**Table 3.** Explorative analysis of gender effects

| | | Multi-SNP haplotype systems | | | | | |
|---|---|---|---|---|---|---|---|
| Gender | SNP ID | 1 SNP | 2 SNP | 3 SNP | 4 SNP | 5 SNP | 6 SNP |
| Female ($n=36$) | SNP1 | 0.55 | | | | | |
| | | | 0.41 | | | | |
| | SNP2 | 0.084 | | **0.017** | | | |
| | | | **0.012** | | **0.012** | | |
| | SNP3 | **0.0011** | | **0.049** | | **0.028** | |
| | | | **0.028** | | 0.084 | | **0.023** |
| | SNP4 | 0.50 | | 0.076 | | 0.057 | |
| | | | 0.67 | | 0.16 | | |
| | SNP5 | 0.34 | | 0.79 | | | |
| | | | 0.57 | | | | |
| | SNPA | 0.73 | | | | | |
| Male ($n=146$) | SNP1 | 0.23 | | | | | |
| | | | 0.13 | | | | |
| | SNP2 | 0.28 | | 0.18 | | | |
| | | | 0.51 | | **0.036** | | |
| | SNP3 | 0.11 | | 0.47 | | 0.053 | |
| | | | 0.26 | | 0.41 | | **0.042** |
| | SNP4 | 0.58 | | 0.19 | | 0.22 | |
| | | | 0.55 | | 0.078 | | |
| | SNP5 | 0.40 | | 0.12 | | | |
| | | | 0.30 | | | | |
| | SNPA | 0.50 | | | | | |

Bold values represent significant $p$ values.

Meth-use disorder were strongly associated with a SNP and haplotypes of AKT1, while male samples were weakly associated. However, because the sample size of female subjects was small ($n=36$), a type I error might occur in this explorative analysis. Even assuming that there are no 'gender effects' of AKT1, the fact remains that AKT1 is associated with Meth-use disorder. In this case, these association analyses of total and divided samples indicate the following interpretations. (1) SNP3 might not be an 'actual' predisposing SNP by itself, nor be in perfect LD with 'actual' predisposing polymorphisms, because male samples with Meth-use disorder were not associated with SNP3 (only total or female samples were associated with it). (2) At least some haplotypes of AKT1 may play a possible role in the development of Meth-use disorder, because two haplotypes of AKT1, including the combination of SNP1-2-3-4 and SNP 1-2-3-4-5-A, are associated with Meth-use disorder both in divided samples and total samples.

Our results had several limitations in terms of interpreting positive associations. (1) The positive associations we detected might be due to type I error, possibly because of population stratification, an unmatched-gender sample and small sample size, described above. Larger sample size and genomic control would be required. (2) Type I error might also have occurred because of multiple testing. We corrected $p$ values by applying a permutation procedure and Bonferroni correction in total samples. However, in individual haplotypic analysis or explorative analysis of gender effects, we did not apply each correction. At this time, an optimal method for resolving this problem (correction among global and individual haplotypic analysis, or explorative-subgroup analysis) has not yet been established. Greater knowledge of genetic models and more useful methods would be required to re-analyse these results. (3) The other confounding factors such as ascertainment bias can account for the apparent association in this study. For example, because subjects with Meth-induced psychosis consisted of the majority of our samples, this condition would be over-represented in our samples of Meth-use disorder. Further explanation is given in Nishiyama et al. (2005).

Our results indicate that AKT1 may play a role in the development of Meth-use disorder. Our findings also support the hypothesis that abnormalities in AKT1 might contribute to the pathophysiology of DA-dependent behaviour such as Meth-use disorder and schizophrenia. Further studies including mutation search to detect 'actual' predisposing polymorphisms and functional analysis of AKT1 (or its cascade) may open the way to elucidation of the pathophysiology of this condition.

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## Statement of Interest

None.

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# Association Analysis of Chromosome 5 $GABA_A$ Receptor Cluster in Japanese Schizophrenia Patients

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***Background:*** *Several investigations suggest that abnormalities in γ-amino butyric acid (GABA) neurotransmission systems may be related to the pathophysiology of schizophrenia. A $GABA_A$ receptor gene cluster on 5q31-35 (β2 [GABRB2], α6 [GABRA6], α1 [GABRA1], and γ2 [GABRG2] subunit genes) is one of the most attractive candidate regions for schizophrenia susceptibility.*

***Methods:*** *We performed 1) systematic polymorphism search of GABRB2, GABRA6, and GABRA1, in addition to our colleague's study of GABRG2; 2) evaluation of linkage disequilibrium (LD) within this cluster with 35 single nucleotide polymorphisms (SNPs); 3) "selection of haplotype-tagging (ht) SNPs"; and 4) two-stage association analysis that comprised first-set screening analysis of all htSNPs (288 Japanese schizophrenia patients and 288 control subjects) and second-set replication analysis of the positive htSNPs (901 schizophrenic patients and 806 control subjects).*

***Results:*** *In the first-set scan, we found a significant association of two htSNPs in GABRA1, but no association of GABRB2, GABRA6, and GABRG2. In the following second-set analysis, however, we could not confirm these significant associations.*

***Conclusions:*** *These results indicate that this gene cluster may not play a major role in Japanese schizophrenia. They also raised an alert with regard to preliminary genetic association analysis using a small sample size.*

**Key Words:** Linkage disequilibrium, haplotypes, single nucleotide polymorphism, multiple testing

Gamma-amino butyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system. Recently, abnormalities in the GABA neurotransmission system have been considered to be a possible factor related to the pathophysiology of schizophrenia, on the basis of the following findings: 1) the alternation of GABA neurons in the prefrontal cortex of schizophrenia patients might contribute to cognitive dysfunction, one of the main features of schizophrenia (Benes and Berretta 2001; Blum and Mann 2002; Weinberger et al 1986); and 2) GABA has an important role in neurodevelopment (Carlsson et al 2001; Owens and Kriegstein 2002), the abnormality of which has been hypothesized in schizophrenia (Weinberger 1995).

The formation of $GABA_A$ receptor requires co-expression of α, β, and γ subunit genes that also form α-β-γ clusters on the same chromosomes (Russek 1999): 4p12, 5q31-35, and 15q11-13. Among them, a cluster on 5q31-35 composed of β2 (GABRB2), α6 (GABRA6), α1 (GABRA1), and γ2 (GABRG2) subunit genes is important because the products of genes of this cluster are abundant in the brain (McKernan and Whiting 1996; Whiting et al 1999) and play key roles in the mechanism of psychotropic drugs, including anxiolytics, anticonvulsants, and hypnotics. Multiple whole genome linkage studies of schizophrenia showed a linkage in 5q31-35 (DeLisi et al 2002; Gurling et al 2001; Kendler et al 2000; Levinson et al 2000; Lewis et al 2003; Paunio et al 2001; Sklar et al 2004) close to the location of the GABA gene cluster. Thus, the 5q cluster genes are attractive candidates for schizophrenia susceptibility. Recently, more interest has been focused on this cluster because a positive association was reported between GABRB2 and schizophrenia in Han Chinese (Lo et al 2004).

We previously performed a systematic polymorphism search and association analysis on the basis of linkage disequilibrium (LD) of GABRG2 in which we found no association between single nucleotide polymorphisms (SNPs) in GABRG2 and schizophrenia in Japanese patients (Nishiyama et al 2005).

In the present study we expanded this strategy to the $GABA_A$ receptor subunit gene cluster on 5q. After a systematic polymorphism search in this region, we evaluated LD with 35 SNPs and selected 21 "haplotype-tagging (ht) SNPs" with relatively strict criteria. We included two-stage association analyses with a different panel of samples, in which the significant htSNPs in the first-set screening analysis were further assessed in the second-set replication analysis. This strategy is powerful for genetic association analysis from the viewpoints of "htSNP" selection and the correction of multiple testing. Thus, the results might be able to reliably rule out type I and type II errors.

## Methods and Materials

### Subjects

The subjects for polymorphism search were 96 patients with various psychiatric disorders (37 schizophrenia, 27 bipolar I disorder, 2 major depressive disorder, 10 obsessive-compulsive disorder, 10 panic disorder, and 10 amphetamine-related disorder). For the evaluation of LD, 96 control subjects were used. In the first-set screening analysis, 288 patients with schizophrenia (148 men and 140 women; 39.6 ± 14.0 years [mean age ± SD]) and 288 control subjects (150 men and 138 women; 33.6 ± 14.0 years) were genotyped. In the second-set replication analysis, a different panel of samples was used that consisted of 901 patients (482 men and 419 women; 49.2 ± 15.0 years) and 806 control subjects (403 men and 405 women; 40.0 ± 14.1 years). All subjects were unrelated to each other and ethnically Japanese.

The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy control subjects, about one-half of whom

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were hospital staff or medical students and one-half recruited from the general population, were also psychiatrically screened on the basis of unstructured interviews. After description of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University, Okayama University, and Nagoya University.

### SNP Identification

Genomic DNA was extracted from peripheral blood of all subjects. Primer pairs were designed with information from the GenBank sequence (accession number: NM-023133.11) and 42 amplified regions that covered all the coding regions, the branch sites, and 5'-flanking regions, which are 500 base pair (bp) upstream from the initial exons of GABRB2, GABRA6, and GABRA1. For GABRG2, because we had already performed a polymorphism search in the coding regions, we added only the 5'-flanking regions 500 bp upstream from the initial exon. A more detailed description of methods can be seen in a previous paper (Suzuki et al 2003). Sequences of primer pairs are available on request.

