A-C-T-C-T-G-C	49.08 (0.1753)	43.2 (0.1543)	0.4429	0.51
C-A-C-T-C-C-G-C	27 (0.09643)	22 (0.07857)	0.56	0.45
G-T-T-T-T-C-G-C	**15.01 (0.05362)**	**28.99 (0.1035)**	**4.829**	**0.028**
G-T-T-C-T-T-G-C	11 (0.03929)	13 (0.04643)	0.1743	0.68
	global $p = 0.61177$			

SNP, Single nucleotide polymorphism.
Haplotype frequencies were estimated to be $>3\%$.
[a] Estimated using UNPHASED software.
Bold face indicates significant haplotypes.

remained statistically significant after Bonferroni correction. Furthermore, we found several major haplotypes for SNPs 1–5, 1–8 and 10–11 which showed significant association with autism after Bonferroni correction. Applying Bonferroni correction for non-independent markers would be too conservative (Clayton and Jones, 1999). Nontheless, our result was the first to indicate an association between the *NRCAM* gene and autism even after Bonferroni correction. Regarding haplotypes SNPs 1–8, significant association with autism was found using both the 170 cases, 214 control samples and 148 TDT samples. Although there are differences in the combinations of alleles presented for associated haplotypes both within our study and between studies of the *NRCAM* gene, taken together with the results of earlier reports, our results might be strong evidence of major variations of the *NRCAM* gene being able to contribute to the aetiology of autism.

The haplotype comprised of SNPs 1–8 was the most remarkable region, which showed an association with autism even after Bonferroni correction or replication study. This haplotype covers the 5′-UTR intron-2 region of 82 kb, and this region is involved in gene expression. The *NRCAM* gene is involved in brain development in embryogenesis (Lane et al., 1996; Lustig et al., 1999, 2001). These results might indicate the possibility that this gene is involved in the central nerve development of the autistic brain. Thus, further studies, including replication studies or investigation of the expression and function of this gene, are desirable.

Each of the haplotype blocks reflects the descent from a single ancient ancestral chromosome (Gabriel et al., 2002). The number of observed haplotypes (especially if the loci are within a haplotype block) is much smaller than the number of all possible haplotypes (Gabriel et al., 2002). The construction of a haplotype block is one way to reduce the complexity of the problem of association mapping of a common complex disease. However, the haplotype block border is not usually stable, blocks can fall into sub-blocks within the border (Nothnagel and Rohde, 2005) and these facts do not contradict the importance of the significant results of the individual haplotypes located across haplotype block borders.

Several limitations of the present study may be acknowledged. First, ADOS or ADI, which may currently be the major tools for the diagnosis of autism in North America and Western Europe, were not available in the Japanese language at the sample collection time in the present study. However, the diagnosis, according to DSM-IV criteria, was cautiously confirmed by very experienced child psychiatrists for the present subjects; patients and parents were interviewed using a widely used questionnaire in Japan (the CBQ-R) and clinical records were carefully reviewed.

In the present study, the significant results in the TDT were not retained after multiple corrections (Bonferroni correction). Thus, the possibility of a false-positive might exist. However, considering our case-control results and several former studies, the results in the TDT in the present study could support the association between the *NRCAM* gene and autism. In addition, the allele combinations of the significant haplotypes were different between the case-control analysis and TDT. Thus, our result might not be direct

evidence for the identical allele combination of the haplotype that was associated with autism. However, it might be possible that several allele combinations of the haplotype participated in the aetiology of autism, and that we could not detect these associations completely in this study. Nonetheless, the *NRCAM* gene region was associated with autism both in the case-control study and TDT. These facts still strongly suggest that this gene might be involved in the aetiology of autism.

The controls in this study were not age-matched to the patients. Twin and family studies indicate that autism might be highly heritable. The heritability estimate, calculated from the sibling recurrence risk and the MZ:DZ concordance ratio, is >90% (Bailey et al., 1995; Szatmari et al., 1998). Autism is a developmental disorder usually apparent by age 3 yr. In making a comparison between autistic patients and normal controls aged >3 yr, this lack of age-matching is not likely to have affected the results, considering the strong effect of genetic factors in autism compared with the rather small effect of environmental factors (Folstein and Rosen-Sheidley, 2001).

Another concern was the population stratification of the sample, which has the potential to affect the results of case-control studies. However, this was unlikely to have affected the present results, because the Japanese population is much more homogeneous, with no major immigration occurring for more than a thousand years, than the European or North American population. No subjects in this study had parents or grandparents of ethnicity other than Japanese. In addition, our former studies, which indicated no associations, may support the non-existence of stratifications in this study (Marui et al., 2005, 2007a,b).

Taken together with the results of earlier reports, the present study is the first to provide evidence for a significant association of the *NRCAM* gene with autism, even after multiple corrections or replication study. This study also replicates the nominal associations reported by the two earlier studies (Bonora et al., 2005; Sakurai et al., 2006). Our results suggest that the *NRCAM* gene plays an important role in the aetiology of autism, and indicates that the *NRCAM* gene is one of the strong candidate genes for autism. Thus, these results may provide helpful information for future investigations of autism. However, the sample size in the present study might be too small to conclude that a definite association exists between the *NRCAM* gene and autism. Further studies are needed to replicate the results of this study.

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Statement of Interest

None.

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