the treatment response in the schizophrenic patients. Gutierrez have reported no association between *HTR3A* polymorphism and clozapine response [8], however, this study did not take haplotype block structure into consideration and did not cover whole genomic region of *HTR3A*.

5-HT4 receptor gene (*HTR4*) also has been reported to be associated with schizophrenia in the Japanese population [25]. Therefore, this gene could also be a candidate gene for TRS.

Thus, the *HTR2A*, *3A*, *4* could be considered as plausible genes related to the development of the TRS. Therefore, in the present study, we performed linkage disequilibrium (LD) analysis of *HTR3A*, followed by the case-control association studies between *HTR3A* polymorphisms and TRS using single-marker association analyses and haplotype analyses. In addition, the association was also examined among *HTR2A* polymorphism, *HTR4* polymorphism and TRS.

This study was initiated after the approval by the Ethics Committee of the Nagoya University School of Medicine. Written informed consent was obtained from all subjects at study entry.

A total of 340 patients with schizophrenia (male = 200; female = 140; age: 54 ± 12.8; duration of illness: 33.6 ± 12.4 years; daily neuroleptic dosage: 1021 ± 1857 mg/day) who had been diagnosed using the criteria of DSM-IIIR [1] were selected in this study. All patients were Japanese descent and had been hospitalized and receiving antipsychotic drugs for more than 1 year.

The definition of TRS is described elsewhere in the previous study [11]. Briefly, information about the neuroleptic therapy that the schizophrenic patients had been receiving was obtained from their clinical records. The daily neuroleptic dosage was calculated from the recent 1-year neuroleptic prescription history. Schizophrenic patients were diagnosed as having TRS when they had been hospitalized for more than 1 year and had been receiving antipsychotic therapy at dosages of at least 1000 mg/day chlorpromazine equivalents for more than 1 year.

Using the information obtained from the HapMap Database and the dbSNP Database, two single nucleotide polymorphisms – rs1062613 and rs1176713 – were selected as haplotype tag SNPs (htSNPs) that covered the whole coding region, 5′ flanking region upstream 500 bp, and 3′ UTR region downstream 500 bp of *HTR3A*. The LD block was defined using HAPLOVIEW Version 3.0 (http://www.broad.mit.edu/mpg/haploview/) as a region of $D' > 0.8$. In each LD block, haplotype frequency was estimated by the expectation-maximization (EM) algorithm and htSNPs were selected using the same program. Additionally, a SNP (rs6313) of *HTR2A* and two SNPs (rs2278392, rs3734119) of *HTR4* which have been reported to be associated with schizophrenia in the previous study [25] were selected. Genotyping was carried out using polymerase chain reaction-restriction fragment length polymorphism assays or direct sequence assays for each SNP. Sequences of each primer pairs are available on request.

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by Chi-square test. Single-marker and haplotype analyses were performed using SPSS Version 11.0J (Tokyo, Japan) and Cocaphase 2.403 (http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/), respectively. Comparison of the daily neuroleptic dosage among each genotype was performed using Mann–Whitney *U*-test. Power calculation was performed by Power Calculator (http://calculators.stat.ucla.edu/powercalc/). The level of significance for all statistical tests was set at 0.05.

A total of 101 schizophrenic patients were identified as the TRS (TRS: male = 67, female = 34, age = 50 ± 10.5, onset age = 20 ± 5.3; NON-TRS: male = 133, female = 106, age = 56 ± 13.1, onset age = 23.5 ± 8.2). The male ratio tended to be higher in the TRS patients ($p < 0.1$), and the age at onset was significantly younger in this group ($p = 0.009$). However, no significant difference was observed in the incidence of any psychiatric symptom between the two groups, such as delusion and hallucination, bizarre behavior, disorganization, and negative symptoms at their first episode, as reported in our previous report [12]. The genotype distributions of the polymorphisms did not deviate significantly from the HWE in each study group for any polymorphism. The genotype and allele frequencies of three kinds of serotonin receptor genes in TRS and NON-TRS groups are shown in Table 1.

No significant association was observed in the single marker analysis of *HTR2A*, *HTR3A*, and *HTR4*, and in haplotype analysis of *HTR3A* and *HTR4* (Table 1).

The characteristics of neuroleptic treatment among the three subgroups showing each SNP polymorphism are shown in Table 2. In rs1062613 of *HTR3A*, the daily neuroleptic dosage during maintenance therapy was significantly higher in patients with the T/T genotype than the others ($p = 0.041$).

When the proportion of TRS was set to be 30% [9], we obtained more than 80% power to detect an association with the SNPs of which the minor frequency is more than 10%.

The results presented here suggest that *HTR3A* may be involved in the development of TRS in the Japanese population. In this study, significant difference in the daily neuroleptic dosage received during maintenance therapy was observed in schizophrenic patients with the T/T genotype of *HTR3A* polymorphism (rs1062613).

The SNP rs1062613 is located on the promoter region of *HTR3A* and has been reported to regulate the expression of this gene [22]. Since presynaptic 5-HT3A receptors modulate the release of several neurotransmitters in various brain regions [15,26], the abnormal expression of *HTR3A* might increase the dopamine concentration in the synaptic cleft. This may lead to increase the therapeutic antipsychotic doses in the schizophrenic patients with this mutation.

