研究成果の刊行に関する一覧表

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ORIGINAL ARTICLE

Dynamin 2 gene is a novel susceptibility gene for late-onset Alzheimer disease in non-*APOE*-ε4 carriers

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Abstract Alzheimer disease (AD) is characterized by progressive cognitive decline caused by synaptic dysfunction and neurodegeneration in the brain, and late-onset AD (LOAD), genetically classified as a polygenetic disease, is the major form of dementia in the elderly. It has been shown that β amyloid, deposited in the AD brain, interacts with dynamin 1 and that the dynamin 2 (*DNM2*) gene homologous to the dynamin 1 gene is encoded at chromosome 19p13.2 where a susceptibility locus has been detected by linkage analysis. To test the genetic association of LOAD with the *DNM2* gene, we performed a case–control study of 429 patients with LOAD and 438 sex- and age-matched control subjects in a Japanese population. We found a significant association of LOAD with single nucleotide polymorphism markers of the *DNM2* gene, especially in non-carriers of the apolipoprotein E-ε4 allele. Even though subjects with the genotype homozygous for the risk allele at rs892086 showed no mutation in exons of the *DNM2* gene, expression of *DNM2* mRNA in the hippocampus was decreased in the patients compared to non-demented controls. We propose that the *DNM2* gene is a novel susceptibility gene for LOAD.

Keywords Alzheimer · Apolipoprotein E · Association · Chromosome 19p · Dynamin 2 · Genetic risk

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Introduction

Alzheimer disease (AD) is the most common form of dementia in the elderly and is characterized by progressive cognitive decline with brain atrophy that is most marked in the temporal lobes. It is thought that β amyloid is a causative molecule in AD by disturbing synaptic function, leading to neuronal death (for review, see Selkoe 2002; Yao 2004). Although both early- and late-onset AD (LOAD) exhibit the same neuropathology in the brain, LOAD is genetically classified as a polygenetic disease and is characterized by more heterogeneous conditions than autosomal dominant early-onset AD. Apolipoprotein E (*APOE*) has been shown to be a major risk factor for LOAD (Corder et al. 1993; Farrer et al. 1997). Genome scans of LOAD detected several susceptibility loci, among

which chromosomes 12, 10 and 9 have been the targets of searches for risk genes (Pericak-Vance et al. 1997; Blacker et al. 2003). Multipoint linkage analysis of LOAD families have also demonstrated a susceptibility locus at 19p13.2 between D19S391and D19S914 (Wijsman et al. 2004).

The major role of the dynamin proteins is in the endocytosis of vesicles, and its functions in vesicle budding have been described as being responsible for the constriction of the lipid neck, fission of lipids and regulation of the scission reaction (for review, see Praefcke and McMahon 2004). Expression of the dynamin 2 (*DNM2*) as well as dynamin 1 (*DNM1*) gene is downregulated by β amyloid in hippocampal neurons (Kelly et al. 2005), suggesting that the dynamin proteins are involved in the cascade of neurodegeneration caused by β amyloid. The dynamin-binding protein (*DNMBP*) gene on chromosome 10 has also been shown to be associated with LOAD (Kuwano et al. 2006). We observed that the *DNM2* gene is located at 19p13.2, within the region where a susceptibility locus was noted (Wijsman et al. 2004). Therefore, the *DNM2* gene could be a positional and functional candidate for a genetic risk for LOAD.

To examine whether the *DNM2* gene is genetically associated with LOAD, we performed an age- and sex-matched case–control study in a Japanese population. We propose herein that the *DNM2* gene is a novel genetic factor for LOAD in non-*APOE*-ε4 carriers.

Subjects and methods

Study subjects

Patients with LOAD were diagnosed as having definite or probable AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) (McKhann et al. 1984). Controls consisted of non-demented elderly subjects obtained from the general population. Written informed consent to participate in this study was obtained, and then peripheral blood was drawn and subjected to DNA extraction. For a definite diagnosis of AD, dissections were carried out at the Choju Medical Institute after obtaining the agreement of the patients' guardians for diagnosis and genomic research. In total, 429 (69.9% female) patients participated in the study, of whom 66 had definite AD and 363 had probable AD. The mean age $\pm$ SD of the patient population at onset was 72.3 $\pm$ 8.1 years (range 60–94 years), and the mean age at blood drawing was 77.4 $\pm$ 8.7 years (range 60–98 years). The controls consisted of 438 individuals (63.7% female). The mean age of the controls at assessment was 74.5 $\pm$ 5.5 years (range 60–99 years). The age at onset of the patient was matched to the age of controls, and the sex composition was not different between the groups. Hippocampal tissue was also obtained from the postmortem brains of 22 patients with AD (age 82.8 $\pm$ 8.5 years, 63.6% female) and 12 controls (age 89.0 $\pm$ 7.0 years, age at onset 72.9 $\pm$ 7.2 years, 58.0% female). DNA was extracted from peripheral blood using a QIAamp DNA Blood Kit (Qiagen, Tokyo, Japan) and from brain tissue by the phenol–chloroform method (Sambrook et al. 1989). The procedure to obtain the specimens was approved by the Genome Ethical Committee of Osaka University Graduate School of Medicine, Ehime University, and the Choju Medical Institute of Fukushimura Hospital.

Genotyping and sequencing

Single nucleotide polymorphisms (SNPs) in the *DNM2* gene regions used in this study are listed in Table 1. Genotyping was performed by a quantitative genotyping method using the TaqMan SNP Genotyping System (Applied Biosystems, Foster City, CA). The genotype of the *APOE* gene was determined by a PCR-restriction fragment length polymorphism (RFLP) method (Wenham et al. 1991). DNA obtained from six patients and three controls homozygous for the risk allele at rs892086 of the *DNM2* gene was subjected to direct sequencing of its exons using the primers listed in Electronic Supplementary Material.

