Table 1
Studies included in meta-analysis for Val/Met polymorphism.

Author	Year	Ethnic	*n*		Diagnostic system
			Case	CON	
Daniels	1996	European	78	78	III-R
Strous	1997	European	54	87	III-R
Karayiorgou	1998	European	157	129	III
Ohmori	1998	Asian	150	150	IV
de Chaldee	1999	European	136	137	III-R
Chen	1999	Asian	177	99	IV
Kotler	1999	European	92	415	ICD-10
Egan	2001	European	175	55	IV
Arinami	2001	Asian	300	300	III-R
Liou	2001	Asian	198	188	IV
Joober	2002	European	104	96	IV
Park	2002	Asian	103	103	IV
Shifman	2002	European	719	2970	IV
Semwal	2002	Asian	535	262	IV
Inada	2003	Asian	100	201	III-R
Kremer	2003	Other	276	77	III-R
Wonodi	2003	Other	96	79	IV
Gallinat	2003	European	49	170	IV
Illi	2003	European	94	94	IV
Rujescu	2003	European	28	328	IV
Herken	2003	European	143	65	IV
Iwata	2003	Asian	51	69	IV
Thaker	2004	Other	62	53	IV
Han	2004	Asian	168	158	IV
Fan	2005	Asian	862	928	III-R
Poyurovsky	2005	European	113	171	IV
Lee	2005	Asian	320	379	IV
Galderisi	2005	European	111	106	IV
Joo	2005	Asian	239	248	IV
Funke	2005	European	196	467	IV
Williams	2005	European	677	684	IV
Szoke	2006	European	66	50	IV
Numata	2006	Asian	158	317	IV
Golimbet	2006	European	146	130	ICD-10
Ohnishi	2006	Asian	47	76	IV
Han	2006	Asian	132	80	IV
Krabbendam	2006	European	23	21	IV
Nicodemus (Germany)	2007	European	501	627	IV
Nicodemus (USA)	2007	European	296	370	IV
Muntjewerff	2007	European	252	405	IV
Yu	2007	Asian	241	290	IV
Diez-Martin	2007	European	177	141	IV
Nunokawa	2007	Asian	399	440	IV
Goghari	2007	European	39	20	IV
Martorell	2007	European	585	615	IV
Talkowski	2008	European	478	501	IV
Sanders	2008	European	1871	2003	IV
Okochi	2008	Asian	1114	1099	IV

ethnically Japanese, from the central area of Japan. The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. Patients with other Axis I diagnoses (such as mood disorder, schizoaffective, anxiety, drug abuse) were excluded. All healthy controls were also psychiatrically screened on the basis of unstructured interviews. To exclude subjects with any brain or psychotic disorder, or who had first-degree relatives with psychotic disorders, a trained psychiatrist interviewed them with a focus on current and/or past mental states (psychotic, mood, anxiety, obsessive–compulsive symptoms) and family history (Ikeda et al., 2005, 2008).

The study was described to, and written informed consent was obtained from, each subject. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University School of Medicine.

2.1.2. Mutation scan

Genomic DNA was extracted from peripheral blood of 96 patients with schizophrenia. We amplified the entire exon and P2 promoter regions (promoter of MB-COMT), which are 500 base pairs (bp) upstream from the initial exon. In the human brain, MB-COMT is dominantly detectable. It is important to detect rare variants in the P2 promoter region, which may change MB-COMT expression. Primers for each region were designed with the use of Primer3 software (Whitehead Institute, Cambridge, Massachusetts).

Denaturing high performance liquid chromatography (dHPLC) analysis was carried out to detect mutation. DNA sequencing was then performed using a 3100-Avant Genetic Analyzer (Applied Biosystems, CA). A more detailed description of the methods can be seen in a previous paper (Suzuki et al., 2003) (Supplementary Table 1).

2.1.3. SNP selection and LD evaluation

We first consulted the HapMap database (release#21a, Jan 2007 www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05) and found 48 SNPs covering the COMT gene (5′-flanking regions including 9130 bp from the initial exon and 1559 bp downstream (3′) from the last exon: HapMap database contig number chr22:18300000... 18336530). Then 16 'tagging SNPs' were selected with the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger), an implement of the HAPLOVIEW software (Barrett et al., 2005).

2.1.4. SNP genotyping

We used TaqMan assays (Applied Biosystems) for all SNPs. Detailed information, including primer sequences, can be seen in Supplementary Table 2.

2.1.5. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the chi-square test (SAS/Genetics, release 8.2, SAS Japan INC, Tokyo, Japan). Marker-trait association was also evaluated by the chi-square test in allele- and genotype-wise analyses. Haplotype frequencies were estimated in a two- to three-marker sliding window fashion and log likelihood ratio tests were performed for global *P*-values with the COCAPHASE program, version 3.0.6 (Dudbridge, 2003). In these haplotype-wise analyses, rare haplotypes (less than 0.05) of cases and controls were excluded. Power calculation was performed using a statistical program prepared by Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purecell//gpc/). The level of significance for all statistical tests was 0.05.

2.2. Meta-analysis of COMT

2.2.1. Search literature

Articles were searched on a database (PubMed) from first date up to 2008 April, using the search words 'schizophrenia', 'COMT', and 'catechol-O-methyltransferase'. In cases when we could not obtain detailed information about allele or genotype

Table 2
Association analysis of tagging SNPs in COMT.

Gene	Marker IDs		Distance to next SNP (bp)	N[a]		MAF[b]		M/M[c]		M/m[d]		m/m[e]		P-values		Haplotype				
				SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	genotype	allele	2 window (P-values)		3 window (P-values)		
COMT	M1	rs2871047	0	1112	1093	0.344	0.35	492	471	473	478	147	144	0.838	0.699					
	M2	rs2075507	1761	1067	1096	0.281	0.287	559	554	415	453	93	89	0.5	0.649	0.957		0.953		
	M3	rs4646310	714	1106	1097	0.065	0.072	969	944	129	148	8	5	0.319	0.396		0.717		0.886	
	M4	rs737865	1315	1106	1087	0.255	0.258	625	600	396	411	85	76	0.569	0.814	0.712		0.967		0.902
	M5	rs1544325	1547	1115	1089	0.3	0.298	551	533	458	462	106	94	0.694	0.884		0.919		0.969	
	M6	rs174675	2383	1110	1084	0.446	0.452	343	331	543	525	224	228	0.884	0.684	0.945		0.992		0.965
	M7	rs5993882	3482	1113	1101	0.123	0.125	861	840	229	246	23	15	0.288	0.855		0.882		0.895	
	M8	rs5992500	4414	1116	1097	0.049	0.055	1008	982	105	109	3	6	0.534	0.418	0.975		0.58		0.811
	M9	rs740603	3230	1114	1096	0.379	0.383	443	420	497	511	174	165	0.637	0.763		0.824		0.612	
	M10	rs4633	5058	1116	1100	0.321	0.307	527	536	460	451	129	113	0.574	0.317	0.811		0.551		0.625
	M11	rs6267	28	1118	1100	0.087	0.086	933	915	175	180	10	5	0.413	0.92		0.561		0.22	
	M12	rs2239393	165	1111	1087	0.264	0.258	612	603	410	407	89	77	0.71	0.619	0.91		0.259		0.481
	M13	rs4680	843	1114	1099	0.341	0.325	497	499	474	485	143	115	0.215	0.264		0.38		0.109	
	M14	rs165774	1290	1115	1091	0.174	0.164	765	759	311	304	39	28	0.438	0.403	0.547				
	M15	rs174696	615	1112	1088	0.429	0.424	357	367	555	518	200	203	0.555	0.748		0.779			
	M16	rs174697	656	1111	1097	0.368	0.391	446	408	511	520	154	169	0.304	0.123	0.156				
	M17	rs165599	2949	1098	1089	0.432	0.457	367	328	512	526	219	235	0.234	0.1		0.305			
	M18	rs165849	1888	1118	1085	0.439	0.461	359	318	536	532	223	235	0.313	0.132	0.119				
	M19	rs165824	697	1104	1097	0.413	0.434	389	357	517	527	198	213	0.369	0.161		0.11			

[a] N = number, SCZ = schizophrenia, CON = control.
[b] MAF = minor allele frequency.
[c] M/M = major allele/major allele.
[d] M/m = major allele/minor allele.
[e] m/m = minor allele/minor allele.

or haplotype frequencies in the article, we tried to contact the author directly or we referred to the 'SzGene database' (http://www.schizophreniaforum.org/res/sczgene/default.asp) (Allen et al., 2008) (however, we could not obtain results on haplotype frequencies in the study by Shifman et al. (2002)).

2.2.2. Criteria for inclusion

From the database search, we selected population-based case-control studies that investigated the genotype and allele frequencies of the Val/Met polymorphism or other *COMT* polymorphisms (in patients diagnosed according to the ICD or DSM criteria and healthy controls). Duplication articles were excluded. Studies in which control allele frequencies deviated from HWE (P< 0.01) were also excluded.

