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# Association of the HSPG2 Gene with Neuroleptic-Induced Tardive Dyskinesia

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Tardive dyskinesia (TD) is characterized by repetitive, involuntary, and purposeless movements that develop in patients treated with long-term dopaminergic antagonists, usually antipsychotics. By a genome-wide association screening of TD in 50 Japanese schizophrenia patients with treatment-resistant TD and 50 Japanese schizophrenia patients without TD (non-TD group) and subsequent confirmation in independent samples of 36 treatment-resistant TD and 136 non-TD subjects, we identified association of a single nucleotide polymorphism, rs2445142, (allelic  $p = 2 \times 10^{-5}$ ) in the *HSPG2* (heparan sulfate proteoglycan 2, perlecan) gene with TD. The risk allele was significantly associated with higher expression of *HSPG2* in postmortem human prefrontal brain (p < 0.01). Administration of daily injection of haloperidol (HDL) for 50 weeks significantly reduced *Hspg2* expression in mouse brains (p < 0.001). Vacuous chewing movements (VCMs) induced by 7-week injection of haloperidol–reserpine were significantly infrequent in adult *Hspg2* hetero-knockout mice compared with wild-type littermates (p < 0.001). Treatment by the acetylcholinesterase inhibitor, physostigmine, was significantly effective for reduction of VCMs in wild-type mice but not in *Hspg2*, probably even after antipsychotic treatment, and may be associated with TD susceptibility.

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#### INTRODUCTION

Antipsychotic-induced tardive dyskinesia (TD) is an involuntary movement disorder that develops in patients who are undergoing long-term treatment with antipsychotic medications. The clinical symptoms most commonly involve orobuccal, lingual, and facial muscles, especially in older individuals. The lingual involvement in the form of fine vermicular movements of the tongue while it is sitting at the base of the oral cavity is a common early feature (Sachdev, 2000). In more severe cases, the movements may involve trunk and limbs (Tarsy and Baldessarini, 2006). Such movements lower the quality of life (QOL) of patients (Gerlach, 2002). Therefore, predicting those patients who are vulnerable to TD remains a high priority for psychiatrists in selecting the best medication for a given individual. Introduction of second-generation atypical antipsychotics has reduced the occurrence of TD to approximately 1% annually compared with the 5% frequency with typical agents (de Leon, 2007; Remington, 2007). Owing to the lack of effective treatments for TD, however, therapeutic management of TD can be problematic for schizophrenia patients receiving antipsychotic medications, especially for those patients who develop severe treatment-resistant TD. Therefore, the strategies to prevent TD are often discussed

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in the context of safety and use of antipsychotic drugs (Inada et al, 2008).

The etiology of TD is complex and remains unclear. Age, gender, and ethnicity are all the suggested risk factors for TD. Smoking, drinking, and using street drugs may also increase the risk of TD (Menza *et al*, 1991). There is some evidence for a genetic component to TD (Muller *et al*, 2004) and molecular genetic studies of TD were conducted to identify genes related to TD (Malhotra *et al*, 2004).

The pathophysiology of TD is not completely understood. The causative role of antipsychotic and other dopamine antagonists resulted in the proposal of the dopamine supersensitivity hypothesis of TD (Klawans *et al*, 1980). However, as the hypothesis explains only some aspects of TD, many other pathophysiological models including changes in other neurotransmitter signaling systems that are affected by neuroleptics have been considered. They include gamma-aminobutyric acid (Gerlach and Casey, 1988), norepinephrine (Saito *et al*, 1986), serotonin (Haleem, 2006), and acetylcholine (Ach) (Tammenmaa *et al*, 2002).

The advent of single nucleotide polymorphism (SNP) chips for genome-wide association analysis has made screening of susceptibility genes for TD possible. We carried out a genome-wide association study of treatment-resistant TD in schizophrenia patients and reported that SNPs associated with TD were aggregated significantly in genes belonging to the gamma-aminobutyric acid receptor signaling pathway (Inada *et al*, 2008). In this study, we analyzed the *HSPG2* gene, which includes SNPs that showed the most significant association with TD in our genome-wide association study.

# MATERIAL AND METHODS

#### **Ethical Considerations**

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

# Human Subjects

Human subjects in this study were 86 Japanese schizophrenia patients with TD and 186 Japanese schizophrenia patients without TD, who have been described elsewhere (Inada et al, 2008). Briefly, subjects were identified at psychiatric hospitals located around Tokyo and Nagoya areas of Japan. All patients satisfied the diagnostic criteria of DSM-IV (Association, 1994) for schizophrenia. All subjects and their parents were of Japanese descent. All subjects had been receiving antipsychotic therapy for at least 1 year and their TD status was monitored for at least 1 year. TD was assessed according to the Japanese version of the Abnormal Involuntary Movement Scale. TD was diagnosed according to the criteria proposed by Schooler and Kane (Schooler and Kane, 1982). Once TD was identified, the patients were followed up and received standard therapeutic regimens for TD to minimize TD symptoms. If TD persisted even after 1 year of therapy, patients were considered potential treatment-resistant TD patients. Treatment-resistant TD patients were defined as those patients with dyskinetic movements that persisted for more than 1 year and did not improve even after 1 year of appropriate treatment after guideline-recommended therapeutic regimens for TD. We hypothesized that treatment-resistant TD, a severe form of TD, was suitable for detection of genetic association with TD. Only treatmentresistant TD patients were included as those affected with TD in this study.

## Genotyping, Resequencing, and Statistics

Association screening was performed using the Illumina Sentrix Human-1 Genotyping 109k BeadChip according to the manufacturer's instructions (Illumina, San Diego CA, USA). All DNA samples were subjected to rigorous quality control to check for fragmentation and amplification. Approximately 750 ng of genomic DNA was used in each sample. Normalized bead intensity data obtained for each sample was entered into the Illumina BeadStudio 3.0 software, which converted fluorescence intensities into SNP genotypes. A GenCall Score of 0.85 was used as a minimum threshold for per-sample genotyping completeness. The mean call rate across all samples was 97%. After removing SNPs with a low genotyping rate (p < 0.95: n = 3952), SNPs deviating from the Hardy-Weinberg equilibrium (p < 0.001: n = 135), SNPs with low minor allele frequency (MAF < 0.05: n = 2762), and SNPs located outside exons and introns, we screened for SNPs associated with TD using 40 573 SNPs. SNPs located within 10 kb from the 5' and 3' ends of known genes were included. SNPs in the linkage disequilibrium (LD) of  $r^2 > 0.8$  with other SNPs were excluded. The call rate was at least 99.4% for the 40 573 SNPs. The concordance rate was evaluated by comparisons of genotypes in the 100 screening samples and this gave concordance of over 98% for each sample. Genotyping using TaqMan probes (Applied Biosystems, Foster City, CA, USA) was carried out twice for each SNP, and genotype concordance was 99.7%. Genotyping completeness was >0.99. We treated these uncalled or discrepant genotypes as missing genotypes.

To screen for novel polymorphisms, we used direct sequencing with a Big Dye Terminator Cycle Sequencing kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All exons and the exon-intron junctions of the *HSPG2* gene were amplified from the genomic DNAs of the 86 TD group patients. The sequences of primers for mutation screening are available on request.

For a more detailed analysis of the associations between SNPs in the *HSPG2* gene and TD, the tag SNPs in the gene were selected using the Haploview program (http:// www.broad.mit.edu/mpg/haploview/) with the condition of an  $r^2$  threshold of 0.8 and a minor allele frequency of 0.1, and genotyped by the TaqMan method. Allelic discrimination was performed using the ABI PRISM 7900HT Sequence Detection System using SDS 2.0 software (Applied Biosystems, Foster City, CA, USA).

Allelic associations between SNPs and TD, and departure from the Hardy–Weinberg equilibrium were evaluated by  $\chi^2$  test or Fisher's exact test. Bonferroni's correction for multiple comparisons was applied.

## Human Postmortem Brains

Brain specimens were from individuals of European (Australian) and Japanese descent. The Australian sample comprised 10 schizophrenic patients and 10 age- and gender-matched controls. The diagnosis of schizophrenia was made according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria (American Psychiatric; Association (1994)) by a psychiatrist and a senior psychologist. Control subjects had no known history of psychiatric illness. Tissue blocks were cut from gray matter in an area of the prefrontal cortex referred to as Brodmann's area 9 (BA9). Japanese samples of BA9 gray matter from Japanese brain specimens consisted of six schizophrenic patients and 11 age- and gender-matched controls. In addition, postmortem brains of 37 deceased Japanese patients with schizophrenia were also analyzed. The Japanese subjects met the DSM-III-R criteria for schizophrenia. Details of the condition of the postmortem brains have been described elsewhere (Ishiguro et al, 2008; Koga et al, 2009).

#### Analysis of Hspg2 Transcription in Human Brain Tissue

Total RNA was extracted from human brain tissues with ISOGEN Reagent (Nippon Gene, Tokyo, Japan). The RNA quality was checked using a Nanodrop ND-1000 spectrophotometer (LMS, Tokyo, Japan) to have an OD 260/280 ratio of 1.8-2 and an OD 260/230 of 1.8 or greater. Expression of the HSPG2 genes was analyzed by the TaqMan real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA). From RNA, cDNA was synthesized with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primers. Expression of the HSPG2 gene was analyzed with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the TaqMan gene expression assays for HSPG2 (Hs01078535\_m1), and normalized to the expression of Human GAPDH Control Reagents (Applied Biosystems).

Genotype effects on HSPG2 expression were analyzed in Australian subjects and replicated in Japanese subjects using analysis of variance followed by Tukey's *post hoc* tests by JMP software version 7.0.1 (SAS Institute, Cary, NC, USA) was used.

#### Animals

Animals were same-sex housed before behavior testing. The same animals were used for all behavior tests.

Four-week-old C57BL/6J male mice (weight: 20–25 g) treated with haloperidol (HDL) or vehicle-saline and 7-week-old male mice (wild type: 8;  $Hspg2^{+/-}$ : 7) with orofacial dyskinesia were housed under 10 h: 14 h light/dark conditions with normal food and water ad libitum, with mice housed separately in groups of 4 or 5 mice.