### SNP Selection

For the evaluation of LD, we included SNPs from databases (dbSNP, NCBI, Bethseda, Maryland; and Celera Discovery Systems, Rockville, Maryland) and other papers (Lo et al 2004; Nishiyama et al 2005) in addition to the SNPs we detected so that the SNPs were nearly evenly distributed. We excluded the minor allele frequencies (MAF) of SNPs less than .1 (Figure 1; Table 1).

First, we determined an "LD block" to be a region in which all pairwise D' values are not lower than .8, with the Genotype2LDblock v0.2 software (Zhang and Jin 2003). Next, "htSNPs" were selected within each "LD block" for 90% haplotype coverage with SNPtagger software (Ke and Cardon 2003). This program requires estimated haplotypes as input, for which we used PHASE version 2.1 (Stephens and Donnelly 2003; Stephens et al 2001). Single nucleotide polymorphisms that might have functional effects (i.e., SNPs in exons, untranslated regions and promoter regions) were selected preferentially, because they were considered potential candidates for predisposing factors.

### SNP Genotyping

For rapid genotyping of SNPs, we used TaqMan assays (Applied Biosystems, Foster City, California), restriction fragment length polymorphism (RFLP) assays, and primer extension methods with denaturing high-performance liquid chromatography. The other SNPs were genotyped by the direct sequencing method. In particular, the genotyping of SNP20 and SNP24, which were positive SNPs in the first-set screening analysis, was done with 192 randomly selected samples (96 cases and 96 control subjects) with direct sequencing to check for genotyping error. TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-μL total reaction volume was used and, after PCR, the allelic specific fluorescence was measured on ABI PRISM 7900 Sequence Detector Systems (Applied Biosystems). The RFLP assays and primer extension methods were described in greater detail previously (Suzuki et al 2003). Detailed information, including primer sequences, is also available on request.

### Statistical Analysis

Genotype deviation from the Hardy-Weinberg equilibrium (HWE) was evaluated by $\chi^2$ test (SAS/Genetics, release 8.2, SAS Institute, Cary, North Carolina).

In the first-set screening analysis, marker-trait association was evaluated allele/genotype-wise with the Fisher exact test (SPSS 10.0J, SPSS Japan, Japan) and haplotype-wise with the program COCAPHASE 2.403 (Dudbridge 2003). The COCAPHASE program performs log-likelihood ratio tests under a log-linear model for global $p$ value. To estimate haplotype frequencies of "htSNP" combinations in each LD block, the expectation-maximization algorithm was used. Rare haplotypes found in less than 3% of both cases and control subjects were excluded from association analysis to provide greater sensitivity and accuracy when the effect is seen in common haplotypes.

In the first-set screening analysis, we also used a recently developed software program, SNPSpD, which is able to reflect the correlation of markers (LD) on corrected $p$ values, to control inflation of the type I error rate (Nyholt 2004).

In the second-set replication analysis, the association was also evaluated with identical allele- and genotype-wise methods, as described in this section.

Power calculation was performed with a statistical program prepared by Ohashi (Ohashi et al 2001). We estimated the power for our sample size under a multiplicative model of inheritance.

The significance level for all statistical tests was .05.

## Results

We identified 6 SNPs in GABRB2, 9 SNPs in GABRA6, and 11 SNPs in GBARA1 (Table 1); however, we could not detect any

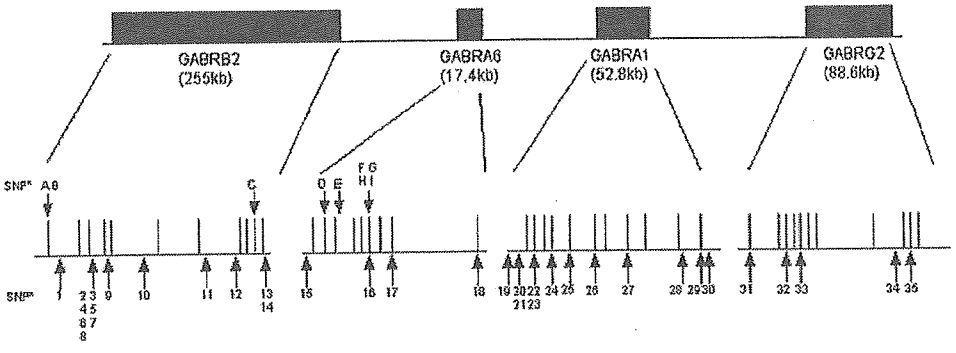


**Figure 1.** Overview of γ-amino butyric acid$_A$ (GABA$_A$) receptor gene cluster on 5q and individual gene structures. GABRB2, β2; GABRA6, α6; GABRA1, α1; and GABRG2, γ2. *shows single nucleotide polymorphism (SNP) ID in Table 1. Vertical bars represent exons.

**Table 1.** SNPs in Polymorphism Search, LD Mapping, and Association Analyses

| Gene Symbol | SNP | ID | LD Block | LD Mapping: Identified SNP | LD Mapping: Selected SNP from Database | LD Mapping: MAF (%) | Genotype Distribution: M/M SCH | M/M CON | M/m SCH | M/m CON | m/m SCH | m/m CON | *p* value[a]: (Allele) | *p* value[a]: (Genotype) | Corrected *p* value[b] (Genotype) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GABRB2 | A | | | rs2229944 | | 1.0 | | | | | | | | | |
| | B | | | 1227C > T | | 2.1 | | | | | | | | | |
| | | 1[c] | I | | rs253017 | 41.1 | 84 | 87 | 149 | 129 | 55 | 72 | .409 | .153 | |
| | | 2[c] | | | rs187269 | 16.7 | 202 | 199 | 75 | 80 | 11 | 9 | 1.000 | .827 | |
| | | 3 | | | rs252944 | 15.6 | | | | | | | | | |
| | | 4 | | | rs194072 | 15.6 | | | | | | | | | |
| | | 5 | | | rs1816072 | 38.0 | | | | | | | | | |
| | | 6 | | | rs1816071 | 31.3 | | | | | | | | | |
| | | 7 | II | | rs6891988 | 16.7 | | | | | | | | | |
| | | 8[c] | | | rs6556547 | 15.6 | 202 | 199 | 74 | 80 | 12 | 9 | 1.000 | .690 | |
| | | 9[c] | | rs2303055 | | 18.2 | 182 | 170 | 94 | 100 | 12 | 18 | .202 | .414 | |
| | | 10[c] | III | | rs1363697 | 27.6 | 152 | 141 | 116 | 118 | 20 | 29 | .216 | .352 | |
| | | 11[c] | | | rs2962407 | 41.1 | 96 | 77 | 134 | 140 | 58 | 71 | .0669 | .177 | |
| | | 12[c] | IV | | hCV1703405 | 35.9 | 114 | 107 | 135 | 136 | 39 | 45 | .467 | .707 | |
| | C | | | −141G > A | | 3.6 | | | | | | | | | |
| | | 13[c] | | rs3816596 | | 47.4 | 63 | 73 | 154 | 147 | 71 | 68 | .444 | .624 | |
| | | 14 | | −218-387G > A | | 11.4 | | | | | | | | | |
| GABRA6 | | 15 | V | | rs3811995 | 33.3 | | | | | | | | | |
| | D | | | 94G > A | | <1.0 | | | | | | | | | |
| | E | | | 225 + 16G > A | | <1.0 | | | | | | | | | |
| | F | | | 276G > A | | <1.0 | | | | | | | | | |
| | G | | | 383A > G | | <1.0 | | | | | | | | | |
| | H | | | 447-20G > A | | <1.0 | | | | | | | | | |
| | I | | | rs3811993 | | <1.0 | | | | | | | | | |
| | | 16 | | 673-121T > C | | 32.3 | | | | | | | | | |
| | | 17[c] | | 1005C > G | | 32.3 | 121 | 141 | 141 | 123 | 26 | 24 | .183 | .242 | |
| | | 18 | | rs3219151 | | 32.3 | | | | | | | | | |
| GABRA1 | | 19 | VI | | hCV478645 | 39.6 | 117 | 110 | 140 | 144 | 31 | 34 | .581 | .831 | |
| | | 20 | | −371-471C > T | | 42.2 | 91 | 123 | 154 | 128 | 43 | 37 | .0249 | .0213 | .41 |
| | | 21[c] | VII | −371-181A > G | | 35.9 | 123 | 117 | 122 | 126 | 43 | 45 | .626 | .870 | |
| | | 22[c] | VIII | rs12658835 | | 18.2 | 162 | 159 | 113 | 114 | 13 | 15 | .784 | .929 | |
| | | 23[c] | | rs4608967 | | 18.2 | 170 | 160 | 102 | 103 | 16 | 25 | .197 | .323 | |
| | | 24[c] | | 74 + 9A > T | | 17.7 | 214 | 242 | 71 | 43 | 3 | 3 | .00830 | .00862 | .166 |
| | | 25[c] | | rs1129647 | | 25.5 | 141 | 148 | 125 | 123 | 22 | 17 | .472 | .659 | |
| | | 26 | | 188-42C > T | | 22.4 | | | | | | | | | |
| | | 27 | | rs11135172 | | 21.9 | | | | | | | | | |
| | | 28 | | rs22790720 | | 42.2 | | | | | | | | | |
| | | 29[c] | | rs998754 | | 40.6 | 70 | 68 | 151 | 158 | 67 | 62 | .860 | .823 | |
| | | 30[c] | | rs2290733 | | 13.0 | 191 | 188 | 86 | 86 | 11 | 14 | .709 | .868 | |
| GABRG2 | | 31[c] | IX | | rs2268583 | 26.6 | 152 | 155 | 113 | 114 | 23 | 19 | .691 | .816 | |
| | | 32 | | | rs11135176 | 26.0 | | | | | | | | | |
| | | 33[c] | | | rs211037 | 38.5 | 85 | 84 | 152 | 142 | 51 | 62 | .515 | .497 | |
| | | 34 | X | | rs211015 | 44.3 | | | | | | | | | |
| | | 35[c] | | | rs211014 | 42.7 | 64 | 80 | 139 | 128 | 85 | 80 | .216 | .317 | |

SNP, single nucleotide polymorphism; LD, linkage disequilibrium; MAF, minor allele frequency of 96 subjects; M, major allele; m, minor allele; SCH, schizophrenic patients; CON, control subjects.
[a]*p* value from Fisher exact test.
[b]calculated using SNPSpD software.
[c]htSNPs for association analyses.