A total of 48 population-based studies (Arinami et al., 2001; Chen et al., 1999; Daniels et al., 1996; de Chaldee et al., 1999; Diez-Martin et al., 2007; Egan et al., 2001; Fan et al., 2005; Funke et al., 2005; Galderisi et al., 2005; Gallinat et al., 2003; Goghari and Sponheim, 2008; Golimbet et al., 2006; Han et al., 2004, 2006; Herken et al., 2003; Illi et al., 2003; Inada et al., 2003; Iwata et al., 2003; Joo et al., 2005; Joober et al., 2002; Karayiorgou et al., 1998; Kotler et al., 1999; Krabbendam et al., 2006; Kremer et al., 2003; Lee et al., 2005; Liou et al., 2001; Martorell et al., 2008; Muntjewerff et al., 2008; Nicodemus et al., 2007; Numata et al., 2006; Nunokawa et al., 2007; Ohmori et al., 1998; Ohnishi et al., 2006; Park et al., 2002; Poyurovsky et al., 2005; Rujescu et al., 2003; Sanders et al., 2008; Semwal et al., 2002; Shifman et al., 2002; Strous et al., 1997; Szoke et al., 2006; Talkowski et al., 2008; Thaker et al., 2004; Williams et al., 2005; Wonodi et al., 2003; Yu et al., 2007) were identified using our search criteria for this meta-analysis (including our case-control study using a Japanese population) (Table 1).

Table 3
Association analysis of attractive haplotypes from other studies global P-values were obtained by the COCAPHASE program rare haplotypes (less than 0.05) were excluded.

Haplotype	Global P-values
rs4680–rs165599	0.396
rs737865–rs4680	0.206
rs6267–rs4680	0.529
rs4633–rs4680	0.274
rs4680–rs165849	0.166
rs737865–rs4680–rs165599	0.301
rs4680–rs165599–rs165849	0.151
rs2075507–rs737865–rs4680–rs165599	0.193
rs737865–rs4633–rs4680–rs165599	0.242
rs737865–rs6267–rs4680–rs165599	0,738

Table 4
Results of meta-analysis for Val/Met polymorphism in COMT.

	OR (95% CI)	P-value (Z)	P-value (Q)
Val/Met (rs4680)			
G/A			
All studies (48)[a]	0.989 (0.942–1.039)	0.667	0.011
European (27)	0.971 (0.909–1.038)	0.392	0.016
Asian (18)	0.984 (0.924–1.048)	0.62	0.378
(GG + GA)/AA			
All studies (48)	0.992 (0.932–1.056)	0.804	0.807
European (27)	0.988 (0.919–1.062)	0.747	0.525
Asian (18)	0.918 (0.81–1.04)	0.677	0.478
GG/(GA + AA)			
All studies (48)	1.020 (0.952–1.092)	0.575	0.043
European (27)	1.030 (0.943–1.124)	0.511	0.192
Asian (18)	0.976 (0.881–1.082)	0.65	0.107

COMT: Catechol-o-methyltransferase, OR: odds ratio, CI: confidence interval. P-value (Z): the significance of the pooled OR was determined using a Z-test. P-value (Q): the heterogeneity was checked using a Q statistic test.
[a] ( ): the number of studies.

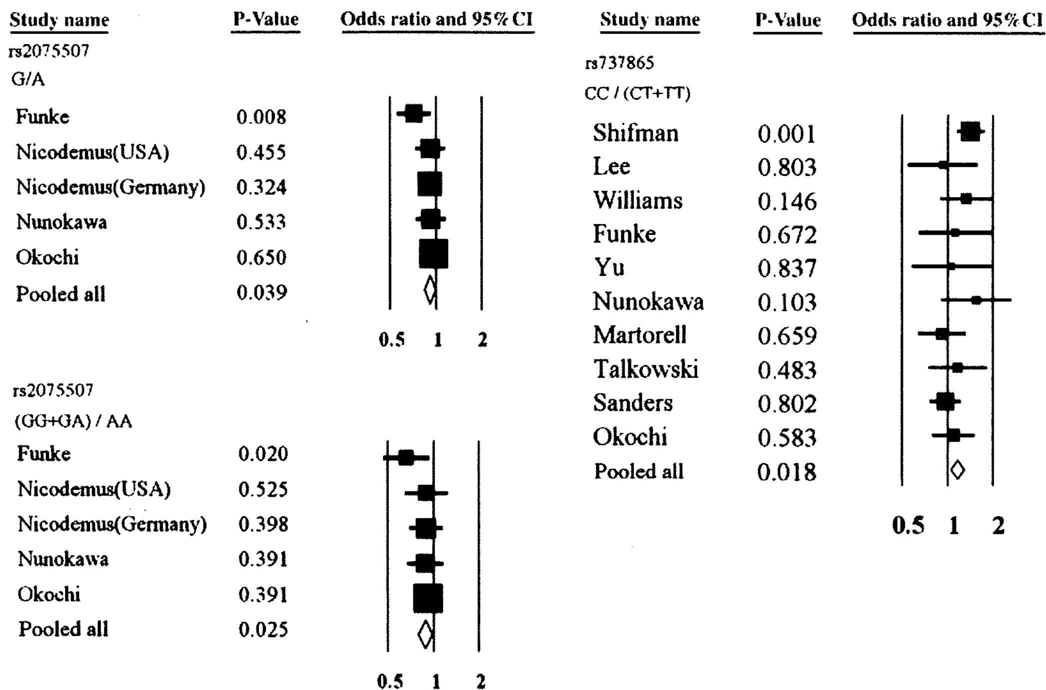

Fig. 3. Forest plots of OR with 95% CI for the rs2075507 (G/A, GG + GA/AA) and rs737865 (CC/CT + TT). Marginal associations were detected in each SNP.

2.2.3. Statistical analyses

A two-by-two table in which subjects were classified by diagnosis and type of allele (additive, dominant and recessive models) was constructed. Random-effect models were adopted to check the heterogeneity using a Q statistic test in the combined studies. Odds ratios (ORs) were pooled using DerSimonian and Laird methods. The significance of the pooled OR was determined using a Z-test. Publication bias was assessed using a funnel plot asymmetry with Egger's test. The statistical significance was set at 0.05. All data were analyzed using Comprehensive Meta Analysis (Version 2.0).

To correct for problems of multiple comparisons, we applied the Benjamini–Hochberg (BH) method, which is a procedure to control for false discovery rate (FDR) (Benjamini and Hochberg, 2000).

3. Result

3.1. Mutation scan and case-control study in the Japanese population

Our mutation scan detected a number of SNPs in this population that are listed in the dbSNP database (rs2020917, rs4633, rs6267, rs4818, rs4680, rs4646316 and rs165774), but did not find any novel SNPs. All SNPs detected in this mutation scan were unlikely to have functional relevance, since they are synonymous or in the branch site regions. We then performed a genetic case-control study using the tagging SNPs from the HapMap database and possible functional SNPs. Genotype frequencies of subjects and controls did not deviate significantly from HWE (Table 2). There was no significant association in the allele/genotype-wise analysis or in the haplotype analysis (Tables 2 and 3). We obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.19–1.57 under a multiplicative model of inheritance.

3.2. Meta-analysis

3.2.1. Val108/158Met polymorphism (rs4680)

All population-based studies that were identified by our search criteria included an analysis for Val/Met polymorphism (total sample sizes were 13088 cases and 16531 controls). No association of this SNP to schizophrenia was found in the allele frequencies (G/A: pooled OR = 0.989, CI = 0.942–1.039, $P(Z) = 0.667$, GG + GA/AA: pooled OR = 0.992, CI = 0.932–1.056, $P(Z) = 0.804$, GG/GA + AA: pooled OR = 1.020, CI = 0.952–1.092, $P(Z) = 0.575$; Table 4).

Heterogeneity among these studies was detected under some models (G/A: $P(Q) = 0.011$, GG/GA + AA: $P(Q) = 0.043$). We therefore divided samples into two groups in accordance with populations: European (27 population-based studies) and Asian (18 population-based studies). In this subgroup analyses, no association was detected from either European or Asian samples (Table 4, Fig. 2), but caution is needed because heterogeneity still existed in the European samples (G/A: $P(Q) = 0.016$ for Europeans).

No publication bias was detected for this SNP (Supplementary Fig. 1).

3.2.2. Other SNPs (rs2075507, rs737865, rs6267, rs165599) and haplotypes

For rs2075507, 5 population-based studies (Funke et al., 2005; Nicodemus et al., 2007; Nunokawa et al., 2007) were

Table 5
Results of meta-analysis for 4 functional SNPs and haplotype in COMT.