Additionally, several antipsychotic drugs reduce the dopaminergic neurotransmission by antagonizing the 5-HT3A receptor [24]. Therefore, reduction in the expression of 5-HT3A receptor may weaken the therapeutic effect of antipsychotics through this pathway; even higher dose of most antipsychotic drugs may not reduce the dopaminergic neurotransmission.

Furthermore, this SNP has been reported to have a critical role in the amygdala activity leading to the facial expression recognition [10], and the defect of facial expression recognition has been reported to be a specific symptom to the schizophrenia including TRS [4,18]. Therefore, this SNP may have a role in

Table 1
Genotype and allele frequencies of *HTR2A*, *HTR3A* and *HTR4* polymorphisms in TRS and NON-TRS

	Genotype			p-Value	Allele frequency (%)		p-Value	Global p-value
HTR2A								
rs6313	C/C	C/T	T/T		C	T		
TRS	19	58	23	0.500	48	52	0.777	
NON-TRS	48	123	68		46	54		
HTR3A								
rs1062613	C/C	C/T	T/T		C	T		
TRS	75	21	5	0.117	85	15	0.400	
NON-TRS	189	47	3		89	11		0.576
rs1176713	A/A	A/G	G/G		A	G		
TRS	49	38	14	0.744	67	33	0.648	
NON-TRS	124	86	27		70	30		
HTR4								
rs2278392	G/G	G/A	A/A		G	A		
TRS	59	36	7	0.867	76	24	0.868	
NON-TRS	148	80	15		77	23		0.863
rs3734119	T/T	T/C	C/C		T	C		
TRS	59	36	8	0.891	75	25	0.869	
NON-TRS	148	80	19		76	24		

the development of TRS based on the effect of the SNP on the impairment of facial expression recognition.

The definition of TRS in the present study is different form that proposed by Kane et al. [14]. Since the polypharmacy is widely prevalent in the antipsychotic treatment of schizophrenia in Japan. In the present study, the psychopathology of TRS was defined by the total antipsychotic doses that the schizophrenic patients had been receiving during the recent 1 year, that is, the severity of illness was extrapolated by the total antipsychotic doses. In addition, they had been hospitalized for more than 1 year, indicating that they had been no good level of functioning over this period. In fact, age at disease onset had been observed to be significantly younger in the TRS subjects, suggesting that the younger onset patients tend to less response to the antipsychotic therapy. Therefore, we consider that virtually no essential difference exists between the present definition of TRS enrolled in Japan and that proposed by Kane et al. [14].

Table 2
Characteristics of NLP treatment among three subgroups showing *HTR2A*, *HTR3A* and *HTR4* polymorphisms

	Genotype		
HTR2A			
rs6313	C/C	C/T	T/T
Daily NLP	575 (2–4042)	603 (4–12893)	372 (3–6283)
HTR3A			
rs1062613	C/C	C/T	T/T
Daily NLP	496 (2–12893)	568 (5–12850)	1179 (281–3048)[a]
rs1176713	A/A	A/G	G/G
Daily NLP	559 (3–8337)	417 (2–12893)	710 (42–4226)
HTR4			
rs2278392	G/G	G/A	A/A
Daily NLP	491 (2–12893)	600 (4–6283)	460 (50–2262)
rs3734119	T/T	T/C	C/C
Daily NLP	491 (2–12893)	605 (4–6283)	439 (30–2262)

Data are expressed as median (min–max).
[a] $p = 0.041$ when compared to the (C/C + C/T) subgroup.

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Letter to the Editor

Large-scale case-control study of a functional polymorphism in the *glutamate receptor, metabotropic 3* gene in patients with schizophrenia

THE A ALLELE of rs6465084, a single nucleotide polymorphism (SNP) of the *glutamate receptor, metabotropic 3* (*GRM3*) gene, has been found to be associated with decreased verbal fluency and reduced prefrontal cortical levels of N-acetylaspartate/creatine.[1] This functional SNP has been shown to be associated with schizophrenia[1] but other studies failed to demonstrate such an association.[2-5] To further investigate the inconsistent results, we conducted a case–control association study.

The Ethics Committee on Genetics of each participating institute approved the present study. All participants provided written informed consent. All participants were unrelated Japanese subjects. The subjects consisted of 2358 patients with schizophrenia, meeting the DSM-IV criteria (1273 men, 1085 women; mean age 46.8 ± 14.7 years), and 2433 control subjects (1313 men, 1120 women; mean age 45.6 ± 13.8 years). We genotyped rs6465084 (C_11245618_10; Applied Biosystems, CA, USA) using the TaqMan 5′-exonuclease assay.[6] We did not examine rs1468412,[7] rs2299225[8] and rs274622[9] because these SNP have not been shown to be associated with schizophrenia in a large Japanese sample,[5] which overlapped the subjects in the present study.

The genotype distributions did not deviate significantly from Hardy–Weinberg equilibrium in either group. The genotype and allele frequencies in patients did not differ from those in controls (Table 1). The results of the present study indicate that the rs6465084 functional polymorphism in *GRM3* does not contribute to genetic susceptibility to schizophrenia.