The generation of Hspg2 knockout mice and the phenotypes of the mice have been described elsewhere (Arikawa-Hirasawa et al, 1999). As Hspg2 null mice are embryonic lethal, timed matings between heterozygotes were carried out to generate homozygous and wild-type mice in this study.

All animal protocols were approved by the Animal Care and Use committee of University of Tsukuba.

# Drugs

Reserpine (methyl reserpate 3,4,5-trimethoxycinnamic acid ester; Wako, Osaka, Japan) and HDL (Wako, Osaka, Japan) were diluted in glacial acetic acid and then diluted in distilled water. Physostigmine (Wako), a reversible cholinesterase inhibitor, was diluted in saline. All solutions were treated subcutaneously in volumes not exceeding 10 ml/kg body weight.

### HDL Treatment

To examine the effects of antipsychotic treatments on gene expression, we made two groups: an acute treatment group: 4-week-old C57BL/6J male mice were treated with intraperitoneal injection (i.p.) of 1.0 mg/kg HDL (n = 10) or vehiclesaline (n = 10) once each day for 4 weeks; and a long-term treatment group: 4-week-old C57BL/6J male mice were treated with intraperitoneal injection of 1.0 mg/kg HDL (n=10) or vehicle-saline (n=10) once each day for 50 weeks. Mice were killed 4 h after the last injection to obtain brain tissues.

#### Induction of Vacuous Chewing Movements

Mice were treated with i.p. of 2 mg/kg HDL and 0.3 mg/kg reserpine every day for 7 weeks to induce the putative TD analogue vacuous chewing movements (VCMs) (Araujo et al, 2004; Burger et al, 2005; Naidu et al, 2003). Before injection and 4 hours after the injection on the 47th day, locomotor activity test and rotarod test were carried out. On the 48th and 49th days, 1, 2, 3, 4, and 24 h after the last injection, the animals were observed for quantification of VCMs for 2 days. On the 50th day, to verify the effects of physostigmine on VCMs, mice were injected with 0.1 mg/kg physostigmine. At 1, 2, 3, 4, and 24 h after the injection of physostigmine, the animals were observed for quantification of VCMs. On the 51st and 52nd day, mice were treated with 2 mg/kg HDL and 0.3 mg/kg reserpine and then observed for quantification of VCMs. On the 53rd day, mice were treated with vehicle-saline, and 1, 2, 3, 4, and 24 h after the injection, the animals were observed for quantification of VCMs.

#### Analysis of Hspg2 Transcription in Brain Tissue of Mice

The prefrontal cortex, midbrain, hippocampus, thalamus, and striatum were taken by dissection, and total RNA was extracted with an RNeasy kit (Qiagen, K.K., Tokyo, Japan). After cDNA synthesis from total RNA samples, the transcription level of cDNA samples was analyzed by a TaqMan Expression assay for *Hspg2* (Mm00464581\_m1; Applied Biosystems) and normalized to that of rodent Gapdh with Rodent Gapdh Control Reagents (Applied Biosystems). The average relative expression levels of five regions were compared with the saline groups by Student's t-test.



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# Table I Allelic p-Values of SNPs for Association with TD in Screening and Replication Samples