Quantitative real-time PCR

Total RNA was isolated from frozen hippocampal tissues using the acid guanidine–phenol–chloroform RNA extraction method provided as ISOGEN (Nippon Gene, Toyama, Japan) and purified using an RNeasy Mini kit (Qiagen, Valencia, CA). RNA samples with an A_{260}/A_{280} absorption ratio over 1.9 were subjected to cDNA synthesis using a High-Capacity cDNA Archive kit (Applied Biosystems). Primers and probe sets for the human *DNM2* and *β-actin* genes were purchased from TaqMan Gene Expression Assay products (Applied Biosystems), and quantitative real-time PCR was carried out in an ABI PRISM 7900HT (Applied Biosystems). All quantitative PCR reactions were duplicated, and the ratio of the amount of *DNM2* cDNA to that of the *β-actin* internal control cDNA was determined at the cycle threshold (CT).

Statistical analysis

Linkage disequilibrium (LD) between all pairs of biallelic loci was measured by Lewontin's D′ (|D′|) (Hedrick 1987)

Table 1 Single nucleotide polymorphism (SNP) markers in the *DNM2* gene

NCBI SNP reference ID	Location in NCBI (build 36.1)	Location	SNP sequence (allele ½)	Strand/ orientation	Minor allele
rs12974306	10691281	Intron 1	CTCTT[G/T]CCTTT	fwd/B	Allele 2
rs714307	10696405	Intron 1	CGCTA[C/T]TGCTG	fwd/B	Allele 1
rs892086	10698677	Intron 1	GTTAG[A/G]TACCA	rev/T	Allele 1
rs34626880	10701428	Intron 1	AGCTC[C/T]ACCTG	fwd/B	Allele 2
rs10775614	10728219	Intron 1	GGCAC[A/ G]TGGCG	fwd/T	Allele 2
rs7246673	10737841	Intron 2	AACCC[G/T]GCTGT	fwd/B	Allele 1
rs3826803	10744126	Intron 2	TTTCT[C/G]ATTTT	fwd/B	Allele 2
rs2043332	10752239	Intron 5	GTGAC[A/C]TCAGG	rev/T	Allele 1
rs873016	10755728	Intron 6	AAATG[A/G]TATTA	rev/T	Allele 1
rs1109376	10775829	Intron 12	AGGAT[A/G]CTTCT	fwd/T	Allele 1
rs3786719	10788100	intron 15	TGGAA[C/G]CTTCC	fwd/T	Allele 2
rs11085748	10788540	intron 15	GTTTT[C/T]CTCAT	fwd/B	Allele 2
rs3760781	10808522	3'UTR	TTGAG[C/T]GCTCA	fwd/B	Allele 2

UTR, Untranslated region; NCBI, National Center for Biotechnology Information

and r^2. Haplotype blocks, defined as segments with strong LD (Gabriel et al 2002), were calculated using HAPLOVIEW (Barrett et al. 2005). Allele and genotype frequencies were assessed for associations by one-sided chi-squared test for both allele and genotype frequencies in dominant and recessive models, where p values less than 0.05 were tentatively judged to be significant. The effective number of independent marker loci in the *DNM2* gene was calculated to correct for multiple testing, using the software SNPSPD (http://www.genepi.qimr.edu.au/general/daleN/SNPSpD/) based on the spectral decomposition of matrices of pair-wise LD between SNPs (Nyholt 2004). The experiment-wide significance threshold required to keep the type I error rate at 5% was used for judging significance to correct for multiple testing. The values obtained by quantitative PCR, having a normal distribution, were compared by Student's t test, and a p value less than 0.05 was considered to be significant.

Results

We genotyped 13 SNPs located from intron 1 to the 3′-untranslated region (UTR) of the *DNM2* gene (Table 1). In total, 429 cases and 438 sex- and age-matched controls were genotyped, and their genotype distributions of both the cases and controls were in Hardy–Weinberg equilibrium. In these datasets, the *APOE*-ε4 allele was associated with LOAD ($p < 1 \times 10^{-10}$): compared to non-*APOE*-ε4 carriers, the odds ratio for carrying one *APOE*-ε4 allele was 4.3 [95% confidence interval (CI) 3.12–6.16] and that for carrying two *APOE*-ε4 allele was 28.4 (95% CI 6.75–119). Linkage disequilibrium statistics indicated more than three haplotype blocks in the *DNM2* gene region (Fig. 1). No validated SNPs were available between rs873016 and rs1109376 at a distance of approximately 20 kb, and no strong evidence of LD was found between these two SNPs. The case–control study showed that p values of less than 0.05 were found in four SNPs located from intron 6 to the 3′UTR in terms of allele distribution, and in seven SNPs from intron 1 to the 3′UTR in terms of genotype frequencies; their odds ratios were between 1.53 and 1.75 (Table 2). Calculations with SNPSPD indicated that the effective number of independent marker loci was 8.3094 and that the experiment-wide significance threshold was 0.006. Therefore, rs3760781 remained significant after the correction for multiple testing ($p = 0.003$). To examine the interaction between the *DNM2* gene and the *APOE* gene, the cases and controls were divided into *APOE*-ε4 carriers and non-*APOE*-ε4 carriers. In non-*APOE*-ε4 carriers, seven markers showed p values of less than 0.05, and the experiment-wide significance threshold (0.0059) supported a significant association at rs892086 ($p = 0.003$) as well as at rs3760781 ($p = 0.004$) (Table 3). However, no association was found in *APOE*-ε4 carriers (data not shown), indicating that the association of the *DNM2* gene is specific for non-*APOE*-ε4 carriers in our dataset.

To examine whether patients with the risk genotype could harbor any mutations in the *DNM2* gene, we sequenced all exons of the *DNM2* gene in patients and controls homozygous for the risk allele at rs892086, but we did not found any mutations, indicating that no particular mutation resulting in amino acid change is linked to the risk genotype of the *DNM2* gene. To examine the expression of the *DNM2* gene in the AD hippocampal tissue, we measured the amount of *DNM2* cDNA normalized to that of *β-actin* cDNA using quantitative PCR. Analysis of ten LOAD and eight control subjects revealed that there was significantly lower amounts of *DNM2* mRNA in the AD hippocampal tissue than in the controls ($p < 0.01$) (Fig. 2).