Table 1 Genotype and allele frequencies of rs6465084 in *GRM3*

	Patients (%)	Controls (%)	*P*
Genotype			0.39
A/A	2045 (86.7)	2132 (87.6)	
A/G	305 (12.9)	289 (11.9)	
G/G	8 (0.4)	12 (0.5)	
Allele			0.46
A	4395 (93.2)	4553 (93.6)	
G	321 (6.8)	313 (6.4)	

GRM3, glutamate receptor, metabotropic 3.

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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.
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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

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		

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	.371	.349
C#3	rs11782671	.472	.474	.296
C#4	rs10103930	.168	.322	.508
C#5	rs10503917	.699	.628	.0935
C#6	rs10107065	.765	.138	**.0244**
C#7	rs6468118	.138	.154	.174
C#8	rs7000590	.0939	.107	.158
MS1	rs7820838	.110	.142	.181
MS2	rs7834206	.149	.0879	.145
C#9	rs4236709	.0786	.187	.352
C#10	rs13260545	.0994	.248	.403
C#11	rs4316112	.948	.144	.0984
C#12	rs2439305	.196	.130	.132
C#13	rs7826814	.715	.851	.129
C#14	rs2466064	.690	.313	.436
MS3	rs3924999	.162	.113	.0699
C#15	rs10954864	.803	.969	.602
C#16	rs2439281	.965	.0725	.301
C#17	rs9642729	.0680	.0988	.137
C#18	rs12547858	.0801	.457	.523
C#19	rs10098373	.801	.835	.654
C#20	rs10095694	.380	.727	.872
MS4	rs3735774	.762	.727	.718
C#21	rs2466058	.372	.526	.587
C#22	rs2466052	.379	.286	.509
C#23	rs2466046	.187	.372	.431
C#24	rs10503923	.546	.473	.203
C#25	rs2466084	.310	.551	.197
C#26	rs2976515	.253	.654	.563
C#27	rs4445183	.702	.484	.500
C#28	rs2919377	.151	.341	.455
C#29	rs2919375	.819	.222	.182
MS5	rs3735776	.740	.758	.129
C#30	rs7007436	.711	.815	.866
C#31	rs3757934	.758	.421	.562
MS7	rs4733376	.379	.336	.357
C#32	rs4360253	.357	.893	.789
C#33	rs7005288	.864	.812	.738
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#5–6–7	1–1–1	9.36	11.8	.0104	.0244
	1–1–2	15.6	13.6	.0896	
	1–2–2	65.8	65.5	.886	
	2–1–1	7.21	6.27	.300	

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

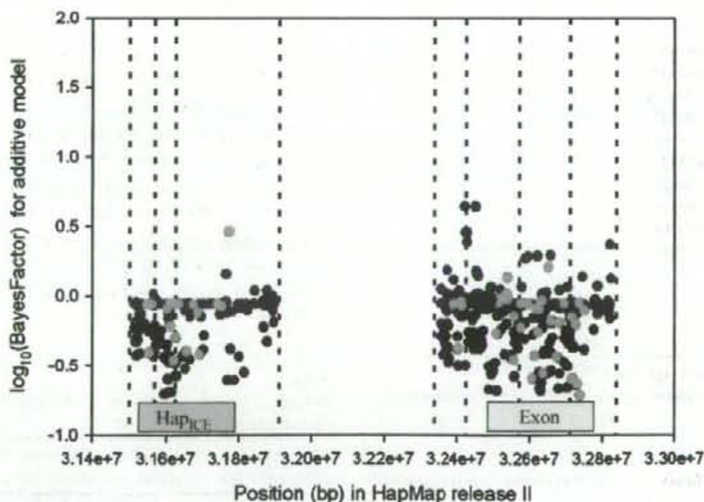

Fig. 1. Results of imputing SNP in the *NRG1* gene. The weights of evidence were calculated using imputed genotypes (red circles) and observed genotypes (black circles). Data from SNPs that constructed the significant haplotype in the first-set samples are shown in blue circles. Dotted lines indicate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

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

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

(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–

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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Pathway-based association analysis of genome-wide screening data suggest that genes associated with the γ-aminobutyric acid receptor signaling pathway are involved in neuroleptic-induced, treatment-resistant tardive dyskinesia

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Objective **Neuroleptic-induced tardive dyskinesia (TD) is an involuntary movement disorder that develops in patients who have undergone long-term treatment with antipsychotic medications, and its etiology is unclear. In this study, a genome-wide association screening was done to identify the pathway(s) in which genetic variations influence susceptibility to neuroleptic-induced TD.**

Methods **Screening with Sentrix Human-1 Genotyping BeadChip (Illumina, San Diego, California, USA) was done for 50 Japanese schizophrenia patients with treatment-resistant TD and 50 Japanese schizophrenia patients without TD. A total of 40 573 single nucleotide polymorphisms that were not in linkage disequilibrium with each other and were located in the exonic and intronic regions of 13 307 genes were analyzed. After gene-based corrections, *P* values for allelic associations were subjected to canonical pathway-based analyses with Ingenuity Pathway Analysis software (Ingenuity Systems, Inc., Redwood City, California, USA).**

