Table 3. The characteristics of the identified SNPs in the *KCNJ6* gene.

Position	SNP name	rs ID	Sample size	Reported allele	Major allele	Minor allele	Minor allele frequency
5′-flanking	A-1361G	–	48	A	A	G	0.010 (N.A.)
	G-1250A	rs6517442	48	G	A	G	0.385 (0.422)
	T-244C	rs7275707	46	T	C	T	0.391 (0.422)
	C-227T	rs7276069	46	C	T	C	0.391 (0.422)
	A-68G	rs11702683	45	A	G	A	0.089 (0.102)
Intron 1	IVS1C75167T	rs2836016	48	C	T	C	0.188 (0.273)
Exon 3	A1032G	rs2070995	48	A	G	A	0.344 (0.432)
Exon 4	C1569T	rs702859	48	C	T	C	0.062 (0.100)
	C1843G	rs56345212	48	C	C	G	0.052 (N.A.)

rs ID, reference SNP ID in the NCBI dbSNP database; Sample size, the number of samples used for genotyping each SNP; Reported allele, the allele appearing in the GenBank reference sequence. The numbers in parentheses represent the minor allele frequencies for the Japanese population described in the NCBI dbSNP database. N.A., not available.

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M. Sato, Japan; current version is available at http://www.vector.co.jp/soft/win95/business/se030917.html) or Simple Interactive Statistical Analysis (free software by Quantitative Skills, The Netherlands; current version is available at http://www.quantitativeskills.com/sisa/) to investigate the deviation of the distributions from those in the theoretical Hardy-Weinberg equilibrium. Analysis of covariance (ANCOVA) was performed to examine the contribution of the SNPs to the subjective pain ratings reported by patients, frequency of 24 h analgesic requirements, and analgesic requirements during the 24 h postoperative period. Bonferroni multiple comparisons were used as *post hoc* tests. Correction of multiple testing was not performed for the results of the G-1250A and A1032G SNPs in this explorative study. The age, height, and weight of the subjects were incorporated as covariables for the ANCOVA because these three factors were found to be significant covariables in a preliminary analysis and may affect analgesic efficacy of opioids and/or NSAIDs. Pearson's correlation coefficient (r) was calculated to examine the correlation between variables. Student's t-test was performed to compare *KCNJ6* expression levels between the A1032G genotype subgroups. For these three analyses, SPSS 12.0J for Windows (SPSS Japan, Inc., Tokyo, Japan) was used. Power analyses were performed using G*Power Version 3.0.5 [26]. gPLINK v. 2.049, PLINK v. 1.01 (http://pngu.mgh.harvard.edu/purcell/plink/) [27], and Haploview v. 4.0 [23] were used for haplotype-specific tests, incorporating gender, age, height, and weight of the subjects as covariables, with the false discovery rate set at 0.05 for correction of multiple testing, based on a previous report [28]. In all statistical tests, the criterion for significance was set at $P<0.05$.

Results

In the first polymorphism search in the whole exon, 5′-flanking, and exon-intron boundary regions of the *KCNJ6* gene, a total of nine SNPs were identified in the 5′-flanking region, intron 1, exon 3, and exon 4. Figure 1 illustrates the relative positions of the SNPs identified in the *KCNJ6* gene. The characteristics of the SNPs are provided in Table 3, where the minor allele frequencies of the SNPs are also shown. The allele frequencies of the SNPs observed in this study were comparable (less than 0.1 difference) to those annotated in the National Center for Biotechnology Information (NCBI) database (Table 3). SNPs for a further association study were selected, considering the LD structure, minor allele frequencies of the SNPs, and the expected impact on gene function. The results of the D' and r^2 calculations for the *KCNJ6* gene are provided in Table 4. Absolute LD ($D'=1$, $r^2=1$) was observed between SNPs G-1250A, T-244C, and C-227T (Table 4),

Table 4. Pairwise D' and r^2 values between the identified SNPs in the *KCNJ6* gene.

		D'								
	SNP name	A-1361G	G-1250A	T-244C	C-227T	A-68G	IVS1C75167T	A1032G	C1569T	C1843G
	A-1361G	–	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	G-1250A	0.017	–	***1.000***	***1.000***	1.000	0.136	0.037	0.518	0.159
	T-244C	0.017	***1.000***	–	***1.000***	1.000	0.114	0.020	0.505	0.104
r^2	C-227T	0.017	***1.000***	***1.000***	–	1.000	0.114	0.020	0.505	0.104
	A-68G	0.000	0.153	0.153	0.153	–	0.191	0.398	0.152	0.093
	IVS1C75167T	0.002	0.007	0.005	0.005	0.014	–	0.078	1.000	0.044
	A1032G	0.020	0.000	0.000	0.000	0.034	0.003	–	0.603	0.401
	C1569T	0.001	0.029	0.028	0.028	0.000	0.015	0.046	–	0.091
	C1843G	0.192	0.002	0.001	0.001	0.004	0.000	0.017	0.007	–

The numbers above and below the hyphens show the results of the pairwise calculations of D' and r^2 between the two SNPs, respectively. The values representing $D'=1.000$ are underlined. The pairs of values representing $D'=1.000$ and $r^2=1.000$ are italicized and bold.

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and Haploview also identified G-1250A as a candidate haplotype-tagging SNP in this LD block structure. The minor allele frequencies for these three SNPs were relatively high (Table 3), and G-1250A was selected for the association study. Among the remaining six SNPs, IVS1C75467T and A1032G are relatively common, with minor allele frequencies greater than 0.1, and could be candidates for an association study. Because IVS1C75467T is in the intron region and thus is less likely to affect the mRNA product or protein levels, it was not selected as a candidate SNP. Therefore, G-1250A and A1032G were selected from the nine *KCNJ6* SNPs for the association study.

The genotype distributions for the two SNPs that were selected were not significantly different from the theoretical Hardy-Weinberg equilibrium values in independent tests of the 48 healthy subjects used in the resequencing procedure or 129 patient subjects used in the association analyses (data not shown). The clinical data of the 129 subjects who were included in the association study are provided in Table 5. Rescue analgesics were required in 59 patients. Doses of rescue analgesics administered to patients are shown in Table 5. More detailed clinical data stratified by each genotype (*KCNJ6* G-1250A and A1032G) are presented as Supporting Information in Table S1.

Two-way ANCOVA was performed to examine the effects of SNPs and gender on the frequency of rescue analgesic administration, the total dose of rescue analgesics administered, and NRS pain scores, incorporating the age, height, and weight of the subjects as covariables. Statistical power analyses for the ANCOVA revealed that the expected power (1 minus type II error probability) was 71% for the Cohen's conventional "medium" effect size 0.25 [29] when the sample size was 129 and type I error probability was set at 0.05. Significant associations were not observed between *KCNJ6* G-1250A and the frequency of rescue analgesic administration or total dose of rescue analgesics administered (frequency of rescue analgesic administration: $F_{2,117} = 1.145$, $P = 0.322$; total dose of rescue analgesics administered: $F_{2,117} = 1.233$, $P = 0.295$). A significant main effect of *KCNJ6* A1032G on the frequency of rescue analgesic administration was observed ($F_{2,120} = 5.336$, $P = 0.006$). *Post hoc* analysis revealed significant differences between the A/A and A/G genotypes ($P = 0.005$) and between the A/A and G/G genotypes ($P = 0.010$), indicating that the carriers of the A/A genotype in the A1032G SNP required rescue analgesics more often compared with carriers of the A/G and G/G genotypes (Figure 2A). The effect of *KCNJ6* A1032G on the total dose of rescue analgesics administered was not significant ($F_{2,120} = 1.332$, $P = 0.268$). However, a significant main effect of the *KCNJ6* A1032G SNP on the total dose of rescue analgesics administered was observed in the female subjects ($F_{2,49} = 3.428$, $P = 0.040$), and differences were

Table 5. The clinical data of the subjects included in the study.

	N	Minimum	Maximum	Mean	SD
***KCNJ6* G-1250A**					
G/G (male/female)	19 (11/8)				
G/A (male/female)	72 (42/30)				
A/A (male/female)	35 (19/16)				
***KCNJ6* A1032G**					
A/A (male/female)	11 (5/6)				
A/G (male/female)	62 (37/25)				
G/G (male/female)	56 (32/24)				
Gender					
male	74				
female	55				
Age	129	28	80	63.57	9.92
Height (cm)	129	133	175	158.21	8.34
Weight (kg)	129	35	80	56.24	10.42
NRS pain score	105	0	4	1.54	1.29
male/female	62/43	0/0	4/4	1.48/1.63	1.21/1.40
Frequency of analgesic administration	129	0	6	0.72	1.01
male/female	74/55	0/0	3/6	0.70/0.75	0.90/1.14
Total dose of rescue analgesics (mg)	129	0	105	12.63	19.16
male/female	74/55	0/0	72/105	13.38/11.62	19.05/19.44
Dose of each rescue analgesic (mg)	129	(0)	(105)	(12.63)	(19.16)
Epidural morphine (mg)	2	0.5 (7.5)	2 (30)	1.25 (18.75)	1.06 (15.91)
Pentazocine (mg)	24	15 (15)	75 (75)	26.25 (26.25)	17.83 (17.83)
Buprenophine (mg)	15	0.2 (18)	0.8 (72)	0.47 (42.00)	0.16 (14.70)
Petidine (mg)	1	35 (8.75)	35 (8.75)	35.00 (8.75)	–
Diclofenac (mg)	14	25 (7.5)	100 (30)	37.50 (11.25)	23.51 (7.054)
Flurbiprofen (mg)	8	50 (15)	100 (30)	62.50 (18.75)	23.15 (6.944)