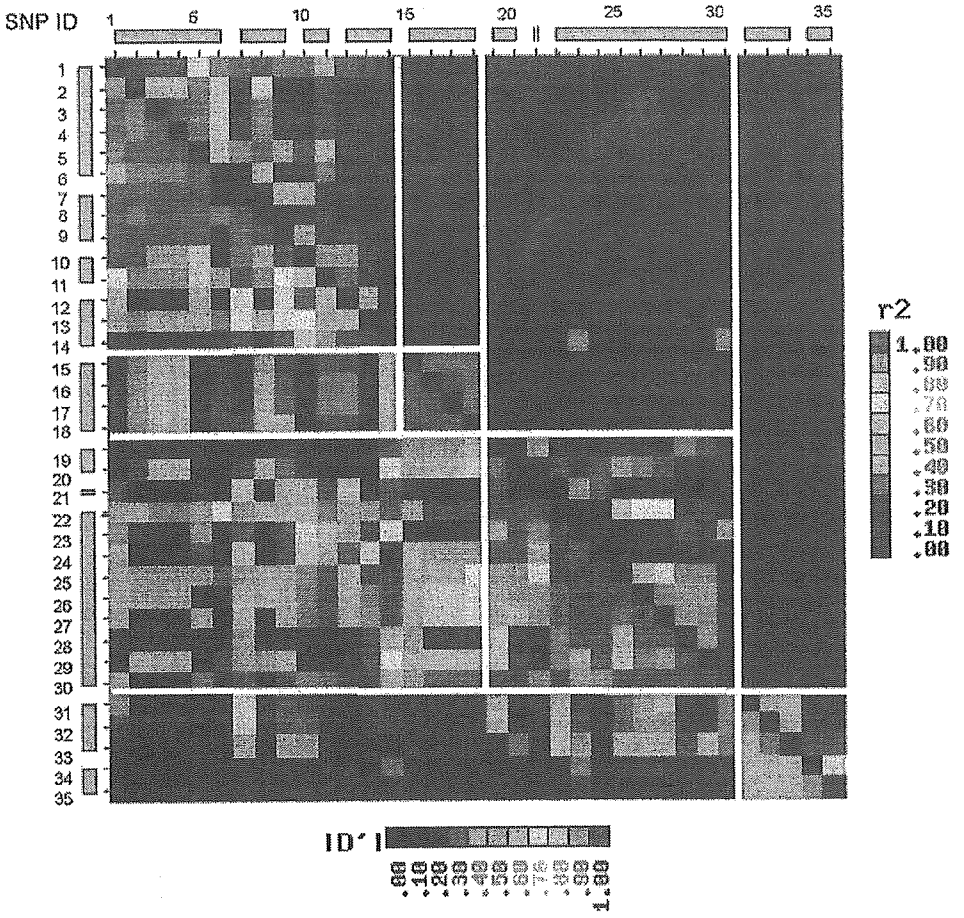


**Figure 2.** Linkage disequilibrium (LD) mapping; LD block are shown both top and left of the diagram. Top right triangles show $r^2$, bottom left triangles show D'. SNP, single nucleotide polymorphism.

functional SNPs such as non-synonymous SNPs. For GABRG2, we could not detect any SNPs within 500 bp upstream of the initial exon.

We genotyped all SNPs for 96 control subjects, because 96 samples were enough to measure LD among SNPs (Reich et al 2001). First, we evaluated the deviations from HWE for all SNPs and found the genotype frequencies to be consistent with HWE. Next, we evaluated pairwise LD between each SNP with MAF > .1, defined "LD blocks", and selected "htSNPs" for association analyses. Consequently, 21 "htSNPs" were selected (Figure 2; Table 1). We genotyped these "htSNPs" for 288 patients with schizophrenia and the remaining 192 control subjects (total 288 control subjects) for the first-set screening analysis. In this step, genotype frequencies were again in HWE.

The SNPs in GABRA6 were in almost perfect LD, and SNP17 was the only "htSNP" among SNPs in GABRA6; thus, we did not perform haplotypic analysis.

For genotype- and allele-wise association, only SNP20 and SNP24 were significantly associated with schizophrenia (genotype-wise $p$ values: .0213, .00862 for SNP20 and SNP24, respectively; allele-wise $p$ values: .0249, .00830; Table 1); however, $p$ values corrected with the SNPSpD software did not reach the significant level (cluster-wide, the effective number of independent loci is 19.25; the experiment-wide significance threshold required that the type I error rate be kept at .05:.002597; Table 1). Furthermore, haplotype-wise analysis did not show significance with any haplotypes constructed from the combination of htSNPs in each LD block (Table 2). We also confirmed our negative results for GABRG2.

To obtain a conclusive result of the positive association in GABRA1, we conducted a second-set replication analysis of these two htSNPs with a larger and different panel of samples. No significant association could be confirmed (Table 3).

We also included a power calculation for second-set replication analysis. We obtained power of more than 80% to detect

**Table 2.** Global Haplotypic Analysis

| Gene Symbol | Block | Combination of SNPs | Global $p$ Values |
|---|---|---|---|
| GABRB2 | I | SNP1–2 | .710 |
| | II | SNP8–9 | .447 |
| | III | SNP10–11 | .151 |
| | IV | SNP12–13 | .633 |
| GABRA1 | VI | SNP19–20 | .0840 |
| | VIII | SNP22–23–24–25–29–30 | .0508 |
| GABRG2 | IX | SNP31–33 | .298 |

SNP, single nucleotide polymorphism.

**Table 3.** Second-Set Association Analysis of Two Positive htSNPs in First-Set Scan

| SNP ID | Phenotype | Number | Genotype Distribution | | | *p* Values | | Power Calculation |
|---|---|---|---|---|---|---|---|---|
| | | | M/M | M/m | m/m | (Genotype) | (Allele) | (GRR) |
| SNP20 | SCZ | 901 | 337 | 429 | 135 | .792 | .503 | 1.22 |
| | CON | 806 | 312 | 381 | 113 | | | |
| SNP24 | SCZ | 901 | 709 | 179 | 13 | .848 | .703 | 1.34 |
| | CON | 806 | 639 | 158 | 9 | | | |

htSNP, haplotype-tagging single nucleotide polymorphism; M, major allele; m, minor allele; GRR, genotype relative risk to obtain 80% power; SCZ, schizophrenic patients; CON, control subjects.

associations when we set the genotype relative risk at each value as shown in Table 3.

## Discussion

Two-stage association analyses with quite high power revealed no association between chromosome 5 $GABA_A$ cluster and Japanese schizophrenia.

We adopted a reasonable strategy for association analyses (van den Oord and Neale 2004) that is more powerful than ones with randomly selected SNPs (Kamatani et al 2004). Furthermore, we carefully treated the inflation of type I error rate due to multiple testing: the independent panel of samples and the adjustment considering dependence between SNPs (by SNPSpD). Our results indicate the clear importance of correcting the inflation of the type I error rate in genetic association analysis when increasing the number of markers examined. A recent simulation showed that increasing the sample size is more powerful than continuously increasing the number of SNPs (Huang et al 2003); thus, we did not genotype additional SNPs in the vicinity of SNP20 and SNP24. Of course, the false positive results in the first-set analysis might be derived from population stratification. Genomic control may be required for conclusive results; however, we speculate that this was not the case, because Japanese population is believed to be homogeneous.

We found a unique LD structure of GABRA1 in initial evaluation of LD, as follows: although the LD matrix between the SNPs of GABRA1 located farthest from each other (SNP19 and SNP30) had strong LD, some pairwise LD matrices within GABRA1 had low D' values (for example, among SNP20/21 and SNP28/29). This structure is unlikely to be in accordance with the genetic model in which LD blocks are dictated by recombination hotspot (Goldstein 2001). Thus, we applied this LD pattern of GABRA1 to stricter criteria of LD (Wall and Pritchard 2003a), although no concept fit this LD pattern. In such a case as this, it is necessary to consider the possibility of genotyping error. At first, we checked the D' values among these markers with first-set samples (288 cases and 288 control subjects). Also, same trends were obtained (D' between SNP20 and SNP29 = .23 [cases], .24 [control]; D' between SNP21 and SNP29 = .16 [cases], .33 [control]). Next, we re-checked the genotyping of "signal" htSNPs, SNP20 and SNP24, with a different method (initial genotyping was by PCR-RFLP), the direct sequencing method (done for 96 cases and 96 control subjects, not all subjects). The results were identical to the initial results. Hence, we speculate that it was unlikely that genotyping error had occurred, and that this unique LD pattern was not related to false positive results in first-set analysis.