brack         brack         brack         brack         brack         brack         brack           n2232452         drl         12622         R/D01         coding         .056         .031         0.001         0.052           n244414         drl         12161         G/U1A         Ninking_JUTR         0.426         0.321         0.001         0.002           n2325444         drl         1212         MANIAZ         Intron         0.426         0.321         0.0001         0.021           n2658557         drl         1414         D/S10         faniking_JUTR         0.216         0.0001         0.021         0.003         0.021           n2658572         drl         1444         Str/D1         Intron         0.412         0.232         0.0003         0.021         <	SNP	Chromosome	Location	Gene	Position relative to gene	Allele frequency			Uncorrected allelic p	
n2729452         chrl         (p3622)         PLODI         coding         0.376         0.381         0.001         0.002         0.00002           n23461142         chrl         (p311)         CDU1AJ         tarking_UTR         0.433         0.331         0.001         0.602         0.60002           n2360444         chrl         (p12)         MANIAZ         tarking_UTR         0.432         0.343         0.0002         0.59           n2669007         chrl         (p11)         CMU1D         tarking_UTR         0.422         0.0002         0.20           n2655652         chrl         (q41)         SMUD2         tarking_UTR         0.223         0.171         0.00002         0.20           n2656272         chrl         (q44)         SMUD2         tarking_UTR         0.325         0.001         0.52           n260027         chrl         2p12         LWND         tarking_UTR         0.325         0.000         0.002         0.76           n197201         chr2         2p13         UVC2D         tarking_UTR         0.351         0.001         0.02         0.76           n197201         chr2         2p13         UVC2D         tarking_UTR         0.454         0.001						TD group	Non-TD group	Screening	Replication	Combined
rix445.0rix41ip21.1COL(1A) Imanminon0.5790.3800.0010.0020.0002ri3320444chiip21.1COL(1A) Imanminon0.4820.320.0030.98ri320544chiip21.2ZAVIAiman0.4820.320.0030.97ri460307chiip21.2ZAVIAiman0.4820.340.00020.97ri460307chiip21.2MiN3.0iman0.2120.100.00020.97ri4536532chiop1.2Zp1.2MiN3.0fiming_SUTR0.2120.100.00070.76ri4536532chi2p1.2MiN4fiming_SUTR0.2120.010.0000.76ri260207chi2p1.2MiN4fiming_SUTR0.370.010.0000.76ri270207chi2p1.3KON+7fiming_SUTR0.570.810.0000.97ri187208chi3p2.3STACimon0.220.990.0010.97ri187208chi3p2.3STACimon0.210.010.211.00ri187209chi3p2.3STACimon0.210.010.210.000.01ri187201chi3p2.3STACimon0.210.010.210.010.21ri187202chi3p2.3STACimon0.220.990.0010.210.02ri187204chi3p2.3STAC	rs7529452	chrl	lp36.22	PLODI	coding	0.396	0.381	0.001	0.05	
ninkn	rs2445142	chrl	Ip36.12	HSPG2	intron	0.579	0.380	0.001	0.002	0.00002
n230644drllp12MANIAZinron0.4620.3720.00050.371n2606367drllp12TBX15faring_JUTR0.4620.3550.0010.37n2643527drllq41DINF0faring_JUTR0.4120.2440.00020.24ne454522drllq41SMVD2inron0.4120.2440.00030.22ne571444drV22012EMRIAfaring_SUTR0.2100.0070.76n2060779drV22013BBIfaring_SUTR0.2200.210.00070.76n1164702dr22.2413BBIfaring_SUTR0.3550.3010.0020.78n1164806dr22.2413BBIfaring_SUTR0.720.0990.0010.27n1164702dr22.2413BEIXfaring_SUTR0.730.010.22n1168866dr43.2622IEUXRIfaring_SUTR0.730.010.21n263328dr43.2623TBLXRIfaring_SUTR0.780.0010.21n263329dr5S.112RCC25/3JUTR0.780.0010.21n233282dr5S.121RCC26/3JUTR0.780.8010.21n233282dr5S.124KMAfaring_SUTR0.640.6010.37n23359dr5S.124KMAfaring_SUTR0.6270.780.0010.21n233592dr5S.124KMAf	rs1934712	chrl	lp21.1	COLIIAI	flanking_3UTR	0.435	0.343	0.0007	0.98	
nesh800neh1Ip12IRX15fanking_SUTR0.7870.1850.00910.97ne6668395neh1Iq14DUS910innorm0.4120.3240.00010.20ne456322neh22p21EM14fanking_SUTR0.2530.1710.00070.26ne4566275dev22p12EM14fanking_SUTR0.2530.7710.00050.52nr3060279dev22p12LRR7M4fanking_SUTR0.2950.2410.0010.76nr1169702dev22p13S051fanking_SUTR0.2950.3110.0010.76nr117886dev22p13S051fanking_SUTR0.2950.5110.0010.76nr131586dev22p13JER252Braking_SUTR0.2730.5110.0010.26nr311587dev13q2632TR1/XR1fanking_SUTR0.2730.5110.0010.27nr30237dev5Sq112JCC25428JUTR0.2710.0310.2010.27nr3115325dev5Sq112JCC2548fanking_SUTR0.2680.00070.41nr3115325dev5Sq112JCC2648fanking_SUTR0.2780.0010.27nr3115325dev5Sq112JCC26748fanking_SUTR0.2680.4070.41nr3115325dev5Sq112JCC26748fanking_SUTR0.2670.0010.41nr3115325dev5Sq113JCC2Imman0.27 <td>rs2306444</td> <td>chrl</td> <td>lpl2</td> <td>MANTA2</td> <td>intron</td> <td>0.482</td> <td>0.392</td> <td>0.0005</td> <td>0.59</td> <td></td>	rs2306444	chrl	lpl2	MANTA2	intron	0.482	0.392	0.0005	0.59	
ne668395         ehr         lq41         DUSP (0         flanking_SUTR         0.418         0.53         0.001         0.59           ne744237         ch1         lq44         SMTD2         intran         0.412         0.23         0.117         0.0003         0.20           ne71444         ch2         2p12         KM14         flanking_SUTR         0.213         0.0007         0.76           s2660277         ch2         2p12         RRTM4         flanking_SUTR         0.329         0.714         0.0007         0.76           s1649702         ch2         2p13         RRTM         flanking_SUTR         0.329         0.511         0.0005         0.45           s1649702         ch2         2p23         STAC         flanking_SUTR         0.27         0.511         0.010         0.22           s1649866         ch3         3p24.21         ICD2S1/2         ITron         0.23         0.030         0.000         0.03           s1115988         ch4         6p14.3         Ep1.4         flanking_SUTR         0.890         0.001         0.21         0.02           s1315252         ch5         Sp11.4         C9         flanking_SUTR         0.641         0.041         0	rs869807	chrl	lpl2	TBX15	flanking_3UTR	0.282	0.185	0.0009	0.97	
net/3237         chrl         Ind         MMD3         immon         0.412         0.23         0.0002         0.20           restS562         ch2         7p1 <i>EM</i> I4         fanking_SUTR         0.23         0.171         0.0003         0.21           restS662         ch2         2p12 <i>IRTM44</i> fanking_SUTR         0.28         0.777         0.0005         0.52           restS662         ch2         2q13 <i>BUR14</i> fanking_SUTR         0.285         0.211         0.001         0.76           restS6864         ch2         2q13 <i>BUR14</i> fanking_SUTR         0.322         0.511         0.001         0.26           restS74927         ch3         3p263         TSL/X1         fanking_SUTR         0.471         0.011         0.21           restS74927         ch5         Sq112 <i>DC225517</i> 3UTR         0.699         0.645         0.001         0.32           restS7424         ch5         Sq112 <i>DC225517</i> 3UTR         0.641         0.53         0.001         0.37           restS7452         ch5         Sq112 <i>DC2251618</i> 0.075         0.486         0.0007         0.41 <td>rs6668395</td> <td>chrl</td> <td>lg41</td> <td>DUSPIO</td> <td>flanking_5UTR</td> <td>0.418</td> <td>0.535</td> <td>0.001</td> <td>0.59</td> <td></td>	rs6668395	chrl	lg41	DUSPIO	flanking_5UTR	0.418	0.535	0.001	0.59	
rs458632ch?2p16M.4fanking_SUTR0.2130.1710.00330.82rs671442ch22p162AS3lanking_SUTR0.2120.1200.00070.76rs167402ch22p162AS3lanking_SUTR0.2200.6410.0010.76rs187201ch22p13BUB1lanking_SUTR0.3950.3010.0020.78rs1873201ch22p13BUB1lanking_SUTR0.3950.3010.0020.78rs174927ch33p23STACinton0.2020.0990.0010.59rs1315988ch44q211I.0C285133UTR0.8740.6450.0010.20rs1315982ch55p131C9lanking_SUTR0.8740.6460.0010.370.13rs131522ch55p14CD2lanking_SUTR0.6770.7030.0010.370.13rs1315232ch55p14CD2inton0.370.4140.0010.370.13rs1315232ch55p14CD2fanking_SUTR0.670.750.0010.410.13rs1315232ch55p14CD2fanking_SUTR0.670.750.0010.370.13rs1315232ch56p14fB192fanking_SUTR0.670.7580.00070.410.002rs1315245ch66p241fJ1924fanking_SUTR0.670.7580.00070.410.002 <td>rs6426327</td> <td>chrl</td> <td>l g44</td> <td>SMYD3</td> <td>intron</td> <td>0.412</td> <td>0.324</td> <td>0.00002</td> <td>0.20</td> <td></td>	rs6426327	chrl	l g44	SMYD3	intron	0.412	0.324	0.00002	0.20	
rs671424dr22p162A893fanking_SUTR0.2120.1200.00070.76rs206077dr22p12LRTM4fanking_SUTR0.650.7770.00050.52rs1169702dr22q13LRTM4fanking_SUTR0.3790.3010.0010.76rs1169802dr22q13UBE23fanking_SUTR0.3750.5110.0010.57rs1169802dr33q2.3T/Linforn0.2020.6010.32-rs144484dr33q5.130.6110.760.32-0.32-rs1315982dr44q2.1L02285133UTR0.470.5780.6010.32-rs30027dr5Sq1.3FLRfanking_SUTR0.470.5780.0010.37-rs315325dr5Sq1.3FLRfanking_SUTR0.5780.0010.37-rs315325dr5Sq1.4FLRfanking_SUTR0.2700.2620.00070.41rs315325dr5Sq1.4FLRfanking_SUTR0.2700.2620.00070.41rs315325dr5Sq1.4FLRfanking_SUTR0.5640.6010.32-rs315325dr5Sq1.4FLRfanking_SUTR0.5710.6000.0070.41rs315352dr5Sq1.4FLRfanking_SUTR0.5810.6000.0010.22rs315353dr6Sq1.4FLRfanking_SUTR	rs4558632	chr2	2p21	EML4	flanking_5UTR	0.253	0.171	0.0003	0.82	
rs2662279         chr2         LRTM4         flanking_SUTR         0.685         0.777         0.0005         0.52           rs1164702         chr2         2q13         BUB1         flanking_SUTR         0.239         0.211         0.001         0.76           rs11648866         chr2         2q13         UBF7E3         flanking_SUTR         0.452         0.581         0.0005         0.45           rs11648866         chr2         2q313         UBF7E3         flanking_SUTR         0.452         0.581         0.0005         0.45           rs1375272         chr3         3p22.3         STLXR         Intron         0.202         0.097         0.001         0.32           rs1375273         chr3         3p12.1         C/202.8571.3         3UTR         0.691         0.691         0.32         0.33           rs103252         chr5         Sq14.3         EDU3         intron         0.375         0.486         0.0002         0.94           rs1315355         chr5         Sq14.3         EDU3         intron         0.27         0.578         0.001         0.37           rs131535         chr5         Sq14.3         EDU3         flanking_SUTR         0.441         Cout         0.002<	rs6714424	chr2	2p16.2	ASB3	flanking 5UTR	0.212	0.120	0.0007	0.76	
rs1163702         chr2         2µ13         BUB1         flanking_SUTR         0.329         0.241         0.001         0.76           rs187301         chr2         2µ13         URC21         flanking_SUTR         0.355         0.301         0.002         0.73           rs167806         chr2         3µ13         URC21         Intron         0.452         0.581         0.0005         0.451           rs3749279         dr3         3µ23         STAC         Intron         0.202         0.099         0.001         0.27           rs644346         dr3         3µ23         STAC         flanking_SUTR         0.481         0.645         0.001         0.32           rs115982         dr4         4µ21         LOC236/36         flanking_SUTR         0.646         0.600         0.600         0.601         0.37           rs131522         dr4         6µ14         FAM46A         flanking_SUTR         0.67         0.57         0.601         0.37         0.661         0.002         0.41           rs256180         chr6         6µ21         CDE7         flanking_SUTR         0.57         0.78         0.001         0.32         0.006           rs315578         chr7         7.3 <td>rs2060279</td> <td>chr2</td> <td>2p12</td> <td>LRRTM4</td> <td>flanking 5UTR</td> <td>0.685</td> <td>0.777</td> <td>0.0005</td> <td>0.52</td> <td></td>	rs2060279	chr2	2p12	LRRTM4	flanking 5UTR	0.685	0.777	0.0005	0.52	
n1873201         chr2         2424.3         KCNH7         flanking_SUTR         0.395         0.301         0.002         0.73           n1168866         chr2         2431.3         UBEZE3         flanking_SUTR         0.452         0.581         0.0005         0.45           n374727         chr3         3p2.33         STAC         intron         0.202         0.099         0.001         0.52           n4443468         chr3         3p2.632         TBLXR1         flanking_SUTR         0.694         0.645         0.001         0.32           n2700237         chr5         Sp111         C/0         sanking_SUTR         0.694         0.643         0.0007         0.83           n332582         chr5         Sq11.3         MCC3364B         flanking_SUTR         0.670         0.537         0.686         0.0007         0.31           n1313252         chr5         Sq14.3         EDL3         intron         0.375         0.686         0.001         0.37           n2591525         chr6         6q14.1         CFD2         flanking_SUTR         0.697         0.378         0.001         0.32         0.006           n2537650         chr6         6q24.1         C/1592         coding<	rs11694702	chr2	2al3	BUBI	flanking 5UTR	0.329	0.241	0.001	0.76	
nr.1164886.         chr.2         23,13         UBE2E3         flanking_SUTR         0.581         0.0005         0.45           re374727         chr3         3p22.3         TAC         intron         0.202         0.091         0.59           re6443468         chr3         3p22.3         TAL         intron         0.202         0.091         0.001         0.59           re6443468         chr3         3p22.3         TBL/IX/I         flanking_SUTR         0.641         0.645         0.001         0.32           re700377         chr5         Sp1.1         CP         flanking_SUTR         0.641         0.543         0.001         0.37         0.31           re3115322         chr5         Sq1.1.3         FER         flanking_SUTR         0.692         0.578         0.001         0.37         0.31           re351525         chr6         6q2.1         CC/C16         intron         0.79         0.800         0.0007         0.41           re391526         chr6         6q2.1         CC/C16         intron         0.79         0.800         0.001         0.32         0.002           re3937550         chr6         6q2.1         R/ZP27/11/2/2         odng         0.057	rs1873201	chr2	2q24.3	KCNH7	flanking 5UTR	0.395	0.301	0.002	0.78	
r374927         chr3         3p22.3         STAC         intron         0.202         0.099         0.001         0.57           rsi414368         chr3         3q2.3.2         TAL/XR         flanking_SUTR         0.373         0.511         0.001         0.26           rsi3115988         chr4         4q2.1         LOC28551.3         SUTR         0.644         0.645         0.001         0.37         0.03           rsi315322         chr5         5q1.1.2         MGC32448         flanking_SUTR         0.641         0.543         0.001         0.37         0.03           rsi315322.         chr5         5q1.1.2         MGC32448         flanking_SUTR         0.641         0.548         0.0007         0.41           rsi375305         chr6         6q1.1         FAM44A         flanking_SUTR         0.207         0.262         0.0007         0.41           rsi375376         chr6         6q2.1         CTED2         flanking_SUTR         0.494         0.604         0.001         0.71           rsi375376         chr6         6q2.1         FLB24         flanking_SUTR         0.495         0.001         0.32         0.006           rsi37547         chr6         6q2.1         FLB24	rs11688866	chr2	2a31.3	UBE2E3	flanking 5UTR	0.452	0.581	0.0005	0.45	
