Table 2. Signficantly differently expressed genes in the prefrontal cortex of *Smarca2* knockout mice and in the postmortem prefrontal cortex of schizophrenia in Stanley database

Genes (human)	Stanley SZ against control Gene expression fold change	StanleyP	Genes (mouse)	accession ID	*Smarca2* −/− against +/+ Gene expression fold change
APPBP2	−1.0335	0.042	*Appbp2*	*NM_025825.2*	−1.54
ARHGEF9	−1.0579	0.008	*Arhgef9*	*NM_025657.2*	−1.46
ARPP-19	−1.0632	0.045	*Arpp-19*	*NM_030562.1*	−2.32
ASPH	1.0313	0.012	*Asph*	*NM_173382*	1.27
ASPSCR1	−1.0471	0.045	*Aspscr1*	*NM_144960.1*	−1.69
BDNF	−1.0523	0.031	*Bdnf*	*NM_007540.3*	−1.82
CDK5R2	−1.0617	0.021	*Cdk5r2*	*NM_177775.2*	−2.11
CLCN3	−1.0562	0.020	*Clcn3*	*NM_183108.1*	−1.89
CPSF6	−1.0377	0.047	*Cpsf6*	*NM_017372.2*	−1.79
CSDA	1.0577	0.030	*Csda*	*NM_028878.1*	1.43
CXCR4	−1.0757	0.002	*Cxcr4*	*XM_130951.1*	1.57
DOCK11	−1.0530	0.003	*Dock11*	*NM_001033349.1*	1.69
DOK5	−1.0952	0.007	*Dok5*	*NM_007386.1*	−1.93
DUSP3	−1.0795	0.001	*Dusp3*	*NM_025657.2*	−3.72
ERCC1	−1.0549	0.045	*Ercc1*	*NM_008701.1*	−1.43
FKBP5	1.0521	0.046	*Fkbp5*	*NM_007386.1*	1.54
GPAA1	−1.0745	0.005	*Gpaa1*	*NM_138648.1*	1.45
GSTT2	−1.0770	0.015	*Gstt2*	*NM_011986.2*	2.07
HAGHL	−1.1255	0.005	*Haghl*	*NM_139064.1*	−1.24
HDLBP	−1.0482	0.007	*Hdlbp*	*NM_013881.3*	−3.00
HGF	1.0233	0.005	*Hgf*	*NM_028094.1*	−2.39
HOMER1	−1.1043	0.017	*Homer1*	*NM_147176.1*	−4.39
HRK	−1.0488	0.040	*Hrk*	*NM_028094.1*	−1.88
IFITM2	1.1655	0.001	*Ifitm2*	*NM_028878.1*	1.79
IGFBP7	1.0935	0.000	*Igfbp7*	*XM_203293.2*	−1.41
KCNK1	−1.1331	0.001	*Kcnk1*	*NM_013881.3*	−1.30
LGI4	1.0299	0.039	*Lgi4*	*NM_011986.2*	1.32
MAPK8	−1.0373	0.033	*Mapk8*	*NM_028094.1*	−1.65
MAPK9	−1.0640	0.038	*Mapk9*	*NM_011719.2*	−2.38
NAP1L1	−1.0396	0.009	*Nap1l1*	*NM_026000.1*	−1.24
OSBP	−1.0512	0.017	*Osbp*	*NM_130859.2*	1.39
PER1	1.0806	0.006	*Per1*	*XM_129848.4*	2.42
PINK1	−1.0958	0.012	*Pink1*	*NM_011960.1*	−1.47
POU2F1	1.0695	0.044	*Pou2f1*	*NM_007386.1*	1.35
PTBP1	1.0286	0.048	*Ptbp1*	*NM_027563.1*	2.81
PTPRB	−1.0340	0.026	*Ptprb*	*NM_001033349.1*	−2.03
RAB1A	−1.0540	0.029	*Rab1a*	*NM_009695.2*	−1.40
RNF14	−1.0818	0.002	*Rnf14*	*NM_153415.1*	−2.32
RTN4	−1.0584	0.050	*Rtn4*	*NM_138648.1*	−1.72
RUFY2	1.0410	0.020	*Rufy2*	*NM_153415.1*	−1.85
SGC2	−1.1655	0.002	*Sgc2*	*NM_009129.1*	−1.36
SDC2	1.0811	0.001	*Sdc2*	*NM_008701.1*	−1.44
SDHC	−1.0471	0.045	*Sdhc*	*NM_011762.2*	−1.14
SFRS11	1.0537	0.011	*Sfrs11*	*XM_135197.4*	−1.82
SNX4	−1.0385	0.034	*Snx4*	*NM_177775.2*	−1.96
SRR	−1.0459	0.028	*Srr*	*NM_030562.1*	−1.17
SYT11	−1.0715	0.024	*Syt11*	*NM_009759.2*	−1.13
TNFRSF25	1.0440	0.014	*Tnfrsf25*	*NM_028878.1*	1.56
TPI1	−1.0590	0.018	*Tpi1*	*NM_001013823.1*	−1.08
UBXD1	−1.0728	0.040	*Ubxd1*	*NM_153415.1*	−1.31
VAMP1	−1.0563	0.034	*Vamp1*	*NM_177775.2*	−1.81
WARS	−1.0604	0.050	*Wars*	*NM_001033349.1*	−3.25

