Associations of common copy number variants in glutathione S-transferase mu 1 and D-dopachrome tautomerase-like protein genes with risk of schizophrenia in a Japanese population

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ABSTRACT

Oxidative-stress, genetic regions of interest (1p13 and 22q11), and common copy number variations (CNVs) may play roles in the pathophysiology of schizophrenia. In the present study, we confirmed associations between schizophrenia and the common CNVs in the glutathione (GSH)-related genes *GSTT1*, *DDTL*, and *GSTM1* using quantitative real-time polymerase chain reaction analyses of 620 patients with schizophrenia and in 622 controls. No significant differences in *GSTT1* copy number distributions were found between patient groups. However, frequencies of characterized CNVs and assumed gain alleles of *DDTL* and *GSTM1* were significantly higher in patients with schizophrenia. In agreement with a previous report, the present data indicate that gains in the CNV alleles *DDTL* and *GSTM1* are genetic risk factors in Japanese patients with schizophrenia, and suggest involvement of micro-inflammation and oxidative stress in the pathophysiology of schizophrenia.

Key words: copy number variants; D-dopachrome tautomerase; glutathione S-transferase; risk factor; schizophrenia

INTRODUCTION

It is accepted that the pathophysiology of schizophrenia involves complex interactions of genetic, environmental, and psychological etiologies. Numerous studies have suggested that environmental oxidative (Ma et al. 2010; Marchbanks et al. 2003; Prabakaran et al. 2004; Yao et al. 2001) and carbonyl stresses (Arai et al. 2010; Katsuta et al. 2014; Miyashita et al. 2013; Takeda et al. 2015), and their interactions, contribute to disease pathogenesis. Oxidative stress reportedly effects dopaminergic neurotransmission via auto-oxidation reactions that produce dopamine quinines (Rabinovic and Hastings 1998; Smythies 1997), and subsequent excesses of dopamine in the brain may contribute to schizophrenia. Glutathione (GSH) is a dominant cellular non-protein antioxidant and redox regulator that protects tissues of the nervous system from reactive oxygen species (Rabinovic and Hastings 1998). Reduced GSH levels have been reported in peripheral blood (Raffa et al. 2009), some brain regions (Do et al. 2000; Yao et al. 2001), and in cerebrospinal fluid (Do et al. 2000) of schizophrenia patients. GSH is synthesized from amino acids such as glutamate, glycine, and cysteine, and several studies have reported altered levels of these substrates in brain (Waziri et al. 1983) and peripheral tissues (Maeshima et al. 2007; Ohnuma et al. 2008) of schizophrenia patients. Thus, low GSH synthesis may also contribute to the

pathophysiology of schizophrenia, warranting investigations of relationships between genes of GSH metabolism and schizophrenia. Recently, we investigated genetic risk factors using Japanese tag single nucleotide polymorphisms (SNPs) for glutathione synthetase (*GSS*) and two GSH synthesis-related genes encoding glutamate cysteine ligase modifier (*GCLM*) and glutamate cysteine ligase catalytic subunit (*GCLC*), but found no significant associations with schizophrenia (Hanzawa et al. 2011). However, glutathione related genetic risk factors for schizophrenia may involve glutathione S-transferase (GSTs) theta 1 (*GSTT1*) and theta 2 (*GSTT2*). The genes for these enzymes are located at the major schizophrenia linked locus 22q11.23 (Strange et al. 2001). This region contains several copy number variations (CNVs), including a 1.5−3 Mb deletion that leads to 22q11.2 deletion syndrome (22q11.2DS), and has been associated with psychotic symptoms in multiple studies (Karayiorgou et al. 1995; Lindsay et al. 1995a; Lindsay et al. 1995b). Moreover, comprehensive surveys of rare *de novo* CNVs in schizophrenia have reported strong associations for CNVs at 22q11 (Bassett et al. 2008; Buizer-Voskamp et al. 2011; Grozeva et al. 2012; Guilmatre et al. 2009; Kirov et al. 2009; Levinson et al. 2011). Although evidence for these rare-variant hypothesis is limited, GSH synthesis-related genes with CNVs are promising candidate schizophrenia risk factors.