No.1.2.1.2.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.	rs3749279	chr3	30223	STAC	intron	0.102	0.099	0.001	0.59	
Instrict         GL32         Curr         Indiring_DT/T         GL33         GL43         GL03         GL33           rs700227         chr5         Sp13.1         C9         flanking_SUTR         0.898         0.830         0.0002         0.80           rs832582         chr5         Sp11.3         C9         flanking_SUTR         0.641         0.543         0.001         0.37         0.03           rs832582         chr5         Sq11.3         FER         flanking_SUTR         0.622         0.578         0.001         0.37           rs5594324         chr5         Sq21.3         FER         flanking_SUTR         0.692         0.578         0.001         0.31           rs2691180         chr6         6q1.1         C7L04         flanking_SUTR         0.644         0.601         0.71           rs375506         chr6         6q2.11         CTED2         flanking_SUTR         0.494         0.604         0.001         0.32         0.006           rs375506         chr6         6q2.1         CTED2         flanking_SUTR         0.494         0.604         0.001         0.32         0.006           rs375506         chr6         8q1.3         KCM2         otr67         0.758	rs6443468	chr3	302632	TRI I XR I	flanking 5LITR	0.202	0.511	0.001	0.37	
ISISTINUS         Clining         Tup         Constraint         Constraint         Constraint         Constraint         Constraint           rs70023         chr5         Spl11         CP         flanking_SUTR         0.641         0.543         0.0001         0.37         0.033           rs8315522         chr5         Spl11         CP         flanking_SUTR         0.641         0.543         0.001         0.37         0.03           rs9315125         chr6         6q14.1         FAW46A         flanking_SUTR         0.207         0.262         0.0007         0.41           rs9376506         chr6         6q21.1         CICL6         intron         0.789         0.890         0.0007         0.41           rs9376506         chr6         6q24.1         CITED2         flanking_SUTR         0.444         0.604         0.001         0.71           rs1832445         chr6         6q24.1         CITED2         flanking_SUTR         0.654         0.415         0.001         0.32         0.006           rs1832445         chr6         6q13.2         SUFI         intron         0.56         0.001         0.32         0.002           rs2307982         chr8         8q13.3         KCN82	rc13115988	chr4	40221	100285513		0.575	0.645	0.001	0.20	
13702.00         101         3p1.31         OP (Marking_DUT)         0.010         0.0001         0.0001         0.037         0.031           rsB32582         chr5         5q11.2         MGC3364B         fanking_DUTR         0.41         0.543         0.001         0.37         0.03           rsB3582         chr5         5q1.3 <i>EDL</i> fanking_DUTR         0.641         0.548         0.0001         0.37         .           rsB5482         chr6         6q21 <i>CDC2L6</i> intron         0.267         0.262         0.0007         0.41           rs9376506         chr6         6q24.1 <i>CDC2L6</i> intron         0.564         0.457         0.001         0.71           rs183245         chr6         6q24.1 <i>CIED2</i> fanking_JUTR         0.564         0.457         0.001         0.32         0.006           rs1047053         chr7         7q36.2 <i>DPF20</i> 3UTR         0.657         0.758         0.001         0.32         0.002           rs1047053         chr8         8q13.3         KCN82         intron         0.571         0.564         0.601         0.37         0.301         0.37           rs233578	rc700237	chr5	5p131	C0	flanking 5LITR	0.898	0.830	0.001	0.32	
Isb.S.2.         OLS         Start         Intersection         Intersection         Outer         OLS         OUTOR         OUT         OUT <th< td=""><td>m022502</td><td>chr5</td><td>50110</td><td>C7 MCC22649</td><td>flanking_SUTR</td><td>0.670</td><td>0.630</td><td>0.0007</td><td>0.80</td><td>0.02</td></th<>	m022502	chr5	50110	C7 MCC22649	flanking_SUTR	0.670	0.630	0.0007	0.80	0.02
N31322         Chr3         Spl.         LDL3         Inton         O.373         Orteo         Orteo         Orteo         Orteo         Orteo         Orteo           rs915125         chr5         6q14.1         FAM46A         flanking_SUTR         0.692         0.789         0.800         0.0007         0.41           rs915125         chr6         6q24.1         CITED2         flanking_SUTR         0.692         0.890         0.0007         0.41           rs9376506         chr6         6q24.1         EITED2         flanking_SUTR         0.644         0.001         0.92         0.006           rs183245         chr6         6q24.1         EIJS9824         flanking_SUTR         0.649         0.001         0.32         0.006           rs183245         chr7         7g362         DP6         3UTR         0.657         0.78         0.001         0.32         0.002           rs2583086         chr8         8q3.3         KCN82         intron         0.169         0.0001         0.01         0.32         0.001         0.32         0.001         0.32         0.001         0.32         0.001         0.32         0.001         0.32         0.001         0.32         0.001         0.32<	12022202	chrS	Eald 2	INGCSS040	indinking_SOTIX	0.275	0.497	0.001	0.37	0.03
NBS-742-4         CHS         SqL13         FER         Interking_SUTR         0.078         0.078         0.001         0.37           rs2691130         chr6         6ql41         FAM46A         flarking_SUTR         0.207         0.262         0.0007         0.41           rs2691180         chr6         6q21         CDC2L6         intron         0.789         0.890         0.001         0.71           rs183245         chr6         6q24.1         FLB2P61/2123         coding_SUTR         0.654         0.457         0.001         0.32         0.006           rs1975353         chr7         7q36         DKFZp761/2123         coding         0.657         0.758         0.001         0.32         0.006           rs2583086         chr8         8q13.2         SULF1         intron         0.169         0.255         0.001         0.95           rs2583086         chr8         8q23.1         STARS         Inking_SUTR         0.629         0.476         0.0004         0.612           rs2927111         chr8         8q2.13         ANXA13         flanking_SUTR         0.629         0.476         0.0014         0.51           rs101292         chr8         8q2.31         ANXA13         <	rs13133232	chro	5q14.3	EDIL3	Intron Academa - ELITR	0.375	0.486	0.0002	0.94	
PS 15.12         Only         Option         Option<	rs6594324	chro	5q21.3	FER	flanking_SUTR	0.692	0.578	0.001	0.37	
Pice/Pilo         One         Op/L         Display bit of the pine pine pine pine pine pine pine pin	rs915125	chr6	6q14.1	FAM46A	TIANKING_SUTK	0.207	0.262	0.0009	0.41	
rss/stools         chr6         6q24.1         CHEDZ         Hanking_SURR         0.544         0.435         0.001         0.71           rs1832445         chr6         6q24.1 <i>FLJ39824</i> flanking_SURR         0.694         0.604         0.001         0.94           rs1632445         chr6         7p13         DKF2p761/2123         coding         0.657         0.758         0.0005         0.32         0.002           rs2583086         chr8         8q13.2         SUEF1         intron         0.169         0.255         0.001         0.95           rs7393478         chr8         8q13.3         KCN82         intron         0.571         0.396         0.0027         0.044         0.0012           rs2927111         chr8         8q2.1         ANCPT1         flanking_SUTR         0.612         0.479         0.0024         0.64         0.001           rs2027113         chr8         8q2.1.1         ANXA13         flanking_SUTR         0.621         0.479         0.0024         0.51           rs2143299         chr10         10p15.3         RBM17         intron         0.622         0.764         0.001         0.21         0.20           rs1032581         chr10	rs2691180	chr6	6q21	CDC2L6	Intron	0.789	0.890	0.0007	0.41	
rs1832445         chr6         6424.1         HJ9924/1         tiaking_SUR         0.494         0.604         0.001         0.94           rs3735478         chr7         7p13         DKF2p76/1/2/23         coding         0.058         0.145         0.001         0.32         0.006           rs1047053         chr7         7q36.2         DP/6         JUTR         0.657         0.758         0.0001         0.95           rs2583066         chr8         8q13.2         SULF1         intron         0.571         0.396         0.0007         0.04         0.0002           rs2927111         chr8         8q2.31         STAR         flanking_SUTR         0.369         0.479         0.0002         0.18<	rs9376506	chr6	6q24.1	CIIED2	flanking_5UTR	0.564	0.457	0.001	0.71	
rs3/38/k         ch/7         / P13         DK/2.p/6/12/2.3         coding         0.058         0.145         0.001         0.32         0.006           rs1047053         chr7         736.2         DPP6         3UTR         0.657         0.758         0.0005         0.3         0.02           rs258306         chr8         8q13.2         SULF1         intron         0.169         0.255         0.001         0.95           rs373826         chr8         8q13.3         KCN82         intron         0.571         0.396         0.0004         0.4         0.001           rs3019982         chr8         8q2.1         ANCPT1         flanking_SUTR         0.612         0.479         0.0004         0.56           rs412329         chr9         9q22.33         COL15A1         intron         0.682         0.744         0.001         0.51           rs193259         chr10         10q1.3         RBM17         intron         0.682         0.543         0.0007         0.51           rs193259         chr10         10q2.13         RG5         coding         0.152         0.290         0.0006         0.21         0.0007           rs193259         chr10         10q2.13         RG5<	rs1832445	chr6	6q24.1	FLJ39824	flanking_3UTR	0.494	0.604	0.001	0.94	
rs104/053       chr/r       /q46.2       DPP6       3UTR       0.657       0.758       0.0005       0.3       0.02         rs258086       chr8       8q13.2       SULF1       intron       0.169       0.255       0.001       0.95         rs4738269       chr8       8q13.3       SULF1       intron       0.571       0.396       0.0007       0.04       0.0002         rs2927111       chr8       8q23.1       STARS       flanking_SUTR       0.612       0.479       0.0002       0.18       0.001         rs3019982       chr8       8q24.13       ANCPT1       flanking_3UTR       0.612       0.764       0.0004       0.56         rs1413299       chr9       9q22.33       COL15A1       intron       0.682       0.746       0.001       0.73         rs125856       chr10       10q21.1       PCDH15       intron       0.628       0.543       0.0007       0.51         rs1058198       chr10       10q24.32       ELOVL3       intron       0.642       0.640       0.0008       0.13       0.002         rs269425       chr10       10q24.32       GBF1       intron       0.646       0.747       0.0009       0.64	rs3/354/8	chr/	/p13	DKFZp/6112123	coding	0.058	0.145	0.001	0.32	0.006
rs2b8086         chr3         8q13.2         SULF         intron         0.169         0.255         0.001         0.95           rs4738269         chr8         8q13.3         KCNB2         intron         0.571         0.366         0.0007         0.04         0.0002           rs2927111         chr8         8q23.1         STARS         flanking_SUTR         0.369         0.487         0.0004         0.4         0.01           rs21927111         chr8         8q23.1         ANGPT1         flanking_3UTR         0.612         0.479         0.0004         0.56           rs1413299         chr8         8q24.13         ANXA13         flanking_3UTR         0.682         0.764         0.0004         0.57           rs1413299         chr9         9q2.33         COL15A1         intron         0.682         0.746         0.001         0.73         r           rs193256         chr10         10q2.1         PCDH15         intron         0.628         0.543         0.0006         0.22         0.0077           rs1058198         chr10         10q2.32         DLG5         coding         0.152         0.290         0.0066         0.20         0.0075           rs2246775         chr10	rs104/053	chr/	/q36.2	DPP6	JUIR	0.657	0.758	0.0005	0.3	0.02
rs4738269         chr8         8q13.3         KCN82         intron         0.571         0.396         0.0007         0.04         0.0002           rs2927111         chr8         8q23.1         STARS         flanking_SUTR         0.369         0.487         0.0004         0.4         0.01           rs3019922         chr8         8q23.1         ANGPT1         flanking_JUTR         0.612         0.479         0.0002         0.18         0.004           rs4242345         chr8         8q24.13         ANXA13         flanking_JUTR         0.659         0.764         0.0004         0.56           rs1413299         chr9         9q22.33         COL15A1         intron         0.682         0.746         0.001         0.73           rs2274359         chr10         10p1.3         RBM17         intron         0.628         0.543         0.0007         0.51           rs1932596         chr10         10q24.32         DLG5         coding         0.152         0.290         0.0006         0.22         0.0007           rs1058198         chr10         10q24.32         BEF1         intron         0.646         0.747         0.009         0.66           rs264755         chr11         I1p1	rs2583086	chr8	8q13.2	SULFT	intron	0.169	0.255	0.001	0.95	
rs2927111       chr8       8q23.1       STARS       flanking_SUTR       0.369       0.487       0.0044       0.4       0.01         rs3019982       chr8       8q23.1       ANGPT1       flanking_3UTR       0.612       0.479       0.0002       0.18       0.004         rs4242345       chr8       8q24.13       ANXA13       flanking_3UTR       0.659       0.764       0.001       0.56         rs113299       chr9       9q22.33       COL15A1       intron       0.682       0.746       0.001       0.73         rs2274359       chr10       10q15.3       RBM17       intron       0.628       0.543       0.0005       0.51         rs1932596       chr10       10q24.32       ELOVL3       intron       0.494       0.634       0.0008       0.13       0.002         rs10748816       chr10       10q24.32       ELOVL3       intron       0.646       0.747       0.0009       0.66         rs765934       chr10       10q24.32       GBF1       intron       0.825       0.696       0.0005       0.22       0.0015         rs286925       chr11       I1p15.1       ABCC8       intron       0.825       0.696       0.0005       0.24       <	rs4738269	chr8	8q13.3	KCNB2	intron	0.571	0.396	0.0007	0.04	0.0002