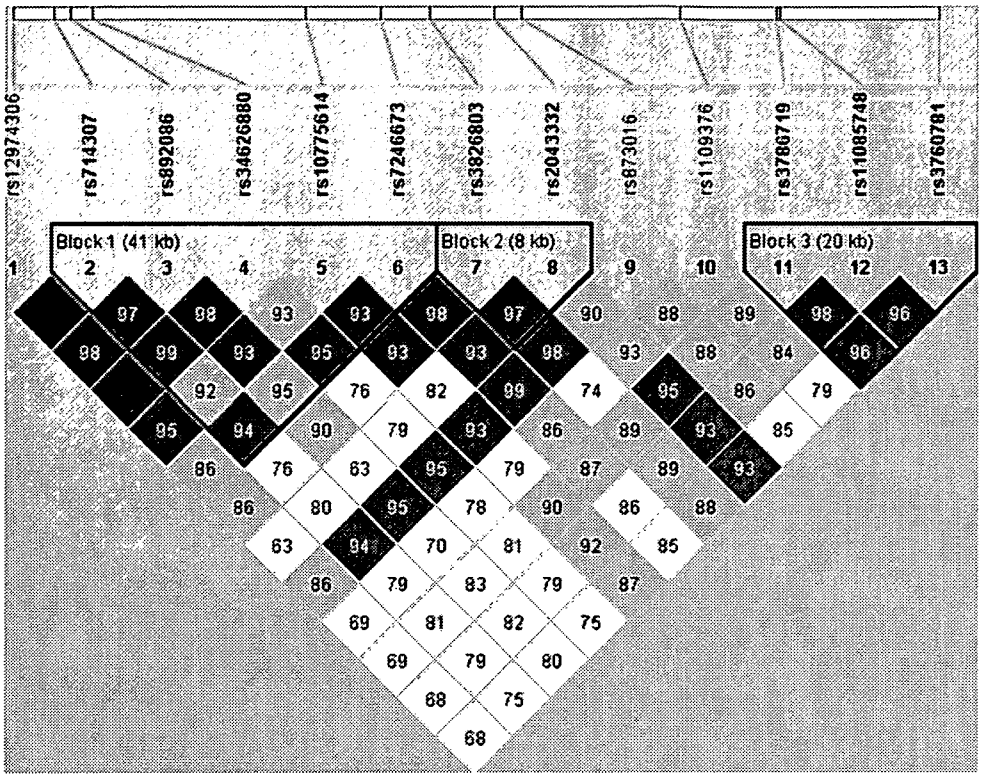

Fig. 1 Linkage disequilibrium coefficients and haplotype blocks in the *DNM2* gene region. Linkage disequilibrium coefficients (ID'I) among *DNM2* single nucleotide polymorphisms (SNPs) and haplotype blocks defined by strong LD are shown

Table 2 Association analysis of late-onset Alzheimer disease in the *DNM2* gene

SNP ID	LOAD				Control				Risk allele	*p* value	Risk genotype	*p* value	O.R (95% CI)
	Genotype number			MAF	Genotype number			MAF					
Genotype	1/1	2/2	½		1/1	2/2	½						
MArs12974306	174	57	196	0.363	204	46	188	0.320	Allele 2	NS	–	NS	
rs714307	16	285	127	0.186	16	304	117	0.170	Allele 1	NS	–	NS	
rs892086	93	134	202	0.452	67	145	226	0.411	Allele 1	NS	1/1	0.015	1.53 (1.08–2.17)
rs34626880	284	16	129	0.188	306	16	116	0.169	Allele 2	NS	–	NS	
rs10775614	306	11	111	0.155	326	14	95	0.141	Allele 2	NS	–	NS	
rs7246673	78	134	215	0.434	55	150	233	0.392	Allele 1	NS	1/1	0.020	1.56 (1.07–2.26)
rs3826803	132	86	209	0.446	145	58	234	0.400	Allele 2	NS	2/2	0.007	1.65 (1.15–2.37)
rs2043332	15	295	118	0.173	11	309	118	0.160	Allele 1	NS	–	NS	
rs873016	80	135	212	0.436	55	153	230	0.388	Allele 1	0.045	1/1	0.012	1.61 (1.11–2.33)
rs1109376	28	256	144	0.234	19	275	144	0.208	Allele 1	NS	–	NS	
rs3786719	145	85	198	0.430	166	57	215	0.376	Allele 2	0.021	2/2	0.007	1.66 (1.15–2.39)
rs11085748	138	81	209	0.433	160	56	222	0.381	Allele 2	0.027	2/2	0.013	1.59 (1.10–2.31)
rs3760781	141	87	199	0.437	157	56	225	0.385	Allele 2	0.028	2/2	0.003*	1.75 (2.21–2.52)

*Significant for experiment-wide significance threshold ($p < 0.006$)

LOAD, Late-onset Alzheimer disease; MAF, Minor Allele Frequency; O.R., odds ratio; 95% CI, 95% confidence interval

Discussion

We found that the *DNM2* gene is genetically associated with LOAD and that this association was specifically significant in non-*APOE*-ε4 carriers. In non-*APOE*-ε4 carriers, two SNPs, not in strong LD, were associated with LOAD. The *DNMBP* gene, which encodes a scaffold protein that binds to DNM1 protein, has been shown to be associated with LOAD in *APOE*-ε3*3 carriers or non-*APOE*-ε4 carriers, but not in *APOE*-ε4 carriers (Kuwano et al. 2006). Therefore, DNM2 protein could interact with proteins encoded in or linked to the *APOE*-ε3 genotype. It is

Table 3 Association analysis of late-onset Alzheimer disease in the *DNM2* gene in non-*APOE*-ε4 carriers