	OR (95% CI)	P-value (Z)	P-value (Q)
rs2075507			
G/A (5)[a]	0.912 (0.835–0.995)	**0.039**	0.341
(GG + GA)/AA	0.882 (0.79–0.984)	**0.025**	0.559
GG/AG + AA	0.92 (0.776–1.090)	0.333	0.421
rs737865			
C/T (10)	1.041 (0.978–1.108)	0.207	0.184
(CC + CT)/TT	1.014 (0.946–1.088)	0.691	0.374
CC/(CT + TT)	1.155 (1.025–1.303)	**0.018**	0.336
rs6267			
T/G (3)	0.781 (0.564–1.081)	0.136	0.031
(TT + TG)/GG	0.765 (0.53–1.103)	0.152	0.021
TT/(GT + GG)	1.49 (0.452–4.913)	0.512	0.176
rs165599			
G/A (11)	1.032 (0.960–1.108)	0.396	0.018
(GG + GA)/AA	1.016 (0.940–1.098)	0.691	0.207
GG/(GA + AA)	1.076 (0.940–1.232)	0.286	0.033
rs737865–rs4680–rs165599			
Haplotype analysis (4)			
CGG[b]	0.904 (0.763–1.071)	0.244	0.456
TGA[c]	0.992 (0.876–1.123)	0.895	0.261
TGG[d]	1.063 (0.942–1.201)	0.321	0.686

COMT: Catechol-o-methyltransferase, OR: odds ratio, CI: confidence interval.
P-value (Z): The significance of the pooled OR was determined using a Z-test.
P-value (Q): The heterogeneity was checked using a Q statistic test.
Bold numbers represent significant P-value (<0.05).
[a] (): the number of studies.
[b] Shifman et al. reported.
[c] J. Chen et al. reported.
[d] Handoko et al. reported.

identified (total sample sizes: 2456 cases and 3000 controls) and there was no evidence for heterogeneity. We found a trend for association between allele frequencies and schizophrenia (G/A: pooled OR = 0.912, CI = 0.835–0.995, $P(Z) = 0.039$, GG + GA/AA: pooled OR = 0.882, CI = 0.79–0.984, $P(Z) = 0.025$; Table 5).

For rs737865, 10 population-based studies (Funke et al., 2005; Lee et al., 2005; Martorell et al., 2008; Nunokawa et al., 2007; Sanders et al., 2008; Shifman et al., 2002; Talkowski et al., 2008; Williams et al., 2005; Yu et al., 2007) were identified (total sample sizes: 6599 cases and 9323 controls) and no heterogeneity was found. Again, a trend for association was detected (CC/CT + TT: pooled OR = 1.155, CI = 1.025–1.333, $P(Z) = 0.018$).

However, two other SNPs (rs6267, rs165599) and haplotype analyses did not find evidence of a significant association (Table 5, Fig. 3).

With further checking of the aforementioned marginal associations in rs2075507 and rs737865, after correcting for multiple testing by FDR, both P-values from these SNPs were found to be larger than the Q-value, indicating the significance for these SNPs was derived from type I error (rs2075507 G/A $P = 0.039 > Q = 0.0062$, GG + GA/AA $P = 0.025 > Q = 0.0041$, rs737865 CC/CT + TT $P = 0.018 > Q = 0.002$).

No publication bias was found for any of the SNPs.

4. Discussion

4.1. LD-based-association analysis of COMT in the Japanese population

In this LD-based case–control study, our data did not show sufficient evidence for an association between possible functional SNPs or tagging SNPs and schizophrenia in the Japanese population. The LD-based strategy we adopted was the minimum required to examine the association of *COMT* with schizophrenia, considering a recent study by Mukherjee et al. (2008) which showed that several haplotypes, but not SNPs by themselves such as Val/Met polymorphism, may be associated with schizophrenia because of LD differences among populations. In fact, they found that haplotype frequencies differed even among European populations in some regions of *COMT*. Therefore, to contain sufficient information and be cost-effective, variants used in association studies should be, at minimum, selected based on information from the HapMap database as appropriate tagging SNPs. Samples should also be from homogeneous population settings. In addition, mutation scans are important in order to detect rare but functional variants in all of COMT. This is partly because it is likely that such rare variants do not overlap among all populations (Pritchard, 2001).

Our results showed a clear lack of association of several tagging SNPs in *COMT* with schizophrenia in the Japanese population. In an explorative analysis, we examined the association of a large number of haplotypes that were constructed by all combinations of SNPs; however, no association was detected (the minimum P-value = 0.151, haplotype rs4680–rs165599–rs165849). Our sample size was one of the largest among genetic association studies for *COMT* to avoid overlooking false negative results, and therefore the results of the analysis should be reliable.

Some limitations should be noted with regard to interpretation of our results. Firstly, we scanned only P2 promoter and exon regions in this mutation scan. We should also note the possibility that far genomic regions or introns affect the gene's expression or splicing patterns. Secondly, our samples were not assessed by standard structured interviews, increasing the chance of false negatives due to misdiagnosis or sampling bias.

4.2. Updated meta-analyses of attractive SNPs and haplotypes

To date, three independent groups have reported meta-analyses of Val/Met polymorphism in *COMT* with schizophrenia; one showed a significant association but the other two showed no evidence for association. Our results indicate that the Val/Met polymorphism and four other functional SNPs may not play a major role in schizophrenia. However, considerable heterogeneity for Val/Met polymorphism was detected among all population-based studies. Therefore, we performed the following subgroup analysis by population, but heterogeneity still existed among the European studies. Heterogeneity is considered to be partly due to population stratification by LD differences and sampling bias.

It is clear that LD differences make interpretation from the results of meta-analysis difficult; positive results may be derived from false positives due to population stratification,

whereas negative results may be induced by simply overlooking population-specific effects of the examined variants. To overcome these difficulties in future meta-analysis, comparison of gene-wide significance from populations considered to have LD differences, adequate sample size, and accurate phenotype and diagnosis definition will be needed (Moskvina et al., 2009).

In conclusion, our results suggest that COMT may not play a major role in schizophrenia. However, there are reported associations of endophenotype with schizophrenia. A recent report showed a meta-analysis of WCST in schizophrenia and controls (Barnett et al., 2007). Further studies will be needed to examine the association between COMT and PFC function.

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Contributors

All authors contributed to and have approved the final manuscript.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.02.019.

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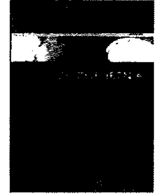

BDNF is not associated with schizophrenia: Data from a Japanese population study and meta-analysis

Kunihiro Kawashima [a,b], Masashi Ikeda [a,c], Taro Kishi [a,b], Tsuyoshi Kitajima [a,b], Yoshio Yamanouchi [a,b], Yoko Kinoshita [a,b], Tomo Okochi [a,b], Branko Aleksic [d], Makoto Tomita [e], Takeya Okada [f], Hiroshi Kunugi [f], Toshiya Inada [g], Norio Ozaki [b,d], Nakao Iwata [a,b,*]

[a] *Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan*
[b] *CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan*
[c] *Department of Psychological Medicine, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, United Kingdom*
[d] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya 466-8850, Japan*
[e] *Risk Analysis Research Center, The Institute of Statistical Mathematics, Tokyo, 106-8569, Japan*
[f] *Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo, 187-8502, Japan*
[g] *Seiwa Hospital, Institute of Neuropsychiatry, Tokyo, 162-0851, Japan*

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ABSTRACT

A variety of evidence suggests brain-derived neurotrophic factor (*BDNF*) as a candidate gene for schizophrenia, and several genetic studies have shown a significant association between the disease and certain SNPs within *BDNF* (specifically, Val66Met and C270T). According to a recent study, the functional microsatellite marker *BDNF*-LCPR (*BDNF*-linked complex polymorphic region), which affects the expression level of *BDNF*, is associated with bipolar disorder. The goals of our current study were to 1) evaluate the quality of HapMap-based linkage disequilibrium (LD) tagging of *BDNF*-LCPR, 2) examine whether these tagging SNPs are associated with schizophrenia in a Japanese population, and 3) conduct a meta-analysis of the two most extensively studied polymorphisms: Val66Met and C270T. We genotyped eight tagging SNPs, including Val66Met and C270T. Our LD evaluation showed that *BDNF*-LCPR could be represented by these tagging SNPs in controls (with 73.5% allelic coverage). However, the functional A1 allele was not captured due to its low minor allele frequency (2.2%). In a case-control study (1117 schizophrenics and 1102 controls), no association was found in single-marker or multimarker analysis. Moreover, in a meta-analysis, the Val66Met polymorphism was not associated with schizophrenia, whereas C270T showed a trend for association in a fixed model ($p = 0.036$), but not in a random model ($p = 0.053$). From these findings, we conclude that if *BDNF* is indeed associated with schizophrenia, the A1 allele in *BDNF*-LCPR would be the most promising candidate. Further LD evaluation, as well as an association study in which *BDNF*-LCPR is genotyped directly, would be required for a more conclusive result.

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1. Introduction

Brain-derived neurotrophic factor (*BDNF*) plays a key role in the central nervous system as a mediator of neuronal survival and plasticity of dopaminergic, cholinergic, and serotonergic neurons (Angelucci et al., 2005). There is a growing body of evidence supporting an association between *BDNF* and schizophrenia: 1) postmortem studies show reduced expression levels of *BDNF* in the anterior cingulate cortex (Iritani et al., 2003) and hippocampus of schizophrenia patients (Durany et al., 2001), 2) a reduced level of *BDNF* was confirmed in the blood serum of schizophrenic patients (Toyooka et al., 2002), and 3) mice in which the *BDNF*

* Corresponding author. Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan. Tel.: +81 562 93 9250; fax: +81 562 93 1831.
E-mail address: nakao@fujita-hu.ac.jp (N. Iwata).

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receptor *trkB* was knocked out show behavioral changes (hyper-locomotion, stereotyped behaviors, and cognitive impairments) related to the symptomatology of schizophrenia (Zorner et al., 2003). In addition, *BDNF* maps to chromosome 11p13, a region with potentially significant linkages to schizophrenia (Suarez et al., 2006).