Results **Eight genes (*ABAT, ALDH9A1, GABRA3, GABRA4, GABRB2, GABRAG3, GPHN,* and *SLC6A11*) contained polymorphisms with gene-based corrected allelic *P* values of less than 0.05. They were aggregated significantly in 33 genes belonging to the γ-aminobutyric acid (GABA) receptor signaling pathway ($P=0.00007$, corrected $P=0.01$). Associations were replicated in an independent sample of 36 patients with TD and 136 patients without TD for polymorphisms in *SLC6A11* (*GABA transporter 3*) ($P=0.0004$ in the total sample), *GABRB2* (*β-2 subunit of GABA-A receptor*) ($P=0.00007$ in the total sample), and *GABRG3* (*γ-3 subunit of GABA-A receptor*) ($P=0.0006$ in the total sample).**

Conclusion **The results suggest that the GABA receptor signaling pathway may be involved in genetic susceptibility to treatment-resistant TD, at least in a subgroup of Japanese patients with schizophrenia. The present results suggest that benzodiazepines may be considered as possible treatment option for TD. *Pharmacogenetics and Genomics* 18:317–323 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Tardive dyskinesia (TD) is an involuntary movement disorder that develops in patients undergoing long-term treatment with antipsychotic medications. Introduction of second-generation atypical antipsychotics has reduced the occurrence of TD to approximately 1% compared with the 5% frequency with typical agents annually [1,2]. Owing to the lack of effective treatments for TD, however, therapeutic management of TD can be problematic for schizophrenia patients receiving antipsychotic medications, especially for those patients who develop severe treatment-resistant TD. Therefore, the strategies to prevent TD are often discussed in the context of the safety and use of antipsychotic drugs.

To identify predisposing factors for TD, extensive epidemiological studies have been conducted, and several risk factors for TD have been identified. These include increased age, female sex, presence of mood disorder, mental retardation, prolonged treatment with antipsychotic drugs, high-dose antipsychotic drugs, high total cumulative dose, poor antipsychotic response, acute extrapyramidal symptoms, use of antiparkinsonian drugs or lithium, and predominance of negative symptoms [3]. TD, however, does not always develop in patients

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deemed to be at high risk. The familial nature of patient subgroups vulnerable to TD indicates that genetic factors could play a critical role in susceptibility to TD [4]. Recently, molecular genetic studies of TD were conducted to identify genes related to TD [5]. Although the pathophysiological mechanisms of TD remain unknown, a number of mechanisms have been proposed, including hypersensitivity of central dopaminergic systems [6], oxidative stress-mediated neurotoxic damage [7], dysfunction of the serotonergic system [8], and γ-aminobutyric acid (GABA) insufficiency [9,10]. The TD susceptibility gene loci likely encode proteins related to these pathways.

The advent of single nucleotide polymorphism (SNP) chips for genome-wide association analysis has made screening of susceptibility genes for TD possible. In this study, we carried out genome-wide association studies of treatment-resistant TD in schizophrenia patients. Treatment-resistant TD cases have been used to study the genetic associations of genes of the central dopaminergic systems [11–14] and the *CYP2D6* genes [15] with TD in patients susceptible and those not susceptible to TD.

The aim of this study was to identify the pathway(s) in which genetic variations influence susceptibility to neuroleptic-induced TD. We used a web-based software, 'Ingenuity Pathway Analysis (IPA)' to help the identification of genes within known networks.

Methods

Ethical considerations

The study was initiated after approval by the ethics committee of each institution. Written informed consent was obtained from all patients after adequate explanation of the study.

Patients

Patients were identified at psychiatric hospitals located around the Tokyo and Nagoya areas of Japan. All patients satisfied the diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition [16] for schizophrenia. All patients and their parents were of Japanese descent. All patients had been receiving antipsychotic therapy for at least 1 year and their TD status was monitored for at least 1 year. TD was assessed according to the Japanese version of the Abnormal Involuntary Movement Scale (AIMS). TD was diagnosed according to the criteria proposed by Schooler and Kane [17]. Once TD was identified, the patients were followed up and received standard therapeutic regimens for TD to minimize TD symptoms. If TD persisted after more than 1 year of therapy, patients were considered potential treatment-resistant TD patients. Treatment-resistant TD patients were defined as those patients with dyskinetic movements that persisted more than 1 year and did not improve after at least 1 year of appropriate treatment following guideline-recommended therapeutic regimens for TD. Dyskinetic movements were judged to be severe on the basis of either of the following conditions: (i) patients with moderate or severe TD in one or more AIMS seven anatomical areas, (ii) patients with mild dyskinetic movements in two or more AIMS seven anatomical areas, whose mild dyskinetic movements developed within 5 years of their initial exposure to antipsychotic agents. Patients in whom TD never developed despite antipsychotic therapy for more than 10 years were recruited as control patients. Dyskinetic movements were videotaped and ratings of TD were assigned by trained raters who had completed the full training process for drug-induced extrapyramidal symptoms and had been working in the project of TD study for more than 5 years. Diagnosis of TD and severity of the observed dyskinetic movements were determined by the complete consensus of trained raters for all patients with TD before genotyping.