SNP ID	LOAD				Control				Risk allele	*p* value	Risk genotype	*p* value	O.R. (95% CI)
	Genotype number			MAF	Genotype number			MAF					
	1/1	2/2	½		1/1	2/2	½						
rs12974306	87	35	97	0.381	174	37	158	0.314	Allele 2	0.019	2/2	0.033	1.71 (1.04–2.80)
rs714307	6	152	63	0.170	12	259	97	0.164	Allele 1	NS		NS	
rs892086	55	64	102	0.480	56	121	192	0.412	Allele 1	0.023	1/1	0.003*	1.85 (1.22–2.81)
rs34626880	152	6	63	0.170	260	12	97	0.164	Allele 2	NS		NS	
rs10775614	164	5	51	0.139	278	11	77	0.135	Allele 2	NS		NS	
rs7246673	41	62	117	0.452	46	127	196	0.390	Allele 1	0.037	1/1	0.041	1.61 (1.02–2.54)
rs3826803	63	42	115	0.452	121	49	198	0.402	Allele 2	NS		NS	
rs2043332	7	149	64	0.177	7	264	98	0.152	Allele 1	NS		NS	
rs873016	42	63	116	0.452	46	130	193	0.386	Allele 1	0.025	1/1	0.031	1.65 (1.04–2.60)
rs1109376	13	138	69	0.216	15	234	120	0.203	Allele 1	NS		NS	
rs3786719	71	45	104	0.441	141	47	181	0.373	Allele 2	0.021	2/2	0.013	1.76 (1.13–2.76)
rs11085748	68	44	109	0.446	136	46	187	0.378	Allele 2	0.022	2/2	0.015	1.75 (1.11–2.74)
rs3760781	73	47	100	0.441	135	46	188	0.379	Allele 2	0.037	2/2	0.004*	1.91 (1.22–2.98)

*Significant for experiment-wide significance threshold ($p < 0.0059$)

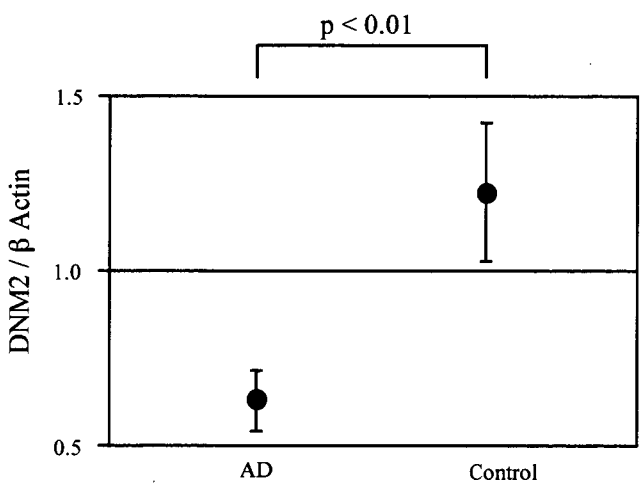

Fig. 2 Expression of *DNM2* mRNA in the hippocampus. The ratio of the amount of *DNM2* cDNA to that of *β-actin* cDNA is shown. *Dots* indicate mean value, *bars* indicate standard error

possible that the causative mechanism of DNM2 for the development of AD could be different from the lipid transfer proteins involved in lipid metabolism, such as the *APOE* (Strittmatter et al. 1993), *LRP* (Kang et al. 1997) and *CYP46* genes encoding cholesterol 24S-hydroxylase (Kolsch et al. 2002). However, the majority of cases genotyped in our study are still living, and the use of still living controls also warrants caution as the incidence of developing dementia increases with age. Therefore, our results could be misrepresented, as the controls may still develop AD, or we may have misdiagnosed AD patients who may actually have another form of dementia.

The *DNM* gene was first identified as the locus for a paralytic phenotype in *Drosophila melanogaster* (Suzuki et al. 1971) and encodes large GTPases that can associate with microtubules in vitro (Shpetner and Vallee 1989; Obar et al. 1990). The dynamin proteins are distinguished from other GTPases by their low GTP-binding affinities and the ability of many members of the dynamin family to interact with lipid membranes (for review, see Praefcke and McMahon 2004). Mutations of the pleckstrin homology domain of the *DNM2* gene, leading to diminished binding of the DNM2 protein to membranes, are responsible for Charcot–Marie-Tooth disease (Zuchner et al. 2005). While Charcot–Marie-Tooth disease is clinically characterized by peripheral neuropathy, the relation between aging and *DNM2* gene expression remains undetermined. Disuse muscle atrophy related to decreased daily activity is commonly found in the elderly, but it is unclear whether exercise is effective for the maintenance of cognitive function.

Kelly et al. (2005) showed that β amyloid induces depletion of the DNM1 as well as DNM2 protein in cultured hippocampal neurons and the hippocampus of a Tg2576 mouse model of AD. On the other hand, dominant-negative DNM1, which selectively inhibits receptor-mediated endocytosis, raises the levels of mature amyloid precursor protein (APP) at the cell-surface, which is consistent with retention of APP on the plasma membrane, and endogenous Aβ secretion was significantly increased (Chyung and Selkoe 2003). It has also been shown that the location of β amyloid can be changed by decreased activity

of the DNM1 protein and that endocytosis affects the precision of PS-dependent epsilon-cleavage in cell culture (Fukumori et al. 2006). Whereas the DNM1 protein is specific for presynaptic terminals in the central nervous system (CNS), the DNM2 protein is ubiquitously expressed and, to our knowledge, does not exist in presynaptic terminals in the CNS. However, DNM2 has a similar structure to DNM1 and might also affect the sequestration and scavenging of β amyloid in relation to its axonal transport in peripheral nervous system.

We found that the expression of hippocampal *DNM2* mRNA was lower in the patients than in the control subjects, but this result should be carefully interpreted. We examined a small number of hippocampal tissue samples and used *β-actin* cDNA as an internal control; however, quantitative PCR revealed that the *β-actin* transcript is differently expressed in brain specimens of AD and control subjects (Gutala and Reddy 2004). Therefore, this decrease should be examined in the other brain areas and also in a larger number of samples using another internal control cDNA, such as *GAPDH* (Gutala and Reddy 2004). Alternatively, *DNM2* gene expression could be depleted in AD due to the widespread devastation of neurons, particularly in the hippocampus, as well as by β amyloid. Therefore, it remains to be determined whether the decrease in *DNM2* expression is the cause or the outcome of AD.