A number of genetic association studies have shown that SNPs in *BDNF* are associated with schizophrenia (Nanko et al., 2003; Szekeres et al., 2003), and a meta-analysis study also showed a weak association between C270T and schizophrenia (Zintzaras, 2007), but not between Val66Met and schizophrenia (Kanazawa et al., 2007; Naoe et al., 2007; Xu et al., 2007; Zintzaras, 2007).

A recent study focused on the complex microsatellite polymorphism *BDNF*-LCPR located ~1.0 kbp upstream of the translation initiation site of *BDNF* (Okada et al., 2006). This polymorphism contained 23 novel allelic variants, including four major alleles (A1–A4). A luciferase assay showed a significantly lower expression level of the A1 allele than the other three alleles of *BDNF*-LCPR. Furthermore, the A1 allele frequency was significantly higher in bipolar disorder patients than in controls. Therefore, *BDNF*-LCPR can be seen as an important schizophrenia-susceptibility factor, but determination of genotype distributions of this polymorphism is difficult due to technical limitations.

Two main goals of the present study were to examine 1) whether tagging SNPs from the HapMap database can represent *BDNF*-LPCR through linkage disequilibrium (LD) evaluation, and 2) whether these tagging SNPs are associated with schizophrenia in a Japanese population. We also performed meta-analysis regarding two polymorphisms (Val66Met and C270T), which have been intensively examined in relation to *BDNF*.

2. Materials and methods

2.1. Subjects

The subjects in the LD evaluation of *BDNF*-LCPR were 66 healthy controls (35 males and 31 females; age 50.33 ± 11.03 (mean ± SD) years) who had participated in a previous study (Okada et al., 2006). The sample used in the association analysis comprised 1117 schizophrenia patients (628 males and 489 females; 47.4 ± 15.3 years) and 1102 healthy controls (504 males and 598 females; 37.1 ± 14.4 years). All participants were unrelated Japanese people living in central areas of Japan. The patients were diagnosed according to DSM-IV criteria with consensus from at least two experienced psychiatrists on the basis of an unstructured interview and review of medical records. Patients with any other axis-I disorder were excluded.

All healthy controls were also psychiatrically screened based on unstructured interviews to exclude subjects with brain/psychotic disorders, or those with first-degree relatives with a psychotic disorder. A trained psychiatrist interviewed each participant with respect to current or past mental states (psychotic, mood, anxiety, obsessive–compulsive symptoms) and family history (Ikeda et al., 2005). Healthy controls were mainly recruited from the staff of participating hospitals.

Written informed consent was obtained from each subject. This study was approved by the Ethics Committee of each institution involved.

2.2. Tagging SNP selection and LD evaluation

First we consulted the HapMap database (release#16.c.1, Jun 2005 www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05) and selected 38 SNPs covering the *BDNF* gene (5′-flanking regions ranging from 9467 bp away from the initial exon to 4454 bp downstream (3′) from the last exon: HapMap database contig number chr11: 27709339..27628562). Then, seven 'tagging SNPs' (rs1491851, rs11030121, rs7934165, rs12291063, rs11030101, rs6265 [Val66-Met], rs1519480: Table 1) were selected with the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (de Bakker et al., 2005) (http://www/broad.mit.edu/mpg/tagger), a tool within the HAPLOVIEW software (Barrett et al., 2005). In addition to these tagging SNPs, we included the C270T polymorphism, which has been intensively examined in other papers. A total of eight SNPs were selected for the following LD evaluation and case-control association analysis.

2.3. Genotyping

Information on genotypic distributions of *BDNF*-LCPR, which had been determined previously (Okada et al., 2006), was used for LD evaluation (66 healthy controls). Genotyping

Table 1
Association analysis of tagging SNPs in *BDNF*.

Gene	Marker IDs		Distance to next SNP (bp)	N[a]		MAF[b]		M/M[c]		M/m[d]		m/m[e]		p-values	
				SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	SCZ	CON	Genotype	Allele
BDNF (minus strand)	M1	rs1491851	0	1114	1100	0.234	0.247	647	622	413	413	54	65	0.491	0.312
	M2	rs11030121	16556	1111	1097	0.047	0.045	1011	999	96	97	4	1	0.409	0.79
	M3	rs7934165	4224	1109	1099	0.46	0.468	332	320	534	529	243	250	0.861	0.581
	M4	C270T	10185	1115	1102	0.032	0.03	1046	1036	67	66	2	0	0.372	0.716
	M5	rs12291063	27697	1117	1095	0.206	0.202	698	695	378	358	41	42	0.842	0.736
	M6	rs11030101	13357	1110	1099	0.344	0.34	484	485	489	480	137	134	0.969	0.813
	M7	rs6265	828	1111	1100	0.413	0.428	394	365	516	529	201	206	0.528	0.326
	M8	rs1519480	4204	1112	1100	0.246	0.235	631	640	414	403	67	57	0.621	0.375

[a] N = number, SCZ = schizophrenia, CON = control.
[b] MAF = minor allele frequency.
[c] M/M = major allele/major allele.
[d] M/m = major allele/minor allele.
[e] m/m = minor allele/minor allele.

of remaining SNPs was performed by allelic discrimination assay (Applied Biosystems, CA).

2.4. Statistical analysis

2.4.1. LD evaluation and gene-based case-control association study

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by χ^2 goodness-of-fit test.

For LD evaluation, multiallelic D' was calculated by the COCAPHASE 2.403 program (Dudbridge, 2003). Haplotype frequencies of tagging SNPs (M6, M7 and M8) and *BDNF*-LCPR were then estimated using PHASE software (http://www.stat.washington.edu/stephens/software.html) (Stephens and Donnelly, 2003). Because this region shows high LD, a block-like structure was conserved, and the sum of haplotype frequencies was calculated to examine whether these haplotypes could capture the specific allele of *BDNF*-LCPR in accordance with the haplotype-tagging method (Kamatani et al., 2004).

Marker-trait association was evaluated with the use of a likelihood ratio test (allele-wise and haplotype-wise analyses) and χ^2 test (genotype-wise analysis). For exhaustive screening, we examined eight-marker haplotype analysis in sliding-window fashion using the COCAPHASE 2.403 program (Dudbridge, 2003). A power calculation was performed using a statistical algorithm implemented in the Genetic Power Calculator program (http://pngu.mgh.harvard.edu/~purcell/gpc/).

We also carried out imputation of SNPs that are not directly genotyped but are present in the HapMap database (JPT + CHB founders, release 23, filtered by MAF greater than 0.01 and genotyping rate greater than 0.95) by PLINK software (version 1.04) (Purcell et al., 2007). A total of 108 SNPs were included for this analysis, from rs12574598 (chr11..27531152) to rs11030149 (chr11..27798413; the location is based on Human, Mar, 2006, hg18 assembly). We used the "-proxy -drop" option and picked up only imputed SNPs with an INFO value greater than 0.8 in accordance with the authors' recommendation.

The level of significance for all statistical tests was set at 0.05.

2.4.2. Meta-analysis

To identify studies eligible for the meta-analysis, we searched PubMed citations through December 2008 using the terms "*BDNF*," "brain-derived neurotrophic factor," and "schizophrenia" as key words.

Regarding selection of studies, we included the case-control genetic association studies of the Val66Met and/or C270T polymorphism. Studies with data for only schizophrenic patients or only healthy controls were excluded, as were family-based studies.

We also carried out quality assessment using the Newcastle–Ottawa Scale (NOS) for case-control studies. The Newcastle–Ottawa Scale uses a "star" rating system to judge the quality based on three aspects of the study: selection of study groups, comparability of study groups and ascertainment of either the exposure or outcome of interest. The maximum number of stars a study may receive in each of these categories is 4, 2 and 3, respectively, for a total of 9 possible stars. The validity of these tools has been previously established (http://www.ohri.ca/programs/clinical_epidemiology/oxford.htm).

We then assessed the HWE in the patients and control subjects of each study using the χ^2 goodness-of-fit test, and we explored whether heterogeneity was present using Q statistics. Finally, we performed a fixed-effects as well as random-effects model meta-analysis within groups of homogeneous odds ratios (ORs). The fixed effects model assumes that all existing studies are included in the meta-analysis, and therefore weights each study only by the inverse of the variance of that study. A random-effect model, in contrast, assumes that between-study variation is due to chance and/or random variation and an individual study effect. Random-effect models are more conservative than fixed effects models and generate a wider confidence interval (CI). The significance of the pooled OR was determined using a Z-test. Fourth, publication bias was assessed using a linear regression analysis to measure funnel plot asymmetry. A probability level of $p < 0.05$ was used as a threshold for statistical significance. Data were analyzed using the "Comprehensive Meta Analysis" (Version 2.2.046) statistical software package.