rs3019922       chr8       8q23.1       ANGP71       flanking_3UTR       0.612       0.479       0.0002       0.18       0.004         rs4242345       chr8       8q24.13       ANXA13       flanking_3UTR       0.659       0.764       0.0004       0.56         rs113299       chr9       9q22.33       COL15A1       intron       0.682       0.746       0.001       0.73         rs2274359       chr10       10p15.3       RBM17       intron       0.628       0.543       0.0007       0.51         rs103596       chr10       10q21.1       PCDH15       intron       0.628       0.543       0.0007       0.51         rs1058198       chr10       10q24.32       ELOVL3       intron       0.494       0.634       0.0008       0.13       0.002         rs2246775       chr10       10q24.32       GBF1       intron       0.494       0.634       0.0009       0.66         rs765934       chr10       10q24.32       GBF1       intron       0.495       0.669       0.0005       0.02       0.0015         rs866292       chr11       I1p15.       ABCG2       intron       0.32       0.611       0.0005       0.75         rs6247	rs2927111	chr8	8q23.1	STARS	flanking_5UTR	0.369	0.487	0.0004	0.4	0.01
rs4242345       chr8       8q24.13       ANXA13       flanking_3UTR       0.659       0.764       0.00004       0.56         rs1413299       chr9       9q22.33       COL15A1       intron       0.682       0.746       0.001       0.73         rs2274359       chr10       10p15.3       RBM17       intron       0.929       0.834       0.0005       0.51         rs1932596       chr10       10q21.1       PCDH15       intron       0.628       0.543       0.0007       0.51         rs1058198       chr10       10q2.3       DLG5       coding       0.152       0.290       0.0006       0.22       0.0007         rs1058198       chr10       10q2.432       ELOVL3       intron       0.494       0.634       0.0008       0.13       0.002         rs2246775       chr10       10q2.432       GBF1       intron       0.646       0.747       0.0009       0.66         rs765934       chr11       Inp15.3       ABCC8       intron       0.825       0.696       0.0015       0.57         rs886292       chr11       Inp13.4       SPCS2       intron       0.738       0.818       0.0005       0.22       0.0015         rs647568	rs3019982	chr8	8q23.1	ANGPTI	flanking_3UTR	0.612	0.479	0.0002	0.18	0.004
rs1413299       chr9       9q22.33       COL15A1       intron       0.682       0.746       0.001       0.73         rs2274359       chr10       10p15.3       RBM17       intron       0.929       0.834       0.0005       0.51         rs1932596       chr10       10q21.1       PCDH15       intron       0.628       0.543       0.0007       0.51         rs1058198       chr10       10q22.3       DLG5       coding       0.152       0.290       0.0006       0.22       0.0007         rs10748816       chr10       10q24.32       EL0VL3       intron       0.494       0.634       0.0008       0.13       0.002         rs246775       chr10       10q24.32       GBF1       intron       0.646       0.747       0.0009       0.66         rs765934       chr10       10q26.3       MGMT       flanking_5UTR       0.732       0.642       0.0012       0.74         rs886292       chr11       Ilp15.1       ABCC8       intron       0.825       0.696       0.0005       0.02       0.0015         rs286925       chr11       Ilp13.4       SPC52       intron       0.738       0.818       0.0009       0.82         rs624786 </td <td>rs4242345</td> <td>chr8</td> <td>8q24.13</td> <td>ANXA13</td> <td>flanking_3UTR</td> <td>0.659</td> <td>0.764</td> <td>0.00004</td> <td>0.56</td> <td></td>	rs4242345	chr8	8q24.13	ANXA13	flanking_3UTR	0.659	0.764	0.00004	0.56	
rs2274359       chrl0       l0p15.3       RBM/7       intron       0.929       0.834       0.0005       0.51         rs1932596       chrl0       l0q21.1       PCDH15       intron       0.628       0.543       0.0007       0.51         rs1058198       chrl0       l0q22.3       DLG5       coding       0.152       0.290       0.0006       0.22       0.0007         rs10748816       chrl0       l0q24.32       ELOVL3       intron       0.494       0.634       0.0008       0.13       0.002         rs2246775       chrl0       l0q24.32       GBF1       intron       0.646       0.747       0.0009       0.66         rs765934       chrl0       l0q26.3       MGMT       flanking_5UTR       0.732       0.642       0.0012       0.74         rs886292       chrl1       l1p15.1       ABCC8       intron       0.825       0.696       0.0005       0.02       0.0015         rs286925       chrl1       l1p13       EHF       SUTR       0.738       0.818       0.0009       0.82         rs624786       chrl1       l1q13.4       NEU3       flanking_SUTR       0.789       0.812       0.0015       0.83         rs144590 </td <td>rs1413299</td> <td>chr9</td> <td>9q22.33</td> <td>COLI 5A I</td> <td>intron</td> <td>0.682</td> <td>0.746</td> <td>0.001</td> <td>0.73</td> <td></td>	rs1413299	chr9	9q22.33	COLI 5A I	intron	0.682	0.746	0.001	0.73	
rs1932596       chr10       I0q21.1       PCDH15       intron       0.628       0.543       0.0007       0.51         rs1058198       chr10       I0q22.3       DLG5       coding       0.152       0.290       0.0006       0.22       0.0007         rs10748816       chr10       I0q24.32       EL0VL3       intron       0.494       0.634       0.0008       0.13       0.002         rs2246775       chr10       I0q26.32 <i>BEI</i> 1       intron       0.646       0.747       0.009       0.66         rs765934       chr10       I0q26.3 <i>MGM</i> T       flanking_5UTR       0.732       0.642       0.0012       0.74         rs886292       chr11       I1p15.1 <i>ABCC8</i> intron       0.825       0.696       0.0005       0.02       0.0015         rs286925       chr11       I1p13 <i>EHF</i> 5UTR       0.738       0.818       0.0009       0.82	rs2274359	chrl0	10p15.3	RBM I 7	intron	0.929	0.834	0.0005	0.51	
rs1058198         chr10         10q22.3         DLG5         coding         0.152         0.290         0.0006         0.22         0.0007           rs10748816         chr10         10q24.32         ELOVL3         intron         0.494         0.634         0.0008         0.13         0.002           rs2246775         chr10         10q24.32         GBF1         intron         0.646         0.747         0.0009         0.66           rs765934         chr10         10q26.3         MGMT         flanking_5UTR         0.732         0.642         0.0012         0.74           rs886292         chr11         11p15.1         ABCC8         intron         0.825         0.696         0.0005         0.02         0.0015           rs86758         chr11         11p13         EHF         5UTR         0.542         0.611         0.0005         0.75           rs647786         chr11         11q13.4         SPC52         intron         0.738         0.818         0.0009         0.82           rs1444590         chr12         12q13.11         SLC38A1         intron         0.789         0.839         0.0013         0.19         0.4           rs1154664         chr12         12q4.32	rs1932596	chrl0	10q21.1	PCDH15	intron	0.628	0.543	0.0007	0.51	
rs10748816chr1010q24.32ELOVL3intron0.4940.6340.000080.130.002rs2246775chr1010q24.32GBF1intron0.6460.7470.00090.66rs765934chr1010q26.3MGMTflanking_SUTR0.7320.6420.00120.74rs886292chr1111p15.1ABCC8intron0.8250.6960.00050.020.0015rs286925chr1111p13EHF5UTR0.5420.6110.00050.75rs568758chr1111q13.4SPCS2intron0.7380.8180.00090.82rs624786chr1111q13.4NEU3flanking_SUTR0.7890.8390.00150.39rs144590chr1212q13.11SLC38A1intron0.6880.5900.00020.80rs1924174chr1313q3.33LIG4flanking_3UTR0.6880.5900.00130.190.04rs1189827chr1414q2.23SEC10L1flanking_3UTR0.7410.6630.00070.45rs11625123chr1414q32.12ITPK1intron0.1240.2250.00090.450.007rs10140345chr1414q32.2VRK1flanking_3UTR0.3000.2730.00110.09	rs1058198	chrl0	10q22.3	DLG5	coding	0.152	0.290	0.0006	0.22	0.0007
rs2246775chr10l0q24.32GBF1intron0.6460.7470.00090.66rs765934chr10l0q26.3MGMTflanking_5UTR0.7320.6420.00120.74rs886292chr11l1p15.1ABCC8intron0.8250.6960.00050.020.0015rs286925chr11l1p13EHF5UTR0.5420.6110.00050.750.82rs568758chr11l1q13.4SPCS2intron0.7380.8180.0090.82rs624786chr11l1q13.4NEU3flanking_5UTR0.7890.8120.00150.83rs1444590chr12l2q13.11SLC38A1intron0.7890.8390.00020.80rs1924174chr13l3q3.3LIG4flanking_3UTR0.6830.5900.00130.190.04rs1189827chr1414q22.3SEC10L1flanking_3UTR0.7410.6630.00070.45rs11625123chr1414q32.12ITPK1intron0.1240.2250.00090.450.007rs10140345chr1414q32.2VRK1flanking_3UTR0.3000.2730.00110.09	rs10748816	chrl0	10q24.32	ELOVL3	intron	0.494	0.634	0.00008	0.13	0.002
rs765934chr10l0q26.3MGMTflanking_5UTR0.7320.6420.00120.74rs886292chr11l1p15.1ABCC8intron0.8250.6960.00050.020.0015rs286925chr11l1p13EHF5UTR0.5420.6110.00050.75rs568758chr11l1q13.4SPCS2intron0.7380.8180.00090.82rs624786chr11l1q13.4NEU3flanking_5UTR0.7350.8120.00150.83rs1444590chr12l2q13.11SLC38A1intron0.7890.8390.00050.39rs154664chr12l2q24.32KIAA1906flanking_3UTR0.6880.5900.00130.190.04rs189827chr14l4q22.3SEC10L1flanking_3UTR0.7410.6630.00070.450.007rs11625123chr14l4q32.12ITPK1intron0.1240.2250.00090.450.007rs10140345chr14l4q32.2VRK1flanking_3UTR0.3000.2730.00110.09	rs2246775	chrl0	10q24.32	GBFI	intron	0.646	0.747	0.0009	0.66	
rs886292chr11l1p15.1ABCC8intron0.8250.6960.00050.020.0015rs286925chr1111p13EHF5UTR0.5420.6110.00050.75rs568758chr1111q13.4SPCS2intron0.7380.8180.00090.82rs624786chr1111q13.4NEU3flanking_5UTR0.7350.8120.00150.83rs1444590chr1212q13.11SLC38A1intron0.7890.8390.00050.39rs154664chr1212q24.32KIAA1906flanking_3UTR0.6880.5900.00130.190.04rs1924174chr1313q3.3LIG4flanking_3UTR0.2820.1950.00130.190.04rs1189827chr1414q2.23SEC10L1flanking_3UTR0.7410.6630.00070.450.007rs11625123chr1414q32.12ITPK1intron0.1240.2250.00090.450.007rs10140345chr1414q32.2VRK1flanking_3UTR0.3000.2730.00110.09	rs765934	chr10	10q26.3	MGMT	flanking_5UTR	0.732	0.642	0.0012	0.74	
rs286925chr11I1p13EHF5UTR0.5420.6110.00050.75rs568758chr11I1q13.4SPCS2intron0.7380.8180.00090.82rs624786chr11I1q13.4NEU3flanking_5UTR0.7350.8120.00150.83rs1444590chr1212q13.11SLC38A1intron0.7890.8390.00050.39rs154664chr1212q24.32KIAA1906flanking_3UTR0.6880.5900.00130.190.04rs1924174chr1313q333LIG4flanking_3UTR0.2820.1950.00130.190.04rs1189827chr1414q22.3SEC10L1flanking_3UTR0.7410.6630.00070.450.007rs11625123chr1414q32.12ITPK1intron0.1240.2250.00090.450.007rs10140345chr1414q32.2VRK1flanking_3UTR0.3000.2730.00110.09	rs886292	chrl I	llpl5.l	ABCC8	intron	0.825	0.696	0.0005	0.02	0.0015
rs568758chr1111q13.4SPCS2intron0.7380.8180.00090.82rs624786chr1111q13.4NEU3flanking_5UTR0.7350.8120.00150.83rs1444590chr1212q13.11SLC38A1intron0.7890.8390.00050.39rs154664chr1212q24.32KIAA1906flanking_3UTR0.6880.5900.00020.80rs1924174chr1313q333LIG4flanking_3UTR0.2820.1950.00130.190.04rs1189827chr1414q22.3SEC10L1flanking_3UTR0.7410.6630.00070.450.007rs11625123chr1414q32.12ITPK1intron0.1240.2250.00090.450.007rs10140345chr1414q32.2VRK1flanking_3UTR0.3000.2730.00110.09	rs286925	chrll	llpl3	EHF	5UTR	0.542	0.611	0.0005	0.75	
rs624786         chrl I         Ilq13.4         NEU3         flanking_5UTR         0.735         0.812         0.0015         0.83           rs1444590         chrl 2         l2q13.11         SLC38A /         intron         0.789         0.839         0.0005         0.39           rs154664         chrl 2         l2q24.32         KIAA / 906         flanking_3UTR         0.688         0.590         0.0002         0.80           rs1924174         chrl 3         l3q3.3         LIG4         flanking_3UTR         0.282         0.195         0.0013         0.19         0.04           rs1189827         chrl 4         14q22.3         SEC10L1         flanking_3UTR         0.741         0.663         0.0007         0.45           rs11625123         chrl 4         14q32.12         ITPK I         intron         0.124         0.225         0.0009         0.45         0.007           rs10140345         chrl 4         14q32.2         VRK I         flanking_3UTR         0.300         0.273         0.0011         0.09	rs568758	chrll	q 3.4	SPCS2	intron	0.738	0.818	0.0009	0.82	
rs1444590         chr12         12q13.11         SLC38A1         intron         0.789         0.839         0.0005         0.39           rs115464         chr12         12q24.32         KIAA1906         flanking_3UTR         0.688         0.590         0.0002         0.80           rs1924174         chr13         13q3.3         LIG4         flanking_3UTR         0.282         0.195         0.0013         0.19         0.04           rs1189827         chr14         14q22.3         SEC10L1         flanking_3UTR         0.741         0.663         0.0007         0.45           rs11625123         chr14         14q32.12         ITPK1         intron         0.124         0.225         0.0009         0.45         0.007           rs10140345         chr14         14q32.2         VRK1         flanking_3UTR         0.300         0.273         0.0011         0.09	rs624786	chrll	q 3.4	NEU3	flanking_5UTR	0.735	0.812	0.0015	0.83	
rs1154664       chr12       12q24.32       KIAA1906       flanking_3UTR       0.688       0.590       0.0002       0.80         rs1924174       chr13       13q3.3       LIG4       flanking_3UTR       0.282       0.195       0.0013       0.19       0.04         rs1189827       chr14       14q22.3       SEC10L1       flanking_3UTR       0.741       0.663       0.0007       0.45         rs11625123       chr14       14q32.12       ITPK1       intron       0.124       0.225       0.0009       0.45       0.007         rs10140345       chr14       14q32.2       VRK1       flanking_3UTR       0.300       0.273       0.0011       0.09	rs1444590	chrl2	2q 3.	SLC38A I	intron	0.789	0.839	0.0005	0.39	
rs1924174         chr13         13q33.3         LIG4         flanking_3UTR         0.282         0.195         0.0013         0.19         0.04           rs1189827         chr14         14q22.3         SEC10L1         flanking_3UTR         0.741         0.663         0.0007         0.45           rs11625123         chr14         14q32.12         ITPK I         intron         0.124         0.225         0.0009         0.45         0.007           rs10140345         chr14         14q32.2         VRK I         flanking_3UTR         0.300         0.273         0.0011         0.09	rs1154664	chrl 2	l 2q24.32	KIAA I 906	flanking_3UTR	0.688	0.590	0.0002	0.80	
rs1189827         chr14         14q22.3         SEC10L1         flanking_3UTR         0.741         0.663         0.0007         0.45           rs11625123         chr14         14q32.12         ITPK I         intron         0.124         0.225         0.0009         0.45         0.007           rs10140345         chr14         14q32.2         VRK I         flanking_3UTR         0.300         0.273         0.0011         0.09	rs1924174	chrl 3	l 3q33.3	LIG4	flanking_3UTR	0.282	0.195	0.0013	0.19	0.04
rs11625123       chr14       14q32.12       ITPK I       intron       0.124       0.225       0.0009       0.45       0.007         rs10140345       chr14       14q32.2       VRK I       flanking_3UTR       0.300       0.273       0.0011       0.09	rs1189827	chrl4	l 4q22.3	SECIOLI	flanking_3UTR	0.741	0.663	0.0007	0.45	
rs10140345 chr14 14q32.2 VRK1 flanking_3UTR 0.300 0.273 <b>0.0011</b> 0.09	rs11625123	chrl4	14q32.12	ITPK I	intron	0.124	0.225	0.0009	0.45	0.007
	rs10140345	chrl4	l 4q32.2	VRKI	flanking_3UTR	0.300	0.273	0.0011	0.09	