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Failure to replicate the association between *NRG1* and schizophrenia using Japanese large sample

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Abstract

Systematic linkage disequilibrium (LD) mapping of 8p12–21 in the Icelandic population identified neuregulin 1 (*NRG1*) as a prime candidate gene for schizophrenia. However, results of replication studies have been inconsistent, and no large sample analyses have been reported. Therefore, we designed this study with the aim of assessing this putative association between schizophrenia and *NRG1* (especially HAP_{ICE} region and exon region) using a gene-based association approach in the Japanese population.

This study was a two-stage association analysis with a different panel of samples, in which the significant association found in the first-set screening samples (1126 cases and 1022 controls) was further assessed in the confirmation samples (1262 cases and 1172 controls, and 166 trio samples). In the first-set scan, 60 SNPs (49 tagging SNPs from HapMap database, four SNPs from other papers, and seven SNPs detected in the mutation scan) were examined.

One haplotype showed a significant association in the first-set screening samples (Global *P*-value=0.0244, uncorrected). However, we could not replicate this association in the following independent confirmation samples. Moreover, we could not find sufficient evidence for association of the haplotype identified as being significant in the first-set samples by imputing ungenotyped SNPs from HapMap database.

Abbreviations: NRG1, neuregulin 1; SNP, single nucleotide polymorphism; GGF2, glial growth factor 2; LD, linkage disequilibrium; dHPLC, denaturing high performance liquid chromatography; MAF, minor allele frequency; TDT, transmission disequilibrium test; UTR, untranslated region.

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These results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover, the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases.

Keywords: Schizophrenia; Neuregulin 1; Association study; False positive; Linkage disequilibrium

1. Introduction

Schizophrenia is a common psychiatric disorder with a lifetime prevalence of 1% worldwide. Family, twin and adoption studies show conclusive evidence of a substantial genetic component in this disorder. Progress towards detecting these genetic elements is now being made (Harrison and Weinberger, 2005).

The neuregulin 1 gene (*NRG1*) was first reported to be a prime candidate gene for schizophrenia in the Icelandic population (Stefansson et al., 2002). The significant association of a haplotype was detected in the 5′-region of glial growth factor 2 (GGF2) isoforms, and this at-risk haplotype, consisting of five single nucleotide polymorphisms (SNPs) and two microsatellites, was named as HAP_{ICE}. Several subsequent studies provided the following evidence to support this association with schizophrenia.

Firstly, the location of this gene corresponds to the linkage regions for schizophrenia (8p12–21, OMIM: SCZ5), which were identified by recent meta-analyses of genome-wide linkage studies (Badner and Gershon 2002; Lewis et al., 2003). Secondly, recent evidence suggests that mutation within the *NRG1* region might give rise to functional alterations that are in line with the neurodevelopmental hypothesis and glutamate/GABA hypothesis of schizophrenia (Corfas et al., 2004).

Thirdly, several independent association studies have replicated the original significant association found by Stefansson et al. (2002). However, the results of replication studies using the identical number or fewer sets of markers have been inconsistent. Thus, while some research groups did not report any association (Iwata et al., 2004), other studies showed a positive association but showed different 'at-risk' haplotypes to be associated with schizophrenia (Harrison and Law, 2006).

These inconsistent results could stem from the possibility that *NRG1* is not involved in the etiology of schizophrenia in all populations. However, this inconsistency could be a consequence of the unique structure of the human genome. In other words, differences in linkage disequilibrium (LD) among populations may also be responsible for the differences in the results, and the negative findings may only indicate a failure to reflect the actual predisposing variants due to the differences in populations.

Therefore, gene-wide (or region-wide) replication analysis based on LD pattern within the *NRG1* region is essential to detect an association in a certain population setting (Neale and Sham, 2004). In such analyses, particular attention should be paid to selection of genetic variants which adequately reflect the LD background in the targeted population (e.g. tagging SNPs).

Although the above-mentioned LD-based association analysis is based on the common disease–common variant hypothesis, one study reported an association between *NRG1* and schizophrenia from the standpoint of the common disease–rare variant hypothesis (Walss-Bass et al., 2006). The authors scanned the whole exon region, detected a non-synonymous SNP in exon 11, and showed a significant association of this SNP with schizophrenia. Detection of rare but potent functional variants relies on large mutation scan samples; however, such rare variants may also differ among populations (Pritchard, 2001).

Thus, in this study, we first focused on two attractive regions: the 5′ regions of GGF2, where the original study showed the association (henceforth referred to as 'HAP_{ICE} region') and the exon region (henceforth referred to as 'exon region'). In the exon region, prior to association analysis of tagging SNPs, we performed a mutation scan in order to detect the existence of possible potent functional variants in the ethnic samples. In addition, this study was a two-stage association analysis with a different panel of samples, in which the significant association in the first-set screening samples (1126 cases and 1022 controls) was further assessed in confirmation samples (1262 cases, 1172 controls, and 166 trio samples). This approach was adopted in order to avoid the possibility of type I or type II error.

2. Methods and materials

2.1. Subjects

Two independent sample sets were used in this study. For the first-set screening analysis, 1126 patients with schizophrenia (627 male and 499 female; mean age± standard deviation (SD) 47.0±15.3 years) and 1022

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healthy controls (530 male and 492 female; 38.8± 14.5 years) were examined. Confirmation analysis was conducted with three samples consisting of: (a) 1262 patients with schizophrenia (662 male and 600 female; 49.1±14.5 years) (b) 1172 controls (576 male and 596 female; 41.7±14.3 years), and (c) 166 family trios samples (of the patients, 91 male and 75 female; 30.0± 8.3 years).

The subjects for mutation search were 96 patients with schizophrenia. These subjects were also included in the first-set samples. 385 cases and 336 controls in the first-set samples, and 349 cases (including 84 cases from family samples) and 424 controls in confirmation samples are identical to those in our previous report (Iwata et al., 2004) and Fukui et al.'s (2006) report, respectively.

Characterization details and psychiatric assessment of these subjects were as follows. The patients were diagnosed according to DSM-IV criteria consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. All subjects were ethnically Japanese.

After the study had been described to subjects, written informed consent was requested from each. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, Osaka University, Niigata University and Nagoya University Graduate School of Medicine.