Values in parentheses for "Dose of each rescue analgesic (mg)" indicate data for dose of each rescue analgesic converted to the equivalent oral morphine dose.
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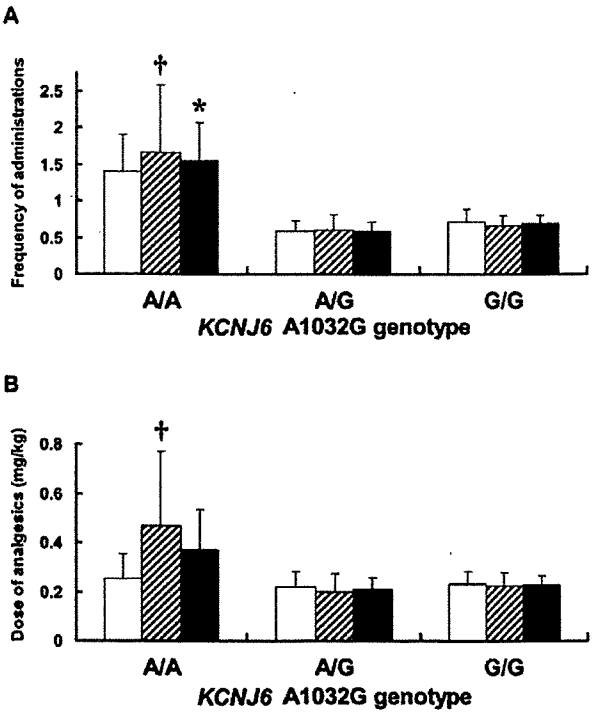

Figure 2. Association analysis between requirements for rescue analgesics and SNPs. The results for the frequency of analgesic administration (A) and the total dose of analgesics administered per weight (B) during the 24 h postoperative period are shown for the *KCNJ6* A1032G SNP. The white, striped, and filled boxes indicate results for male, female, and all subjects, respectively. (A) *: significantly more frequent administration for the A/A genotype compared with the A/G and G/G genotypes in all subjects, †: significantly more frequent administration for the A/A genotype compared with the A/G and G/G genotypes in female subjects. (B) †: significantly greater dose of analgesic administration for the A/A genotype compared with the A/G genotype in female subjects.
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significant between the A/A and A/G genotypes ($P=0.040$) and marginally significant between the A/A and G/G genotypes ($P=0.061$) in the *post hoc* analysis (Figure 2B), whereas such differences were not observed in male subjects ($F_{2,68}=0.032$, $P=0.969$). Neither the main effect of gender nor the SNP×gender interaction was significant (data not shown). Significant associations were not observed between the two SNPs and NRS pain scores (G-1250A: $F_{2,94}=1.455$, $P=0.239$; A1032G: $F_{2,96}=0.115$, $P=0.892$), although significant positive correlations were found between NRS pain scores and frequency of rescue analgesic administration ($r=0.281$, $P=0.004$) and between NRS pain scores and total dose of rescue analgesics administered ($r=0.266$, $P=0.006$), indicating that the patients who received analgesics more frequently felt more pain, possibly attributable to insufficient analgesic effects.

To examine in more detail the association between *KCNJ6* SNPs and rescue analgesic requirements, a haplotype-based test was performed. As shown in Table 6A, a significant association was found between the −1250G/1032A haplotype and the increased frequency of rescue analgesic administration in all patient subjects ($R^2=0.120$, adjusted $P=0.015$). Although no significant associations were observed between each of the *KCNJ6* haplotypes and total dose of rescue analgesics administered in all patient subjects and male subjects ($R^2=0.080$, $P=0.328$; $R^2=0.028$, $P=0.765$, respectively), the −1250G/1032A haplotype was significantly associated with total dose of rescue analgesics administered in female subjects ($R^2=0.277$, adjusted $P=0.038$; Table 6B). Associations between each of the G-1250A/A1032G haplotypes and NRS pain scores were not significant (data not shown).

To estimate the impact of the *KCNJ6* A1032G polymorphism on gene expression level, the relative *KCNJ6* mRNA expression level was compared between the genotype subgroups of the SMRI samples in the real-time qPCR. The relative expression level (mean±SEM) was 1.59±0.17, 2.07±0.08, and 1.99±0.06 for the A/A, A/G, and G/G genotypes in the A1032G SNP, respectively (Figure 3), demonstrating that the expression level was 0.76–0.80 fold lower in the A/A genotype than in the A/G and G/G genotype. The difference was significant between the A/A genotype and combined A/G and G/G genotypes ($t_{98}=2.265$, $P=0.026$).

Table 6. Association of *KCNJ6* haplotypes with postoperative analgesia.

A						
Haplotype	**Frequency**	**Beta**	**R^2**	**F**	***P***	***P****
−1250G/1032A	0.1517	0.6313	0.1197	8.8080	0.0036†	0.0145*
−1250A/1032A	0.1736	−0.0216	0.0546	0.0101	0.9203	0.9203
−1250G/1032G	0.2848	−0.0754	0.0563	0.2211	0.6391	0.8521
−1250A/1032G	0.3898	−0.2179	0.0711	2.1290	0.1472	0.2944
B						
Haplotype	**Frequency**	**Beta**	**R^2**	**F**	***P***	***P****
−1250G/1032A	0.1394	0.3139	0.2771	7.3160	0.0094†	0.0377*
−1250A/1032A	0.2032	−0.0160	0.1673	0.0192	0.8903	1.0000
−1250G/1032G	0.2865	−0.0273	0.1687	0.0995	0.7538	1.0000
−1250A/1032G	0.3709	−0.1054	0.1951	1.6770	0.2015	0.8903

Association of the haplotype composed of the G-1250A/A1032G SNPs with (A) the frequency of analgesic administration in all patient subjects or (B) the total dose of analgesics administered during the 24 h postoperative period in female patient subjects. Frequency, haplotype frequency; Beta, regression coefficient; R^2, coefficient of determination; F, *F* statistic; *P*, crude *P* value; P^a, adjusted *P* value for multiple testing. †$P<0.05$, *$P^a<0.05$.
doi:10.1371/journal.pone.0007060.t006

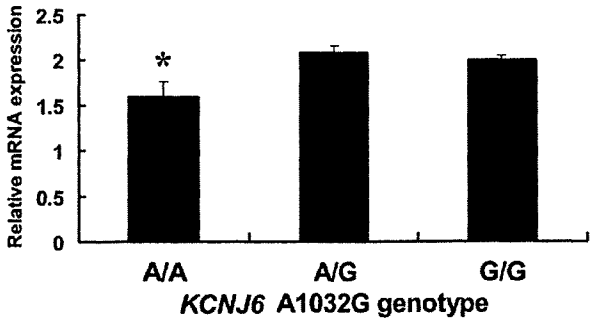

Figure 3. Relative *KCNJ6* mRNA expression level between each genotype subgroup of the SMRI samples. *: significantly lower expression level between the A/A genotype and combined A/G and G/G genotypes in all subjects.
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Discussion

The present study comprehensively examined *KCNJ6* genetic variations in humans and explored the associations between these variations and outcomes in clinical pain management. To our knowledge, the present study is the first to explore SNPs of the *KCNJ6* gene with regard to associations between these SNPs and postoperative analgesic requirements in humans. We found that carriers of the A/A genotype in the A1032G SNP or −1250G/1032A haplotype required rescue analgesics more often and tended to require higher doses of rescue analgesics, especially in female subjects, compared with carriers of other genotypes or haplotypes, respectively, after major open abdominal surgery (Figure 2A and B, Table 6). Although we did not show all of the results of multiple testing for the G-1250A and A1032G SNPs with the Bonferroni correction because this study was explorative, the *P* value for the main effect of *KCNJ6* A1032G on the frequency of rescue analgesic administration was 0.012 after the Bonferroni correction. This suggests that this SNP is likely to affect sensitivity to analgesics, although we must concede that this significance might possibly occur by chance alone. Patients who experienced more severe pain, evaluated by NRS pain scores, required higher-dose and more frequent rescue analgesics, although significant associations were not observed between the A1032G SNP or −1250G/1032A haplotype and NRS pain scores. Moreover, *KCNJ6* gene expression levels in the 1032A/A subjects was significantly decreased compared with 1032A/G and 1032G/G subjects in the real-time qPCR analysis using human brain tissues, suggesting that the 1032A/A subjects required more analgesics because of lower *KCNJ6* gene expression levels and consequently insufficient analgesic effects. Altogether, these results suggest that subjects carrying the A/A genotype in the A1032G SNP or −1250G/1032A haplotype, especially in females, had lower sensitivity to analgesics and, therefore, required more rescue analgesics than subjects carrying other genotypes or haplotypes to achieve a similar degree of pain relief.

For our ANCOVA analyses, the desirable sample size was calculated as 158 for the effect size 0.25 to achieve 80% power. This might suggest that a sample size of 129 subjects in our study was somewhat insufficient to reliably detect moderate differences between the SNP genotypes, and a far greater sample may be required to detect smaller differences. Considering this caveat and relatively small effect size observed in the haplotype analysis (e.g., R^2 for haplotype effect on the frequency of analgesic administration was 0.120), future studies with larger sample sizes may reveal additional associations between polymorphisms and opioid sensitivity.