3. Results

3.1. LD evaluation of BDNF in the Japanese population

Sixteen variants of *BDNF*-LCPR were found in our sample ($N_{allele} = 132$): three samples for the Al allele [$(CG)_{del}(CA)_{12}(GA)_3$], 21 samples for the A2 allele [$(CG)_4(CA)_{12}(GA)_3$], 39 samples for the A3 allele [$(CG)_5(CA)_{12}(GA)_2$], 42 samples for the A4 allele [$(CG)_5(CA)_{13}(GA)_3$], and 27 samples for the A5 allele [combination of remaining rare alleles]. The results for LD between *BDNF*-LCPR and our eight tagging SNPs are

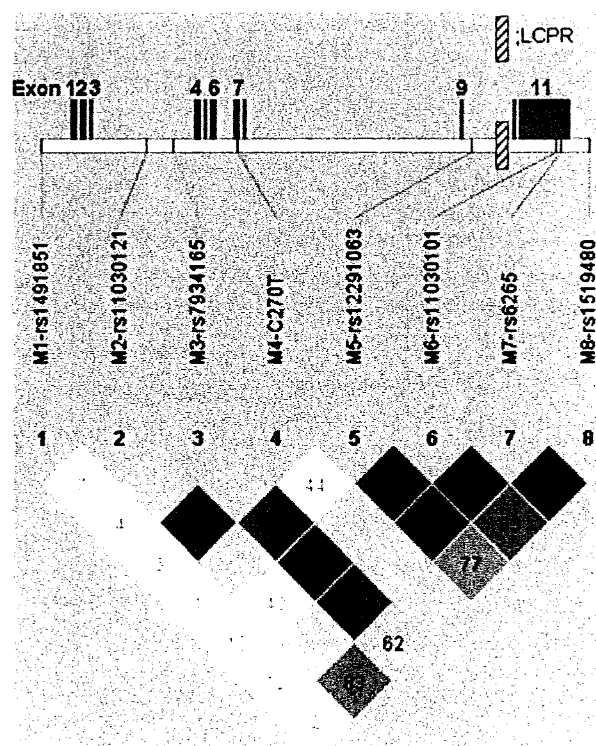

Fig. 1. LD structure in the *BDNF* gene.

Table 2
Sliding-window analysis of tagging SNPs in *BDNF*.

Gene	Marker IDs	*p*-values						
		2-window	3-window	4-window	5-window	6-window	7-window	8-window
BDNF	M1							
		0.4111						
	M2		0.7189					
		0.8163		0.7133				
	M3		0.9294		0.9801			
		0.6334		0.9513		0.9883		
	M4		0.8741		0.9962		0.9851	
		0.7537		0.9594		0.9697		0.975
	M5		0.9088		0.9827		0.8971	
		0.9304		0.8325		0.9167		
	M6		0.8204		0.7188			
		0.5905		0.6972				
	M7		0.4048					
		0.4796						
	M8							

shown in Supplementary Table 1. The LD analysis of *BDNF*-LCPR and M6–M8 polymorphisms showed *D′* values ranging from 0.955 to 0.975, indicating tight linkage between M6, M7 and M8 and *BDNF*-LCPR (Supplementary Table 1).

On the other hand, single marker tests may be inefficient when each single marker carries a small to moderate amount of association information about the trait. In this situation, the association might not be detected when markers are analyzed individually, whereas combining genotypes from neighboring markers may provide a more powerful test. Therefore, in order to perform exploratory multimarker association testing, we estimated the haplotype frequencies and summed the haplotype frequencies to examine whether the haplotypes could capture the specific alleles of *BDNF*-LCPR in accordance with the haplotype-tagging method (Kamatani et al., 2004) (Supplementary Table 2).

3.2. Population-based study in a Japanese population

Genotype frequencies of all SNPs were in HWE. The LD structure can be seen in Fig. 1. We detected no significant association in the allele/genotype-wise analyses (Table 1) or in the haplotype analysis (Table 2). We then performed stratified analysis by gender, since a recent paper reported the effect of gender on *BDNF* in major depressive disorder (Verhagen et al., 2008). Again, we could not find any association in this subgroup analysis (Supplementary Tables 3 and 4).

The A1 allele of *BDNF*-LCPR is a low-frequency allele (around 2.3%). Therefore, considering both the low frequency of A1 and the number of alleles in the LCPR polymorphism, we included imputation analysis around *BDNF*, because additional SNPs might provide better coverage of this very polymorphic repeat. 35 SNPs passed our quality control, but no evidence for association could be detected between imputed SNPs and schizophrenia in the Japanese population (Fig. 2).

We obtained more than 80% power for the detection of an association when we set the genotype relative risk at 1.19–1.57 in schizophrenia under a multiplicative model of inheritance.

3.3. Meta-analysis

3.3.1. Val66Met polymorphism (rs6265, high-resolution plot is shown in Supplementary Figure 1)

An updated meta-analysis (total of 22 population-based association studies), in which three studies (Donohoe et al., 2007; Han et al., 2008; Takahashi et al., 2008) and our current

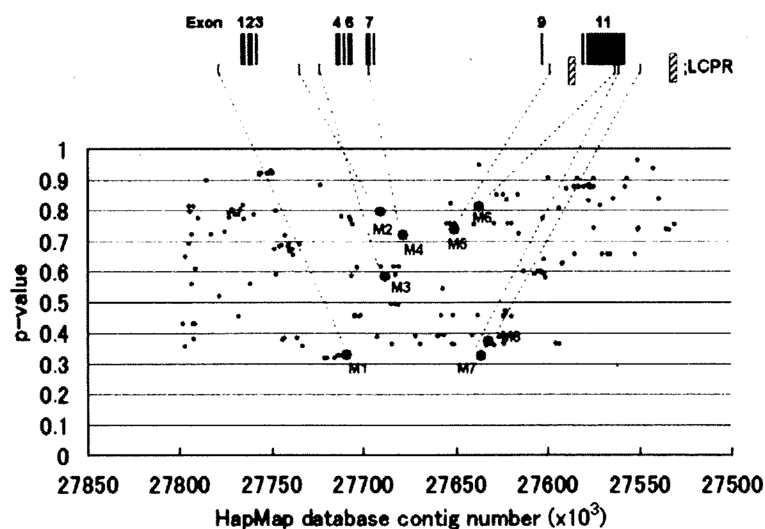

Fig. 2. Results of imputing SNP in the *BDNF* gene. The weights of evidence were calculated using imputed genotypes (small circles) and observed genotypes (big circles). The SNP position from the HapMap database is plotted on the *X* axis.

results were added to the last meta-analysis (Kanazawa et al., 2007), can be seen in Table 4 (Egan et al., 2003; Hong et al., 2003; Nanko et al., 2003; Skibinska et al., 2004; Anttila et al., 2005; de Krom et al., 2005; Neves-Pereira et al., 2005; Schumacher et al., 2005; Tan et al., 2005; Chen et al., 2006; Jonsson et al., 2006; Numata et al., 2006; Tochigi et al., 2006; Watanabe et al., 2006; Zhang et al., 2006; Donohoe et al., 2007; Naoe et al., 2007; Qian et al., 2007; Xu et al., 2007; Han et al., 2008; Takahashi et al., 2008). All studies were independent and in HWE. We did not observe significant heterogeneity among ORs ($Q=18.99$, $df=21$, $p=0.586$). The pooled OR derived from all studies comprising 6568 patients and 8824 control subjects was not significant in each model (fixed model: pooled OR = 0.976, 95% CI = 0.928–1.026, $p=0.345$, random model: OR = 0.976, 95% CI = 0.928–1.026, $p=0.345$; Table 3). Next, in order to limit the ethnic heterogeneity, we analyzed the Caucasian and Asian samples separately. These subgroup analyses also showed no significant

Table 3
Meta-analysis of case-control studies between the rs6265 and schizophrenia.