Stanley-*P*-values are by the SMRI database.

genomic DNA was used in each sample. Normalized bead intensity data obtained for each sample were 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 97.0% for Human-1 and 99.8% for HumanHap370; the call rate was at least 99% for 47021 SNPs for Human-1 and 235868 SNPs for HumanHap370 and at least 95% for 60 568 SNPs for Human-1 and 244 337 for HumanHap370. Concordance rate between Human-1 and HumanHap370 platforms was evaluated by comparisons of genotypes in the 100 screening samples and this gave concordance of over 98.0% for each sample. One thousand one hundred and fifty-two subjects were genotyped twice for each SNP using TaqMan genotyping (Applied Biosystems, Foster City, CA, USA),

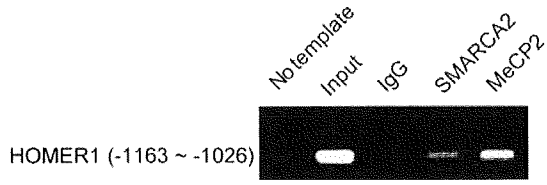

Figure 6. Chromatin immunoprecipitation (ChIP) assays on DNA harvested from T98G cells. DNA that interacts with SMARCA2 and MeCP2 was evaluated using antibodies against to them. Normal rabbit immunoglobulin G (IgG) was used as negative controls. DNA was detected by PCR using primers for the region $-1163 \sim -1036$ from the *HOMER1* exon 1. 1st lane: no template; 2nd lane: DNA template before immunoprecipitation for PCR; 3rd lane: immunoprecipitated DNA template with IgG for negative control. 4th and 5th lanes: immunoprecipitated DNA template with antibodies against SMARCA2 and MeCP2.

and genotype concordance was 99.7%. Genotyping completeness was >0.99.

For a more detailed analysis of the associations of the *SMARCA2* gene, 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).

Cell and animal experiments

Experimental procedures used in cell and animal experiments, including plasmid: construction, knockdown of *SMARCA2* by siRNA, cell culture and transfection, RNA and cDNA preparation, northern and western blot analysis, whole-genome expression analysis, real-time PCR, ChIP assay and behavioral pharmacological analyses of mice are described in the Supplementary Material.

Statistical analysis

Initial screening for association with schizophrenia was done for 11 883 SNPs and, in subsequent analysis, 259 SNPs from the HumanHap370 BeadChip and 34 tag SNPs from *SMARCA2* were added. Therefore, a *P*-value corrected by Bonferroni's method for 12 176 pair-wise comparisons, $P < 4 \times 10^{-6}$, was considered as significant for overall evidence for association. In this study, genotypic *P*-values or haplotype *P*-values were not evaluated to avoid inflation of the *P*-values due to multiple testing.