A genome-wide association screening was done in 50 patients with TD and 50 patients without TD. Some SNPs were genotyped in an independent population consisting of 36 patients with TD and 138 patients without TD. Ages and sex ratios did not differ significantly between the TD group and the non-TD group in the screening and confirmation samples. Clinicopathological features of the patients are summarized in Table 1.

Table 1 Clinical characteristics of patients in the TD group and non-TD group

	Genome-wide sample		Confirmation sample	
	TD (n=50)	Non-TD (n=50)	TD (n=36)	Non-TD (n=138)
Male : female ratio	29 : 21	29 : 21	18 : 18	88 : 50
Age (years)	56.6 ± 17.7	58.5 ± 11.5	58.0 ± 15.7	55.5 ± 1.0
Duration of illness (years)	36.0 ± 19.0	32.9 ± 11.3	37.3 ± 14.1	35.3 ± 1.02
Current neuroleptic dosage (chlorpromazine-eq. mg/day)	364 ± 543	1241 ± 2531	363 ± 236	1116 ± 116
Recent 1-year cumulative neuroleptic dose (chlorpromazine-eq. mg/year)	132 952 ± 198 042	452 784 ± 923 763	132 550 ± 86 292	407 456 ± 42 245

Values are expressed as mean ± SD or number of patients. The patients had been receiving various kinds of typical and/or atypical antipsychotics depending on the clinical situation at the study entry. Chlorpromazine-eq, chlorpromazine equivalents; TD, tardive dyskinesia.

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Although most of the patients have been studied earlier to determine the associations of dopaminergic and *CYP2D6* genes with TD [11–15], no correction for prior statistical testing was made for this study.

Genotyping and statistics

The Sentrix Human-1 Genotyping BeadChip (Illumina) was used for genome-wide association analysis. The SNPs on the chip are exon-centric and 57 979 SNPs were in exons and introns. In this study, we analyzed SNPs in the exons and introns of genes encoding known proteins or predicted to encode proteins. Some SNPs on the chip were in linkage disequilibrium with each other; therefore, before association analysis, we selected tag SNPs by analyzing the genotype data with the Haploview software (*http://www.hapmap.org/index.html*) and running the tagger procedure with the threshold of $r^2 > 0.8$. In addition, we excluded 135 SNPs because their genotype distributions deviated significantly from the Hardy–Weinberg expectancy ($P < 0.05$). A total of 40 573 tag SNPs from the exons and introns of 13 307 genes were analyzed in this study.

We evaluated only allelic associations because genotype associations require further correction for multiple testing, which makes interpretation more difficult. A P value of 1.3×10^{-6} was considered significant for genome-wide association after Bonferroni corrections by the number of SNPs analyzed (40 573).

Canonical pathway analysis was performed as follows. First, the gene-based associations were evaluated. We corrected the allelic P value for each SNP by the number of tag SNPs in the gene where each SNP is located (Bonferroni correction). We refer to these corrected allelic P values as gene-based corrected allelic P values. We next assigned the lowest gene-based corrected allelic P value to each gene. We then performed canonical pathway analysis for these genes with the lowest gene-based corrected allelic P values using IPA 5.0 (Ingenuity Systems, Inc., *http://ingenuity.com/*). IPA is a robust and expertly curated database containing up-to-date information for more than 20 000 mammalian genes and proteins, 1.4 million biologic interactions, and more than 100 canonical pathways incorporating more than 6000 discreet gene concepts. This information is integrated with other relevant databases such as EntrezGene (*http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene*) and Gene Ontology (*http://www.geneontology.org/*). Genes with gene-based corrected allelic P values of less than 0.05 or 0.01 were used as the input data set to query the IPA canonical pathway analysis. Canonical pathway analysis identified those pathways that were most significant to the input data set. The significance of the association between the data set and the canonical pathway was determined based on a P value calculated with Fisher's exact test by calculating the probability that the association between the genes in the data set and the canonical pathway is due to chance alone [18]. Genes listed in the canonical pathway analysis are referred to as canonically analyzed genes. In the IPA content version 1002 dated 30 October 2007, 8640 genes were canonically grouped and SNPs analyzed in this study were located in the exonic or intronic regions of 6212 genes among the 8640 genes. The 6212 genes belonged to 147 canonical pathways. A significantly associated canonical pathway was defined as a pathway with significantly more genes containing SNPs with gene-based corrected allelic P values less than 0.05 or less than 0.01 than the other pathways as calculated by Fisher's exact test and corrected by the number of canonical pathways (147).

We genotyped 50 patients with TD and 50 patients without TD. The power of this study was 0.78 assuming the prevalence of TD of 0.2, frequency of risk allele of 0.2, genotypic relative risk (additive) of 2, and α level of 0.05. When the α level was set at 1.3×10^{-6}, however, the power was less than 0.1. The power was 0.8 to detect polymorphisms with genotype relative risk of 4, even if the α level was set at 1.3×10^{-6}. In the confirmation sample with 36 patients with TD and 138 patients without TD, the power was more than 0.8 to confirm associations with genotypic relative risk of 2 and risk allele frequency of 0.2 when the α level was set at 0.05 (one tailed).