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Table I	Continued
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SNP	Chromosome	Location	Gene	Position relative to gene	Allele frequency			Uncorrected allelic p	
					TD group	Non-TD group	Screening	Replication	Combined
rs2061051	chrl5	15q12	GABRG3	intron	0.206	0.350	0.0014	0.04	0.0006
rs3764211	chrl5	5q 3.	APBA2	flanking_3UTR	0.726	0.815	0.0013	0.12	0.005
rs1036673	chrl5	5q24.	PML	3UTR	0.721	0.592	0.0006	0.64	
rs3809729	chrl7	17p12	DNAH9	flanking_5UTR	0.867	0.869	0.0007	0.11	
rs4630608	chrl7	17p11.2	FBXW10	intron	0.250	0.274	0.0010	0.04	
rs2287352	chrl7	17q12	ACACA	flanking_5UTR	0.247	0.306	0.0014	0.85	
rs3744165	chrl7	17q25.3	FLJ I 384 I	5UTR	0.093	0.130	0.0010	0.15	
rs474122	chrl8	18p11.31	DLGAPI	flanking_5UTR	0.404	0.330	0.0002	0.38	
rs   2460403	chrl9	19p13.3	HMG20B	flanking_3UTR	0.285	0.194	0.0011	0.55	
rs437168	chrl9	19q13.12	NPHSI	coding	0.223	0.139	0.0007	0.9	
rs10419669	chrl9	19q13.31	CBLC	intron	0.094	0.179	0.0003	0.84	
rs8112223	chrl9	19q13.41	HASI	flanking_5UTR	0.314	0.219	0.0003	0.43	
rs2328500	chr20	20p11.23	C20orf26	intron	0.376	0.324	0.001	0.12	
rs7281019	chr2l	21q22.11	TCPIOL	intron	0.924	0.862	0.00008	0.57	0.04
rs2056965	chr22	22q12.3	LOC91464	flanking_5UTR	0.422	0.348	0.0002	0.49	