2.2. Mutation scan

We performed denaturing high performance liquid chromatography (dHPLC) analysis, details of which can be seen in a previous paper (Ikeda et al., 2005). Primer sequences were designed in accordance with another report (Walss-Bass et al., 2006).

2.3. Tagging SNP selection

We included the three signal SNPs (SNP8NRG221533, SNP8NRG241930 and SNP8NRG243177) from the report of Stefansson et al. (2002) (we excluded SNP8NRG221132 and SNP8NRG433E1006 from the first-set analysis due to low minor allele frequencies (MAFs) in the Japanese population), one positive SNP from the report of Walss-Bass et al. (2006), and SNPs we detected in the mutation scan. Next we consulted the HapMap database (release#19, population: Japanese in Tokyo (JPT), MAF: more than 0.05). In this step, we determined the boundaries of the 'HAP_{ICE} regions' that cover 5′ regions including 19,425 bp and 155,564 bp downstream (3′) from the significant SNPs (SNP8NRG221132 and SNP8NRG433E1006, respectively) in Stefansson's report (Table 1 and Supplementary Fig. 1) (Stefansson et al., 2002), and of the 'exon regions' that cover 5′ regions including 120,576 bp from the first exon and 3510 bp downstream 3′ from the last exon (GenBank accession No. NT_007995: Table 2 and Supplementary Fig. 2). Then fifteen and thirty-four 'tagging SNPs' for the HAP_{ICE} regions and exon regions, respectively were selected with the criterion of an r^2 threshold greater than 0.8 in 'Aggressive tagging: use 2- and 3-markers haplotypes' mode of the 'Tagger' program (de Bakker et al., 2005), a function of HAPLOVIEW software (Barrett et al., 2005).

2.4. SNP genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo).

The genotyping of C#5, C#6, C#7 (which were positive SNPs in the first-set screening analysis) was done with 768 randomly selected samples (384 cases and 384 control subjects) with direct sequencing to check for genotyping error. Detailed information including primer sequences of custom TaqMan SNP genotyping assays can be seen in Supplementary Tables 1 and 2.

Table 1
First-set case control analysis of HAP_{ICE} region

Markers	SNP ID	*P*-values		
		1-window	2-windows	3-windows
HAP_{ICE}#1	rs12674974	.0794	.181	
HAP_{ICE}#2	rs4513929	.846	.384	.196
HAP_{ICE}#3	SNP8NRG221533	.188	.397	.620
HAP_{ICE}#4	rs10096573	.200	.414	.462
HAP_{ICE}#5	rs4733263	.310	.616	.267
HAP_{ICE}#6	rs4733263	.274	.399	.578
HAP_{ICE}#7	SNP8NRG241930	.724	.113	.326
HAP_{ICE}#8	SNP8NRG243177	.288	.520	.492
HAP_{ICE}#9	rs4733267	.769	.889	.190
HAP_{ICE}#10	rs13277456	.862	.736	.847
HAP_{ICE}#11	rs13274954	.457	.670	.255
HAP_{ICE}#12	rs12677942	.312	.271	.128
HAP_{ICE}#13	rs4403369	.0803	.268	.548
HAP_{ICE}#14	rs4566990	.625	.628	.525
HAP_{ICE}#15	rs13270788	.541	.730	.699
HAP_{ICE}#16	rs1503491	.813	.866	.0960
HAP_{ICE}#17	rs2202262	.704	.324	.0653
HAP_{ICE}#18	rs10087212	.682		
HAP_{ICE}#4–#5		.414		
HAP_{ICE}#14–#16		.247		
HAP_{ICE}#15–#16		.730		

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Table 2
First-set case control analysis of exon region

Markers	SNP ID	*P*-values[a]		
		1-window	2-windows	3-windows
C#1	rs10503915	.116		
			.0603	
C#2	rs7016691	.231		.349
			.371	
C#3	rs11782671	.472		.296
			.474	
C#4	rs10103930	.168		.508
			.322	
C#5	rs10503917	.699		.0935
			.628	
C#6	rs10107065	.765		**.0244**
			.138	
C#7	rs6468118	.138		.174
			.154	
C#8	rs7000590	.0939		.158
			.107	
MS1	rs7820838	.110		.181
			.142	
MS2	rs7834206	.149		.145
			.0879	
C#9	rs4236709	.0786		.352
			.187	
C#10	rs13260545	.0994		.403
			.248	
C#11	rs4316112	.948		.0984
			.144	
C#12	rs2439305	.196		.132
			.130	
C#13	rs7826814	.715		.129
			.851	
C#14	rs2466064	.690		.436
			.313	
MS3	rs3924999	.162		.0699
			.113	
C#15	rs10954864	.803		.602
			.969	
C#16	rs2439281	.965		.301
			.0725	
C#17	rs9642729	.0680		.137
			.0988	
C#18	rs12547858	.0801		.523
			.457	
C#19	rs10098373	.801		.654
			.835	
C#20	rs10095694	.380		.872
			.727	
MS4	rs3735774	.762		.718
			.727	
C#21	rs2466058	.372		.587
			.526	
C#22	rs2466052	.379		.509
			.286	
C#23	rs2466046	.187		.431
			.372	
C#24	rs10503923	.546		.203
			.473	
C#25	rs2466084	.310		.197
			.551	
C#26	rs2976515	.253		.563
			.654	
C#27	rs4445183	.702		.500
			.484	
C#28	rs2919377	.151		.455
			.341	
C#29	rs2919375	.819		.182
			.222	
MS5	rs3735776	.740		.129
			.758	
C#30	rs7007436	.711		.866
			.815	
C#31	rs3757934	.758		.562
			.421	
MS7	rs4733376	.379		.357
			.336	
C#32	rs4360253	.357		.789
			.893	
C#33	rs7005288	.864		.738
			.812	
C#34	rs6992642	.569		
MS6 (C#24–#30)[b]	rs17731664	.772		
C#5–#11–#14		1.00		
C#5–#14		.180		
C#16–#27		.751		
C#23–#26–#28		.245		

[a] Bold number represents significant *P*-value.
[b] MS6 could be represented by the haplotypes constructed by C#24–30.