First author	Year	Ethnicity	No.		No. of G(Val) major allele		No. of A(Met) minor allele		Diagnostic system	OR	95% CI	*p*-value	NOS
			SCZ	CON	SCZ	CON	SCZ	CON					
Egan	2003	American	203	133	332	218	74	48	DSM-IV	1.012	0.68–1.51	0.952	7 (selection 3/4 comparability 2/2 exposure 2/3)
Skinbinska	2004	Polish	336	375	565	613	107	137	DSM-IV	0.847	0.64–1.12	0.242	7 (selection 3/4 comparability 2/2 exposure 2/3)
Antilla	2005	Finnish	94	98	156	166	32	30	DSM-IV	1.135	0.66–1.96	0.648	7 (selection 3/4 comparability 2/2 exposure 2/3)
Neves-Pereira	2005	Scotish	321	350	541	547	101	153	DSM-IV	0.667	0.51–0.88	0.004	8 (selection 3/4 comparability 2/2 exposure 3/3)
Shumacher	2005	German	533	1097	842	1777	224	417	DSM-IV	1.134	0.95–1.36	0.176	8 (selection 3/4 comparability 2/2 exposure 3/3)
de Krom	2005	Dutch	273	580	437	928	109	232	DSM-IV	0.998	0.77–1.29	0.986	7 (selection 3/4 comparability 2/2 exposure 2/3)
Jonsson	2006	Swdish	187	275	312	452	60	98	DSM-III-R	0.887	0.62–1.26	0.504	7 (selection 3/4 comparability 2/2 exposure 2/3)
Zhang	2006	American	84	250	135	406	33	94	DSM-IV	1.056	0.68–1.64	0.810	8 (selection 3/4 comparability 2/2 exposure 3/3)
Donohoe	2007	Irish	359	745	598	1241	120	249	DSM-IV	1.000	0.79–1.27	0.999	7 (selection 3/4 comparability 2/2 exposure 2/3)
Hong	2003	Han Chinese	93	198	85	189	101	207	DSM-IV	1.085	0.76–1.54	0.648	7 (selection 3/4 comparability 2/2 exposure 2/3)
Nanko	2003	Japanese	178	332	209	382	147	282	DSM-IV	0.953	0.73–1.24	0.716	7 (selection 3/4 comparability 2/2 exposure 2/3)
Tan	2005	Han Chinese	108	145	117	165	99	125	DSM-IV	1.117	0.78–1.59	0.541	8 (selection 3/4 comparability 2/2 exposure 3/3)
Chen	2006	Han Chinese	560	576	573	607	547	545	DSM-IV	1.063	0.90–1.25	0.465	7 (selection 3/4 comparability 2/2 exposure 2/3)
Numata	2006	Japanese	159	318	198	364	120	272	DSM-IV	0.811	0.62–1.07	0.137	7 (selection 3/4 comparability 2/2 exposure 2/3)
Tochigi	2006	Japanese	401	569	487	675	315	463	DSM-IV	0.943	0.78–1.13	0.533	7 (selection 3/4 comparability 2/2 exposure 2/3)
Watanabe	2006	Japanese	349	423	407	491	291	355	DSM-IV	0.989	0.81–1.21	0.914	7 (selection 3/4 comparability 2/2 exposure 2/3)
Naoe	2007	Japanese	211	205	249	258	173	152	DSM-IV	1.179	0.89–1.56	0.247	7 (selection 3/4 comparability 2/2 exposure 2/3)
Qian	2007	Han Chinese	604	650	616	657	592	643	DSM-IV	0.982	0.84–1.15	0.820	7 (selection 3/4 comparability 2/2 exposure 2/3)
Xu	2007	Han Chinese	275	297	292	297	258	297	DSM-IV	0.884	0.70–1.11	0.296	7 (selection 3/4 comparability 2/2 exposure 2/3)
Han	2008	Korean	96	79	97	84	95	74	DSM-IV	1.112	0.11–0.22	0.622	7 (selection 3/4 comparability 2/2 exposure 2/3)
Takahashi	2008	Japanese	33	29	39	37	27	21	ICD-10	1.220	0.20–0.37	0.592	7 (selection 3/4 comparability 2/2 exposure 2/3)
Current study	2007	Japanese	1111	1100	1304	1259	918	941	DSM-IV	0.942	0.86–1.06	0.326	7 (selection 3/4 comparability 2/2 exposure 2/3)
Pooled (22)[a] fixed		All	6568	8824	8591	11813	4543	5835		0.973	0.93–1.02	0.293	
Pooled (22)[a] random		All								0.973	0.93–1.02	0.293	
Pooled (9)[b] fixed		Caucasian	2390	3903	3918	6348	860	1458		0.967	0.88–1.06	0.480	
Pooled (9)[b] random		Caucasian								0.956	0.85–1.08	0.444	
Pooled (13)[c] fixed		Asian	4178	4921	4673	5465	3683	4377		0.980	0.92–1.04	0.501	
Pooled (13)[c] random		Asian								0.980	0.92–1.04	0.501	

SCZ, schizophrenia patients; CON, control.
OR, odd ratio; CI, confidence interval; NOS, The Newcastle–Ottawa Scale.
[a] $Q=18.99$, $df=21$, $p=0.586$ for heterogeneity.
[b] $Q=11.54$, $df=8$, $p=0.173$ for heterogeneity.
[c] $Q=7.39$, $df=12$, $p=0.831$ for heterogeneity.

association in either ethnic sample (Table 3). No publication bias was found ($t = 0.369$, $p = 0.716$).

3.3.2. C270T polymorphism (high-resolution plot is shown in Supplementary Figure 2)

For C270T polymorphism, nine population-based association studies and our current study met our criteria for the updated meta-analysis (Watanabe et al., 2007). (Table 4) (Nanko et al., 2003; Szekeres et al., 2003; Anttila et al., 2005; Galderisi et al., 2005; Szczepankiewicz et al., 2005; Jonsson et al., 2006; Watanabe et al., 2006; Zhang et al., 2006; Xu et al., 2007). Total sample sizes for patients and control subjects were 2887 and 3336, respectively. All studies were independent and in HWE. We did not observe significant heterogeneity among ORs ($Q = 13.57$, $df = 9$, $p = 0.139$). The ORs and 95% CIs for the 10 population-based studies are shown in Table 3. When the analysis was run within a fixed model, the T allele was significantly associated with schizophrenia (pooled OR = 1.219, 95% CI = 1.013–1.468, $Z = 2.100$, $p = 0.036$). However, in a random effects model, there was no evidence of a significant association (pooled OR = 1.268, 95% CI = 0.997–1.614, $Z = 1.934$, $p = 0.053$). When the study that showed the most significant results (Szekeres et al., 2003) was removed, the association in the fixed model did not remain significant (pooled OR = 1.165, 95% CI = 0.966–1.407, $Z = 1.596$, $p = 0.110$).

The subgroup analyses based on the ethnic differences were then analyzed separately. The pooled OR derived from the six Caucasian studies comprising 970 patients and 1182 control subjects was not significant (fixed model: pooled OR = 1.210, 95% CI = 0.922–1.589, $p = 0.169$, random model: OR = 1.273, 95% CI = 0.884–1.834, $p = 0.195$), nor was the pooled OR derived from the four Asian studies comprising 1917 patients and 2154 control subjects (fixed model: pooled OR = 1.227, 95% CI = 0.953–1.580, $p = 0.112$, random model: pooled OR = 1.293, 95% CI = 0.894–1.870, $p = 0.173$). No publication bias was found ($t = 1.89$, $p = 0.101$).

4. Discussion

In this study, we carried out a detailed LD evaluation of the region harboring *BDNF*-LCPR, a gene-based association study in the Japanese population, and we updated the meta-analysis of two functional SNPs, Val66Met and C270T. From these results, we conclude that the commonly identified variants in *BDNF* are not associated with schizophrenia.

Regarding single marker based LD tagging, our data showed that *BDNF*-LCPR could be represented well. On the other hand, as for multimarker tagging, our data showed that *BDNF*-LCPR could be represented moderately well by the haplotypes constructed with our tagging SNPs (73.5% coverage) since the most important functional allele in *BDNF*-LCPR, the A1 allele [$(CG)_{del}(CA)_{12}(GA)_3$], could not be captured adequately by our tagging SNPs. These included C270T, which showed a trend for significance in the meta-analysis ($p_{fix\ model} = 0.036$, $r^2 = 0.000945$). In other words, we could not evaluate the tagging SNPs haplotype coverage (M6–M7–M8)

Table 4
Meta-analysis of case-control studies between the C270T polymorphism and schizophrenia.

First author	Year	Ethnicity	No.		No. of major (C) allele		No. of minor (T) allele		Diagnostic system	OR	95% CI	p-value	NOS
			SCZ	CON	SCZ	CON	SCZ	CON					
Szekeres	2003	Finnish	101	68	174	132	28	4	DSM-IV	5.310	1.82–15.51	0.002	7 (selection 3/4 comparability 2/2 exposure 2/3)
Antilla	2005	Finnish	94	98	171	182	17	14	DSM-IV	1.292	0.62–2.70	0.495	8 (selection 3/4 comparability 2/2 exposure 3/3)
Galderisi	2005	Itarian	107	111	201	211	13	11	DSM-IV	1.241	0.54–2.83	0.609	8 (selection 3/4 comparability 2/2 exposure 3/3)
Szczepankiewicz	2005	Polish	397	380	755	725	39	35	DSM-IV	1.070	0.67–1.71	0.777	8 (selection 3/4 comparability 2/2 exposure 3/3)
Jonsson	2006	Swedish	187	275	354	521	20	29	DSM-IV	1.015	0.57–1.82	0.96	7 (selection 3/4 comparability 2/2 exposure 2/3)
Zhang	2006	American	84	250	158	470	10	30	DSM-IV	0.992	0.47–2.07	0.982	8 (selection 3/4 comparability 2/2 exposure 3/3)
Nanko	2003	Japanese	178	332	339	649	17	15	DSM-IV	2.170	1.07–2.13	0.032	8 (selection 3/4 comparability 2/2 exposure 3/3)
Watanabe	2006	Japanese	349	423	672	827	26	19	DSM-IV	1.684	0.92–3.07	0.089	7 (selection 3/4 comparability 2/2 exposure 2/3)
Xu	2007	Han Chinese	275	297	534	574	16	20	DSM-IV	0.860	0.44–1.68	0.658	7 (selection 3/4 comparability 2/2 exposure 2/3)
Current study	2007	Japanese	1115	1102	2159	2138	71	66	DSM-IV	1.065	0.76–1.50	0.716	7 (selection 3/4 comparability 2/2 exposure 2/3)
Pooled (10)[a] fixed		All	2887	3336						1.219	1.01–1.47	0.036	
Pooled (10)[a] random		All								1.268	0.997–1.614	0.053	
Pooled (6)[b] fixed		Caucasian	970	1182						1.210	0.92–1.59	0.169	
Pooled (6)[b] random		Caucasian								1.273	0.88–1.83	0.195	
Pooled (4)[c] fixed		Asian	1917	2154						1.227	0.95–1.58	0.112	
Pooled (4)[c] random		Asian								1.293	0.89–1.87	0.173	