In the replication study, 5 SNPs from the initial screening, 3 SNPs from genes related to chromatin remodeling and 34 tag SNPs in the *SMARCA2* gene were selected to replicate associations with schizophrenia in the replication samples. A $P = 0.05/(5 + 3 + 34) < 0.001$ was considered as significant in the replication samples. Association and Hardy–Weinberg equilibrium were calculated using chi-test. Haplotype frequencies were estimated using the expectation maximization algorithm. The Haploview program (http://www.broad.mit.edu/mpg/haploview/) was used to detect the haplotype block.

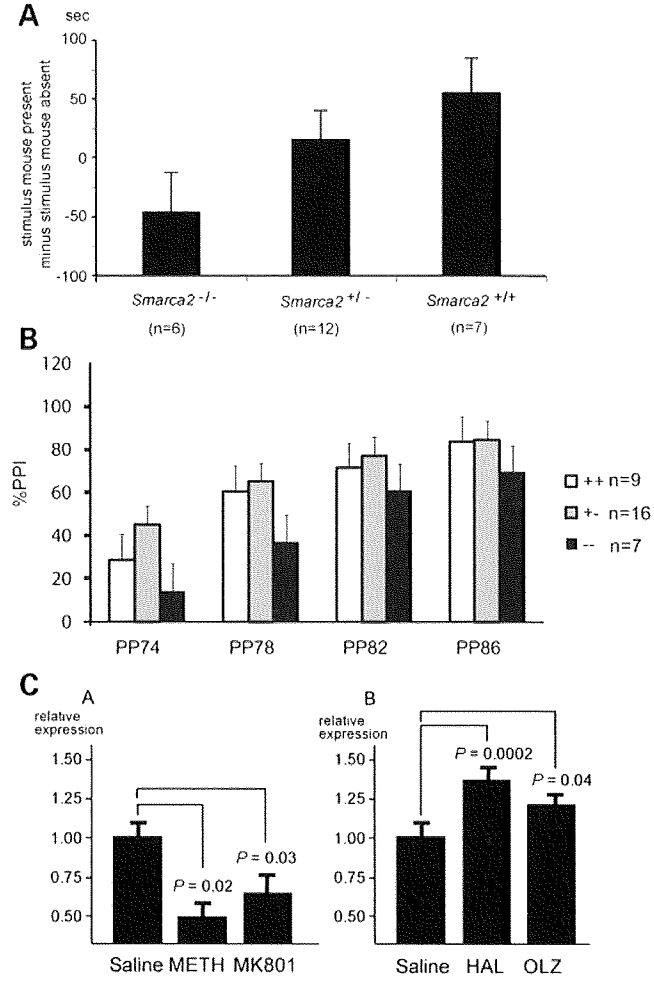

Figure 7. Social interaction and PPI in *Smarca2* −/−, −/+ and +/+ mice and effects of psychotogenic and antipsychotic drugs on *Smarca2* gene expression in the mouse brain. (**A**) The vertical axis is the difference between the time that the test mouse spent sniffing the cylinder where a stimulus mouse was present and the time when stimulus mouse was absent. Genotype was coded as 0, 1 or 2 depending on the number of *Smarca2* copies and a simple regression model was fitted ($P = 0.03$). (**B**) PPI was recorded for *Smarca2* −/−, −/+ and +/+ mice using a conditioning, prepulse noise burst of 74, 78, 82 or 86 dB. *Smarca2* −/− mice had impaired PPI in comparison to heterozygous and wild-type litter-mate mice at 78 dB prepulse noise (F-test, $P = 0.02$), at 82 and 86 dB ($P = 0.07$). (**C**) Effects of psychotogenic and antipsychotic drugs on *Smarca2* gene expression in the mouse brain. *Smarca2* expression levels in the mice brain after treatment with methamphetamine (METH) ($n = 5$), MK-801 ($n = 5$) or saline (control) ($n = 5$) for 12 days (a), haloperidol (HAL) ($n = 10$), olanzapine (OLZ) ($n = 10$) or saline ($n = 10$) for 7 weeks (b). Administration of drugs was by once daily intraperitoneal injection to 4-week-old C57BL/6J male mice. The average relative expression level from the prefrontal cortex, midbrain, hippocampus, thalamus and striatum of the treated group was compared with the saline groups by *t*-test.