In the initial polymorphism screening for *KCNJ6*, a total of nine SNPs were identified in the whole exon, 5′-flanking, and exon-intron boundary regions (Figure 1). Polymorphisms that might cause significant functional changes, such as nonsynonymous or insertion/deletion polymorphisms, were not found in the polymorphism screening of the human *KCNJ6* gene in the present study, possibly attributable to the importance and high conservation of mammalian GIRK channels. A possible explanation derives from studies in *weaver* mice, in which only a single missense mutation in the pore region of the mouse *Kcnj6* gene causes various aberrant changes in cerebellar granule cells [30], membrane permeability [31], loss of K^+ selectivity [32,33], significantly lower analgesia compared with wildtype mice [3], and lack of activating effects of ethanol [34]. The *Kcnj6* gene orthologs might be under purifying selection over many generations of the species, including human and mouse, because of the profound functional constraints attributable to the importance of these orthologs in these organisms.

The A/A genotype in the A1032G SNP was significantly associated with increased postoperative analgesic requirements in our study. The G allele appears to be dominant in mediating the transmission of intensified opioid signaling compared with the A allele. However, this particular SNP is synonymous and causes no amino acid change; therefore, the protein structure encoded by this gene may not be altered by this SNP. Nevertheless, local structural difference in the 1023–1059 position was observed between the sequences, including 1032A and 1032G, in our prediction of the *KCNJ6* mRNA secondary structure (Figure S1). Whereas the 1032A mRNA formed an interior loop, a hairpin loop, and a 6 bp helix, the 1032G mRNA formed a bulge loop as well as an interior loop, a hairpin loop, and a 7 bp helix in the local structure. Although the role of this difference in gene function remains to be determined, the SNP may actually influence mRNA expression level. Indeed, recent studies measuring allelic expression imbalances [35] have demonstrated that even a synonymous SNP could affect mRNA and protein levels [36], possibly by altering mRNA stability and protein synthesis [37]. Similar mRNA and protein levels, but altered conformations, were found for synonymous polymorphisms [38].

To further infer the precise mechanism underlying the increased requirements for rescue analgesics in the A/A subjects in the A1032G SNP, we compared the relative *KCNJ6* mRNA expression level between the genotype subgroups of the SMRI samples in the real-time qPCR. A significant difference in expression level was observed between the A/A genotype and the combined A/G and G/G genotypes, consistent with the results of the association study, in which only the subjects with the A/A genotype in this SNP demonstrated significantly higher requirements for rescue analgesics than the other genotypes. The 1032A/A subjects required more analgesics, probably because of lower *KCNJ6* gene expression levels and consequently insufficient analgesic effects.

We do not have any evidence to explain how the −1250G/1032A haplotype contributes to increased requirements for rescue analgesics compared with other haplotypes. One might infer that the G-1250A SNP in the putative regulatory region could be related to some moderate functional alteration, and the −1250G and 1032A alleles could be risk factors for decreased sensitivity to analgesics. Both alleles might combine synergistically to cause profound decreases in sensitivity to analgesics. Future functional studies focusing on both the G-1250A and A1032G SNPs are required to investigate this hypothesis.

In conclusion, the A/A genotype in the *KCNJ6* A1032G SNP and −1250G/1032A haplotype were significantly associated with increased analgesic requirements after major open abdominal surgery. Furthermore, *KCNJ6* gene expression levels in the 1032A/A subjects was significantly decreased compared with the 1032A/G and 1032G/G subjects. Although the association might be restricted to the Japanese population, and the mechanism by which individual sensitivity to postoperative analgesics is altered by the G-1250A and A1032G SNPs remains to be fully elucidated, the outcome indicates that the A1032G SNP and G-1250A/A1032G haplotype could serve as markers that predict increased analgesic requirements. Our findings will provide valuable information to better modulate individual analgesic dosages required to achieve satisfactory pain control and open new avenues for personalized pain treatment.

Supporting Information

Table S1 The clinical data of the subjects stratified by genotype
Found at: doi:10.1371/journal.pone.0007060.s001 (0.12 MB DOC)

Figure S1 Predicted secondary structure for the KCNJ6 mRNA based on the nucleotide sequences of the GenBank database (accession number: NM_002240.2). The sequences for *KCNJ6* mRNA position 1012–1072 are presented for the 1032A (A) and 1032G (B) mRNA. The numbers next to the sequences indicate relative positions from position 982.
Found at: doi:10.1371/journal.pone.0007060.s002 (0.99 MB TIF)

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Author Contributions

Conceived and designed the experiments: DN SK NS IS MH KI. Performed the experiments: DN JH. Analyzed the data: DN YO. Contributed reagents/materials/analysis tools: DN YO WH JH. Wrote the paper: DN MH KI. Collection of DNA: YO KI. Collection of clinical data and DNA: MN RK YS MT MH.

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Reduced CYP2D6 activity is a negative risk factor for methamphetamine dependence

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Abstract

Because methamphetamine (METH) is metabolized by CYP2D6 at the first step of hydroxylation and demethylation, it is possible that functional variants of CYP2D6 alter susceptibility to methamphetamine-induced dependence. We genotyped *CYP2D6*1*, **4*, **5*, **10*, and **14* for 202 patients with METH dependence and 337 controls in a Japanese population and found a significant association of the *CYP2D6* gene with METH dependence ($p = 0.0299$). The patients had fewer **10* and **14* alleles, which are hypofunction alleles, than the controls. *CYP2D6* genotypes were divided into three phenotypes: extensive metabolizers, intermediate metabolizers, and poor metabolizers. There was no poor metabolizer among our Japanese subjects, and intermediate metabolizers of CYP2D6 were significantly fewer in methamphetamine-dependent subjects than in controls ($p = 0.0212$), with an odds ratio of 0.62 (95% confidence interval: 0.51–0.76). The present study demonstrated that reduced CYP2D6 activity was a negative risk factor for methamphetamine dependence.

Keywords: CYP2D6; Methamphetamine; Amphetamine; Dependence; Intermediate metabolizer; Case-control association study

Methamphetamine (METH) is an addictive stimulant drug used all over the world, and METH has long been the most popular substance of abuse in Japan [33,40]. Genetic factors may contribute substantially to the development of substance dependence. Family and twin studies have shown that predisposition to drug-taking behaviors and psychological dependence on substances including amphetamines has a strong hereditary component [24,38]. Several genetic risk factors for METH dependence and psychosis, e.g., the dopamine transporter gene [39], μ-opioid receptor gene [18], prodynorphin gene [31], $GABA_A$ receptor γ2 subunit gene [30], and AKT-1 gene [19], have been identified by our

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group, but more, currently unknown, genetic factors are likely.

Several previous studies showed that genetic variability in the enzyme that metabolizes a certain drug was associated with dependence on that drug [17], e.g., alcohol dehydrogenase and aldehyde dehydrogenase genes for alcoholism [5,16,27], *CYP2A6* for nicotine dependence [2,28], and *CYP2D6* for codeine dependence [14]. METH is metabolized by 4-hydroxylation or *N*-demethylation as the first step and then converted to 4-hydroxy METH (4OH-METH) or amphetamine (AMPH); the latter is subsequently metabolized to 4-hydroxy AMPH (4OH-AMPH) by 4-hydroxylation. These metabolites of METH, 4-OH METH, AMPH, 4-OH AMPH, and norephedrine [4,26], are all active and have psychostimulating actions [12]. An experimental study showed that chronic but not acute treatment with AMPH produced accumulation of hydroxylated metabolites in the striatum [10]. Studies of patients with AMPH psychosis showed that the intensity of the psychosis was positively correlated with the amount of basic polar metabolites of AMPH, including 4-OH AMPH and norephedrine, excreted in the urine but not with the AMPH plasma level [1]. The two initial metabolic pathways of METH in humans, 4-hydroxylation and *N*-demethylation, are catalyzed by CYP2D6 [26]. Therefore, it is possible that an alteration of CYP2D6 activity changes the metabolism of METH and synthesis of hydroxylated metabolites and may affect susceptibility to METH dependence.

CYP2D6 is one of the best-known of the polymorphic drug-metabolizing enzymes. CYP2D6 is involved in the metabolism of 20–25% of clinically used drugs and exhibits a clinically relevant gene polymorphism that modifies the pharmacokinetics of nearly 50% of the drugs [20]. Approximately 5–6% of Caucasians are CYP2D6 deficient due to inactivating mutations of the CYP2D6 gene (*CYP2D6*), *CYP2D6*3* (5%), **4* (75%), and **5* (15%) and are termed poor metabolizers (PM) [3,6,7]. In comparison, the ratio of PM among Japanese is less than 1%, comprising mostly *CYP2D6*5* with a few *CYP2D6*4* and **14* genotypes [25,29,36]. However, the allele frequency of *CYP2D6*10* in Japanese (38–51%) [25,29,36] is much higher than that in Caucasians (1–5%), black Africans (6%), and Ethiopians and Saudi Arabians (3–9%) [6]. Individuals with *CYP2D6*10/*10*, which causes decreased CYP2D6 activity, are termed intermediate metabolizers (IM) [22] and account for approximately 15% of Japanese. Therefore, we examined *CYP2D6*1*, **4*, **5*, **10*, **14* in patients with METH dependence in a Japanese population to test our hypothesis that genetic variants of the *CYP2D6* gene could be a genetic factor in susceptibility to METH dependence.