Abbreviations: SNP, single nucleotide polymorphism; TD, tardive dyskinesia.

*p*-Values with bold emphasis indicate p < 0.05 in 1st *p* and 2nd *p*, and combined p < 1st *p*.

#### **Evaluation of VCMs**

Mice were placed individually in observation cages  $(16 \times 17 \times 19 \text{ cm}^3)$  without food. Hand-operated counters were used to quantify VCMs continuously for 5 min. VCMs were referred to as single mouth openings in the vertical plane not directed toward physical material. If VCMs occurred during a period of grooming, they were not taken into account. Mirrors were placed under the floor and behind the back wall of the cage to permit observation of oral movements when the animal faced away from the observer. The observations were made by two observers who were blind to the animal's group assignment. The observation criteria were not subjective, because an excellent inter-observer agreement was found in a previous pilot experiment (Pearson's correlation = 0.98). All behavioral experiments were conducted between 1000 and 1800hours.

#### Locomotor Activity

The locomotor activity test was conducted between 1200 and 1700 hours in a dimly lit testing room. Mice were habituated to the room for at least 30 min before testing. The locomotor activity test was videotaped with a Sony Digital Video Camera (Sony, Tokyo, Japan). The behavioral testing apparatus was a black Plexiglas rectangular box (41 cm long  $\times$  22 cm wide  $\times$  20.5 cm tall) and activity was recorded for 20 min. The total distance traveled (locomotion) was scored.

#### **Rotarod Test**

The rotarod test was conducted between 1200 and 1700 hours in a dimly lit testing room. All mice were brought to

the testing room in their home cages and were allowed to sit undisturbed in the testing room for at least 5 min before the start of behavioral testing. Motor performance was assessed by rotarod (Med Associates, St Albans, VT). A 1-min training session was given to each mouse on the rotarod (diameter 8 cm, 7 rpm) 5 min before the first measurement. Motor performance (time until the first fall) was registered during a 2-min session.

# Statistical Analysis for Behavioral Data and Gene Expression in Animal Experiments

Effects of genotype, drug treatment, and time were analyzed using analysis of variance) followed by Tukey's *post hoc* tests or using Student's *t*-test. Individual differences of the number of VCMs between before and after injection of physostigmine and saline were tested by nonparametric test for one sample test of mean = 0.

# RESULTS

#### Association Study

We screened for SNPs associated with TD using 40 573 tag SNPs on the Sentrix<sup>®</sup> Human-1 Genotyping BeadChip (Illumina) to identify loci associated with susceptibility to TD in 50 TD and 50 non-TD subjects (Inada *et al*, 2008). The potential impact of population structure on this association study was evaluated by using the genome-wide  $\chi^2$  inflation factor,  $\lambda$ , as a genomic control (Devlin and Roeder, 1999; Devlin *et al*, 2001). The estimated value of  $\lambda$  was 1.04, by which genome-wide association *p*-values were corrected. The lowest uncorrected allelic *p*-value for association with TD was  $1 \times 10^{-5}$ . Therefore, no SNP was

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significantly associated with TD after Bonferroni correction. An attempt was made to replicate the association of 63 SNPs, which were allelic *p*-values < 0.002 and located within 10 kb from known genes with the TaqMan genotyping assay (Table 1). A potential association was found for four SNPs (allelic p < 0.05) (Table 1). However, no significant association was found after correction for multiple testing of 63 SNPs in the replication sample only. Among these four SNPs, an association between *GABRG3* SNP and TD has already been reported (Inada *et al*, 2008). The lowest allelic *p*-value for the association was found for rs2445142 ( $p = 2 \times 10^{-5}$ ) when the initial genome-wide sample and replication sample were combined. The SNP is located in the *HSPG2* gene.

Next, we tested associations of 24 tag SNPs including rs2445142 in the *HSPG2* gene with TD and found a nominal significant association for five SNPs in addition to rs2445142 (Table 2). Other than rs2445142, we found a significant association of rs2124368 located in intron 43 of the *HSPG2* gene with TD even after applying Bonferroni's correction for multiple testing (uncorrected allelic p = 0.0003, corrected p = 0.007). The SNP rs2124368 was not in LD with rs2445142, which was located in intron 1  $(D' = 0.13, r^2 = 0.01)$ .

Subsequently, we genotyped the SNPs of rs2501255 (intron 1), rs2501257 (intron 1), rs897474 (intron 3), rs2254357 (exon 6), rs2254358 (exon 6), and rs2497632 (intron 9), because of the expected complete LD between these SNPs and rs2445142 based on the HapMap database. We confirmed that they were in complete LD with rs2445142 ( $r^2 = 1.00$ ). These SNPs were located in introns 1-9 of the HSPG2 gene. Age, sex, and age of onset were not associated with these SNPs. Acute extrapyramidal symptoms were associated with rs2445142 and the six SNPs in complete LD with rs2445142 (allelic p = 0.00002). Resequencing all exons of the HSPG2 gene in patients with TD did not reveal novel SNPs. Finally, we genotyped missense SNPs of rs3736360, rs2229493, s2291827, rs2228349, rs2229491, rs2229490, rs2229489, rs2229475, rs897471, rs2229481, and rs989994, which were listed in dbSNP and did not find significant associations of these SNPs with TD (data not shown). Thus, we tested a total of 103 SNPs, including 41 SNPs in the HSPG2 gene, in our total subjects of 86 TD and 136 non-TD patients.

# Association Between *Hspg2* Expression Levels in the Postmortem Prefrontal Cortex and Rs2445142

The transcription level in the postmortem prefrontal cortex, as measured by TaqMan real-time polymerase chain reaction, was not significantly different by diagnosis, age, sex, postmortem intervals, or pH of brain samples. A significant genotype effect on *HSPG2* gene expression levels was observed in 20 Australian subjects (F(2, 17) = 4.9, p = 0.02) and replicated in 54 Japanese subjects (F(2, 51) = 3.5, p = 0.04). The association was significant in the combined subjects (F(2, 71) = 7.6, p = 0.001). Tukey's *post hoc* tests showed that *HSPG2* expression levels were significantly higher in the subjects with the GG genotype than in those with the CC genotype (Figure 1). Unfortunately, information about TD in the brains we analyzed was not available.

**Table 2** Allelic *p*-Values of Tag SNPs in the HSPG2 Gene for

 Association with TD

	Location	Allele frequency*						
		Allele	TD group	Non-TD group	Allelic p			
rs3736360	exon 96 (N4331S)	A/G	0.19	0.20	0.8715			
rs3767137	intron 77	A/G	0.23	0.19	0.2759			
rs10917053	intron 71	A/G	1.00	0.99	0.3308			
rs7355045	intron 64	G/A	0.84	0.81	0.4235			
rs2290501	intron 60	C/A	0.22	0.21	0.7134			
rs1563370	intron 52	A/G	0.35	0.27	0.0687			
rs2229475	exon 47 (11967V)	G/A	0.01	0.01	0.9477			
rs2305562	intron 43	A/G	0.61	0.49	0.0117			
rs4654991	intron 42	G/A	0.39	0.36	0.5605			
rs2124368	intron 42	G/A	0.77	0.60	0.0003			
rs897472	intron 36	C/A	0.09	0.05	0.1098			
rs897471	exon 36 (VI503A)	A/G	0.88	0.87	0.7005			
rs2229478	exon 8 (L248L)	A/G	0.53	0.42	0.0273			
rs3767141	intron 6	G/A	0.66	0.60	0.1811			
rs2445142	intron I	G/C	0.58	0.38	0.00002			
rs878949	intron I	A/G	0.22	0.20	0.5867			
rs1545593	intron I	C/A	0.41	0.30	0.0122			
rs1002480	intron I	G/C	0.41	0.32	0.0368			
rs6698486	intron I	G/A	0.46	0.38	0.0754			
rs10799719	intron I	G/A	0.80	0.75	0.1789			
rs9426785	intron I	A/G	0.57	0.55	0.7389			
rs4654773	intron I	A/G	0.45	0.45	0.9165			
rs11587857	intron I	G/A	0.50	0.46	0.3465			
rs4233280	5′ flanking	A/G	0.07	0.03	0.0588			

Abbreviations: SNP, single nucleotide polymorphism; TD, tardive dyskinesia \*The frequency of the first allele.