In real-time PCR experiments, correlation of SMARCA2 gene expression and diagnosis, ethnicity, age, sex, PMI and the pH of brain samples was analyzed by one-way analysis of variance (ANOVA) tests or regression analyses by JMP computer software version 7. In linear multiple regression analysis, genotypes of the SNPs as qualitative variables, age, sex, PMI, pH, diagnosis and ethnicity of brain samples were included as variables. The genotypes of the SNPs were

assigned to 0, 1 and 2. Differences of *SMARCA2* expression levels between genotypes were analyzed by Student's *t*-tests. A $P < 0.05$ was considered as significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Supportive evidence for reduced expression of *GNB1L* in schizophrenia

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***Background*: Chromosome 22q11 deletion syndrome (22q11DS) increases the risk of development of schizophrenia more than 10 times compared with that of the general population, indicating that haploinsufficiency of a subset of the more than 20 genes contained in the 22q11DS region could increase the risk of schizophrenia. In the present study, we screened for genes located in the 22q11DS region that are expressed at lower levels in postmortem prefrontal cortex of patients with schizophrenia than in those of controls. *Methods*: Gene expression was screened by Illumina Human-6 Expression BeadChip arrays and confirmed by real-time reverse transcription-polymerase chain reaction assays and Western blot analysis. *Results*: Expression of *GNB1L* was lower in patients with schizophrenia than in control subjects in both Australian (10 schizophrenia cases and 10 controls) and Japanese (43 schizophrenia cases and 11 controls) brain samples. *TBX1* could not be evaluated due to its low expression levels. Expression levels of the other genes were not significantly lower in patients with schizophrenia than in control subjects. Association analysis of tag single-nucleotide polymorphisms in the *GNB1L* gene region did not confirm excess homozygosity in 1918 Japanese schizophrenia cases and 1909 Japanese controls. Haloperidol treatment for 50 weeks increased *Gnb1l* gene expression in prefrontal cortex of mice. *Conclusions*: Taken together with the impaired prepulse inhibition observed in heterozygous *Gnb1l* knockout mice reported by the previous study, the present findings support assertions that *GNB1L* is one of the genes in the 22q11DS region responsible for increasing the risk of schizophrenia.**

Key words: 22q11DS/haloperidol/prefrontal cortex/postmortem brain

Introduction

Schizophrenia, a devastating mental disorder that affects approximately 1% of the world's population, is a genetically complex disorder. The multifactorial polygenic model has received the most support as the mode of inheritance that underlies the familial distribution of schizophrenia; therefore, a variety of genetic, environmental, and stochastic factors are likely involved in the etiology. However, it is also possible that specific genes play major roles in susceptibility to schizophrenia. Genes involved in 22q11.2 deletion syndrome (22q11DS) substantially increases susceptibility to schizophrenia. 22q11DS is associated with several diagnostic labels including DiGeorge syndrome, velocardiofacial (or Shprintzen) syndrome (VCFS), conotruncal anomaly face, Cayler syndrome, and Opitz GBBB syndrome. Schizophrenia is a late manifestation in approximately 30% of 22q11DS cases, which is comparable to the risk to offspring of 2 parents with schizophrenia. The 22q11 deletion is detected relatively frequently in patients with schizophrenia;

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a number of studies have shown that 22q11DS schizophrenia is a true genetic subtype of schizophrenia[1,2].