Genotyping was performed on 202 patients with METH dependence (167 males and 35 females; mean age, 36.9 ± 11.8 years; ICD-10-DCR criteria; F15.2), and 337 control subjects (271 males and 66 females; mean age, 37.2 ± 13.1 years) who were age-, gender-, and geographically matched to patients. All subjects in the present study were Japanese, born and living in restricted regions of Japan. The patients were inpatients or outpatients at medical institutions participating in the Japanese Genetics Initiative for Drug Abuse (JGIDA) [39]. The clinical diagnosis was made by two trained psychiatrists according to ICD-10-DCR on the basis of unstructured medical interviews and records. Healthy volunteers were recruited mainly from the medical staff and had no present or past history of major psychiatric disorders, e.g., schizophrenia, bipolar disorder, and drug dependence. This study was approved by the ethics committee of each JGIDA institution. After a complete description of the study to the subjects, written informed consent was obtained.

Genomic DNA was extracted from peripheral leukocytes using standard procedures. The *CYP2D6*5* genotype was identified using long-PCR analysis according to the method of Johansson et al. [21]. Those of *CYP2D6*4* (G1840A), *CYP2D6*10* (C188T), and *CYP2D6*14* (G169A) were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to the methods of Heim and Meyer [15] and Wang et al. [41,42]. We did not examine other CYP2D6 alleles, e.g., **3*, and **2xN* alleles, in the present study because it has been demonstrated that the frequency of those alleles is below 0.7% in the Japanese population [25,29,36]. The frequency of *2 in the Japanese population is high, but we did not identify it because it has no effect on CYP2D6 activity. We carried out genotyping in a blinded fashion with control and patient samples randomly mixed. Allele frequencies were calculated using the allele-counting method. Genotype deviation from Hardy–Weinberg equilibrium was assessed by χ^2 goodness-of-fit test. The statistical significance of case-control associations was evaluated by χ^2 test, Fisher's extract test, and the CLUMP program (ver 2.3) [34]. A T4 value was adopted as the *p*-value by CLUMP analysis. Statistical significance was accepted at $p < 0.05$.

The genotype distribution of patients with METH dependence and controls is shown in Table 1. We identified six *CYP2D6* genotypes in the Japanese subjects in the present study, *CYP2D6*1/*1*, **1/*5*, **1/*10*, **5/*10*, **10/*10*, and **10/*14*, whereas no *CYP2D6*4* allele or no *CYP2D6*1/*14*, **5/*5*, **5/*14*, **14/*14* genotype was found in either patients with METH dependence or controls. Genotype distributions of the patients and controls did not deviate from Hardy-Weinberg equi-

Table 1
CYP2D6 genotypes of patients with methamphetamine dependence and controls

Subjects	*N*	CYP2D6 genotypes						*p*
		*1/*1	*1/*5	*1/*10	*5/*10	*10/*10	*10/*14	
Patients	202	74(36.6)	6(3.0)	77(38.1)	4(2.0)	41(20.3)	0(0.0)	0.121
Controls	337	98(28.9)	16(4.7)	121(36.0)	12(3.6)	88(26.2)	2(0.6)	

p-Value was generated by CLUMP ver 2.3 using 10,000 simulations.

Table 2
CYP2D6 allele frequencies of patients with methamphetamine dependence and controls

Subjects	N	CYP2D6 alleles				p
		*1	*5	*10	*14	
Patients	404	231(57.2)	10(2.5)	163(40.3)	0(0.0)	0.0299
Controls	672	329(48.9)	28(4.2)	313(46.6)	2(0.3)	

p-Value was generated by CLUMP ver 2.3 using 10,000 simulations.

librium (patients: $G=2.75$, d.f. $=4$. $p=0.60$; controls: $G=7.92$, d.f. $=6$, $p=0.24$). The frequency of *CYP2D6*1/*1*, a wild-type genotype, was 36.9% in patients with METH dependence, and was higher than that in controls, 28.9%. In contrast, *CYP2D6*10/*10* and **5/*10* genotypes were observed in 20.3% and 2.0% of patients, respectively, which were lower than those in controls, 26.2% and 3.6%, respectively. However, the difference in the *CYP2D6* genotype distribution between controls and patients with METH dependence was not statistically significant ($p=0.121$).

The allele distributions of *CYP2D6* in patients and controls are shown in Table 2. The frequency of the wild-type allele, **1*, was 48.9% in controls, which was less than that of patients, 57.2%. The frequencies of **5* and **10* alleles in controls were 4.2% and 46.6%, respectively, which were higher than those in patients, 2.5% and 40.3%, respectively. Only two subjects had the **14* allele, and they were controls (0.3%). The distribution of allele frequencies of the *CYP2D6* gene differed significantly between patients with METH dependence and controls ($p=0.0299$).

Then we divided *CYP2D6* genotypes into three functional phenotypes, EM, IM, and PM, according to as the level of enzyme activity. The definition of the three phenotypes follows the modified method described by Someya et al. [35]. They defined the heterozygote of a wild and inactivating allele, e.g., *CYP2D6*1/*5* as IM because they did not find a significant difference in *CYP2D6* activity between *CYP2D6*10/*10* and *CYP2D6*1/*5*. Briefly, EM comprise *CYP2D6*1/*1* and **1/*10*, IM comprise of *CYP2D6*10/*10*, **1/*5*, **5/*10*, *10/*14, and PM comprise *CYP2D6*5/*5*, **5/*14*, and **14/*14*. No subject with the PM phenotype was observed in the subjects of the present study. The patients with METH dependence had significantly fewer IM and more EM ($p=0.0212$, Table 3). The odds ratio of IM for METH dependence was 0.62 (95% confidence interval: 0.51–0.76).

We found that the distributions of *CYP2D6* allele and phenotype frequency were significantly associated with susceptibility to METH dependence. Hypofunction alleles of *CYP2D6*, *10 and *14, and IM phenotypes comprising *CYP2D6*10/*10*, **1/*5*, **5/*10*, *10/*14 were fewer in the patients than in the controls. Intermediate metabolism of CYP2D6 was identified as a negative risk factor for development of METH dependence, and reduced the risk of METH dependence to about six out of ten. CYP2D6 catalyzes the first step in the metabolism of METH, 4-hydroxylation of the aromatic ring and *N*-demethylation [26], which produces several kinds of metabolites, e.g., 4-OH METH, 4-OH AMPH, and norephedrine. The ratios of these metabolites excreted in urine were different among human, rat, and guinea pig species [8]. In humans, the major metabolites of METH in urine are the unchanged drug and 4-OH METH, and the minor ones are hippuric acid, norephedrine, 4-OH AMPH, and 4-OH norephedrine. Hydroxylated metabolites of METH were shown to be active neurochemically and behaviorally like the parent compound. OH-METH and OH-AMPH inhibited uptake of noradrenaline in chopped cerebral cortex [43], uptake of dopamine into striatal homogenates [9], and induced release of noradrenaline and dopamine from striatal homogenates [13]. The potency of 4-OH AMPH in inhibition and release of dopamine from the striatum was almost equivalent to that of AMPH. These hydroxylated metabolites themselves had propensity to induce hyperlocomotion and stereotyped behaviors [37], indicating psychostimulating and psychotomimetic activities, and also to enhance abnormal behaviors induced by the parent drugs [11]. These metabolites were taken up by dopaminergic terminals [23] and accumulated greatly in synaptic terminals of the striatum and hypothalamus after chronic administration of METH or AMPH because the half life of hydroxylated AMPH is 1.5 days in rat brain, whereas the half-life of AMPH is 45 min. Therefore, hydroxylated metabolites of METH could preferentially contribute to the development of dependence or the psychotic disorder induced by METH in the chronic phase of METH abuse. This speculation seems to be consistent with a clinical observation by Anggard et al. [1], who noted that the intensity of the psychosis of patients with AMPH dependence is more closely related to the urinary levels of hydroxylated metabolites than to the plasma levels of AMPH.

Ramamoorthy et al. [32] showed that the intrinsic clearance rate of (+)- and (−)-METH in 4-hydroxylation by *10 of CYP2D6 was 30- and 67-fold slower than the wild-type *1 *in vitro*. Thus, it can be expected that, *in vivo*, an individual who is a CYP2D6 IM, for example, the *10/*10 genotype, would display much lower levels of hydroxylated metabolites of METH after abuse of METH, resulting in less accumulation of the hydroxylated metabolites of METH in the brain and more rapid excretion of unchanged METH in the urine. This pharmacokinetic change of METH by CYP2D6 IM could result in relative insuscepti-

Table 3
Phenotypes of patients with methamphetamine dependence and controls

Subjects	N	CYP2D6 phenotypes		p
		EM	IM	
Patients	202	151(74.8)	51(25.2)	0.0212
Controls	336	218(64.9)	118(35.1)	

No poor metabolizer was found. EM, extensive metabolizer; IM, intermediate metabolizer.

bility to development of dependence on the drug. However, our findings need to be confirmed in larger samples because our sample size may not be large enough to exclude possibilities of population stratification and type 1 and 2 errors. Confirmation in different populations, e.g., Caucasian should be very useful because there are poor metabolizers of CYP2D6, who must show the least dependence to methamphetamine. In addition, examination of the *CYP2D6* gene in patients who abuse methylendioxymethamphetamine, another widely abused drug, should be informative because it is also a substrate for CYP2D6.

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The Dysbindin Gene (*DTNBP1*) Is Associated with Methamphetamine Psychosis

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Background: The dysbindin (*DTNBP1* [dystrobrevin-binding protein 1]) gene has repeatedly been shown to be associated with schizophrenia across diverse populations. One study also showed that risk haplotypes were shared with a bipolar disorder subgroup with psychotic episodes, but not with all cases. *DTNBP1* may confer susceptibility to psychotic symptoms in various psychiatric disorders besides schizophrenia.

Methods: Methamphetamine psychosis, the psychotic symptoms of which are close to those observed in schizophrenia, was investigated through a case (n = 197)–control (n = 243) association analyses of *DTNBP1*.