We could not replicate a previously reported positive association of SNPs (SNP2-SNP6 and SNP8 in Table 1) in GABRB2 in Han Chinese (Lo et al 2004). To avoid redundant results, in this study we did not genotype all positive SNPs, because our sample showed tight LD patterns different from those of the Han Chinese sample and our "htSNP" might represent these positive SNPs. Our data support a difference of LD structure in study populations (Wall and Pritchard 2003b), and for confirmation we consulted the HapMap data (http://www.hapmap.org/: HapMap public release #15; accessed February 18, 2005) around this region in Japanese and in Chinese populations. Unfortunately, these data did not contain the positive SNPs; however, the regions included in these SNPs (between rs252942 and rs967771) were strong LD (D'=1) in both populations. We assume that LD patterns among these SNPs in Chinese might also be complex, whereas the LD patterns of Japanese were similar to those of HapMap data (when we excluded SNP7, which were not analyzed by Lo et al [2004]).

The most recently reported association study between GABRA1 and Caucasian schizophrenia showed a significant association (Petryshen et al 2004). Our results could not support Petryshen's findings, but further replication study will be required with other samples from different populations.

A few points of caution should be noted in interpreting our results. First, the lack of association may be due to biased samples, especially unmatched age samples and ascertainment bias of control subjects. Second, we must consider the interaction of other candidates related to this $GABA_A$ receptor gene cluster, such as other $GABA_A$ receptor genes on 4p and 15q, glutamic acid decarboxylase and others.

In conclusion, these results, obtained with one of the optimal strategies for genetic association analysis, indicate that this gene cluster may not play a major role in Japanese schizophrenia. They also raise an alert with regard to preliminary genetic association analysis with a small sample size, and indicate that a replication analysis using large samples is required for reliable results that avoid type I and type II errors.

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# No Association of GSK3β Gene (*GSK3B*) With Japanese Schizophrenia

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**Several lines of evidence indicate that glycogen synthase kinase-3β (GSK3β) is one of the candidates for schizophrenia-susceptibility factor. However, it has not been reported the association analysis between GSK3β gene (*GSK3B*) and Japanese schizophrenia based on linkage disequilibrium (LD). We provide an association analysis using relatively large samples (381 schizophrenia, and 352 controls) after determination of "tag single nucleotide polymorphisms (SNPs)." In this LD mapping, we selected and genotyped for eight polymorphisms (seven SNPs and one diallelic $(CAA)_n$ repeat), which covered the entire region of GSK3B, and determined two "tag SNPs." In the following association analysis using these two "tag SNPs," we could not find association with Japanese schizophrenia. Furthermore, we also include subgroup analysis considering age-at-onset and subtypes, neither could we find associations. Because our samples provided quite high power, these results indicate that GSK3B may not play a major role in Japanese schizophrenia.**


**KEY WORDS: linkage disequilibrium; tag SNP; association analysis**

## INTRODUCTION

Glycogen synthase kinase-3β (GSK3β) is one of the attractive candidate molecules for schizophrenia-susceptibility factor based on the following findings. (1) GSK3β is a key component of many signal transduction cascades including the phosphatidylinositol 3-kinase cascade and the Wnt cascade. (2) GSK3β is a critically important regulator of several transcriptional factors, and can influence the expression of numerous genes [Grimes and Jope, 2001]. (3) GSK3β levels were decreased in the prefrontal cortex of schizophrenia [Kozlovsky et al., 2000, 2001], and phosphorylation of GSK3β levels were also decreased in peripheral lymphocytes and brains of schizophrenia [Emamian et al., 2004]. (4) AKT1, one of the mediators of GSK3β, was reported to be associated with schizophrenia [Emamian et al., 2004].

Thus, we provide an association analysis based on linkage disequilibrium (LD) between the GSK3β gene (*GSK3B*) and Japanese schizophrenia.

## MATERIALS AND METHODS

### Subjects

Subjects for LD mapping consisted of 96 controls. For association analysis, total 381 patients with schizophrenia and 352 controls were genotyped. And the patients were divided into subgroups considering age-at-onset (AAO; early-onset schizophrenia (EOS) with onset younger than age 19) and subtypes of schizophrenia. The general characterization of these subjects and description of their psychiatric assessment are in Supplementary on line material. After description of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University and Nagoya University Graduate School of Medicine.

### SNP Selection

For LD mapping, we selected seven SNPs and one diallelic $(CAA)_n$ repeat to make polymorphisms distributed appropriate intervals (Fig. 1). Then we determined "tag SNPs," which were the highest minor allele frequencies (MAFs) from "LD block," or which were independent from "LD block." More details are in Supplementary on line material.

### Statistical Analysis

A detailed description can be seen in Supplementary on line material (see the online Supplementary Material at http://www.interscience.wiley.com/jpages/1552-4841/suppmat/index.html.).

## RESULTS

For LD mapping, we genotyped eight polymorphisms for 96 controls. First, we evaluated the deviations from HWE for all polymorphisms. Each genotype frequency was in HWE. Next, we evaluated the pairwise LD matrices and determined "tag SNPs" from the LD blocks. One LD block was detected, and SNP6 was determined as a "tag SNP" for this block. Another SNP (SNP8) were in tight LD with this "LD block," however, the LD matrices among this "LD block" and SNP8 were not fit the criteria. Consequently, we determined two SNPs (SNP6 and SNP8) as "tag SNPs" (Fig. 2).

For association analysis, we expanded genotyping of these two "tag SNPs" for the rest samples. In this step, also genotype frequencies were in HWE. There were no significant associations between each "tag SNP" and schizophrenia. In the subgroup analysis considering AAO (EOS = 81), nor could we find associations between EOS and controls. And in another

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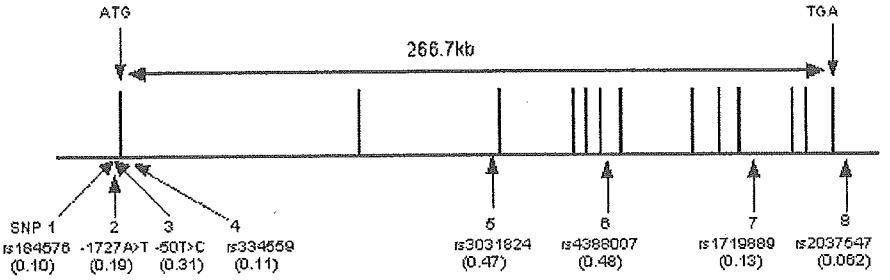


Fig. 1. Genomic structure of GSK3B with single nucleotide polymorphisms (SNPs) used in linkage disequilibrium (LD) mapping. Vertical bars represent exons. Numbers under arrows represent SNP ID. Parenthetic numbers represent minor allele frequencies (MAFs) of 96 controls.

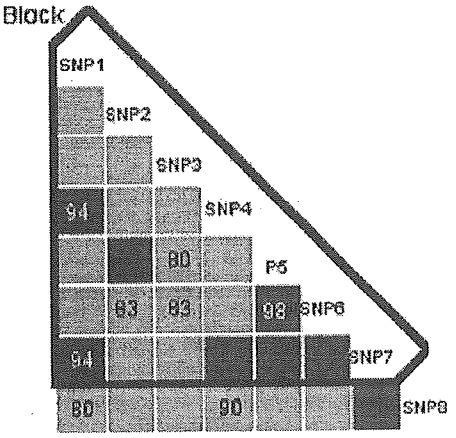


Fig. 2. LD mapping of GSK3B for control subjects. Numbers in box represent D' values after decimal point. D' values of 1.0 are not shown. The other information is described in Haploview's website.

subgroup analysis by subtypes, we did not analyze the association between catatonic type and controls because of small sample size. Neither could we find associations between each subtype (paranoid, disorganized, and residual type) and controls (Table I).

## DISCUSSION

In this study, we could not find association of "tag SNPs" in GSK3B with Japanese schizophrenia in accordance with common disease-common variant (CD-CV) hypothesis [Reich and Lander, 2001].

This initial LD mapping using control samples showed that GSK3B was typical LD pattern. Although SNP8 and one LD block were in tight LD each other, SNP8 was not included in this LD block. This might be derived from low MAF of SNP8.

And P5, which was reported the positive association with "paranoid type" [Scassellati et al., 2004], was involved in this LD block. This indicates that P5 was represented by SNP6, which was the highest MAF in this LD block with the highest power for association analysis. Considering these polymorphisms in the LD mapping might be just markers [Scassellati et al., 2004], we did not genotype all of these, thereby avoiding redundant results.

Aside from this, the LD pattern among SNP2, SNP3, and P5 were different from the previous reports [Russ et al., 2001; Scassellati et al., 2004]. These discrepancies might be derived from the difference of population [Wall and Pritchard, 2003].

The power of our analysis was quite high, more than 80% for susceptibility gene whose genotype relative risk (GRR) (multiplicative model) set 1.34 (SNP6) and 1.73 (SNP8). And the power of "paranoid type" were more than 80% when GRR set 1.49 (SNP6).