**Figure 1** *HSPG2* expression levels in the postmortem prefrontal region by genotype. The vertical scores show the average (SEM) of relative expression levels in each of the three genotype groups, compared with the mean gene expression in the total samples. \*Indicates p < 0.05 by Tukey's *post hoc* tests.

# *Hspg2* Gene Expression in the Mouse Brains by HDL Treatment

Hspg2 expression levels were evaluated in the mouse brain after treatment with the antipsychotic drug, HDL. The expression of Hspg2 levels did not alter after a 4-week treatment of HDL except for the striatum where Hspg2 was expressed significantly higher than after the saline



**Figure 2** Effects of haloperidol (HDL) on HSPG2 gene expression in the mouse brains. Relative expression levels of Hspg2 from the prefrontal cortex, midbrain, hippocampus, thalamus, and striatum in the mouse brains after treatment with HDL for 4 weeks (n = 10) and HDL for 50 weeks (n = 10) were compared with the saline groups for 4 weeks (n = 10) and 10 weeks (n = 10) by Student's t-test.

treatment. Significantly lower expression of *Hspg2* was observed in all brain regions after a 50-week treatment with HDL than after a 50-week treatment with saline (Figure 2) (F(1,18) = 42.9, p < 0.0001 at the prefrontal cortex; F(1,18) = 20.1, p = 0.0003 at the hippocampus; F(1,18) = 15.9, p = 0.0009 at the striatum; F(1,17 = 19.3, p = 0.0004 at the midbrain; F(1,18) = 16.5, p = 0.0007 at the thalamus).

# Analysis of VCMs Induced by Haloperidol-Reserpine in *Hspg2* Knockout Mice

As we could not induce VCMs by administration of HDL only to mice, VCMs induced by long-term treatment with HDL and reserpine in female Hspg2 hetero-knockout mice and female wild-type gene litters were measured to evaluate the relationship between expression levels of Hspg2 and TD (Figure 3a). *Hspg2*-null knockout mice were embryonic lethal. The relative expression levels of Hspg2 in Hspg2 hetero-knockout mouse brains were almost half of that in the wild littermates (data not shown). Body weight, locomotor activities, and performance in the rotarod test before and after 48 days of administration of HDL and reserpine were not significantly different between Hspg2 hetero-knockout and wild litters (data not shown). There was a significant effect of genotype (F(1, 545) = 36.8), *p* < 0.0001), post-treatment time (F(4, 495) = 6.15,p < 0.0001), and treatment (F(3, 543) = 5.7, p = 0.0008) for the number of VCMs for 5 min. Post hoc analysis showed that the number of VCMs were significantly lower

in hetero-knockout mice than in wild-type mice after the last injection of HDL and reserpine after 48 or 49 consecutive days of administration of HDL and reserpine, and subsequent injection of physostigmine on the 50th day, or saline on the 53rd day (Figure 3b). The response of VCMs to physostigmine was subsequently evaluated (Figure 3c). There was a significant effect of genotype (F(1, 128) = 36.9,p < 0.0001), but not post-treatment time (F(4, 125) = 1.03, p = 0.39) for individual differences in the number of VCMs between pre-injection and post-treatment time. As for saline treatment, there was no significant effect of genotype (F(1, 118) = 0.13, p = 0.72) and post-treatment time (F(4, 115) = 0.31, p = 0.87). The numbers of VCMs were significantly reduced by injection of physostigmine compared with those before the injection at 24 h after HDL and reserpine injection in the wild-type mice but the differences in the numbers of VCMs before and after injection of physostigmine were not significant in hetero-knockout mice. The number of VCMs did not significantly alter after injection of saline in hetero-knockout mice and wild-type mice.

# DISCUSSION

From a genome-wide association analysis, this study identified the role of *HSPG2* in neuroleptic-induced TD. The association was not significant in the initial screening and second confirmation after correction for multiple testing. However, screening with the tag SNPs for *HSPG2*,

а Evaluation of locomotor and rota-rod activities Evaluation of VCMs 1st day 47th day 48th day 49th day 50th day 51st day 52nd day 53rd day \*\*\*\*\*\* saline physostigmine 0.1 mg/kg haloperidol 2 mg/kg + reserpine 0.3 mg/kg b 70 physostigmine Number of VCMs / 5min 60 +/saline 50 40 30 20 10 0 24h 1h 2h 3h 4h 24h 1h 2h Зh 24h 24h 48h 1h 2h 3h 4h haloperidol-reserpine haloperidol-reserpine Hour after injection 48th and 49th days 50th day 51st and 53rd day 52nd day 47 or 48 days consecutive injection of haloperidol-reserpine С 10 10 0 0 Difference -10 -10

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Figure 3 Analysis of vacuous chewing movements (VCMs) induced by haloperidol-reserpine in Hspg2 knockout mice. (a) Schematic presentation of schedule of injections and measurements. (b) The average number (SEM) of VCMs for 5 min after injection. The abscissa axis shows the time after injection of HDL and reserpine, physostigmine, or saline. Significant difference between Hspg2 hetero-knockout and wild-type mice is shown as \* at p < 0.05, \*\* at p < 0.01, and \*\*\* at p < 0.001. (c) Reduction of the number of VCMs after physostigmine or saline injection. Individual differences of the number of VCMs before injection (50th day for physostigmine or 53rd day for saline) to each time after injection grouped by the genotype are shown. Significant difference from mean = 0 is shown as \* at p < 0.001.

where the SNP (rs2445142) with the smallest p-value for association with TD in our genome-wide association study was located, identified one SNP (rs2124368) associated with TD even after correction for multiple testing. These two SNPs, which were found to be associated with TD, one identified by a genome-wide screening and another identified by screening with the tag SNPs, were not in LD. However, it is not obvious whether the finding for genetic association with TD of these SNPs in the HSPG2 gene can be interpreted as significant, because of two steps of genomewide association analyses before the step of screening of tag SNPs. Furthermore, the Human-1 BeadChip used in our initial screening is far from a complete genome coverage. This may affect the credibility of the results. Confirmation of associations in other populations is necessary.

ㅗ/ㅗ

4h

24h

The SNP rs2445142 that showed the lowest association *p*-value in this study was associated with the expression levels of HSPG2 in the human postmortem prefrontal cortex. The risk allele was associated with increased expression of HSPG2. The SNP rs2445142 is located in intron 1 of the HSPG2 gene and is in complete LD with at least six SNPs located from introns 1-9. Among the SNPs associated with TD found in this study, the program TFSEARCH (http:// www.cbrc.jp/research/db/TFSEARCH.html) predicts alteration of the transcription factor, LYF-1, binding affinity between the T and C alleles of rs897474 in intron 3. Synonymous SNPs, rs2254357 (exon 6), and rs2254358 (exon 6) that were associated with TD might affect mRNA decay rates. Unfortunately, the mechanism of the association between these SNPs and HSPG2 expression levels could not be elucidated in this study.

From findings in human postmortem brain samples, we speculated that increased expression of *HSPG2* is a risk factor for TD and interpreted that decreased expression of *Hspg2* in mouse brains after chronic administration of HDL was a compensatory or adaptive response to neuroleptic drugs. We, therefore, hypothesized that decreased expression level of *HSPG2* is protective for TD. We examined our hypothesis using hetero-knockout mice and confirmed it after finding lower numbers of VCMs in hetero-knockout mice than in the wild-type littermates after chronic administration of HDL and reserpine. We carried out the experiment using only female mice; therefore, we do not have the data on the sex difference.

The mechanism behind our hypothesis that increased expression levels of HSPG2 may induce a susceptibility to neuroleptic-induced TD is not known at present. A potential efficacy of cholinergic drugs in the treatment of TD has been reported (Caroff et al, 2001; Tammenmaa et al, 2004). AChE terminates neurotransmission at cholinergic synapses by hydrolyzing acetylcholine. At the neuromuscular junction, AChE is in the basal lamina, where AChE tetramers bind the collagen ColQ, which interacts in turn with the dystroglycan complex through perlecan (Peng et al, 1999). Perlecan is an essential component of the ColQ-AChE localization in neuromuscular junction (Rotundo et al, 2005). At central synapses, AChE tetramers bind directly to the PRiMA (Perrier et al, 2002). Although ColQ also anchors AChE in brain and heart in addition to skeletal muscle (Feng et al, 1999), the role of perlecan in acetylcholine receptor signaling in central synapses is unclear. In this study, we tested the effect of the AChE inhibitor, physostigmine, on HDL- and reserpon-induced VCMs in mice. We found significant reduction in the number of VCMs only in wild-type mice and the number of VCMs was not reduced in hetero-knockout mice. These findings indicate that perlecan may be involved in the role of AChE in TD and the genotyping and/or levels of HSPG2 may provide useful information about the effectiveness of treatment of TD with AChE.

The other important molecule to which perlecan and TD may be related is FGF2. Perlecan promotes FGF2–FGFR1 binding (Whitelock *et al*, 1996) and HSPGs including perlecan were upregulated by responding to injury and may have a role in intracellular trafficking of FGF2 in neurons and glia in the adult rat cerebral cortex (Leadbeater *et al*, 2006). Clozapine increases FGF2 expression and, on the basis of the neuroprotective activity of FGF2, a potential use of clozapine in TD was proposed (Riva *et al*, 1999).

Perlecan is expressed at the capillary endothelial cells in the brain and perlecan at the blood-brain barrier (BBB) may have a role in maintaining the blood-brain barrier function because of acceptance of the FGF2 secreted from astrocytes (Deguchi *et al*, 2002). It is reported that neuroleptics, such as HDL and chlorpromazine, alter the blood-brain barrier function and increase brain iron levels, which affect neuroleptic-induced dopamine receptor supersensitivity (Ben-Shachar *et al*, 1993).

Although the exact mechanisms of the association between HSPG2 and TD are unclear, this study identified the role of *HSPG2* in neuroleptic-induced TD.

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#### DISCLOSURE

The authors declare that no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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