Although the deleted region is approximately 3 Mbp in most patients with 22q11DS, the critical region is approximately 1.5 Mbp.[3,4] Less than 30 genes are located in the 22q11DS region. Studies of 22q11DS patients without the common chromosomal deletion suggested that the *TBX1* is a major contributor to the conotruncal malformations of 22q11DS.[5] One of the mutations in the *TBX1* was found to be a loss-of-function mutation.[6] Mice heterozygous for a null mutation in *Tbx1* develop conotruncal defects.[7] Deletion of one copy of the *Tbx1* affects the development of the fourth pharyngeal arch arteries, whereas the homozygous mutation severely disrupts the pharyngeal arch artery system.[8] The contribution of the *TBX1* haploinsufficiency to psychiatric disease was suggested by the identification of a family with VCFS in a mother and her 2 sons. These 3 patients all had a null mutation of the *TBX1*, and one of the sons was diagnosed with Asperger syndrome after psychiatric assessment.[9]

Contribution of genes in the 22q11DS region to susceptibility to schizophrenia has been examined mainly by genetic association studies. Associations between schizophrenia and nucleotide variations in the *ZNF74*,[10] *DGCR*, [11] *DGCR14*,[12] *PRODH*,[13] *ZDHHC8*,[14] *COMT*,[15–18] and *CLDN5*[19,20] genes have been reported. These associations, however, have not been confirmed in other populations[19–22] or by meta-analyses.[19–24]

Studies of genetically engineered mice have provided supporting evidence for roles of the genes located in the human 22q11DS region in schizophrenia. *Prodh* knockout mice exhibited deficits in learning and responses to psychomimetic drugs.[25] Observation of overlapping loci across 5 heterozygous mice strains with different deletion sites revealed that a 300-kb locus, which contains the *Gnb1l*, *Tbx1*, *Gp1bb*, and *Sept5* genes, is crucial for impaired sensorimotor gating measured by prepulse inhibition test (PPI).[9] In that study, the authors speculated that the *GP1BB* was unlikely to be related to schizophrenia because it is expressed only in platelets. The *GP1BB* causes Bernard-Soulier disease, which has no associated psychiatric disorders. The *Sept5* heterozygous knockout mice did not show impaired PPI. *Gnb1l* or *Tbx1* heterozygous knockout mice showed reduced PPI.[9] Therefore, the authors concluded that the *Tbx1* and *Gnb1l* are strong candidates for psychiatric disease in patients with 22q11DS.[9] In another study, however, *Tbx1* heterozygous knockout mice showed normal locomotor activity, habituation, nesting, and locomotor responses to amphetamine.[25]

Recently, Williams et al[26] reported associations between polymorphisms in the *GNB1L* gene region and schizophrenia in the United Kingdom, German, and Bulgarian population. They found excess homozygosity at rs5746832 and rs2269726 in male schizophrenia subjects and that the markers associated with male schizophrenia were related with cis-acting changes in *GNB1L* expression. These mouse and human studies indicated a correlation between *GNB1L* gene expression and psychosis.

The working hypothesis of the present study was that genes in the 22q11DS region involved in the susceptibility to schizophrenia were likely to be expressed at lower levels in patients with schizophrenia than in control subjects. We performed a scan of expressional changes of the genes in the 22q11DS region in schizophrenic and control prefrontal cortex and found that the *GNB1L* gene was compatible with our hypothesis.

Materials and methods

Human Postmortem Brains

Brain specimens were from individuals of European descent Australian and Japanese. Australian sample comprised 10 schizophrenic patients and 10 age- and gender-matched controls (Supplementary Table S1). 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 6 schizophrenic patients and 11 age- and gender-matched controls (Supplementary Table S1). In addition, postmortem brains of 37 deceased Japanese patients with schizophrenia were also analyzed (Supplementary Table S1). The Japanese subjects met the DSM-III-R criteria for schizophrenia. The study was approved by the Ethics Committees of Central Sydney Area Health Service, University of Sydney, Niigata University, University of Tsukuba, Tokyo Metropolitan Matsuzawa Hospital, and Tokyo Institute of Psychiatry.