Results: *DTNBP1* showed significant associations with methamphetamine psychosis at polymorphisms of P1635 (rs3213207, $p = .00003$) and SNPA (rs2619538, $p = .049$) and the three-locus haplotype of P1655 (rs2619539)-P1635-SNPA (permutation $p = .0005$). The C-A-A haplotype, which was identical to the protective haplotype previously reported for schizophrenia and psychotic bipolar disorders, was a protective factor ($p = .0013$, odds ratio [OR] = .62, 95% confidence interval [CI] .51–.77) for methamphetamine psychosis. The C-G-T haplotype was a risk for methamphetamine psychosis ($p = .0012$, OR = 14.9, 95% CI 3.5–64.2).

Conclusions: Our genetic evidence suggests that *DTNBP1* is involved in psychotic liability not only for schizophrenia but also for other psychotic disorders, including substance-induced psychosis.

Key Words: Akt1, *DTNBP1*, dysbindin, methamphetamine psychosis, substance dependence

A genetic variation of the dystrobrevin-binding protein 1 (*DTNBP1*) gene has recently been shown to be associated with schizophrenia in several independent studies. Straub *et al.* (1) revealed original evidence for a positive genetic association between schizophrenia and variants in a gene on 6p22.3, dysbindin (*DTNBP1*), which is located within one of several promising loci revealed by a genomewide linkage scan. Many replication studies showed consistent findings in different populations, for example, German (2), Irish (3), Chinese (4), Swedish/German/Polish (5), UK/Irish (5), Bulgarian (6), American (7), Scottish/Chinese (8), and Japanese (9), although the significantly associated alleles and haplotypes were not always consistent among populations. Two postmortem studies also revealed that dysbindin protein or its mRNA level was reduced in the dorsolateral prefrontal cortex and in presynaptic glutamatergic terminals of the hippocampus of schizophrenia patients (10,11). These findings suggest that the dysbindin is involved in the pathogenesis of schizophrenia.

Recently, Raybould *et al.* (12) examined three loci of the *DTNBP1* gene in a large sample of patients with bipolar disorder, another endogenous psychosis, in UK Caucasians, and found that the *DTNBP1* gene was not associated with all cases of bipolar disorder but was associated with a subgroup of bipolar disorder characterized by the complication of psychotic features during episodes. The risk and protective haplotype were identical to those found in their previous schizophrenia study (13). Therefore, they speculated that the *DTNBP1* genetic variation influences susceptibility to schizophrenia and bipolar psychosis across the Kraepelinian dichotomy.

Abuse of large amounts of methamphetamine for long periods easily produces psychotic symptoms, such as delusions of reference, persecution, and poisoning, as well as auditory and visual hallucinations (14–16). Further consumption of methamphetamine may result in severe psychosis, liability to relapse with reconsumption of methamphetamine or psychological stress, and a gradually worsening prognosis. Clinical similarities between methamphetamine psychosis and schizophrenia in a cross-section of clinical features have been noted; these include auditory hallucination and delusion, the longitudinal process of progressive exacerbation with acute relapses, relatively good response to neuroleptics, and enduring vulnerability to relapse to stressors, especially in the paranoid type of schizophrenia. Indeed, methamphetamine psychosis has long been considered a pharmacologic model of schizophrenia (17,18), and shared molecular mechanisms could be involved in these psychotic disorders. Based on this rationale, it is possible that the *DTNBP1*

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gene may influence susceptibility to substance-induced psychoses in the same manner that influence susceptibility to schizophrenia and bipolar psychosis disorders. To examine this hypothesis, we investigated the association between *DTNBP1* and methamphetamine psychosis in a case–control analyses.

Methods and Materials

Subjects

The subjects consisted of 197 patients (162 male, 35 female; mean age ± SD, 38.1 ± 12.6) with methamphetamine psychosis (MAP) and 243 age-, gender-, and geographic-origin-matched healthy control subjects (193 male, 50 female; mean age ± SD, 37.2 ± 12.0) who had no individual or family history of drug dependence or major psychotic disorders such as schizophrenia and bipolar disorders. All the subjects were unrelated Japanese who were born and lived in relatively restricted areas of Japan. All patients were outpatients or inpatients in psychiatric hospitals of the Japanese Genetics Initiative for Drug Abuse (JGIDA). Consensus diagnoses of methamphetamine psychosis were made by two trained psychiatrists according to the ICD-10 criteria on the basis of unstructured interviews and medical records. All healthy control subjects were also psychiatrically screened based on unstructured interviews. The study protocol and purpose were explained to all subjects participating in the study, and written informed consent was obtained from all subjects. This study was approved by the Ethics Committee of each participating institute of JGIDA.

The patients with methamphetamine psychosis were divided into subgroups according to three clinical phenotypes that may indirectly indicate the severity of and liability to psychosis:

1. *Latency to onset of psychotic state after initial methamphetamine consumption:* Median latency was 3 years; 99 (54.4%) of patients developed psychotic symptoms within 3 years of the first methamphetamine abuse, and 83 (45.6%) patients did after 3 or more years.
2. *Duration of the psychotic state after therapy:* Methamphetamine-induced psychosis (transient type) will usually subside within 10 days to 1 month following discontinuance of consumption and beginning of pharmacologic therapy with antipsychotics such as haloperidol or risperidone. Some patients show sustained (longer than 1 month) psychotic symptoms (prolonged type), however, regardless of detoxification from methamphetamine and adequate antipsychotic therapy (16,19). In our study, 107 (56.6%) patients showed the transient type of psychosis, and 82 (43.4%) patients showed the prolonged type of psychosis.
3. *Complication of spontaneous psychosis:* Once methamphetamine psychosis has developed, some remitted patients may experience spontaneous relapse due to nonspecific stresses, such as severe fatigue or life problems, without consumption of methamphetamine. The observation period for the presence or absence of spontaneous relapse was at least 1 year and averaged 12.3 ± 11.1 years. Eighty-three patients (42.8%) experienced spontaneous relapse, and 111 (57.2%) did not.

As to multisubstance abuse status, 37.2% patients concurrently abused other illicit drugs in addition to methamphetamine. Cannabinoids were most frequently abused (34.0%), followed by LSD (14.1%), cocaine (13.1%), opioids (12%), and hypnotics (9.9%). More than 60% of patients abused only methamphetamine, but about half had a past history of organic solvent abuse in their teenage years. All clinical data were obtained from interviews with patients and their families. Urine examination was not applied.

DNA Analysis

We genotyped the three single nucleotide polymorphisms (SNPs), P1655 (rs2619539), P1635 (rs3213207), and SNPA (rs2619538) of the *DTNBP1* gene that were examined previously by O'Donovan's group and were shown to have a significant association with both schizophrenia and psychotic bipolar disorders (12,13). They showed in the schizophrenia study that these three locus haplotypes showed the most significant results among 26 significantly associated haplotypes constructed by combinations of 9 SNPs of *DTNBP*. P1655 and P1635 were two of the markers that had provided the most significant results in the study by Straub *et al.* (1), and SNPA was reported to be significantly associated with schizophrenia in a Japanese population (9).

The genomic DNA was extracted from peripheral leukocytes using the phenolchloroform method. Genotyping was performed by the polymerase chain reaction (PCR)–restriction fragment length polymorphism method. Each polymorphic site was amplified by PCR in a volume of 15 mL containing 3% dimethyl sulfoxide and .75 units of Taq DNA polymerase (Promega, Japan) using a unique primer set (P1655 [mismatch]; 5′-ATCAGGCAAAATGATGTACTGC-3′, 5′-GCCTTTTAAATAATCCTATTAGCTATGAGAGT-3′, P1635; 5′-CTTTATGCAATAAGTATTCCTG-3′, 5′-GTATACCCTGTTTTAAGCAGAC-3′, SNPA; 5′-CCTGTTTCTCAACTTAGTACAC-3′, 5′-CCTTTATCTTATTTAACTCCTG-3′). PCR reaction was performed under the following conditions: 95°C for 5 min, then 35 denaturing cycles of 30 sec each at 95°C, 1 min of annealing at the appropriate temperature, and 30 sec of extension, and final elongation at 72°C for 10 min. The PCR products were digested with the corresponding restriction enzyme for each polymorphism, *Hin*fI for P1655, *Bse*NI for P1635, and *Cai*I for SNPA, and then electrophoresed on 3.0% agarose gels and stained with ethidium bromide. All genotyping was performed in a blinded fashion, with the control and case samples mixed randomly. Part of the genotyping of P1655, P1635, and SNPA was confirmed by direct sequencing and a TaqMan SNP genotyping assay (C_16036968_10), respectively.

Statistical Analysis

Statistical analysis of association was performed using SNPAlyze software (Dynacom, Mobara City, Chiba, Japan). Deviation from Hardy–Weinberg equilibrium and the case–control study were tested using the χ^2 test. Linkage disequilibrium (LD) was tested using the χ^2 test, and D' and r^2 values were made the index in the authorization of LD. Case–control haplotype analysis was performed by the permutation method, and permutation p values were calculated based on 100,000 replications.

GenBank/EMBL Accession Numbers

Genome; NC_000006.10, NT_007592.14, MIM; 607145.