A positive association between schizophrenia and a homozygous *GSTT1* deletion (null type, *GSTT1**0) was previously reported, and although the concomitant absences of enzyme activities have been reported in schizophrenia patients (Kashani et al. 2011), several reports show contradicting results (Gravina et al. 2011; Raffa et al. 2013; Saadat et al. 2007).

Glutathione S-transferase mu 1 (*GSTM1*) is located at the putative schizophrenia susceptibility locus 1p13.3 (1p21.2–1p13.2), which was identified using genome-wide high-density SNP linkage analyses of Japanese families (Arinami et al. 2005). Moreover, a functional CNV in *GSTM1* (*GSTM1**0 null deletion) was significantly associated with schizophrenia alone and via gene–gene interactions with other GSH related genes (Harada et al. 2001; Matsuzawa et al. 2009; Nafissi et al. 2011; Pae et al. 2004; Rodriguez-Santiago et al. 2010). Finally, The 22q11 region included genes encoding D-dopachrome tautomerase (*DDT*) and DDY-like protein (*DDTL*), which are associated with micro-inflammatory pathophysiology of schizophrenia (Kirkpatrick and Miller 2013), and is likely associated with oxidative stress.

In the present study, we investigated the roles of 1) oxidative-stress, 2) genetic regions of interest (1p13 and 22q11), and 3) common CNVs in the pathophysiology of

schizophrenia and demonstrated relationships between common CNVs in *GSTT1*, *DDTL*, and *GSTM1* and schizophrenia.

MATERIALS AND METHODS

Participants

The present case-controlled genetic association study was performed in 640 unrelated Japanese schizophrenia in- or out-patients enrolled from the Juntendo Koshigaya Hospital (Saitama) or the Juntendo University Hospital (Tokyo). All patients met the criteria for schizophrenia at structured clinical interviews that were performed according to the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV). Furthermore, a total of 622 healthy controls who did not meet current or past criteria for any Axis I disorders from the DSM-IV were analyzed for comparison. All participants met the following criteria: (1) no evidence of systemic or neurological disease, (2) no prior head trauma with loss of consciousness, and (3) no lifetime history of alcohol or substance dependence. Patients and controls were recruited from the two Japanese geographic regions of Saitama and Tokyo.

Written informed consent was obtained from all subjects after full explanation of the procedures. The present study was conducted in compliance with the World Medical

Association Declaration of Helsinki and was approved by the Research Ethics Committee of Juntendo University (2012083).

Determination of relative copy numbers using quantitative real-time polymerase chain reaction (QRT-PCR)

Genomic DNA was extracted from peripheral white blood cells using a QIAamp® DNA Blood Maxi kit (Qiagen, Courtaboeuf, France). CNVs of *GSTT1*, *DDTL*, and *GSTM1* were determined using quantitative real-time PCR (QRT-PCR) with TaqMan® Copy Number Assays (Covault et al. 2003) and validated probes and primers (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes for duplex QRT-PCR were designed to specifically amplify target genes without detecting paralogous or allelic sequence variants. A schematic of locations targeted by the present QRT-PCR CNV probes is presented in Figure 1, and further details of amplicons are presented in Supplementary Table 1.