RNA Isolation and Gene Expression Microarray

Total RNA was extracted from brain tissues with ISOGEN Reagent (Nippon Gene Co, 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. Microarrays were used to screen for differential gene expression between Australian schizophrenic patients and controls. In brief, 500 ng of total RNAs were reverse transcribed to synthesize first- and second-strand complementary DNA (cDNA), purified with spin columns, then in vitro transcripted to synthesize biotin-labeled complementary RNA (cRNA). A total of 1500 ng of biotin-labeled cRNA was hybridized on Sentrix® Human-6 Expression BeadChip (Illumina Inc., San Diego, CA) at 55°C for 18 h. The hybridized BeadChip was washed and labeled with streptavidin-Cy3, then scanned with an Illumina BeadStation 500 System

(Illumina Inc). Scanned image was imported into BeadStudio (Illumina Inc) for analysis. Forty-six thousand transcripts can be analyzed by a single BeadChip.

Real-time Quantitative RT-polymerase chain reaction

Expression of the *GSCL*, *HIRA*, *SEPT5*, *GNB1L*, *TBX1*, and *CDC45L* genes was analyzed by TaqMan Real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA). From RNA, cDNA was synthesized with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primer. Expression of these 6 genes was analyzed with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the TaqMan gene expression assays for *GSCL* (Hs00232019_m1), *HIRA* (Hs00983699_m1), *SEPT5* (Hs00160237_m1), *GNB1L* (Hs00223722_m1), *TBX1* (Hs00271949_m1), and *CDC45L* genes (Hs00185895_m1) and normalized to expression of Human *GAPDH* Control Reagents (Applied Biosystems). *GNB1L* expression was analyzed in Australian samples and replicated the analysis in Japanese subjects.

Protein Isolation and GNB1L Protein Levels in Brain

Protein was extracted from prefrontal cortex tissues with Laemmli Buffer. Western blotting method was used to compare GNB1L protein levels between schizophrenics and controls. Each of 2 μg protein was run on Pro-Pure™ SPRINT NEXT GEL (Amresco, Solon, OH) and transferrd to BioTrace™ PVDF (Nihon Pall Ltd, Tokyo, Japan). Polyclonal antibodies against the human GNB1L protein (OTTHUMP00000028644) were generated by injecting rabbits with the following peptide: CAGSKDQRISLWSLYPRA (MBL, Nagoya, Japan). Mouse polyclonal antibody against beta-actin (Sigma Aldrich Japan, Tokyo, Japan) was also used for normalization purpose. The bound primary antibodies were detected with goat anti-rabbit or anti-mouse IgG antibody HRP conjugate (MBL) and Immobilon™ Western, Chemiluminescent HRP Substrate (Millipore, Billerica, MA) on X-film (Fujifilm Medical, Tokyo, Japan). The signals of GNB1L or beta-actin of each subject on X-films were quantitated by computer software, ImageJ 1.40g (http://rsb.info.nih.gov/ij/), and GNB1L protein levels were normalized to beta-actin and compared.

Peripheral Blood and Brain DNA Sample and Genotyping

The subjects comprised 1918 unrelated Japanese patients with schizophrenia (1055 men, 863 women; mean age ± standard deviation [SD], 48.9 ± 14.5 years) diagnosed according to DSM-IV with consensus from at least 2 experienced psychiatrists and 1909 mentally healthy unrelated Japanese control subjects (1012 men, 893 women; mean age ± SD, 49.0 ± 14.3 years) of whom the first- and second-degree relatives were free of psychosis as self-reported by the subjects. The association analysis was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University, and Teikyo University, National Center of Neurology and Psychiatry, University of Tokyo, and all participants provided written informed consent. DNAs were extracted from these blood samples and the same brain tissues used for gene expression analysis. The tag single-nucleotide polymorphisms (SNPs) comprising rs5746832, rs5746834, rs2269726, rs748806, rs29807124, rs5993835, rs13057609, rs4819523, rs2073765, rs7286924, rs10372, rs3788304, and rs11704083 at the *GNB1L* gene region were selected by Haploview program using HapMap Project Japanese data set (http://www.hapmap.org/), as the previously reported schizophrenia-associated SNPs, rs5746832 and rs2269726, were forced included. The TaqMan reaction was performed in a final volume of 3 µl consisting of 2.5 ng genomic DNA and Universal Master Mix (EUROGENTEC, Seraing, Belgium), and genotying was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Genotyping quality control consisted of $\geq$98% successful calls. We confirmed concordance among repeat genotyping in $\approx$10% of genotypes.