Results

The genotype distribution and allele frequencies for each polymorphism of patients with methamphetamine psychosis and control subjects are shown in Table 1. The genotype distributions of patients and control subjects did not deviate from the Hardy–Weinberg equilibrium at any of the three SNPs. We found a significant difference between patients and control subjects in the frequencies of the genotype or allele at P1635 and SNPA of

Table 1. Genotype and Allele Distribution of Three Single Nucleotide Polymorphisms of the *DTNBP1* Gene in Control Subjects and Patients with Methamphetamine (MAP) Psychosis

			Genotype				Allele		
		N	C/C	C/G	G/G	*p*	C	G	*p*
P1655	rs2619539								
Control		240	118 (49.2)	107 (44.6)	15 (6.2)		343 (71.5)	137 (28.5)	
MAP Psychosis		190	78 (41.0)	94 (49.5)	18 (9.5)	.17	250 (65.8)	130 (34.2)	.076
P1635	rs3213207		A/A	A/G	G/G		A	G	
Control		243	239 (98.4)	4 (1.6)	0 (.0)		482 (99.2)	4 (.8)	
MAP Psychosis		197	175 (88.8)	22 (11.2)	0 (.0)	.000025	372 (94.4)	22 (5.6)	.000030
SNPA	rs2619538		A/A	A/T	T/T		A	T	
Control		232	225 (97.0)	7 (3.0)	0 (.0)		457 (98.5)	7 (1.5)	
MAP Psychosis		197	182 (92.4)	15 (7.6)	0 (.0)	.046	379 (96.2)	15 (3.8)	.049

Numbers in parentheses indicate percentages.

the *DTNBP1* gene (P1635: genotype, $\chi^2 = 17.74$, $df = 1$, $p = .000025$; allele $\chi^2 = 17.20$, $df = 1$, $p = .000030$; SNPA: genotype $\chi^2 = 4.63$, $df = 1$, $p = .046$; allele $\chi^2 = 4.51$, $df = 1$, $p = .049$). The minor alleles of P1635 and SNPA, G and T alleles, respectively, were in excess in methamphetamine psychosis when compared with control subjects. To avoid a type I error due to multiple comparison, the Bonferroni correction was applied to the results. The G allele of P1635 was still significantly more frequent in the methamphetamine psychosis patients than in control subjects, but SNPA was not significantly different after correction. P1655 did not show significant differences in distribution of allele and genotype between groups.

Comparison between subgroups of the patients according to clinical phenotypes showed a significant difference in allelic and genotypic distribution of P1635 between the two subgroups divided by duration of psychotic state after therapy, transient and prolonged types (Table 2). The frequency of the minor allele G of P1635 was only 0.8% in control subjects, whereas it was 3.3% in patients with transient psychosis and 8.5% in patients with prolonged psychosis ($p = .027$, compared with transient psychosis). After Bonferroni correction, this was not significant. The other clinical phenotypes, psychosis latency and spontaneous relapse, were not associated with any SNP examined.

Estimation of the pairwise LD between the three SNPs of the *DTNBP1* gene using the D′ and r^2 values as an index showed that P1655, P1635, and SNPA have strong LD (D′ ranging between 0.65 and 1.0) with each other (Table 3). We then analyzed the three-marker haplotypes (Table 4) and found significant differences in patients and control subjects at P1655-P1635-SNPA ($\chi^2 = 27.8$, $df = 6$, global permutation $p = .0005$).

Table 2. Association of the *DTNBP1* Gene with Subgroups of Patients Divided by Clinical Phenotypes

		Genotype				Allele		
	N	C/C	C/G	G/G	*p*	C	G	*p*
P1655								
Latency to Onset of Psychosis, <3Y	96	35 (36.5)	50 (52.1)	11 (11.4)		120 (62.5)	72 (37.5)	
Latency to Onset of Psychosis, ≥3Y	79	36 (45.6)	37 (46.8)	6 (7.6)	.41	109 (69.0)	49 (31.0)	.20
Transient MAP Psychosis	103	44 (42.7)	50 (48.6)	9 (8.7)		138 (67.0)	68 (33.0)	
Prolonged MAP Psychosis	79	29 (36.7)	42 (53.2)	8 (10.1)	.71	100 (63.3)	58 (36.7)	.46
Spontaneous Relapse; No	108	41 (38.0)	54 (50.0)	13 (12.0)		136 (63.0)	80 (37.0)	
Spontaneous Relapse; Yes	77	34 (44.1)	38 (49.4)	5 (6.5)	.40	106 (68.8)	48 (31.2)	.24
P1635		A/A	A/G	G/G		A	G	
Latency to Onset of Psychosis, <3Y	99	89 (89.9)	10 (10.1)	0 (.0)		188 (94.9)	10 (5.1)	
Latency to Onset of Psychosis, ≥3Y	81	71 (87.7)	10 (12.3)	0 (.0)	.63	152 (93.8)	10 (6.2)	.64
Transient MAP Psychosis	107	100 (93.5)	7 (6.5)	0 (.0)		207 (96.7)	7 (3.3)	
Prolonged MAP Psychosis	82	68 (82.9)	14 (17.1)	0 (.0)	.022	150 (91.5)	14 (8.5)	.027
Spontaneous Relapse; No	111	98 (88.3)	13 (11.7)	0 (.0)		209 (94.1)	13 (5.9)	
Spontaneous Relapse; Yes	82	73 (89.0)	9 (11.0)	0 (.0)	.87	155 (94.5)	9 (5.5)	.88
SNPA		A/A	A/T	T/T		A	T	
Latency to Onset of Psychosis, <3Y	99	91 (91.9)	8 (8.1)	0 (.0)		190 (96.0)	8 (4.0)	
Latency to Onset of Psychosis, ≥3Y	82	75 (91.5)	7 (8.5)	0 (.0)	.91	157 (95.7)	7 (4.3)	.910
Transient MAP Psychosis	108	102 (94.4)	6 (5.6)	0 (.0)		210 (97.2)	6 (2.8)	
Prolonged MAP Psychosis	82	73 (89.0)	9 (11.0)	0 (.0)	.170	155 (94.5)	9 (5.5)	.18
Spontaneous Relapse; No	110	104 (94.5)	6 (5.5)	0 (.0)		214 (97.3)	6 (2.7)	
Spontaneous Relapse; Yes	82	74 (90.2)	8 (9.8)	0 (.0)	.26	156 (95.1)	8 (4.9)	.27

Number in parentheses indicate percentages.

Table 3. Pairwise Linkage Disequilibrium Between Single Nucleotide Polymorphisms of the *DTNBP1* Gene

	P1655	P1635	SNPA
P1655		.9643	1.0000
P1635	.0128		.6519
SNPA	.0114	.3522	

Right upper and left lower diagonal showed D′ and r^2 values, respectively.

The estimated haplotype frequency of C-A-A of P1655-P1635-SNPA was significantly lower in patients with methamphetamine psychosis than in control subjects (p = .0013). Conversely, the C-G-T haplotype was significantly higher in patients than in control subjects (p = .0012). Permutation p values of these haplotypes remained significant even after Bonferroni correction. Odds ratios were .62 (95% confidence interval [CI] .51–.77) and 14.9 (95% CI 3.5–64.2), respectively, indicating that the C-A-A haplotype protected against development of methamphetamine psychosis. On the other hand, the C-G-T haplotype was a significant risk factor for development of methamphetamine psychosis.

Discussion

We found a significant association between the *DTNBP1* gene and methamphetamine psychosis in individual marker and haplotype-based case–control analyses. The G allele of P1635 was shown to be a risk factors for methamphetamine psychosis. Numakawa *et al.* (9) reported that the G allele of P1635 was a risk factor for schizophrenia in Japanese; other reports have shown that it was also overtransmitted in Irish (1) but not in German schizophrenia (2). We also found that the G allele of P1635 was in excess in a subgroup showing a prolonged psychotic state, indicating that the allele was a risk for a worse prognosis of psychosis or refractoriness to antipsychotic therapy in patients with methamphetamine psychosis. The T allele of SNPA also showed a nominally significant risk for methamphetamine psychosis. Although it did not remain significant after multiple comparison correction, one study of schizophrenia showed that it was a significant risk (9), whereas another did not (13). The most striking findings in our study were that analyses of a haploype constructed by P1655-P1635-SNPA of the *DTNBP1* gene revealed a strong association with methamphetamine psychosis (p = .0005). The C-A-A haplotype was significantly more common in control subjects than patients with methamphetamine psychosis (p = .0013), implying a substantial protective factor given the odds ratio of .62. The protective haplotype found in our study of methamphetamine psychosis was identical with that previously reported in studies of schizophrenia and psychotic bipolar disorders (12,13). This evidence may indicate that the C-A-A haplotype of *DTNBP1* reduces the liability of individuals who suffer from endogenous psychoses or substance abuse to complications of psychotic symptoms such as delusions and hallucinations. Another possibility should be also considered, however; the C-A-A haplotype may be associated with methamphetamine dependence but not methamphetamine psychosis because all the patients examined in our study suffered not only from methamphetamine psychosis but also dependence. Accordingly, these hypotheses should be examined in other psychotic disorders—for example, psychotic depression, organic psychoses, and cocaine paranoia—as well as in other dependence disorders. In contrast, the C-G-T haplotype was a significant risk for development of methamphetamine psychosis. The frequency of the C-G-T haplotype was small at about 3% in methamphetamine psychosis but almost absent in control subjects, resulting in a strong risk and an odds ratio of 14.9. This haplotype was absent in the UK/Irish studies. In these studies, the C-A-T haplotype was a risk for both schizophrenia and psychotic bipolar disorder; however, this haplotype was rare (<1%) in Japanese samples and was not a significant factor for methamphetamine psychosis. In addition, the UK/Irish studies showed the G-G-T haplotype was as rare as 3% in control subjects but completely absent in schizophrenia, indicating a potent protective factor against schizophrenia. Again, this haplotype was absent in our samples. Such inconsistencies between the present study and UK/Irish studies indicate that the influence of genetic variation of *DTNBP1* on susceptibility to psychiatric disorders differs among the three distinct disorders (i.e., methamphetamine psychosis, schizophrenia, and psychotic bipolar disorder), although the protective C-A-A haplotype was common to all of them. In addition, population differences in SNP frequencies may also affect results. For example, the minor allele frequency of SNPA was .02, which was consistent with another Japanese study (9), but UK/Irish samples showed a frequency of .45 (13). The P1655 frequency was .28 in our samples, which was similar to another Japanese sample (.31) but different from Caucasian samples (.47 in Straub's study [1] and .49 in Williams's study [13]).