SCZ, schizophrenia patients; CON, control.
OR, odd ratio; CI, confidence interval; NOS, The Newcastle–Ottawa Scale.
[a] $Q = 13.57$, $df = 9$, $p = 0.139$ for heterogeneity.
[b] $Q = 8.24$, $df = 5$, $p = 0.143$ for heterogeneity.
[c] $Q = 5.32$, $df = 3$, $p = 0.150$ for heterogeneity.

for the A1 allele appropriately, due to the small size of the LD evaluation sample and the low population frequency of A1 (only 3 subjects had the A1 allele). Therefore, we included the imputation analysis of SNPs around *BDNF* to examine whether unobserved SNPs would be associated with schizophrenia. This is because we expected to increase the possibility of capturing the A1 allele in *BDNF*-LCPR by multi-marker information in the HapMap database. Nevertheless, we could find no evidence for association from imputed SNPs with schizophrenia. Thus, taking these findings together, we speculate that: 1) Variants in *BDNF* including *BDNF*-LCPR may not be associated with schizophrenia, and 2) we merely overlooked the association of the A1 allele in *BDNF*-LCPR in our analysis. The aforementioned scenario also implies that the LD structure between the Al allele and common SNPs examined in the LD evaluation (or the imputation) may be unique. Therefore, further LD evaluation and a mutation scan will be needed to obtain conclusive results and in order to capture the A1 allele, considering the technical difficulty regarding direct genotyping of this complex polymorphic region.

Our gene-based association study and meta-analysis of Val66Met did not show an association with schizophrenia. Our sample size in these analyses was large enough to rule out type II error. Meanwhile, meta-analysis of C270T showed a trend for significance in the fixed model. In general, the meta-analytic strategy has the advantage of increasing the statistical power; however the interpretation of positive results from meta-analysis in genetic association studies is difficult. Speaking conservatively, the pooling of results can be truly reasonable only when the causal variants have been identified (i.e. biological evidence for such variants has been established clearly) (Sand, 2007). Of course we cannot safely say in every case, therefore we have to try to reduce the possible confounding effects, such as LD differences among populations; it is well known that LD structures are often unstable and cannot be generalized across populations of different origin (Ingles et al., 1997). In relation to this, the author proposed that the MAFs among populations must be examined for meta-analysis (Sand, 2007). In our case, a two-fold difference in C270T allele frequency was found between Caucasian samples (5.2%) and Asian samples (2.7%), suggesting that there is genetic heterogeneity among populations. Thus, we assume from the viewpoint of this heterogeneity or just from multiple testing that this trend for association may be derived from false-positives. To correct the p-value using the Benjamini–Hochberg (BH) method, which is a type of false discovery rate (FDR) controlling procedure, the Q-value in the C270T meta-analysis ($Q = 0.0136$) is higher than the p-value in the fixed model ($p = 0.036$). This gap suggests no significance in the C270T meta-analysis. Therefore, our case-control analysis based on LD and meta-analyses does not provide evidence for an association of the *BDNF* gene with schizophrenia.

Several caveats should be noted. Firstly, we did not include a systematic mutation scan in the 5′ flanking region or exon regions. Secondly, our phenotypic diagnosis is not based on structured interviews and control samples are significantly younger than case samples.

To conclude, our results indicate that common SNPs in the *BDNF* gene do not play a major role in patients with schizophrenia. However, if there is a possibility for an association of *BDNF* with schizophrenia, the A1 allele in *BDNF*-LCPR is the most attractive candidate variant. Further LD evaluation or an association study in which *BDNF*-LCPR is genotyped directly will be required for a conclusive result.

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Contributors

KK and TK performed laboratory assays and the data-analysis. KK, TK and MI drafted the manuscript. MI, TK, YY, YK, NT, SS, KO, YY, RH, BA, MT and TI advised on data-analysis. NO and NI participated in the design of the study, interpretation of the data, and drafting of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.03.040.

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Genetic Investigation of Some Behaviors and Impairments with Autism Spectrum Disorders Using Structural Equation Modeling*

Makoto Tomita[1], *Takeshi Nishiyama*[2], *Hiroko Taniai*[3], *Sungho Moon*[4], *Taishi Miyachi*[5], *Satoshi Sumi*[6]

Abstract

Autism is a severe developmental disorder of unknown etiology but with evidence for genetic influences. The current study combined 47 twins in order to improve statistical power. At least one proband in each pair was diagnosed as having autism spectrum disorder(ASD), using the DSM-IV category of pervasive developmental disorder. An investigation of genetic structure underlying autistic traits was performed. We use bivariate coefficient for this study. Among all possible values of the parameters, we found the maximum-likelihood estimate(MLE), the value that maximizes the log-likelihood for the model. A best fitting model of influences on autistic traits, incorporating additive genetic (A), shared environmental (C) and non-shared environmental influences (E), was generated using this model framework. We got the AE model as the best model by Akaike's information criterion(AIC), then the estimated heritability was 0.74 for restricted and repetitive behaviors and interests(RRBI), whereas for social impairments(SI) it was 0.96. They are very high values of heritability, respectively.

Keywords : autism, autism spectrum disorder, structural equation modeling, twin study.

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[1]Associate Professor, Risk Analysis Research Center, The Institute of Statistical Mathematics, 106-8569, Japan. E-mail : mtomita@ism.ac.jp, Tel: +81-3-3446-1501

[2]Researcher, Department of Biological Information, Nagoya City University, Mizuho-ku, Nagoya, 467-8501, Japan. E-mail : nishiyama@minos.ocn.ne.jp

[3]Medical Doctor, Nagoya Child Welfare Center, 6-4, Kawanayama-cho, Showa-ku, Nagoya, 466-0827, Japan. E-mail : taniai@kjd.biglobe.ne.jp

[4](Corresponding Author) Professor, Department of Data Management, Pusan University of Foreign Studies, Uam-Dong, Nam-Gu, Busan, 608-768, Korea. E-mail : shmoon@pufs.ac.kr

[5]Assistant Professor, Osaka-Hamamatsu Joint Center for Child Mental Development, 1-20-1, Handayama, Hamamatsu, 431-3192, Japan. E-mail : tai.miya@med.nagoya-cu.ac.jp

[6]Professor, Nagoya West District Care Center for Disabled Children, 1-20-48, Komoto, Nakagawa-ku, Nagoya, 454-0828, Japan. E-mail : satoshi-sumi@mbn.nifty.com

1. Introduction

Autism is a behaviorally defined syndrome, characterized by pervasive impairment of social interaction and communication, and the presence of stereotypical characteristics. The diagnostic criteria of autism have been expanded, from a strictly defined category to a range of autism spectrum disorders(ASDs)(Wing, 1996). Using DSM-IV criteria(American Psychiatric Association 1994), ASDs are characterized as pervasive developmental disorders(PDD). This diagnostic criteria consists of three domains : (1) impairment in social interaction(SI), (2) restricted and repetitive behaviors and interest(RRBI), (3) impairment in communication. It is the very effective model "structural equation modeling(SEM)" which can estimate the heritability for a disease without genome-wide genetic information, so that we can study a preliminary analysis before a large scale genome-wide association study. Bailey et al.(1995) reported that the heritability of autism was 0.91, in their combined sample 60% of monozygotic(MZ) pairs were concordant for autism versus no dizygotic(DZ) pairs. However, autistic patients whose condition may be due to non-genetic factors have also been studied(Gilberg and Colman, 2000). Consequently, autism is a behaviorally defined syndrome with a variety of both genetic and non-genetic causes. In the past decade a lot of genome wide investigations have been carried out in Europe and the United States; however their results have often proved contradictory (Shastry, 2003). There was a possibility that different genes or factors may have been involved in causing the effects in different families. Therefore, it seems to be necessary to select families with a homogeneous etiology. Further epidemiological studies are also important for a successful genome wide investigations, after checking high heritability for a disease, which can be estimated without typing genome informations. So, it can be estimated if there are twins or siblings informations. In our most recent twin study we demonstrated that heritability was higher for females than for males. The genetic influence on each domain is still unknown. In this study we examine the genetic influences on the domains, RRBI(restricted and repetitive behaviors and interests) and SI(social impairments).

2. Subjects and Methods

This study was conducted using a regional routine screening system in the catchment area, as described in the previous report(Taniai et al., 2008). Twin samples were the same as in their twin study. Diagnosis and zygosity were also the same as for the previous reports. The sample size was 47 twins.

The Childhood Autism Rating Scale(CARS) was used for evaluating the severity of

restricted and repetitive behaviors and interests(RRBI) and social impairments(SI). The CARS-Tokyo Version(CARS-TV) is an objective, behaviorally based rating scale with demonstrated reliability and validity in Japanese(Kurita et al, 1989). The CARS comprises 15 items; (1) relationships with people, (2) imitation, (3) affect, (4) use of body, (5) relation to non-human objects, (6) adaptation to environmental change, (7) visual responsiveness, (8) auditory responsiveness, (9) near receptor responsiveness, (10) anxiety reaction, (11) verbal communication, (12) nonverbal communication, (13) activity level, (14) intellectual functioning, and (15) general impressions, rated ranging from one to four with a score of one indicating age-appropriate behavior and a score of four representing severely abnormal behavior with subunits of 0.5. The total score of items (4)(5)(6) is used as an index to RRBI and that of items (1)(2)(11)(12) is as an index to SI.