Two points of caution must be noted in interpreting these negative results. (1) We must perform a systematic mutation

TABLE I. Association Analysis Between GSK3B and Japanese Schizophrenia Using Tag SNPs'

| SNP ID | Samples | Subgroups[a] | N[b] | Genotype[c] M/M | M/m | m/m | *P*-value |
|---|---|---|---|---|---|---|---|
| SNP6 | Control | | 352 | 103 | 180 | 69 | |
| | Schizophrenia | | 381 | 100 | 203 | 78 | 0.66 |
| | | EOS | 81 | 19 | 45 | 17 | 0.58 |
| | | Paranoid | 136 | 35 | 73 | 28 | 0.74 |
| | | Disorganized | 107 | 30 | 58 | 19 | 0.84 |
| | | Residual | 87 | 24 | 48 | 15 | 0.78 |
| SNP8 | Control | | 352 | 311 | 40 | 1 | |
| | Schizophrenia | | 381 | 341 | 38 | 2 | 0.69 |
| | | EOS | 81 | 71 | 10 | 0 | 0.88 |
| | | Paranoid | 136 | 122 | 14 | 0 | 0.91 |
| | | Disorganized | 107 | 98 | 9 | 0 | 0.60 |
| | | Residual | 87 | 76 | 9 | 2 | 0.16 |

[a]EOS, early onset of schizophrenia.
[b]N, numbers.
[c]M, major allele; m, minor allele.

search to detect "true" predisposing polymorphism. (2) Other candidate genes in this cascade must be studied in considering locus heterogeneity [Schork et al., 2001]. Recently, two controversial replications between AKT1, a mediator of GSK3β, and Japanese schizophrenia have been reported [Ikeda et al., 2004; Ohtsuki et al., 2004]. This concept might explain the discrepancy of these results, and suggest that combined analysis of this signaling cascade would be required for more concrete conclusions.

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## *Research Articles*

# A Functional Glutathione S-Transferase P1 Gene Polymorphism Is Associated With Methamphetamine-Induced Psychosis in Japanese Population

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**Several lines of evidence suggest that oxidative stress plays a role in the mechanisms of action of methamphetamine (MAP) in the human brain. Given the role of glutathione *S*-transferases (GSTs) in the protection against oxidative stress, genes encoding the GSTs have been considered as candidates for association studies of MAP abuse. This study was undertaken to investigate the role of the functional polymorphism of *GSTP1* gene exon 5 (Ile105Val) in the pathogenesis of MAP abuse. Genotyping for *GSTP1* gene polymorphism exon 5 (Ile105Val) in 189 MAP abusers and 199 normal controls was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). Association between *GSTP1* gene polymorphism and clinical features (prognosis of psychosis (transient-type and prolonged-type), spontaneous relapse (positive and negative), and poly-substance abuse) of MAP abusers was evaluated. Significant differences in the frequency of both alleles ($P = 0.026$, odds ratio: 1.70, 95% confidence intervals (CI) 1.06–2.72) and genotypes ($P = 0.029$) between MAP abusers and controls were detected. In particular, a significant difference in both genotype frequency ($P = 0.013$) and allele frequency ($P = 0.014$, odds ratio: 1.84, 95% CI 1.13–2.97) between MAP abusers with psychosis (transient-type and prolonged-type) and controls was detected. Our findings suggest that the polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene may contribute to a vulnerability to psychosis associated with MAP abuse in Japanese population.**


**KEY WORDS: methamphetamine; psychosis; drug abuse; genetic factor; polymorphism**

## INTRODUCTION

Abuse of methamphetamine (MAP) is a growing problem worldwide. Some lines of evidence suggest that both environmental and genetic factors might contribute to drug abuse vulnerability [Merikangas et al., 1998; Kendler et al., 2000; Rawson et al., 2002; Uhl et al., 2002; Cami and Farre, 2003]. It is well known that MAP induces a strong psychological dependence, and that repeated further consumption of MAP results in psychotic states, the symptoms of which resemble those of the paranoid type of schizophrenia [Sato et al., 1983, 1992].

Positron emission tomography (PET) imaging studies of the brains of MAP abusers have demonstrated that the density of dopamine (DA) transporters is significantly decreased in the caudate/putamen of MAP abusers [Sekine et al., 2001; Volkow et al., 2001]. Such findings suggest that the long-term use of MAP leads to the damage of dopaminergic neurons in the human brain. It has been shown that MAP-induced neurotoxicity in the brain requires the involvement of striatum DA and

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also involves mechanisms associated with oxidative stress, further suggesting that oxidative stress in dopaminergic pathways might be implicated in MAP-induced neurotoxicity [Cadet et al., 2003]. There are a number of papers demonstrating the neuroprotective effects of glutathione or its related compounds on MAP- or DA-induced neurotoxicity [Choi et al., 2002; Shimizu et al., 2002; Fukami et al., 2004; Hashimoto et al., 2004]. In addition, it is also well known that dopaminergic pathways in the mesocorticolimbic systems can play an important role in drug reward [Kalivas, 2002]. Therefore, polymorphisms in genes that regulate dopaminergic pathways may contribute to interindividual differences as regards a vulnerability to drug abuse [Koob and Le Moal, 1997].

The glutathione *S*-transferases (GSTs: EC 2.5.1.18) belong to a family of multifunctional enzymes that catalyze the conjugation of reduced glutathione with electrophilic groups of a wide variety of compounds including carcinogens, environmental contamination, and products of the oxidative process [Mannervik, 1985; Smythies and Galzigna, 1998; Hayes and Strange, 2000]. Because of their important role in the cellular defense against oxidative stress, GSTs are of interest in the context of association studies of MAP abuse. The genes encoding three classes of GSTs, i.e., GSTM (mu, chromosome 1p13.3), GSTP (pi, chromosome 11q13), and GSTT1 (theta, chromosome 22q11.2), are known to be polymorphic [Watson et al., 1998; Stucker et al., 2002; De Roos et al., 2003; Kelada et al., 2003; Wang et al., 2003]. Recently, we reported an association between *GSTM1* gene deletion and female MAP abusers, suggesting that *GSTM1* gene deletion may contribute to a vulnerability to MAP abuse in Japanese subjects [Koizumi et al., 2004]. Furthermore, it has been reported that genetic polymorphisms of GSTP1 exon 5 (rs947894, Ile105Val (A > G)) and exon 6 (rs1799811, Ala114Val (C > T)) have functional relevance to the GST gene product resulting in reduced GST enzyme activity (~30%) [Board et al., 1989; Zimniak et al., 1994; Ali-Osman et al., 1997; Watson et al., 1998]. Taken together, such findings appear to suggest that individuals with these variant GSTP1 genotypes which result in reduced GSTP1 enzymatic activity may be at greater risk of MAP abuse. In order to verify the potential role of the *GSTP1* gene in the pathogenesis of MAP abuse, we analyzed a polymorphism of the *GSTP1* gene in subjects with a diagnosed MAP-related disorder.

## METHODS

This study was performed after obtaining the approval of the ethics committees of each affiliated institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA). All subjects provided written informed consent for the use of their DNA samples for this study. The subjects were 189 patients (149 males and 40 females, age: 36.9 ± 11.9 years (mean ± SD), age range: 18–69 years) with MAP dependence and a psychotic disorder meeting the ICD-10-DCR criteria (F15.2 and F15.5) who were outpatients or inpatients of psychiatric hospitals of the JGIDA (Table I). The control subjects were 199 age-, gender-, and geographical origin-matched normal controls (157 males and 42 females, age: 37.2 ± 10.5 years (mean ± SD), age range: 19–73 years), the majority of whom were with no past history and no family history of drug dependence or psychotic disorders. Diagnoses were made by two trained psychiatrists by interview and available information including hospital records. Patients were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder, or an organic mental syndrome as reported previously [Ujike et al., 2003]. All subjects were Japanese, born and living in restricted areas of Japan including northern Kyushu, Setouchi, Tyukyou, Toukai, and Kantou.

The patients were divided into subgroups by some characteristic clinical features (Table I). The patients were divided by poly-substance abuse status, 55 patients abuse MAP only in their lifetime, and 116 patients abuse some other drugs besides MAP in present or past. Organic solvent was most frequently abused besides MAP, followed by marijuana. Cocaine and heroine were rarely abused in the present study. Prognosis of MAP psychosis was various among patients, and some patients showed continuous psychotic symptoms even after MAP discontinuance as previously reported [Sato et al., 1983, 1992]. Therefore, patients were divided into two categories of prognosis, transient-type and prolonged-type, based on duration of psychotic state after MAP discontinuance. Thus, patients with transient-type whose psychotic symptoms improves within 1 month after discontinuance of MAP consumption and beginning of treatment with antipsychotic drugs, and those with prolonged-type whose psychosis continues for more than 1 month even after discontinuance of MAP consumption and beginning of treatment. In this study, patients with transient- and prolonged-types of MAP psychosis were 94 and 65, respectively (Table I). It has been well documented that once MAP psychosis has developed, patients in remission state becomes reliable to spontaneous relapse without re-consumption of MAP [Sato et al., 1983, 1992]. It is postulated that sensitization phenomenon induced by repeated consumption of MAP should be developed in the brain of MAP psychosis patients which result in neural basis for enhanced susceptibility to relapse. Therefore, the patients were divided into two groups according to presence or absence of spontaneous relapse. The patients with and without spontaneous relapse were 62 and 111, respectively (Table I).