All qRT-PCR reactions were performed on an ABI Prism 7500 Instrument (Applied Biosystems) with Sequence Detection Software version 1.3.1 as previously reported with some modifications (Covault et al. 2003). Briefly, Ribonuclease P (*RNase P*) that targeted the RNA component H1 (H1RNA) gene (*RPPH1*) on chromosome 14 was used

as a single copy number (CN) control (because there is only one copy of this gene per genome; Hebbring et al. 2007). QRT-PCR reaction mixtures were prepared by adding 4 μl of ddH_2O, 5 μl of 2 × TaqMan® Genotyping Master Mix, 0.5 μl of 20 × working stock TaqMan® Copy Number Assay mixture containing forward primer, reverse primer, and FAM dye-labeled MGB probe specific for the target CNV, and 0.5 μl of 20 × TaqMan® Copy Number Reference Assay mixture containing forward primer, reverse primer, and VIC dyelabeled TAMRA probe specific for *RNase P*. Subsequently, 10-mg samples of genomic DNA were added to each tube to a total reaction volume of 10 μl. Amplifications of target CNVs and control *RNase P* were performed in the same tube and determinations were performed in quadruplicate. Thermal cycling conditions included a pre-run at 95°C for 10 min followed by 40 cycles of denaturation for 10 s at 95°C and annealing and extension for 60 s at 60°C. QRT-PCR reactions were performed on 384-well plates containing samples from patients and normal controls, and template-free controls. Copy numbers were assessed using CopyCaller Software (Applied Biosystems, version 2.0) with a calibrator sample (*RNase P*) according to the manufacturer's protocol (Applied Biosystems, Part Number 4400042C). Estimates of confidence and absolute z-scores were calculated by the software and were used to determine correct assignments and intra-assay variations.

Statistics

Differences in mean age and sex ratios between healthy controls and patients were identified using two-tailed Student-*t* tests and chi-square (χ^2) tests, respectively, using SPSS Statistics Version 21 (IBM, Chicago, IL, USA). For the case-control association study, Hardy–Weinberg equilibrium (HWE) tests of CNVs were performed using SNPAlyze Ver. 7.0 Pro (Dynacom, Yokohama, Japan). Differences in genotype and allele frequencies were identified using χ^2 difference tests. Linkage disequilibrium (LD, denoted *D*') for *GSTT1* and *DDTL* (separated by only 100 kb) was calculated from haplotype frequencies using an expectation-maximization algorithm. Differences in CNs between study groups were identified using Fisher's exact tests with SPSS Statistics Version 21. All reported *P*-values are two-tailed. Comparisons of the three genes were performed using Bonferroni correction and differences were considered significant when $P < 0.016$. Power calculations were performed using the Power Calculator for Two Stage Association Studies (http://csg.sph.umich.edu/abecasis/CaTS/index.html) under a prevalence of 0.01, and were executed separately for additive and multiplicative models based on measured allele frequencies of the associated markers 0.34 (*GSTT1*), 0.02 (*DDTL*), and 0.27

(*GSTM1*). The resulting odds ratios with an alpha level of 0.05 were 1.03 (*GSTT1*), 2.03 (*DDTL*), and 1.31 (*GSTM1*).

RESULTS

Three CNVs were genotyped in 640 schizophrenia patients (307 females and 333 males) and 622 controls (321 females and 301 males). Sex ratios did not differ between groups (χ^2 = 1.67, P = 0.215), although patients were significantly younger than controls (39.7 ± 13.8 years vs. 45.6 ± 18.1 years; t = −6.47, P < 0.001). Powers for detecting associations based on additive and multiplicative models were 6% and 6% for *GSTT1*, 85% and 86% for *DDTL,* and 82% and 87% for *GSTM1*, respectively. No deviations from Hardy–Weinberg equilibriums were observed in either patient or control samples (χ^2 = 1.26−2.78, all P > 0.05). Authentic CNs including rare CN gains are shown in Table 1, and those of *DDTL* and *GSTM1* differed between study groups, whereas CNs in *GSTT1* did not vary significantly. In accordance with genotypic models from early studies that investigated null genotypes of *GSTM1*, we included rare gain CNVs including *GSTT1* (CN of 3; 0.2%), *DDTL* (CNs of 4 and 5; 0.5%), and *GSTM1* (CNs of 3 and 4; 1%) as maximal gain genotypes and allele types for each CNV (Table 2). The highest frequency CNV genotype (assumed to be the neutral genotype) differed for each