We wish to determine the best fitting models of causation for autistic traits-restricted and repetitive behaviors and interests(RRBI) and social impairments(SI). The data were subjected to structural equation modeling(SEM) using the statistical package Mx(Neale et al., 1994; Posthuma and Boomsma, 2005). We examined the nature of the discrepancies in the causal structure of autistic traits without sex differences, so that it is compared the model in which causative influences were constrained to be equal across the two genders. The first model tested(the full model) incorporated the effects of three parameters, additive genetic (A), shared environmental (C), and non-shared environmental causal influences (E), within the general sex limitation model framework in a continuous data analysis. Usually, for evaluated genetic effects, univariate or bivariate genetic analyses showed that the relative effects of genetic(additive genetic) vs. environmental(common shared environment and non-shared environment) factors. For monozygotic/dizygotic twins, successive models in which one or more of these variables were then dropped from the full model were tested to quantify the extent to which each model fit the observed data. The likelihood ratio χ^2 test was used to compare nested models and the Akaike's information criterion(AIC) was employed to compare unnested models.

Additionally, as we ascertained twin pairs via probands, an ascertainment correction was applied for continuous raw data using the option for user-defined fit function in the Mx Script according Wada(1999). In brief, the likelihood of 2 persons pairs not being in the ascertained sample can be expressed as a double integral of the bivariate normal distribution. In detail, see Nishiyama et al.(2009). We use the same equation as previous report.

Recently, the prevalence of ASDs is much higher than about 1%, reported by Baird et al.(2006). Similarly, we also found that the prevalence of ASDs in males was 3.3% and that in females was 0.82% in our population(Sumi et al., 2006), corresponding to the standard normal deviate of 1.84 and 2.40, respectively and these threshold values were used for ascertainment

correction for males and for females.

3. Results

Among all possible values of the parameters, we found the maximum-likelihood estimate (MLE) using Mx, the value that maximized the log-likelihood for the model. A best fitting model of influences on autistic traits, incorporating additive genetic and non-shared environmental influences, was generated. We have considered a full model, the ACE model with common additive genetic (Ac), common shared environmental (Cc), common non-shared environmental causal influences (Ec), specific additive genetic (As), specific shared environmental (Cs) and specific non-shared environmental causal influences (Es) since Akaike's information criterion(AIC) had a value of 153.04. On the other hand, AIC is 150.82 when (full-Ac) model, 149.04 when (full-Cc) model, 146.72 when (full-Cc-Ec) model, 148.88 when (full-Cc-Ec-As-Cs) model, and so on(See Table 3.1). We got the best AIC value 140.72 when (full-Cc-Ec-As-Cs|s) model, then the estimated parameters are shown in Figure 3.1 and Figure 3.2. the estimated heritability was 0.74(0.55-0.87) for restricted and repetitive behaviors and interests(RRBI), whereas for social impairments(SI) it was 0.96(0.88-0.99) which are shown in Figure 3.3. They are very high values of heritability, respectively.

Independent ACE model has additive genetic (A), shared environmental (C) and non-shared environmental influences (E). Common ACE model has common additive genetic (Ac), common shared environmental (Cc) and common non-shared environmental influences (Ec). As is specific additive genetic influences, Cs is specific common shared environmental influences and Es is specific non-shared environmental influences.

Table 3.1 Akaike's information criterion (AIC) on independent/common ACE models

	Akaike's information criterion (AIC)	d.f.
Full model (independent ACE)	153.04	54
(Full-Ac) model	150.82	56
(Full-Cc) model	149.04	56
(Full-Cc-Ec) model	146.72	58
(Full-Cc-Ec-As) model	142.72	60
(Full-Cc-Ec-As-Cs|s) model	140.72	61
(Full-Cc-Ec-As-Cs) model	148.88	54
Full* model (common ACE)	167.41	56
(Full*-Ac) model	165.41	57
(Full*-Ac-Ec) model	163.41	58
(Full*-Ac-Cc-Ec) model	161.41	59

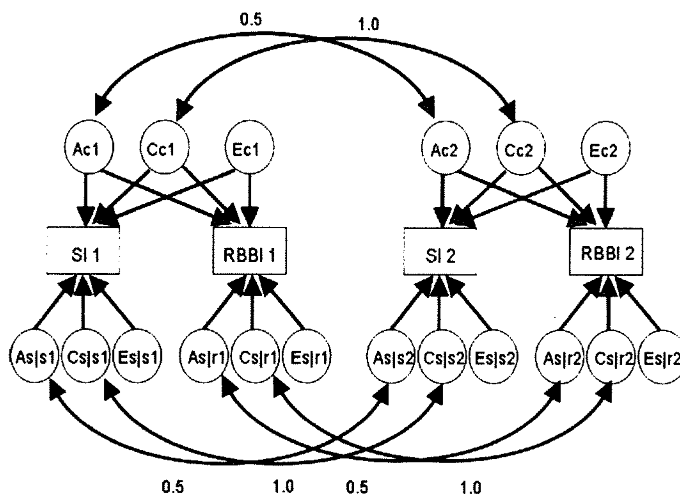

Figure 3.1 Independent ACE model

SI 1(2): SI score of each twin, RBBI 1(2): RBBI score of each twin,
Ac: common additive genetic influences between SI and RBBI,
Cc: common shared environmental influences between SI and RBBI
Ec: common non-shared environmental influences between SI and RBBI,
As|s1(2): specific additive genetic influences for SI,
Cs|s1(2): specific common shared environmental influences for SI
Es|s1(2): specific non-shared environmental influences for SI,
As|r1(2): specific additive genetic influences for RBBI
Cs|r1(2): specific common shared environmental influences for RBBI,
Es|r1(2): specific non-shared environmental influences for RBBI

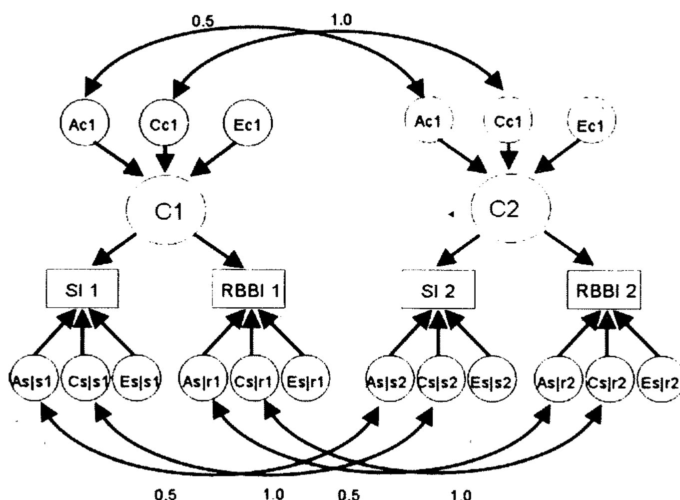

Figure 3.2 Common ACE model

Notations are same as Figure 3.1. C1: common factor SI1 and RBBI1, C2: common factor SI2 and RBBI2

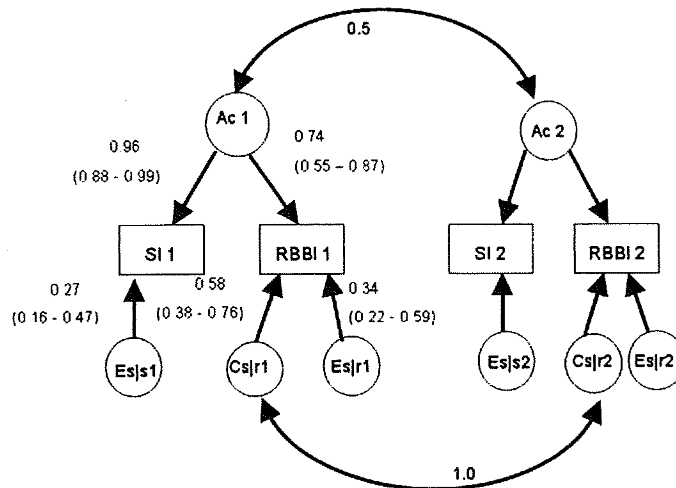

Figure 3.3 A result of best fitting model (dizygotic twin) by AIC

Notations are same as Figure 3.1.

4. Discussion

This study has also indicated that restricted and repetitive behaviors and interests(RBBI) and social impairments(SI) have high heritabilities, we've gotten similar results in our previous study(Taniai et al., 2008; Nishiyama et al., 2009). Especially, Nishiyama et al.(2009) had studied about bivariate IQ values and CARS scores, it is a good result because we have selected the same model as their study.

We think it would be accurate to say that genetic influences are responsible for the autism spectrum disorders strongly. However, it is not clearly that how much influences are responsible for each domain, respectively.

We know that there are the strong additive genetic influences for two phenotypes-RRBI and SI. Second, there are the specific common shared environmental influences for RRBI and SI. Third, it may be that there are some biases of heritability in this study, since we didn't think the difference between male and female, non-shared additive genetic influences and the correlation of ascertainment bias. Because, the sample size is so small, then the this study should have more large sample size and the further discussion(e.g. the considering sex differences) for an exact evaluation. However, further investigation might be necessary to verify that the model is well founded.

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