Two polymorphisms on exon 5 and exon 6 of the *GSTP1* gene have previously been reported. We analyzed exon 5 (rs947894, Ile105Val) of the *GSTP1* gene in this study, since no minor

TABLE I. Characteristics of Control Subjects and MAP Abusers

| Variable | Controls | Abusers | *P*-value |
|---|---|---|---|
| Sex, M/F | 157/42 | 149/40 | 0.989[a] |
| Age, mean ± SD, years | 37.2 ± 10.5 (19–73) | 36.9 ± 11.9 (18–69) | 0.813[b] |
| Prognosis of psychosis | | | |
| Transient type | | 94 | |
| Prolonged type | | 65 | |
| Spontaneous relapse | | | |
| Positive | | 62 | |
| Negative | | 111 | |
| Poly-substance abuse | | | |
| No | | 55 | |
| Yes | | 116 | |

[a]The comparison between two groups was performed using the $\chi^2$ test.
[b]The comparison between two groups was performed using the *t*-test.

allele frequency of the polymorphism of exon 6 (rs1799811, Ala114Val) was detected among Japanese normal subjects [Ishii et al., 1999]. Genotyping for this gene was performed by PCR-RFLP analysis. The polymorphic site in exon 5 (Ile105-Val) was amplified as reported previously [Wang et al., 2003]. The primers of exon 5 of the *GSTP1* gene were GSTP1-5F (5′-GTAGTTTGCCCAAGGTCAAG-3′) and GSTP1-5R (5′-AGC-CACCTGAGGGGTAAG-3′). After performing PCR, a 433 bp DNA fragment was amplified for GSTP1 exon 5, followed by 2 hr digestion with BsmA I (New England Biolabs, Inc., Beverly, MA). The fragments were separated on 2% agarose gel stained with ethidium bromide. The wild-type (A/A), heterozygous genotype (A/G), and mutant genotype (G/G) yielded two bands (328 and 105 bp), four bands (328, 222, 106, and 105 bp), and three bands (222, 106, and 105 bp), respectively.

The differences between groups were evaluated by Fisher's exact test. The odds ratio and 95% confidence intervals (CI) between two variables were calculated as an estimate of risk. Differences were considered significant at $P < 0.05$.

## RESULTS

The frequencies of allele and genotypes in MAP abusers and controls are shown in Table II. The genotype distribution in both MAP abusers and controls was in the Hardy–Weinberg equilibrium. The differences in both genotype frequency ($P = 0.029$) and allele frequency ($P = 0.026$) between MAP abusers and controls were found to be significant (Table II). The frequency of carrying the G allele in MAP abusers was significantly higher ($P = 0.026$, odds ratio: 1.70, 95% CI 1.06–2.72) than that of controls.

Next, we examined the association between the clinical features of MAP abusers (i.e., prognosis of psychosis, spontaneous relapse, and poly-substance abuse) and normal controls. A significant difference in both genotype frequency ($P = 0.013$) and allele frequency ($P = 0.014$, odds ratio: 1.84, 95% CI 1.13–2.97) between MAP abusers with psychosis (transient-type and prolonged-type) and controls was detected (Table II). There was a significant difference in genotype frequency ($P = 0.045$) between MAP abusers with transient-type psychosis and controls, and was a trend toward difference in allele frequency ($P = 0.052$, odds ratio: 1.75, 95% CI 1.01–3.06) between MAP abusers with transient-type psychosis and controls. There was also a significant difference in both genotype frequency ($P = 0.028$) and allele frequency ($P = 0.039$, odds ratio: 1.96, 95% CI 1.07–3.59) between MAP abusers with prolonged-type psychosis and controls. Furthermore, a significant difference in terms of both genotype frequency ($P = 0.009$) and allele frequency ($P = 0.009$, odds ratio: 2.00, 95% CI 1.19–3.35) between MAP abusers with negative spontaneous relapse and controls was detected (Table II). Moreover, there was a trend toward difference in both genotype frequency ($P = 0.052$) and allele frequency ($P = 0.053$, odds ratio: 1.70, 95% CI 1.00–2.88) between MAP abusers with poly-substance abuse and controls (Table II).

## DISCUSSION

Our findings suggest that a functional polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene may contribute to MAP abuse vulnerability in Japanese subjects. Since a polymorphism (Ile105Val) on exon 5 has been shown to be of functional significance in terms of enzyme activity [Zimniak et al., 1994; Watson et al., 1998], individuals with the G allele (valine) would be expected to have decreased GST detoxification. Based on the role of GSTs in the antioxidant system preventing MAP-induced neurotoxicity, variant *GSTP1* genes might lead to an excess of metabolic products (e.g., DA-quinone) of the oxidative process induced by the administration of MAP, and may furthermore lead to MAP-induced neurotoxicity in the brain, including damage of the dopaminergic neurons, as compared to the products associated with the A allele (isoleucine) of *GSTP1* gene. We also found that the frequency of the G allele in MAP abusers with psychosis (transient-type and prolonged-type) was significantly higher than that of controls, suggesting that this *GSTP1* gene polymorphism may be associated with MAP-induced psychosis in Japanese subjects. Thus, it appears to be the case that the GSTP1 polymorphism (Ile105Val) on exon 5 may contribute to a susceptibility to MAP-induced psychosis among Japanese subjects. In contrast, we found an association between GSTP1 polymorphism (Ile105Val) and negative spontaneous relapse, whereas no association between this polymorphism and positive spontaneous relapse was detected. Taken together, it seems that GSTP1 polymorphism (Ile105Val) may be implicated in MAP-induced psychosis, but not spontaneous relapse, although further studies using a large sample are necessary.

It has been suggested that DA-quinones synthesized by auto-oxidation of DA might play a role in MAP-induced neurotoxicity in the brain, and that glutathione and GST might play a role in the detoxification against DA-quinone induced neurotoxicity [Smythies and Galzigna, 1998; LaVoie and Hastings, 1999; Whitehead et al., 2001; Shimizu et al., 2002; Asanuma et al., 2003]. Thus, DA auto-oxidation results in the formation of DA-quinones, which readily participate in nucleophilic addition reactions with sulfhydryl groups on free cysteine, glutathione, or cysteine found in proteins including DA transporters [Smythies and Galzigna, 1998;

TABLE II. Genotype and Allele Frequencies of the GSTP1 Exon 5 Gene Polymorphism in Controls and MAP Abusers

| Ile105Val (A > G) | | Genotype | | | | Allele | | |
|---|---|---|---|---|---|---|---|---|
| rs947894 | n | AA | AG | GG | *P* | A | G | *P* |
| Control | 199 | 167 (83.9%) | 32 (16.1%) | 0 (0%) | | 366 (92.0%) | 32 (8.0%) | |
| Abuser | 189 | 144 (76.2%) | 41 (21.7%) | 4 (2.1%) | 0.029* | 329 (87.0%) | 49 (13.0%) | 0.026* |
| Prognosis of psychosis | 159 | 119 (74.8%) | 36 (22.6%) | 4 (2.5%) | 0.013* | 274 (86.2%) | 44 (13.8%) | 0.014* |
| Transient | 94 | 71 (75.5%) | 21 (22.3%) | 2 (2.1%) | 0.045* | 163 (86.7%) | 25 (13.3%) | 0.052 |
| Prolonged | 65 | 48 (73.8%) | 15 (23.1%) | 2 (3.1%) | 0.028* | 111 (85.4%) | 19 (14.6%) | 0.039* |
| Spontaneous relapse | | | | | | | | |
| Positive | 62 | 50 (80.6%) | 11 (17.7%) | 1 (1.6%) | 0.255 | 111 (89.5%) | 13 (10.5%) | 0.463 |
| Negative | 111 | 81(73.0%) | 27 (24.3%) | 3 (2.7%) | 0.009** | 189 (85.1%) | 33 (14.9%) | 0.009** |
| Poly-substance abuse | | | | | | | | |
| No | 55 | 44 (80.0%) | 9 (16.4%) | 2 (3.6%) | 0.065 | 97 (88.2%) | 13 (11.8 %) | 0.254 |
| Yes | 116 | 87 (75.0%) | 28 (24.1%) | 1 (0.9%) | 0.052 | 202 (87.1%) | 30 (12.9%) | 0.053 |

*$P < 0.05$.
**$P < 0.01$ as compared to control (Fisher's exact test).

Whitehead et al., 2001]. Based on the known role of GSTs in the process of antioxidant defense, we considered the possibility that MAP abusers with the G allele of GSTP1 polymorphism were more susceptible to MAP-induced psychosis or to a spontaneous relapse of MAP abuse. In this study, we found significant differences in the distribution of genotype and allele frequencies between MAP abusers with psychosis and controls. Furthermore, we found a significant difference between MAP abusers with negative spontaneous relapse and controls. Taken together, it is likely that the polymorphism (Ile105Val) on exon 5 of the *GSTP1* gene could be a risk factor for the development of MAP-induced psychosis in Japanese subjects.