gene as follows: *GSTT1*, 0/1; *DDTL*, 1/1; and *GSTM1*, 0/0 (Table 2). Genotype and allele frequencies of *GSTT1* did not differ significantly between groups. However, the frequencies of *DDTL* gain genotypes (1/2 and 2/2) and gain alleles (2) were significantly higher in patients than in controls. The frequencies of *GSTM1* gain genotypes (0/1 and 1/1) and gain alleles (1) were also significantly higher in patients (Table 2) than in controls. Finally, linkage equilibrium analyses of *GSTT1* and *DDTL* yielded *D*' = 0.16, suggesting no strong LD block between these CNVs.

DISCUSSION

Based on previous pathophysiological and genome-wide studies of associations between schizophrenia, glutathione-related genes, and genetic regions of interest, we performed genetic case-control CNV analyses to test for possible associations between schizophrenia and CNVs for the three genes *GSTT1*, *DDTL*, and *GSTM1* in Japanese patients. The present results are consistent with previous CNV analyses (Rodriguez-Santiago et al. 2010) that show associations between schizophrenia and *GSTM1* CNVs in a Japanese population. However, our results conflict with several studies of CNVs, including studies of Japanese patients, warranting further studies of differences in CNVs between ethnic groups and disease subtypes.

Nonetheless, whereas null CNVs of *GSTT1* were reported in Iranian patients (Kashani et al. 2011), associations between the corresponding gain CNVs and schizophrenia were reported in Iranian (Saadat et al. 2007), Italian (Gravina et al. 2011), and Tunisian (Raffa et al. 2013) patients. Moreover, a previous case-control study in a Japanese population found no association between schizophrenia and GSH-related genes, including *GSTT1* (Matsuzawa et al. 2009). Accordingly, we found no association between *GSTT1* CNVs and schizophrenia in much larger patient and control groups (both approximately 3 times larger). However, differences in CNV distributions of *GSTT1* have been shown between Japanese and other ethnic groups (Katoh et al. 1996), indicating that the present data may not be globally representative. Nonetheless, these data indicate that CNVs of *GSTT1* are not a significant genetic risk factor for schizophrenia in Japanese subjects.

The genes *DDT* and *DDTL* are tightly linked to *GSTT2*, and are separated by only 50 kb in a head-to-head orientation (Coggan et al. 1998). The region containing these genes harbors an inverted repeat that forms a pseudogene with a premature stop code and a splice site abnormality. However, the present distributions of CNVs of *DDTL* were not consistent with those of *GSTT2* (Rodriguez-Santiago et al. 2010). Specifically, the most frequent *DDTL* genotype in the present patients had a CN of 2 (92.2% of patients; Table

1), whereas Rodriguez-Santiago et al. (2010) reported that the most common *GSTT2* CN in schizophrenia was 0 (74.7% of patients). In contrast, CNV frequencies of *DDTL* were similar to those of *DDT* (CN of 2, 98.0% in schizophrenia), reflecting multiple segmental duplications (paralogs of *DDTL*; Table 1 in Rodriguez-Santiago et al., 2010). Thus, increased *DDTL* and/or *DDT* CNs, rather than *GSTT2* CNs, may contribute to schizophrenia in Japanese patients. Nonetheless, Rodriguez-Santiago et al. (2010) showed variants with the same basic genetic duplication at this region in patients, and the predominant schizophrenia related genetic feature in this region (22q11.22−23) was a CN gain. In the present study, CNVs of *GSTT2* were not determined due to the lack of a suitable validated CNV probe. Although further studies are required to investigate gene interactions using simultaneous determinations of *GSTT2* CNVs, the largest study of CNVs in schizophrenia reports large rare duplication CNVs in this region, and protective effects against schizophrenia (Rees et al. 2014). Hence, future studies are needed to resolve these contrasting effects of common and rare CNVs on schizophrenia risk.