Results

As shown in Table 1, there was no significant difference in the duration of illness between the TD group and the non-TD group in the screening and confirmation samples. Recent 1-year cumulative dosage was significantly lower in patients with TD than in those without TD. This may have been as a result of efforts to reduce the neuroleptic dosage in patients with TD in line with guideline-recommended therapeutic regimens for this condition.

To identify loci associated with susceptibility to TD, we screened for SNPs associated with TD using 40 573 tag SNPs on the Sentrix Human-1 Genotyping BeadChip (Illumina). The lowest uncorrected allelic P value for association with TD was 1×10^{-5}. Therefore, no SNP was significantly associated with TD after Bonferroni correction. As shown in Table 2, the distributions of gene-based corrected allelic P values of the SNPs analyzed for association with TD were similar to those expected when no association was assumed, though a slightly higher number of SNPs showed lower P values than expected when no association is assumed. Thus, as most low P values are chance findings, some SNPs may actually be associated with TD, and genes containing one or more SNPs with low P values may be candidates for genetic susceptibility to TD. True associations, however, cannot be determined without studies of additional populations.

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We tried to confirm the associations of 14 SNPs with gene-based corrected allelic P values less than 0.001 in the confirmation samples (Table 3). No significant association was, however, observed for any of the SNPs examined.

As shown in Table 4, the numbers of genes with gene-based corrected allelic P values of less than 0.05 and 0.01 were 652 and 132, respectively. These numbers were almost 5 and 1% of the 13 307 genes analyzed. Of these 13 307 genes, IPA assigned 2612 genes to 147 canonical pathways. As many genes belong to more than one canonical pathway, the number of overlapping genes belonging to these 147 canonical pathways in IPA was 8640, and of these, 6212 had gene-based corrected allelic P values in this study. We examined whether genes with gene-based corrected allelic P values of less than 0.05 and 0.01 aggregated in specific canonical pathways. As shown in Table 4, genes with gene-based corrected allelic P values of less than 0.05 were found more frequently in three canonical pathways, and those with P values of less than 0.01 were found more frequently only in the GABA receptor signaling pathways. Among the 6212 overlapping genes, 268 had gene-based corrected allelic P values of less than 0.05, whereas among the 33 genes in the GABA receptor signaling pathway, eight had gene-based corrected allelic P values below 0.05 ($268/6212 = 4.8\%$ vs. $8/33 = 24\%$, nominal $P < 0.00007$, Fisher's exact test). As there were 147 canonical pathways, the P value corrected for the number of canonical pathways in this study was 0.01. Although genes with gene-based corrected P values of less than 0.05 also aggregated in the synaptic long-term potentiation pathway and calcium signaling pathway, the aggregations were not significant after correction for the number of canonical pathways.

Genotype and allele distributions of eight SNPs in eight genes associated with the GABA receptor signaling pathway are shown in Table 5. We examined these SNPs in the confirmation samples (Table 5). Among eight SNPs, the associations were replicated in the confirmation samples for SNPs in *SLC6A11* (*GABA transporter 3*), *GABRB2* (*β-2 subunit of GABA-A receptor*), and *GABRG3* (*γ-3 subunit of GABA-A receptor*).

Table 2 Distributions of allelic P values of SNPs associated with TD by genome-wide association analysis

Gene-based corrected allelic P values	Genes analyzed	SNPs analyzed
Range		
$P<0.0001$	3	3
$P<0.001$	11	11
$P<0.01$	132	172[a]
$P<0.05$	652	846[b]
Total	13307	40573

[a, b]We considered these genes as the top 1 and 5% likely susceptibility genes for TD in this study. SNPs, single nucleotide polymorphisms; TD, tardive dyskinesia.

Discussion

To our knowledge, this is the first report of genome-wide association analysis for neuroleptic-induced TD. A number of limitations, however, do exist. First, the Sentrix Human-1 Genotyping BeadChip (Illumina) used in this study is one of the first commercially available chips for genome-wide association analysis. This chip contains a relatively small number of SNPs. One advantage of this chip is that SNPs contained on the chip are relatively concentrated in the gene region of interest. The chip contains 6680 nonsynonymous polymorphisms. We were able to examine associations between some polymorphisms earlier reported to be associated with TD. The G9S polymorphism (rs6280) in the *DRD3* gene and the *Taq*I A polymorphism (rs1800497) near the *DRD2* gene, which were found to be associated with TD in several studies [19], are included on the Illumina chip. Associations between these SNPs and TD, however, were not found in this

Table 3 List of SNPs with gene-based corrected allelic P values of less than 0.001 associated with TD by genome-wide association analysis