It is reported that the frequency (18%) of the G allele in Asians such as Taiwanese is lower than that in African-American (42%) and European-American (33%) [Watson et al., 1998]. The frequency (8%; our study) of the G allele in Japanese control subjects is significantly ($\chi^2 = 13.3$, $P = 0.0003$) lower than that (18%) of Taiwanese, suggesting the ethnic difference between Asians and European- and African-Americans for the polymorphism (Ile105Val) on exon 5 of GSTP1. Therefore, it may be of interest to examine the association between the *GSTP1* gene polymorphism and methamphetamine abusers in European- and African-Americans. If replication studies are confirmed, the polymorphism (exon 5 Ile105Val) of GSTP1 would be only the known specific mechanism by which genetic variation leads to a risk for the development of MAP-induced psychosis. Interestingly, our recent PET study demonstrated that the antioxidant *N*-acetyl-L-cysteine (a precursor for glutathione synthesis) could attenuate significantly the reduction of DA transporter in monkey striatum after repeated administration of MAP [Hashimoto et al., 2004]. In addition, we reported that *N*-acetyl-L-cysteine attenuated hyperlocomotion, development of sensitization, and neurotoxicity after administration of MAP [Fukami et al., 2004], suggesting that *N*-acetyl-L-cysteine would be a suitable drug for treatment of MAP abuse. As described in "Introduction," it is likely that endogenous antioxidant glutathione plays a role in the behavioral changes and neurotoxicity associated with MAP abuse. Taken together, our findings may shed light on some of the neurobiological mechanisms and pathways that lead to the development of MAP abuse, and could thereby facilitate the development of novel treatments and prevention strategies for MAP abuse.

In conclusion, our findings indicate that a polymorphism (exon 5 Ile105Val) of the *GSTP1* gene may contribute to a vulnerability to MAP abuse among Japanese subjects. Furthermore, it is likely that this polymorphism (exon 5 Ile105Val) of the *GSTP1* gene could be a risk factor for the development of MAP-induced psychosis in Japanese subjects.

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# Genomewide High-Density SNP Linkage Analysis of 236 Japanese Families Supports the Existence of Schizophrenia Susceptibility Loci on Chromosomes 1p, 14q, and 20p

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**The Japanese Schizophrenia Sib-Pair Linkage Group (JSSLG) is a multisite collaborative study group that was organized to create a national resource for affected sib pair (ASP) studies of schizophrenia in Japan. We used a high-density single-nucleotide–polymorphism (SNP) genotyping assay, the Illumina BeadArray linkage mapping panel (version 4) comprising 5,861 SNPs, to perform a genomewide linkage analysis of JSSLG samples comprising 236 Japanese families with 268 nonindependent ASPs with schizophrenia. All subjects were Japanese. Among these families, 122 families comprised the same subjects analyzed with short tandem repeat markers. All the probands and their siblings, with the exception of seven siblings with schizoaffective disorder, had schizophrenia. After excluding SNPs with high linkage disequilibrium, we found significant evidence of linkage of schizophrenia to chromosome 1p21.2-1p13.2 (LOD = 3.39) and suggestive evidence of linkage to 14q11.2 (LOD = 2.87), 14q11.2-q13.2 (LOD = 2.33), and 20p12.1-p11.2 (LOD = 2.33). Although linkage to these regions has received little attention, these regions are included in or partially overlap the 10 regions reported by Lewis et al. that passed the two aggregate criteria of a meta-analysis. Results of the present study—which, to our knowledge, is the first genomewide analysis of schizophrenia in ASPs of a single Asian ethnicity that is comparable to the analyses done of ASPs of European descent—indicate the existence of schizophrenia susceptibility loci that are common to different ethnic groups but that likely have different ethnicity-specific effects.**

## Introduction

Schizophrenia (MIM 181500) is a common disorder, with a lifetime morbidity risk of 1%. A large number of family, twin, and adoption studies have revealed that individual differences in susceptibility are predominantly genetic, with a heritability of 0.70–0.85 and a 10-fold increased risk in siblings of probands (Levinson and Mowry 2000). More than 20 genome scans for susceptibility loci for schizophrenia have been completed, and evidence satisfying genomewide significance levels for linkage to schizophrenia was obtained for chromosome regions 6p24-p22 (MIM 600511) (Straub et al. 1995), 1q21-q22 (MIM 604906) (Brzustowicz et al. 2000), 13q32-q34 (MIM 603176) (Blouin et al. 1998), 10p14 (DeLisi et al. 2002*b*), and 10q25.3-q26.3 (Williams et al. 2003). Linkage for other regions—including 8p22-p21 (MIM 603013) (Kendler et al. 1996; Blouin et al. 1998), 6q21-q25 (MIM 603175) (Cao et al. 1997; Lindholm et al. 2001), 22q11-q12 (MIM 600850) (Pulver et al. 1994; Schizophrenia Linkage Collaborative Group

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for Chromosomes 3 and 8 1996), and 5q21-q33 (MIM 181510) (Bassett et al. 1988; Paunio et al. 2001)—has been reported multiple times. However, none of the above-named regions has been identified consistently in a majority of the genome scans. It is possible that loci with small populationwide effects hinder confirmation of linkage because replication of linkage data requires a larger sample population than the original data set (Suarez et al. 1994) and because the samples for most genome scans of schizophrenia have been small, typically 20–100 families.

Some problems of power and replication can be addressed by meta-analysis. Lewis and colleagues (2003) used the rank-based genome-scan meta-analysis (GSMA) method to analyze 20 complete genome scans for susceptibility loci for schizophrenia. In GSMA, the autosomes were divided into 30-cM bins, and the evidence of linkage in each study was rank ordered across bins with and without weights for sample size. The average ranks across studies were evaluated for statistically significant evidence of linkage in several ways. Lewis et al. (2003) concluded that schizophrenia loci are highly likely to be present in one or more of the following regions: 1p13.3-q23.3, 2p12-q23.3, 3p25.3-p22.1, 5q23.2-q34, 6pter-p21.1, 8p22-p21.1, 11q22.3-q24.1, 14pter-q13.1, 20p12.3-p11, and 22pter-q12.3, all of which met two aggregate criteria for linkage. Another meta-analysis found significant results only for chromosomes 8p, 13q, and 22q (Badner and Gershon 2002). However, meta-analysis has limitations (Levinson et al. 2003). One is that meta-analysis methods may not distinguish several weakly linked loci in the same region. This issue can be addressed by pooling the raw genotyping data for meta-analysis. Analysis of a multicenter sample of 779 pedigrees did not yield significant evidence of linkage of 22q to schizophrenia (Mowry et al. 2004); however, those authors suggested that collaborative pooling of data sets was limited by intersite differences in sampling frames, population ethnicity, and genotyping methods.

The largest genomewide linkage findings to date were reported by DeLisi and colleagues (2002*b*), who studied 294 families with 382 nonindependent affected sib pairs (ASPs) with schizophrenia or schizoaffective disorder from the United States, the United Kingdom, Italy, Chile, and Belgium. Williams and colleagues (2003) described linkage findings in 272 families with 353 nonindependent ASPs from the United Kingdom, Sweden, and the United States. Among these ASPs, 287 nonindependent ASPs in 231 families received a diagnosis of schizophrenia or schizoaffective disorder. Straub and colleagues (2002) described linkage findings in 270 families with 261 nonindependent ASPs with schizophrenia or poor-outcome schizoaffective disorder from Ireland and Northern Ireland. The Irish families were ethnically homogeneous, and most of the pedigrees in the other two studies were of European origin. The narrow ethnic distributions of these sample populations could have influenced the results, because an ethnically diverse study population has increased potential for variation, which could result in heterogeneity at certain susceptibility loci. A recent study found ethnic heterogeneity between European and East Asian populations in allelic association of the 102T/C polymorphism of the *HTR2A* gene (MIM 182135) with schizophrenia (Abdolmaleky et al. 2004). This type of heterogeneity compounds the recognized difficulty in characterizing genetically complex diseases for which the magnitude of the effect of any one locus is unknown.

The Japanese Schizophrenia Sib-Pair Linkage Group (JSSLG), a multisite collaborative study group, was established in 1997 as a national resource for genetic studies of schizophrenia. An initial genomewide linkage study was performed with 417 STR markers in 130 families; however, no loci with significant linkage to schizophrenia were detected (JSSLG 2003). We recruited additional families to participate in the JSSLG study and analyzed 236 families with 268 nonindependent ASPs with a high-density SNP linkage mapping set. High-density SNP linkage mapping systems provide significantly improved levels of information extraction with extremely high accuracy, particularly when founder genotypes are unavailable (Sawcer et al. 2004).

## Material and Methods

### *Subjects*

Linkage of genetic loci to schizophrenia was analyzed in Japanese families with at least two available siblings who had received the diagnosis of schizophrenia or schizoaffective disorder. A total of 236 families with 602 individuals were recruited at 24 centers across Japan (table 1). Of these, 122 families with 315 individuals comprised the same subjects analyzed by STR markers that we reported elsewhere (JSSLG 2003). Each family member received the diagnosis on the basis of the DSM-IV structured clinical interview. Each face-to-face interview was conducted by two experienced interviewers. In addition to direct interviews, all available medical records and information from relatives and hospital staff were considered. Inclusion criteria for this collaborative sample recruitment were DSM-IV-defined schizophrenia for probands and schizophrenia or schizoaffective disorder for affected siblings. Seven siblings with schizoaffective disorder were included. All participants and their parents were of Japanese descent. The study protocol was approved by the ethics committee of each institution, and written informed consent was obtained from all subjects.