The relationship between abnormal dysbindin function and methamphetamine psychosis is unclear. The *DTNBP1* gene encodes a 40-Kd coiled-coil-containing protein that binds to β-dystrobrevin to form dystrophin-associated protein complex (DPC), which is found in postsynaptic densities of the brain (20). *DTNBP1*, however, is particularly expressed in certain axon terminals, notably, mossy fiber synaptic terminals in the cerebellum and hippocampus independent of DPC (20). Talbot *et al.* (10) found that patients with schizophrenia displayed a presynaptic DTNBP1 reduction in the hippocampus, and an inversely correlated increase in vesicular glutamate transporter-1 occurred in the same schizophrenia cases, suggesting a relationship between glutamatergic neurotransmission and DTNBP1. Evidence in vitro showed that overexpression of DTNBP1-enhanced glutamine release accompanied by an increase of presynaptic machinery SNAP25 and synapsin 1 and a knockdown of DTNBP1 by siRNA-reduced glutamate release. Reduced expression of DTNBP1 in schizophrenic brains may result in hypofunction of the glutamatergic system in the brain, which has been promising hypothesis for the pathophysiology of schizophrenia (21,22). Based on the clinical similarity between methamphetamine psychosis and schizophrenia, it has been assumed that shared neural mechanisms, not only dopamine systems but also gluta-

Table 4. Haplotype Frequencies of the *DTNBP1* Gene of Control Subjects and Methamphetamine (MAP) Psychosis

Haplotype P1655-P1635-SNPA	Controls Frequency	MAP Psychosis Frequency	Permutation p
C-A-A	.7101	.6046	.0013
G-A-A	.2741	.3315	.076
C-G-T	.0022	.0318	.0012
C-G-A	.0023	.0178	.11
C-A-T	.0073	.0055	.83
G-G-A	0	.0089	.15
G-A-T	.0039	0	.18

Haplotype analysis was performed by the permutation method. The global permutation p value was .0005.

mate systems, may be involved in the two psychotic disorders. Many lines of evidence from experimental studies using behavioral sensitization by repeated psychostimulant treatment, which has been recognized as an animal model of methamphetamine psychosis (18), showed pivotal roles of N-methyl-D-aspartate (NMDA) receptors and glutamate systems in the development of behavioral sensitization. Thus repeated administration of amphetamine or cocaine produces behavioral sensitization with enhanced efflux of glutamate in the ventral tegmental area (VTA) and accumbens, which are key brain structures for sensitization phenomena (23,24). NMDA receptor antagonists, including the noncompetitive antagonist MK-801, prevent behavioral sensitization to amphetamines when administered systemically or microinjected into the VTA (25–28). In contrast, phencyclidine, another NMDA antagonist, exacerbates amphetamine-induced abnormal behaviors and a hyperdopaminergic state in the prefrontal cortex and striatum (29–31). Amphetamines can also directly inhibit the NMDA receptor complex (32). Although the roles of NMDA receptors and glutamatergic systems in animal models of methamphetamine psychosis seem to be complex, our findings may indicate that variants of *DTNBP1* affect susceptibility to methamphetamine psychosis by implication of glutamatergic neurotransmission. In addition, DTNBP1 was shown to enhance phosphorylation of AKT protein by PI3-kinase and protect against neuronal cell death. Impaired PI3-kinase-Akt signaling and a genetic association with the *AKT1* gene were found in schizophrenia (20,33,34). Previously, we also found a significant association of the *AKT1* haplotype with the same patients of methamphetamine psychosis (35). It is possible that DTNBP1 confers susceptibility to methamphetamine psychosis via the PI3-kinase-Akt signaling cascade. In vitro evidence of interaction between dysbindin and dopamine system was recently reported. Kumamoto *et al.* (36) found that mRNA of disbindin expressed in the mouse substantia nigra, that suppression of dysbindin expression in PC 12 cells resulted in an increase of dopamine release, and that overexpression of dysbindin produced a tendency to decrease dopamine release. This finding suggests that dysbindin dysfunction may induce susceptibility to methamphetamine psychosis through interaction with dopamine systems.

Alternatively, the effect of DTNBP1 on cognitive ability should be considered. In an analysis of the phenotype–haplotype relationship, Williams *et al.* (13) found that the C-A-A protective haplotype was significantly associated only with higher educational attainment. A longitudinal study of childhood and adolescent antecedents of drug and alcohol problems in adulthood showed that, for both males and females, educational attainment was directly associated with a reduced risk for substance use problems (37). In this respect, higher educational attainment due to carrying the C-A-A haplotype might be involved in a reduced risk for methamphetamine psychosis, and the phenotype of higher educational attainment might be a common protective factor in methamphetamine psychosis and schizophrenia. Further studies are required to confirm this possibility.

Although our results remained significant after Bonferroni correction, it is possible that this was a chance finding resulting from reduced power due to small sample size. Analysis showed, however, that our sample size for the three SNPs had powers of .9994, 1.0000, and .9594 to detect an effect size (w = .1892, .5388, and .1263, respectively), with a significance level of .05 to detect significant associations in allelic analysis between control subjects and subjects with methamphetamine psychosis. Our total sample size is therefore large enough statistically, and it is unlikely that our positive findings results from reduced power. When methamphetamine psychosis patients are divided into subgroups according to clinical phenotypes, however, the statistical power may be reduced. It is possible that a rare haplotype C-G-T as a risk for methamphetamine psychosis may result from a chance fluctuation. In addition, a false-positive association owing to population stratification could not be excluded in this study despite careful matching of control subjects and patients. Our findings should be confirmed in larger samples and in different populations.

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The Glycine Transporter 1 Gene (*GLYT1*) Is Associated With Methamphetamine-Use Disorder

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Glycine transporter (GlyT)-1 plays a pivotal role in maintaining the glycine level at the glutamatergic synapse. Glycine is an allosteric agonist of *N*-methyl-D-aspartate (NMDA) receptors. Because activation of NMDA receptors is an essential step for induction of methamphetamine dependence and psychosis, differences in the functioning of GlyT-1 due to genetic variants of the *GlyT-1* gene (*GLYT1*) may influence susceptibility. A case-control genetic association study of the *GLYT1* gene examined 204 patients with methamphetamine-use disorder and 210 healthy controls. We examined three single nucleotide polymorphisms (SNPs), SNP1, IVS3 + 411C > T, rs2486001; SNP2, 1056G > A, rs2248829; and SNP3, IVS11 + 22G > A, rs2248632, of the *GLYT1* gene and found that SNP1 showed a significant association in both genotype ($P = 0.0086$) and allele ($P = 0.0019$) with methamphetamine-use disorder. The T-G haplotype at SNP1 and SNP2 was a significant risk factor for the disorder ($P = 0.000039$, odds ratio: 2.04). The present findings indicate that genetic variation of the *GLYT1* gene may contribute to individual vulnerability to methamphetamine dependence and psychosis.

KEY WORDS: substance dependence; glycine transporter (GlyT); association study

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INTRODUCTION

Abuse of methamphetamine induces a strong psychological dependence, and further consumption produces highly psychotic states, such as auditory hallucinations and persecutory delusions [Tatetsu, 1963; Ujike and Sato, 2004]. Conditioned place preference (CPP) and behavioral sensitization induced by methamphetamine treatment in rodents have been recognized as animal models of methamphetamine dependence and psychosis. Many lines of experimental evidence have shown that A10 dopamine neurons in the ventral tegmentum area (VTA) projecting into the accumbens, amygdala, and prefrontal cortex play central roles in the induction of CPP and sensitization to methamphetamine [Kalivas and Stewart, 1991; McBride et al., 1999; Ujike, 2002]. It was also demonstrated that activation of glutamate neurons in the prefrontal cortex projecting into the accumbens, amygdala, and VTA and activation of *N*-methyl-D-aspartate (NMDA) receptors are essential to the development of CPP and sensitization [Wolf, 1998]. For example, bilateral lesions of the prefrontal cortex prevented the induction of sensitization to psychostimulants [Wolf et al., 1995], and systemic treatment or intra-VTA injection of NMDA antagonists also prevented it [Karler et al., 1989; Vezina and Queen, 2000]. NMDA receptors are multimeric protein complexes that are activated by glutamate binding to the NR2 subunit [Laube et al., 1997]. Glycine also activates the NMDA receptors by binding to allosteric sites of the NR1 subunit [Johnson and Ascher, 1987]. Recent studies indicated that glycine concentrations around NMDA receptors in the forebrain are efficiently regulated and maintained at a subsaturated level by glycine transporters (GlyTs), which belong to a superfamily of 12 transmembrane Na^+/Cl^--dependent transporters [Sato et al., 1995]. GlyT antagonists, e.g., *N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)] propylsarcosine (NFPS), inhibited glycine uptake [Harsing et al., 2003]

and robustly enhanced the NMDA receptor functions [Bergeron et al., 1998; Kinney et al., 2003]. GlyTs are divided into two subtypes, GlyT-1 and GlyT-2 encoded by *GLYT1* (SLC6A9, MIM 601019) and *GLYT2* (SLC6A5, MIM 604159), respectively. GlyT-1 is known to be predominantly expressed in glial cells in the central nervous system, especially the frontal cortex, and hippocampus [Borowsky et al., 1993; Zafra et al., 1995]. However, GlyT-1 was recently reported in neurons as well [Cubelos et al., 2005], where it is closely associated with NMDA receptors and regulates the extracellular glycine concentration at synapses [Smith et al., 1992]. GlyT-2 is mainly expressed in the spinal cord and brainstem, where it belongs to the family of Na^+/Cl^--dependent plasma membrane transporters [Liu et al., 1993], and is colocalized with inhibitory glycine receptors [Gomeza et al., 2003]. Therefore, it is possible that altered function of GlyT due to genetic variants of the *GLYT1*, but not the *GLYT2* gene, may affect individual susceptibility to methamphetamine-use disorder. We investigated the association between the *GLYT1* gene polymorphisms and the disorder by a case-control study.