In functional studies, *DDT* inhibited adipogenesis and suppressed macrophage migration (Ishimoto et al. 2012; Lubetsky et al. 2002), which may both contribute to the pathogenesis of schizophrenia via oxidative and carbonyl stresses (Arai et al. 2010;

Katsuta et al. 2014; Takeda et al. 2015), and micro-inflammation (Kirkpatrick and Miller 2013), respectively. However, the functions of *DDTL* CNVs remain uncharacterized.

Numerous studies of *GSTM1* CNVs have been conducted following the discovery of the common null genotype as an early risk factor for some forms of cancer (Yang et al. 2015). Furthermore, several studies have investigated relationships between the *GSTM1* null genotype and schizophrenia. However, these studies report conflicting observations, with no reported associations in Tunisian (Raffa et al. 2013) or Japanese (Matsuzawa et al. 2009; Watanabe et al. 2010) patients, but positive associations in Iranian (Kashani et al. 2011), Japanese (Harada et al. 2001), Caucasian (Gravina et al. 2011), and Korean patients (Pae et al. 2004). Moreover, a positive association with the gain CNV genotype was reported in Spanish patients (Rodriguez-Santiago et al. 2010), and the present data indicate that *GSTM1* gains could be a genetic risk factor for schizophrenia. Contradictions with a previous Japanese study remain unresolved, particularly because sample sizes and methods were similar and the same assay (Hs02575461_cn) was used (Watanabe et al. 2010). It is possible that differences in clinical features such as severity and clinical responses may have contributed to this disparity, warranting further studies of correlations between CNVs and the pathogenesis of schizophrenia.

The pathogenic mechanisms of the present deletions and gains remain speculative. However, GSH deficiencies have been associated with oxidative stress in brains and peripheral tissues of schizophrenia patients, indicating that increased CNs may elevate GST enzyme activity, leading to excessive GSH conjugation and consumption.

This study is limited by low statistical power for analyses of *GSTT1*, heterogeneity of the patient population, and the absence of subtype analyses. Moreover, further studies are required to investigate whether these CNVs are *de novo* or inherited.

In conclusion, gains of *DDTL* and *GSTM1* CNs are genetic risk factors for schizophrenia in Japanese subjects. Overexpression of these genes may deplete endogenous GSH, leading to oxidative stress and micro-inflammation, and possible contributions to the pathogenesis of schizophrenia.

Supplementary material cited in this article is available online.

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Figure Legends

Figure 1. Genome target locations of qPCR-CNV probes

*Locations of investigated CNVs 1, 2, and 3 are indicated in white boxes. Locations of gain CNVs of *GSTT2* and *GSTM1* that were associated with schizophrenia in a previous CNV study by Rodriguez-Santiago et al. (2010) are indicated in gray boxes.

Probe information and genomic loci were unified by Assembly of Feb. 2009 (GRCh37/hg19).