**Table 1**

**JSSLG Subjects**

| Center[a] | No. of Families | No. of JSSLG Families: Both Parents[b] 2 Affected Sibs | Both Parents[b] 3 Affected Sibs | One Parent[b] 2 Affected Sibs | One Parent[b] 3 Affected Sibs | No Parent[b] 2 Affected Sibs | No Parent[b] 3 Affected Sibs |
|---|---|---|---|---|---|---|---|
| Hakkaido University | 2 | 0 | 0 | 0 | 0 | 1 | 1 |
| Hirosaki University | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Minami Hanamaki National Hospital | 18 | 5 | 0 | 8 | 0 | 5 | 0 |
| Tohoku University | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| Fukushima Medical University | 4 | 0 | 0 | 2 | 0 | 2 | 0 |
| Niigata University | 19 | 3 | 0 | 2 | 0 | 14 | 0 |
| University of Tsukuba | 16 | 0 | 0 | 3 | 2 | 10 | 1 |
| Teikyo University Ichihara Hospital | 23 | 0 | 0 | 2 | 0 | 21 | 0 |
| RIKEN Brain Science Institute | 7 | 5 | 0 | 2 | 0 | 0 | 0 |
| Juntendo University | 3 | 2 | 0 | 1 | 0 | 0 | 0 |
| Toho University | 10 | 0 | 0 | 0 | 0 | 10 | 0 |
| Tokyo Institute of Psychiatry | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Nihon University | 11 | 1 | 1 | 0 | 0 | 9 | 0 |
| Teikyo University | 4 | 0 | 0 | 2 | 0 | 2 | 0 |
| National Center of Neurology and Psychiatry | 5 | 0 | 0 | 0 | 0 | 5 | 0 |
| Fujita Health University | 7 | 0 | 0 | 0 | 0 | 7 | 0 |
| Osaka Medical College | 4 | 0 | 0 | 0 | 0 | 4 | 0 |
| Okayama University | 33 | 0 | 0 | 2 | 0 | 27 | 4 |
| University of Occupational and Environmental Health | 10 | 1 | 0 | 0 | 0 | 9 | 0 |
| Kyushu University | 2 | 0 | 0 | 0 | 0 | 2 | 0 |
| Kurume University | 7 | 0 | 0 | 0 | 0 | 7 | 0 |
| Saga Medical School | 22 | 5 | 1 | 5 | 1 | 10 | 0 |
| Nagasaki University | 19 | 7 | 0 | 7 | 1 | 3 | 1 |
| Kagoshima University | 7 | 0 | 1 | 1 | 0 | 5 | 0 |
| Total | 236 | 29 | 3 | 37 | 4 | 154 | 9 |

[a] In order of location from north to south.

[b] Available for genotyping.

### *Genotyping*

The Illumina SNP-based Linkage Panel IV was used for genotyping. The panel includes 5,861 SNP markers distributed evenly across the genome. The average and median intervals between markers were 503 kb (0.64 cM) and 301 kb (0.35 cM), respectively. The largest interval between successfully genotyped markers was 4.9 Mb (8.8 cM) on chromosome Xp21. The Illumina markers were typed with the Illumina BeadStation 500G, in accordance with the manufacturer's standard recommendations.

### *Statistical Analysis*

Multipoint linkage analysis was performed along the entire length of each chromosome with the MERLIN program (Center of Statistical Genetics) developed by Abecasis et al. (2002). Both the nonparametric linkage (NPL) Z score and nonparametric LOD score, calculated with the Kong and Cox (1997) linear model, were extracted from the MERLIN runs and were used to generate graphic plots of the whole-genome scan results. Because linkage disequilibrium (LD) between closely spaced SNP markers can falsely inflate linkage statistics, we used the SNPLINK program (Webb et al. 2005; Institute of Cancer Research), which removes LD from the marker sets in an automated fashion. Because the program considers LD between pairs of adjacent SNPs, the possibility of high LD between nonadjacent SNPs but low LD between adjacent SNPs, such as a situation in which there was high LD between SNPs 41 and 43 and low LD between SNPs 41 and 42 and between SNPs 42 and 43, was examined with the Haploview program. Because no empirical justification to remove LD by any criteria has been published, we tested the significant and suggestive regions, using a range of criteria from $r^2 = 0.4$, and gradually decreased the thresholds to $r^2 = 0.05$. The linkage panel includes 28 SNPs from the pseudoautosomal regions of the X chromosome (20 from the short arm; 8 from the long arm). Because no currently available multipoint linkage program can integrate data from X-linked and pseudoautosomal markers in a single analysis, each pseudoautosomal region was analyzed separately, as though it were an independent autosomal chromosome. The results of these analyses were then combined with those from the standard X-linked markers. Empirical $P$ values were calculated for the NPL Z and LOD scores via simulation. MERLIN was used to

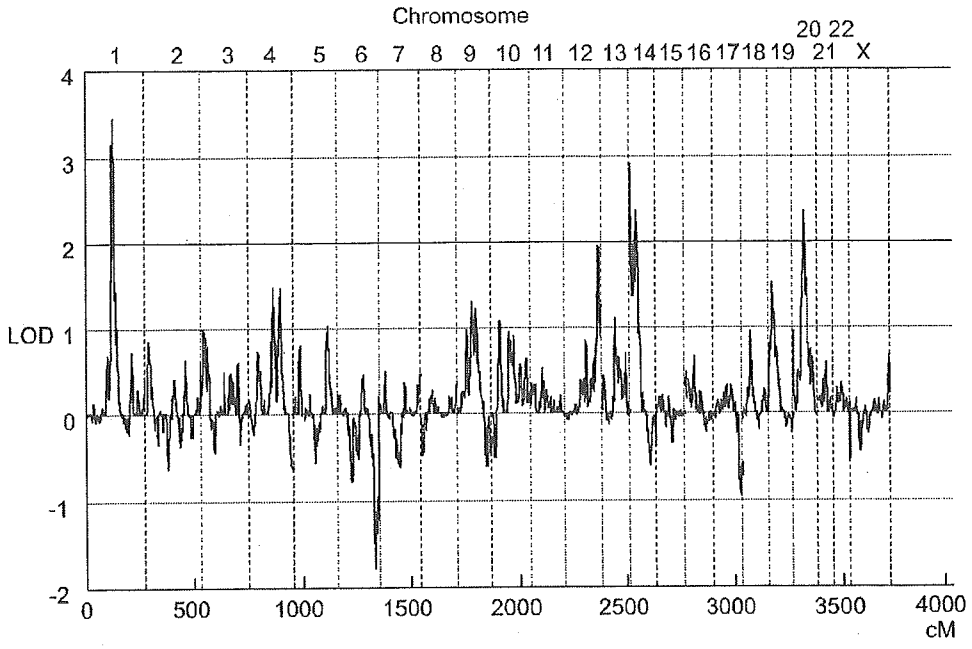


**Figure 1** Multipoint nonparametric LOD score (Kong and Cox 1997) of genomewide scan for JSSLG ASPs with schizophrenia

generate 50,000 replicates of families identical to those in our sample. Markers with similar allele frequencies were also generated under the assumption of no linkage. Linkage analyses were then performed on these unlinked replicates, and peaks of NPL Z and LOD scores separated by at least 30 cM on each chromosome were recorded for each simulation. Simulation studies of our genome scan suggested that, on average, an NPL Z of 2.87 and a LOD of 2.06 per genome scan would have been expected, whereas an NPL Z of 3.48 and a LOD of 3.07 would have been expected to occur only once in every 20 genome scans in the absence of linkage. Therefore, these values correspond to "suggestive" and "significant" thresholds for genomewide significance, as defined by Lander and Kruglyak (1995). The GeneFinder program (Liang et al. 2001; Chiu et al. 2002; Glidden et al. 2003) was used to obtain 95% CIs for the locations of linked loci. The information content of the genotypes was estimated by MERLIN, with use of entropy information described by Kruglyak et al. (1996). Simulations suggested that our study had a power of >0.99, 0.79, 0.38, and 0.05 to detect a susceptibility locus of $\lambda s$ = 3, 2, 1.5, and 1.25 for schizophrenia, with a genomewide significance of $P = .05$.

## Results

Among our Japanese family members, we observed an average minor-allele frequency of 0.29 and a mean heterozygosity of 0.37. These values were identical to those in Asian populations in the Illumina Linkage IV Panel. In our Japanese population, 125 SNPs were not polymorphic. The call rate (percentage of successful genotype calls among subjects) was used as a measure of quality. The average call rate was 98.5%, and we excluded 10 SNPs with call rates of <90%. The rate of Mendelian inconsistency or impossible recombination identified by the MERLIN program was 0.027% in the families with parents available for genotyping. Because the low heterozygosity of SNPs means that only 37% of genotyping errors will appear as Mendelian inconsistencies (Abecasis et al. 2002), the approximate genotyping error rate was estimated to be 0.073%.

Results of the linkage analysis are presented in figure 1. One region, 1p21.1, showed genomewide significance ($P < .05$) on the basis of simulation studies (LOD = 3.39; NPL Z = 3.96) with a 95% CI of 102.0–111.9 Mb (National Center for Biotechnology Information [NCBI] build 35). We also obtained suggestive evidence of linkage to chromosome 14q11.2 (LOD = 2.87; NPL Z = 3.14), with a 95% CI of 19.4–34.9 Mb; chromosome 14q12 (LOD = 2.33; NPL Z = 2.95), with a 95% CI of 19.4–34.9 Mb; and chromosome 20p11.2 (LOD = 2.33; NPL Z = 3.10), with a 95% CI of 16.0–33.2 Mb (table 2). Notable results were also obtained for chromosomes 4q24 (LOD = 1.44; NPL Z = 2.32), 4q31.3 (LOD = 1.44; NPL Z =