MATERIALS AND METHODS

Subjects

The subjects were 204 patients with methamphetamine-use disorder (167 males and 37 females; mean age, 37.4 years; SD 11.9 years) who met the ICD-10-DCR criteria (F15.2) and who were outpatients or inpatients in psychiatric hospitals of the Japanese Genetics Initiative for Drug Abuse (JGIDA), and 210 age-, gender-, and geographical origin-matched normal controls (163 males and 47 females; mean age, 36.5 years; SD 10.6 years), who were mostly medical staff members without a past individual or family history of drug dependence or major psychotic disorders. Patients who had a comorbidity of any other major psychiatric disorders, e.g., schizophrenia and bipolar disorder, were excluded. Assessment for the diagnosis of methamphetamine-use disorder and controls were performed by trained psychiatrists on the basis of all available information, including hospital notes. These assessments were done by unstructured interview.

The patients started methamphetamine abuse at 21.0 ± 5.5 years. As to multi-substance abuse status, 37.2% patients concurrently abused other illicit drugs besides methamphetamine. Cannabinoids were most frequently abused (34.0%), and followed by LSD (14.1%), cocaine (13.1%), opioids (12%), and hypnotics (9.9%). More than 60% patients abused methamphetamine solely, but about half of them had a past history of organic solvent abuse in their teenage. One hundred eighty-eight patients have or had the comorbidity of methamphetamine psychosis (F15.50). More details about the backgrounds of the patients have been published elsewhere [Ujike et al., 2003]. After the study was described, written informed consent was obtained from all participants. This study was approved by the Ethics Committee at each institute of the JGIDA.

Genotyping

The genomic DNA was extracted from peripheral leukocytes using the standard phenol/$CHCl_3$ method. Over 100 single nucleotide polymorphisms (SNPs) spanning the *GLYT1* gene were listed in the NCBI SNP database and the International HapMap project data. Since there was no non-synonymous SNP in the gene which should have potential physiological effects, we selected SNPs which fulfilled the following conditions: (1) those allele frequencies in a Japanese population were already known by the database and their minor allele frequencies were over 10%, (2) they were recognized by inexpensive restriction enzymes, (3) they were not located in deep introns, (4) they were located apart from each other to cover the entirety of the gene. Finally, we selected three SNPs, rs2486001 (IVS3 + 411C > T, SNP1), rs2248829 (1056G > A, SNP2), rs2248632 (IVS11 + 22G > A, SNP3). The three SNPs of the *GLYT1* gene were individually amplified by polymerase chain reaction (PCR) using the primers listed in Table I. PCR was carried out in a total volume of 15 μl with 3% dimethyl sulfoxide and 0.75 units of Taq DNA polymerase in the reaction mixture. Initial denaturation was performed for 5 min at 95°C; 35 cycles were then performed (30 sec of denaturing at 95°C, 30 sec of annealing at the appropriate temperature, and 30 sec of extension at 72°C), followed by a final extension at 72°C for 5 min. The PCR products were then analyzed on 3.0% agarose gels after digestion with Eco47I (SNP1), HincII (SNP2), and MnlI (SNP3), respectively (Table I). Genotyping of SNP1, SNP2, and SNP3 were confirmed by direct sequencing of a part of the samples. To ensure the positive association for SNP1, we confirmed the genotypes of all samples by a different PCR mismatch primer set to produce a recognition site for a different restriction enzyme, Eco0109I (Table I).

Statistical Analysis

Deviation of the genotype counts from Hardy–Weinberg equilibrium was tested using a chi-square goodness-of-fit test. The statistical significance of difference was assessed by a chi-square test (genotype comparison) or log likelihood ratio test (allele comparison) at a significance level of 0.05. The pairwise linkage disequilibrium (LD) and haplotype frequencies were estimated by the EH algorithm using the SNPalyze program (Dynacom Co., Mobara-shi, Chiba, Japan).

RESULTS

Both genotype and allele frequency distributions of patients with methamphetamine-use disorder and control subjects are shown in Table II. The genotype distributions of SNP1, 2, and 3 of patients and controls did not deviate significantly from Hardy–Weinberg equilibrium. We found significant differences in the frequency of genotypes and alleles of SNP1 (genotype, $\chi^2 = 9.52$, $P = 0.0086$; allele, G = 9.66, $P = 0.0019$) and SNP2 (genotype, $\chi^2 = 6.04$, $P = 0.048$; allele, G = 4.14, $P = 0.042$) between patients with methamphetamine-use disorder and control subjects. SNP3 showed no significant differences in allele or genotype between groups. After Bonferroni correction, the genotype and allele frequencies of SNP1, but not SNP2, remained significant. The odds ratio of the T allele of SNP1 for methamphetamine-use disorder was 1.69 (95% confidence interval: 1.22–2.36).

We calculated the pairwise LD between SNP1, SNP2, and SNP3. D′ (absolute value) and r^2 for pairwise LD are shown in Table III. High LD was detected between SNP1 and SNP2 ($D' = 0.76$, $r^2 = 0.064$), SNP1 and SNP3 ($D' = 0.92$, $r^2 = 0.087$), and SNP2 and SNP3 ($D' = 0.87$, $r^2 = 0.70$).

Because SNP1, SNP2, and SNP3 were shown to be located on the same LD block, the global haplotypic association was analyzed for every combination of the three SNPs (Table IV). Haplotypes consisting of SNP1–SNP2, SNP2–SNP3, and SNP1–SNP2–SNP3 showed significant association with patients with methamphetamine-use disorder. Among them, the haplotype comprising SNP1–SNP2 showed the smallest P value ($P = 0.000011$). The frequency of each haplotype consisting of SNP1–SNP2 is shown in Table V. The T-G haplotype of SNP1–SNP2 showed a significant excess in patients with methamphetamine-use disorder over control subjects ($P = 0.000039$), indicating that this haplotype was a risk factor for methamphetamine-use disorder, with an odds ratio of 2.04 (95% confidence interval, 1.45–2.86). These associations were still significant even after Bonferroni correction.

TABLE I. PCR Primers for Single Nucleotide Polymorphisms in the Glycine Transporter-1 (*GLYT1*) Gene

SNP		Primer sequence	Product size (bp)	Annealing temp. (°C)	Restriction enzyme
SNP1 (IVS3 + 411C > T, rs2486001)	Forward-1	5′-CACCCCCAGCCTGTCTTTCAAC-3′	122	61	Eco471
	Reverse-1	5′-CCCCTAAATGGTTCAGGGATGT-3′			
	Forward-2	5′-AGCCTGGGCTGAGGCACACCAC-3′	188	65	Eco01091
	Reverse-2	5′-TTCTATTCCCTGGGGTTCAGCA-3′			
SNP2 (1056G > A, rs2248829)	Forward	5′-TGGGCAGAGGCAGGCACCTC-3′	141	61	HincII
	Reverse	5′-GAGTGACCCTGGAGGGAGCCGTTGA-3′			
SNP3 (IVS11 + 22G > A, rs2248632)	Forward	5′-AGAAGAGGGGTGGTGGGAATCC-3′	131	63	MnlI
	Reverse	5′-TACGGTGAGCACTCGAGCGTCC-3′			

TABLE II. Genotype and Allele Frequencies of SNPs of the *GLYT1* gene

Group	N	Genotype			*P*	Corrected *P*	Allele		*P*	Corrected *P*
SNP1 (rs2486001)		C/C (%)	C/T (%)	T/T (%)			C (%)	T (%)		
Control	210	139 (66.2)	63 (30.0)	8 (3.8)			341 (81.0)	79 (19.0)		
METH-use disorder	204	106 (51.9)	82 (40.2)	16 (7.8)	0.0086	0.026	294 (72.1)	114 (27.9)	0.0019	0.0057
SNP2 (rs2248829)		G/G (%)	G/A (%)	A/A (%)			G (%)	A (%)		
Control	210	110 (52.4)	76 (36.2)	24 (11.4)			296 (70.5)	124 (29.5)		
METH-use disorder	204	119 (56.7)	75 (35.7)	10 (4.8)	0.048	—	313 (76.7)	95 (23.3)	0.042	—
SNP3 (rs2248632)		G/G (%)	G/A (%)	A/A (%)			G (%)	A (%)		
Control	210	113 (53.8)	81 (38.6)	16 (7.6)			307 (73.1)	113 (26.9)		
METH-use disorder	204	122 (58.1)	70 (33.3)	12 (5.7)	0.45	—	287 (76.3)	89 (23.7)	0.20	—

METH, methamphetamine.