			Genome-wide sample			Confirmation sample
Gene	Chromosome	SNP ID	Uncorrected P	Number of tag SNPs	Gene-based corrected P	Uncorrected P
ELOVL3	10q24.32	rs10748816	0.00002	2	0.00005	0.12
BCOR	Xp11.4	rs6609051	0.00001	4	0.00006	0.34
TCP10L	21q22.11	rs7281019	0.00004	2	0.00008	0.34
CBLC	19q13.31	rs10419669	0.00003	14	0.0004	0.48
SLC38A1	12q13.11	rs1444590	0.00002	22	0.0005	0.25
EHF	11p13	rs286925	0.00002	22	0.0005	0.32
TBCD	17q25.3	rs3744165	0.00007	8	0.0006	0.11
RBM17	10p15.1	rs2274359	0.0002	3	0.0006	0.32
DLG5	10q22.3	rs1058198	0.0001	5	0.0006	0.14
ABCC8	11p15.1	rs886292	0.00005	14	0.0007	0.09
MAN1A2	1p12	rs2306444	0.0002	4	0.0008	0.35
EDIL3	5q14.3	rs13153252	0.00002	39	0.0008	0.52
ANXA13	8q24.13	rs4242345	0.00004	24	0.0009	0.32
SMYD3	1q44	rs6426327	0.00001	91	0.0009	0.07

SNPs, single nucleotide polymorphisms; TD, tardive dyskinesia.

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Table 4 Canonical pathway analysis of genome-wide association findings

Canonical pathway	Total number of genes assigned to the specific pathway	Genes with analyzed SNPs	Genes with SNPs with gene-based corrected allelic P values of less than 0.05		Genes whose SNP with gene-based corrected P values of less than 0.01		Genes[a]
		Number	Number	P value	Number	P value	
GABA receptor signaling	40	33	8	0.00007[*]	2	0.03	*ABAT, ALDH9A1*, **GABRA3**, *GABRA4, GABRB2*, **GABRAG3**, *GPHN, SLC6A11*
Synaptic long term potentiation	67	56	8	0.003	2	0.08	*ADCY1*, **CALM1, CAMK2G, GRIN2B, ITPR1, ITPR2, RPS6KA**
Calcium signaling	172	133	15	0.0008	2	0.32	*CACNA1D, CACNB1, CACNB2, CACNG3, CALM1*, **CAMK2G**, *GRIA3, GRIN2B, GRIN3A, HDAC8, ITPR1, ITPR2, NFATC1, PRKAG2*, **TRDN**
Total	8640	6212	268		54		

GABA, γ-aminobutyric acid; SNPs, single nucleotide polymorphisms.
[a]Genes with gene-based corrected allelic P values of less than 0.01 are shown in bold.
[*]$P = 0.01$ after Bonferroni correction for the number of canonical pathways (147) examined in this study ($0.00007 \times 147 = 0.01$).

study ($P > 0.05$). The P value was 0.03 for rs2296973 in the *HTR2A* gene, which is in linkage disequilibrium with 102T/C (rs6313) ($D' = 1$ and $r^2 = 0.73$). The 102T/C was found to be associated with TD in several studies [19]. We could not evaluate polymorphisms in the *CYP1A2* gene in this study.

The second limitation is the small sample size. We exclusively enrolled patients with clinically severe, treatment-resistant TD that did not respond to recommended therapies for TD. For this reason, the number of patients was limited. Limited detection power for associations and insufficient control for multiple testing owing to the small sample size are the major limitations. Concerning detection power, the aim of this study was to detect polymorphisms with relatively strong influence on the development of TD, that is, with a genotype relative risk of greater than 2. The type 2 errors are, however, expected to be huge. In general, genome-wide significance level requires P values of less than 10^{-7} to control for multiple testing [20]. In this study, the lowest P value was 1×10^{-5} at rs6426327, which is located in an intron of *SMYD3*. Therefore, detection of significant association needs replication analysis in independent sample sets. We examined 14 SNPs with low P values for association in the confirmation sample. We, however, did not find significant association for any of these SNPs.

The third limitation is that the patients with severe TD in this study may not have been a homogeneous group. In fact, the patients with TD showing severe tardive dystonia were observed in 15 cases in the genome-wide samples and three cases in the confirmation samples. In addition, mild tardive dystonias were also observed in some of the remaining patients with TD. These patients with TD showing tardive dystonia may be a subgroup among TD patients.

In this study, we used an alternative method of analysis to evaluate associations – canonical pathway analysis. We hypothesized that variations in multiple genes in certain pathways contribute to neuroleptic-induced TD and that P values for association of SNPs in genes belonging to certain pathways may distribute disproportionately toward lower values. We found potential association of the GABA receptor signaling pathway with neuroleptic-induced TD. We attempted to confirm eight SNPs identified by canonical pathway analysis in a second independent sample of cases, and the association was confirmed for three of these eight SNPs. Odds ratios greater than 2 for association with TD were obtained for SNPs in *SLC6A11, GABRB2, GABRG3*, and *GABRA3* (Table 5).

Reduced activity in a subgroup of striatal GABA neurons has been suggested as the basis of TD [6]. Gunne *et al.* [10] reported that decreased GABAergic activity in the substantia nigra correlates with enhanced oral activity in rats and with neuroleptic-induced dyskinesia in monkeys. Delfs *et al.* [21] reported an increase in levels of the mRNA that encodes glutamic acid decarboxylase, the rate-limiting enzyme in GABA synthesis, in the striatum and pallidum of adult rats after long-term haloperidol treatment, suggesting that decreased GABAergic transmission may play a critical role in the motor side effects associated with long-term antipsychotic therapy. Our present results suggest that genetic variations in GABAergic systems may contribute to the pathophysiology of TD.