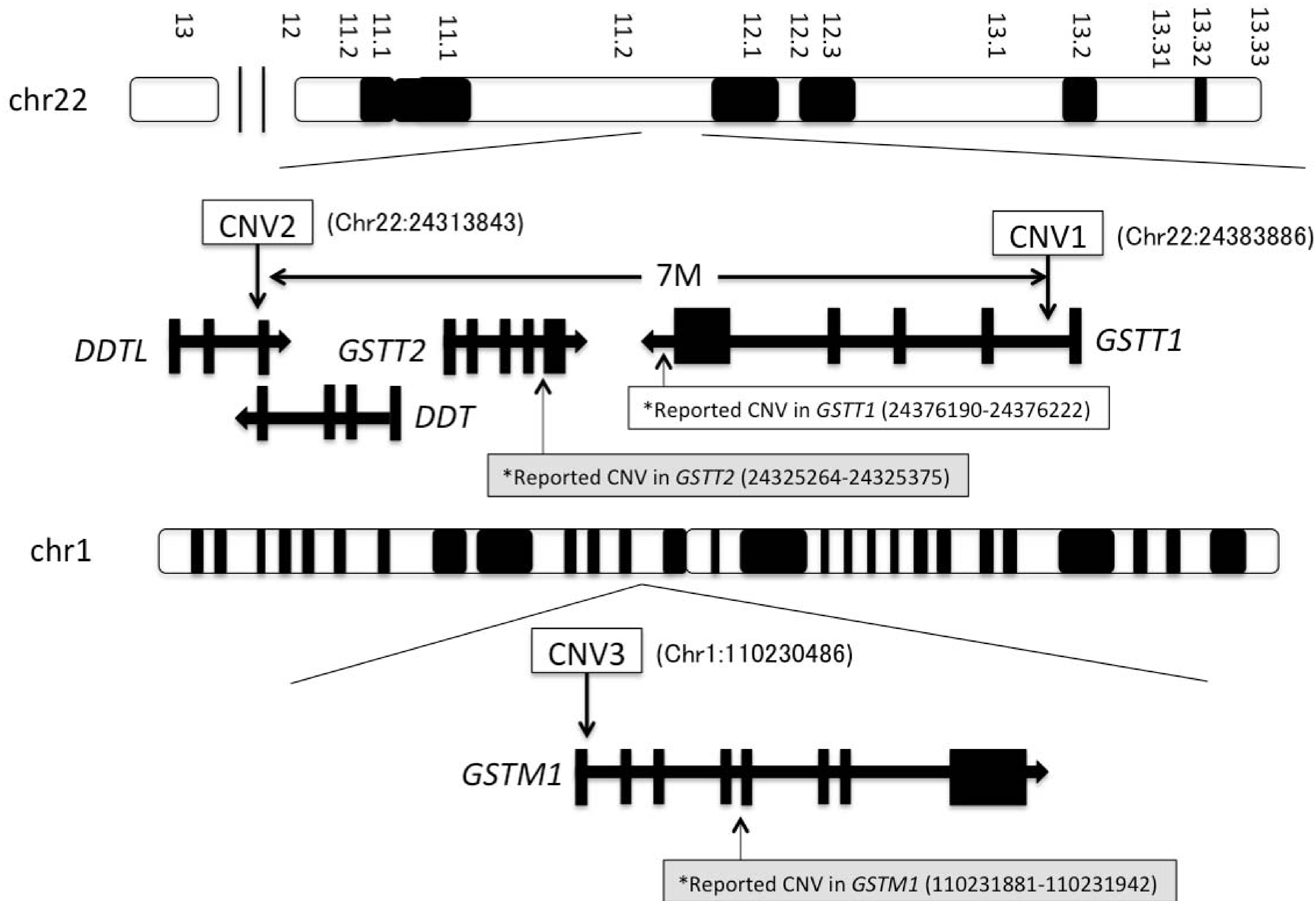
chr22
13
12
11.2
11.1
11.1
11.2
12.1
12.2
12.3
13.1
13.2
13.31
13.32
13.33
CNV2
(Chr22:24313843)
CNV1
(Chr22:24383886)
7M
DDTL
GSTT2
GSTT1
DDT
*Reported CNV in GSTT1 (24376190-24376222)
*Reported CNV in GSTT2 (24325264-24325375)
chr1
CNV3
(Chr1:110230486)
GSTM1
*Reported CNV in GSTM1 (110231881-110231942)