2.5. Statistical methods for conventional association analysis

In the case–control samples, the marker–trait association was evaluated with the χ^2 test in allele- and genotype-wise analyses. Haplotype frequencies were estimated in a 2- to 3-marker sliding window fashion by EM algorithm and Log likelihood ratio tests were performed for Global *P*-values with COCAPHASE program version 3.06 (Dudbridge, 2003). In the family samples, the transmission disequilibrium test (TDT) and 3-marker haplotype analyses were performed with the TDTPHASE program version 3.06 (Dudbridge, 2003). In these haplotype-wise analyses, rare haplotypes (less than 0.05) of cases and controls were excluded from the association analysis in order to provide greater sensitivity and accuracy.

The significance level was set at $P<0.05$.

2.6. Imputation of ungenotyped SNPs

Our conventional haplotype-wise analysis was done in a sliding window fashion, since our selection for tagging SNPs was not based on the haplotype block concept. Although this type of haplotype-wise analysis does not adapt to the degree of LD, so that it is unclear which markers should be considered jointly, it results in a higher level of statistical power since it can reflect unknown SNPs that were not included in the analysis. Considering this, we included a recently developed method, imputation, to test for any SNPs that reflect the significant haplotypes (Marchini et al., 2007). The IMPUTE program imputes the genotypic distribution of un-observed SNPs using observed SNP information (60 SNPs used in the screening scan) and the HapMap database (fine-scale recombination map, haplotype for JPT/CHP).

The targeted region for imputation was limited to within known recombination hot spots, because our data targeted only the HAP_{ICE} and exon regions.

After imputation, we applied a Bayesian test with an additive model to assess the association using SNPTEST software (Marchini et al., 2007). Default values were used in all settings needed in IMPUTE and SNPTEST (e.g. effective population size for JPT/CHP, buffer, call threshold for calling genotyped SNPs and number of samples of genotypes that should be used for Bayesian tests).

Table 3
Individual haplotype analyses from significant Global *P*-values in first-set samples

	haplotypes	Case Freq (%)	Con Freq (%)	*P*-value	Global *P*-value
C#5–	1–1–1	9.36	11.8	.0104	.0244
6–7	1–1–2	15.6	13.6	.0896	
	1–2–2	65.8	65.5	.886	
	2–1–1	7.21	6.27	.300	

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2.7. Power calculation

Power calculation was performed with a web-based statistical program, Genetic Power Calculator (Purcell et al., 2003). Power was estimated under a multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values observed in control samples.

3. Results

3.1. Mutation scan and first-set association analysis

We detected seven SNPs through dHPLC analysis of the exon region (MS1–7: Table 2). One of them, MS3 (rs3924999), is a non-synonymous SNP (Gly38Arg) and had shown a significant association in the Chinese population (Yang et al., 2003). The other SNPs were located in an untranslated region (UTR) or branch site, and may therefore have a functional effect (Table 2).

Next, 49 SNPs and 7 haplotypes were selected as Tagging SNPs from the HapMap database. These SNPs are located in the HAP_{ICE}- and coding regions based on the HapMap database (Tables 1 and 2).

Consequently, by involving 11 SNPs (the 7 SNPs we detected and 4 SNPs reported in other papers (Stefansson et al., 2002; Walss-Bass et al., 2006)), a total of 60 SNPs were genotyped in the first-set screening samples (however, since we were unable to design a genotyping method for one SNP that we detected (MS6) by TaqMan Assay by Design (Applied Biosystems), we determined the genotype distribution of some samples (192 cases and 192 controls) using a direct sequencing method. With these samples we confirmed that MS6 could be represented by the haplotypes constructed by C#24–30 in LD evaluation.).

The SNP for which significance was shown in the report of Walss-Bass et al. (2006) was not polymorphic in our samples.

Allele- and genotype-wise analyses did not show association either the HAP_{ICE} region or the exon region. In this haplotype-wise analysis, 3-marker haplotypes of C#5–6–7 were associated with schizophrenia (Global P-value=0.0244, uncorrected: Tables 1, 2 and 3, Supplementary Tables 3 and 4). The genotyping of C#5, C#6, C#7 in a subset of the screening samples was re-confirmed by direct sequencing, and the results were perfectly identical to those shown by TaqMan assay. Hence, we speculate that it was unlikely that genotyping error had occurred.

3.2. Imputation of ungenotyped SNP for first-set samples

Data for ungenotyped SNPs could not provide sufficient evidence for association in either region (Fig. 1). In particular, the weights of evidence for the regions near the significant haplotypes in first-set samples were less than one. Since weights of evidence of at least four are required for evidence for association

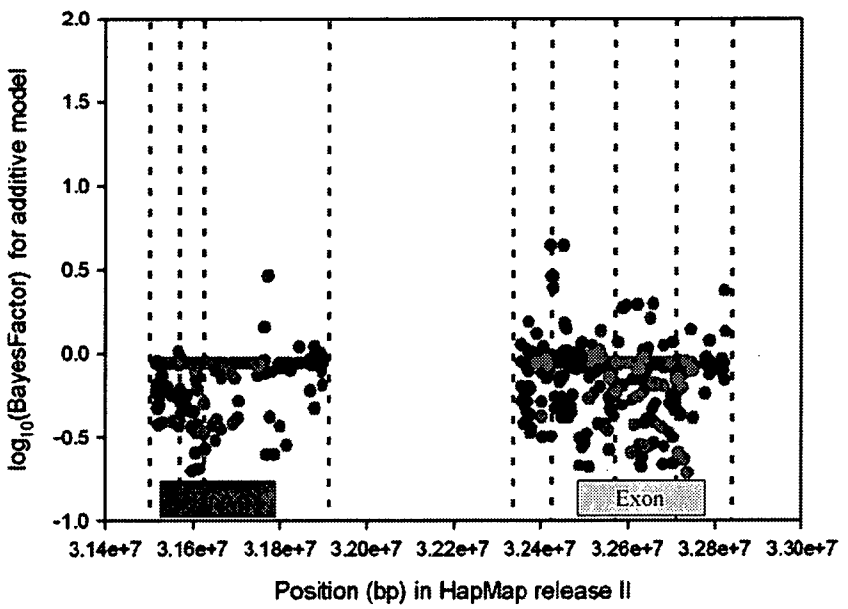

Fig. 1. Results of imputing SNP in the *NRG1* gene. The weights of evidence were calculated using imputed genotypes (red circles) and observed genotypes (black circles). Data from SNPs that constructed the significant haplotype in the first-set samples are shown in blue circles. Dotted lines indicate the estimated hot spots from the HapMap database. The SNP position from the HapMap release II database is plotted on the X axis.