In previous genetic case–control studies, schizophrenia patients were enrolled as soon as TD was identified. TD, however, is considered a complex condition comprising several heterogeneous traits that show various natural courses and responses to therapeutic regimens for TD [11]. In fact, even dyskinetic movements diagnosed as TD sometimes disappear after appropriate therapy. In

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Table 5 Genotype and allele distributions of the tag SNPs in the genes in the GABA receptor signaling pathway

Gene	Chromosome	SNP	Population		Genotype counts in						Allele frequency				
					Patients with TD			Patients without TD			With TD	Without TD	Allelic P*	OR	95% CI
ALDH9A1	1q24.1	rs12670			AA	AC	CC	AA	AC	CC	A				
			First		2	22	26	0	13	36	0.24	0.13	0.02	2.3	1.1–4.79
			Second		0	12	24	5	35	98	0.17	0.16	0.53	1.03	0.51–2.06
			Sum		2	34	50	5	48	134	0.22	0.16	0.07	1.55	0.98–2.44
SLC6A11	3p25.3	rs4684742			AA	AG	GG	AA	AG	GG	A				
			First		10	28	11	4	20	25	0.49	0.29	0.003	2.4	1.33–4.33
			Second		8	9	11	19	42	66	0.45	0.31	0.04	1.75	0.97–3.16
			Sum		18	37	22	23	62	91	0.47	0.31	0.0004	2.04	1.38–3.00
GABRA4	4p12	rs953380			AA	AG	GG	AA	AG	GG	A				
			First		28	15	7	36	13	1	0.71	0.85	0.02	2.31	1.15–4.65
			Second		20	11	4	84	47	7	0.73	0.78	0.23	1.31	0.72–2.39
			Sum		48	26	11	120	60	8	0.72	0.80	0.04	1.55	1.02–2.36
GABRB2	5q34	rs918528			AA	AC	CC	AA	AC	CC	A				
			First		0	14	36	3	24	23	0.16	0.30	0.01	2.63	1.30–5.35
			Second		2	15	18	23	61	54	0.27	0.39	0.04	1.7	0.95–3.03
			Sum		2	29	54	26	85	77	0.19	0.36	0.00007	2.38	1.54–3.67
GPHN	14q23.3	rs6573744			AA	AG	GG	AA	AG	GG	A				
			First		9	22	18	2	19	27	0.41	0.24	0.01	2.19	1.18–4.06
			Second		6	13	17	18	56	64	0.35	0.33	0.46	1.06	0.62–1.84
			Sum		15	35	35	20	75	91	0.38	0.31	0.09	1.38	0.95–2.02
GABRG3	15q12	rs2061051			AA	AG	GG	AA	AG	GG	A				
			First		1	19	30	13	16	21	0.21	0.42	0.001	2.72	1.46–5.08
			Second		1	12	22	15	59	63	0.20	0.32	0.03	1.92	1.02–3.64
			Sum		2	31	52	28	75	84	0.21	0.35	0.0006	2.08	1.36–3.19
ABAT	16p13.2	rs1641022			AA	AC	CC	AA	AC	CC	A				
			First		2	24	24	12	22	16	0.30	0.46	0.01	2.19	1.22–3.94
			Second		5	17	13	15	71	52	0.39	0.37	1	0.92	0.54–1.58
			Sum		7	41	37	27	93	68	0.32	0.39	0.13	1.34	0.92–1.97
GABRA3	Xq28	rs11795489			AA	AG	GG	AA	AG	GG	A				
			First	Male	21		8	29		0	0.77	0.96	0.002	6.59	1.83–23.8
			First	Female	13	8	0	18	3	0					
			Second	Male	17		1	81		7	0.88	0.89	0.51	1.1	0.42–2.89
			Second	Female	13	3	1	38	11	1					
			Sum	Male	38		9	110		7	0.82	0.91	0.02	2.24	1.19–4.19
			Sum	Female	26	11	1	56	14	1					

95% CI, 95% confidence interval; GABA, γ-aminobutyric acid; OR, odds ratio; SNPs, single nucleotide polymorphisms; TD, tardive dyskinesia.
*By Fisher's exact test.

some cases, the severity of TD fluctuates in response to changes in the psychopharmacologic treatment. Treatable cases of TD may not be considered as clinically problematic as cases of treatment-resistant TD. In this study, we enrolled only those patients who had treatment-resistant TD for at least 1 year. Once TD developed, we carefully managed and treated these patients for at least 1 year to exclude those patients with treatable TD from the study. Our present findings suggest that GABA-mimetic drugs may be useful for treating patients with treatment-resistant, neuroleptic-induced TD.

The present results suggest that the GABA receptor signaling pathway may be involved in the pathophysiology of TD, at least in a subgroup of Japanese schizophrenia patients. If the present results are confirmed in other populations, the GABA receptor signaling pathway may be an important target for the prevention and treatment of TD.

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