Table 1. Observed copy number (N) for each investigated gene

	0 N (%)	1 N (%)	2 N (%)	3 N (%)	4 N (%)	5 N (%)	χ^2	*P*
GSTT1								
schizophrenia	273 (42.7)	305 (47.6)	62 (9.7)	0 (0.0)			3.73	0.292
controls	268 (43.1)	284 (45.6)	67 (10.8)	3 (0.5)				
DDTL								
schizophrenia			590 (92.2)	46 (7.2)	4 (0.6)	0 (0.0)	14.3	**0.003**
controls			598 (96.2)	22 (3.5)	0 (0.0)	2 (0.3)		
GSTM1								
schizophrenia	303 (47.3)	261 (40.8)	71 (11.1)	5 (0.8)	0 (0.0)		12.9	**0.012**
controls	337 (54.2)	239 (38.4)	39 (6.3)	6 (1.0)	1 (0.1)			

P values with statistical significance are in **bold**.

Table 2. Genotype and allele frequencies of copy number variations (CNVs) of *GSTT1*, *DDTL*, and *GSTM1* in schizophrenia patients and controls

	CNV frequency (%)			χ^2	P	
GSTT1						
genotype	0/0	0/1*	1/1**			
schizophrenia	273 (42.7)	305 (47.6)	62 (9.7)	1.023	0.600	
controls	268 (43.1)	284 (45.6)	70 (11.3)			
allele	0	1**				Odds ratio (95%CI)
schizophrenia	851 (66.5)	429 (33.5)		0.091	0.763	1.03 (0.87–1.21)
controls	820 (65.9)	424 (34.1)				
DDTL						
genotype	1/1*	1/2	2/2**			
schizophrenia	590 (92.2)	46 (7.2)	4 (0.6)	8.936	**0.011**	
controls	598 (96.2)	22 (3.5)	2 (0.3)			
Allele	1	2**				Odds ratio (95%CI)
schizophrenia	1226 (95.8)	54 (4.2)		9.315	**0.002**	2.03 (1.26–3.26)
controls	1218 (97.9)	26 (2.1)				
GSTM1						
genotype	0/0*	0/1	1/1**			
schizophrenia	303 (47.3)	261 (40.8)	76 (11.9)	9.897	**0.007**	
controls	337 (54.2)	239 (38.4)	46 (7.4)			
allele	0	1**				Odds ratio (95%CI)
schizophrenia	867 (67.7)	413 (32.3)		9.714	**0.002**	1.31 (1.10–1.56)
controls	913 (73.4)	331 (26.6)				

*Genotypes with the highest frequencies for each CNV are assumed neutral; **Rare gain copy numbers (CNs); *GSTT1*, CN of 3 (0.2%); *DDTL*, CNs of 4 and 5 (0.5%); *GSTM1*, CNs of 3 and 4 (1%) are included in these genotypes and allele types. CI; confidence interval

Significant *P* values are shown in **bold**.

Supplementary Table 1. Details of copy number variations (CNVs)

	Target Gene	Assay ID*	Gene Symbol	Cytoband	Amplicon Length	DGV Variation ID	DGV Locus Location	Assay Location
CNV1	*GSTT1*	Hs00010004_cn	*GSTT1*	22q11.23	81	36017, 7338, 36016, 79512, 67856, 32452, 38980, 36015, 104519, 107996, 107828, 73793, 23327, 104217, 73782, 90982, 38796, 36014, 3238, 107606, 59068, 79510, 79514, 22926, 31077	Chr22:24257337–24722933	Chr22:24383886
CNV2	*DDTL*	Hs01725969_cn	*DDTL*	22q11.23	106	3238, 73792, 67856, 0548, 32452, 36014, 104216, 36013, 73794	Chr22:24257337–24722933	Chr22:24313843
CNV3	*GSTM1*	Hs02575461_cn	*GSTM1*	1p13.3	82	30400, 84466, 0385, 74641, 111787, 103597, 110600, 64584, 107666, 1549, 74638, 34680, 107536, 97468, 111776, 64585, 23202, 74639	Chr1:110152918–110260150	Chr1:110230486

*Assay ID for Applied Biosystems

Genomic locus and assay locations were unified by Assembly of Feb. 2009 (GRCh37/hg19).