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Table 4
Confirmation analysis of significant haplotypes from first-set analysis

Samples	SNPID	1-window	2-windows	3-windows
Case–control	C#5	.408	.101	.120
	C#6	.362	.601	
	C#7	.371		
Family samples	C#5	.107	.323	.505
	C#6	.964	.846	
	C#7	.499		
Combined samples	C#5	.976	.591	.478
	C#6	.389	.303	
	C#7	.801		

(if 1000 SNPs of 10,000,000 common human SNPs might be associated with a disease, we may assign a prior odds of association of 1/10,000. Therefore, a Bayes factor more than 10,000 (or $\log_{10}$ [Bayes factor] more than 4) is required (Balding, 2006)). Thus, these results indicate a low probability for association in our sample.

3.3. Confirmation analysis of the positive haplotypes using different case–control samples and family samples

To confirm the significance of exon region C#5–6–7 in the first-set samples, we conducted a confirmation analysis using independent case–control samples and family samples. In these analyses, we could not replicate this association. To increase the power, we combined samples (first-set and confirmation samples) but again we could not detect an association in this explorative analysis (Table 4).

4. Discussion

In the present study, using three large and independent samples, our data did not provide sufficient evidence for associations between tagging SNPs in the HAP_{ICE} and exon regions of *NRG1* and schizophrenia in the Japanese population.

We could not replicate previous reports for the HAP_{ICE} region (Stefansson et al., 2002; Stefansson et al., 2003); however, the results of this study are in concordance with our previous replication study in the Japanese population (schizophrenia=607, controls=515) (Iwata et al., 2004). Another study (Fukui et al., 2006), however, examined independent Japanese samples (belonging to one-third of confirmation case–control samples) and reported a positive association. Specifically, that study reported a significant association of haplotypes constructed by three core SNPs from Stefansson et al. (SNP8NRG221533 (HAP_{ICE}#3), SNP8NRG241930 (HAP_{ICE}#7) and SNP8NRG243177 (HAP_{ICE}#8)), and one more intronic SNP (rs1081062), as well as a trend for association of rs1081062. Since our tagging SNPs could not involve this SNP (rs1081062), we found by consulting the latest HapMap database (release#21a) that rs1081062 is tagged by rs13274954 (HAP_{ICE}#11); moreover, neither HAP_{ICE}#10 nor its haplotypes (HAP_{ICE}#3–7–8–11) were associated with schizophrenia (Global *P*-value= 0.540). Therefore, the aforementioned positive report could have been the result of type I error due to inadequate sample size (schizophrenia=349, controls=424) (Fukui et al., 2006). Or, as the authors speculated (Fukui et al., 2006), the different clinical backgrounds (e.g. genetic loading) in each sample could have led to inconsistent results. In this regard, a recent study reported that *DAOA/G30*, which is also a strong candidate gene for schizophrenia, influences susceptibility to the symptomatology of psychiatric disorders including schizophrenia and bipolar disorder, but not to diagnosis itself (Williams et al., 2006).

In the coding region, our results indicated the importance of controlling inflation of the type I error rate due to multiple testing, when a significant association is obtained in an analysis that involves several markers. In this study we found significant associations only from haplotype-wise analysis, not from allele- or genotype-wise analysis. It is generally accepted that a haplotype-wise analysis gives high power. At the same time, haplotype-wise analysis, especially multi-marker analysis or sliding-window analysis, tends to increase the chance of false positive results, since numerous hypotheses are examined. Bonferroni correction is typically used for solving multiple testing problems; however, since markers are not independent due to the existence of LD, Bonferroni correction is thought to be too conservative.

Therefore, we adopted two methods to validate the observed association; firstly, we imputed ungenotyped SNPs that might reflect a significant haplotype based on observations including our genotypic distribution of tagging SNPs and LD structure from the HapMap database. However, our simulation suggests that results for ungenotyped SNPs do not provide sufficient evidence for association. In other words, there was no SNP which could reflect a significant haplotype in the current data in HapMap release II. Secondly, we examined independent sets of samples for which a significant association was obtained in the initial screening analysis. We considered this to be the best strategy at present; however, the former significance of the exon region haplotype could not be replicated though independent case–control and family trios samples.

It is unlikely that negative results are due to type II error since a large sample size was used in this study; moreover, power analyses showed that the power was more than 80% when genotype relative risk (GRR) was set at 1.2–1.65 and 1.6–3.1 for confirmation case–

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control samples and family samples, respectively (MAF=2.4% and 47%), under a multiplicative model of inheritance in first-set screening samples.

Regarding interpretation of the results from this study, several limitations should be mentioned: Firstly, we did not screen the entire region of *NRG1*. On that point, Corvin et al. showed an independent 'at-risk' haplotype close to an EST cluster of unknown function (*Hs*.97362) within intron 1 of *NRG1* (Corvin et al., 2004). Secondly, our samples were not assessed with the use of the standard structured interview, and therefore have the possibility of false negatives due to misdiagnosis or sampling bias. Detailed association analyses with dense markers in the entire region of *NRG1* in well-phenotyped samples, including symptomatology, are essential in future study.

In conclusion, these results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases; independent samples for examination of significance found in screening results should be an integral part of experimental design in genetic association analysis. Imputation methods should also be used when only haplotype association shows significance, in order to check for possible causal SNPs that can reflect the haplotype.

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Contributors

MI and NT designed the study, wrote the protocol and drafted the manuscript. MI, NT, SS, BA, YW, AN, YY, TK, YK, TK, and KK performed laboratory assays and the data-analysis. RH, HU, TI, TS, and MT 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.2008.01.010.

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