Brain GNB1L Expression and Genotyping

The correlations between *GNB1L* expression and 13 SNPs, rs5746832, rs5746834, rs2269726, rs748806, rs29807124, rs5993835, rs13057609, rs4819523, rs2073765, rs7286924, rs10372, rs3788304, and rs11704083, were examined in Australian and Japanese brain tissues, respectively.

Mice Experiments

Mice treated with haloperidol were studied to examine the effects of antipsychotic treatments on *Gnb1l* gene expression. Thirty-nine C57/BJ6 male mice (age, 8 weeks; weight, 20–25 g) were housed under 10 h/14 h light/dark conditions with normal food and water ad libitum, where groups of 5 or 6 mice were housed separately, and 0.5 mg/kg haloperidol or saline was injected intraperitoneally once each day for 4 weeks or for 50 weeks. The dosage of haloperidol was at maximum clinically used, and 4 or 50 weeks for treatment term correspond to several years or half a lifetime in human terms, respectively. We used extreme but likely condition to clear up the effect of the medication. We determined the dosage of haloperidol according to the previous studies.[27–31] Mice were sacrificed 4 h after the last injection to obtain brain tissues.

The prefrontal cortex was taken, and RNA was extracted with RNeasy kit (Qiagen, K.K., Tokyo, Japan). A cDNA was synthesized with Revertra Ace (Toyobo) and oligo dT primer. Expression of *Gnb1l* was analyzed by TaqMan real-time polymerase chain reaction (PCR) with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with the TaqMan gene expression assay for *Gnb1l* (Mm00499153_m1). Expression of

Gnb1l was normalized to that of rodent *Gapdh* with Rodent *Gapdh* Control Reagents (Applied Biosystems).

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

Statistics

Microarray analysis was performed with GeneSpring software version 7.3.1 (Silicon Genetics, Redwood, CA). The mean background noise level was first corrected in each sample, and then per-chip normalization was applied to eliminate systematic differences between chips. Two-tailed Student's *t*-test was used to examine the difference between schizophrenic patients and controls. In real-time PCR experiments, *GAPDH* or *Gapdh* was used as an internal control, and measurement of threshold cycle (Ct) was performed in triplicate. Data were collected and analyzed with Sequence Detector Software version 2.1 (Applied Biosystems) and the standard curve method. Relative gene expression was calculated as the ratio of expression of the target gene to the internal control (*GAPDH* or *Gapdh*). Correlations of *GNB1L* gene expressions and 2 quality parameters, postmortem interval (PMI) and pH, of brain samples were analyzed with analysis of variance (ANOVA) one-way tests by JMP computer software version 5.1. The density of images reflecting GNB1L protein levels was also compared between schizophrenics and controls with the Wilcoxon test implemented in JMP computer software version 5.1. Deviation from Hardy-Weinberg equilibrium (HWE), allelic associations, and linkage disequilibrium (LD) between SNPs were evaluated with Haploview software version 3.11. A nominal association was defined when the given P value for allelic or genotypic tests was less than 5% (uncorrected $P < .05$). If a nominal significant association was found in the analysis, permutation test was also performed with Haploview software version 3.11. Correlations of *GNB1L* gene expressions and either protein expression or genotypes of the tag SNPs were analyzed with ANOVA one-way tests by JMP computer software version 5.1.

Results

Human-6 Expression BeadChip demonstrated that *GSCL* (GI_48885362-S) and *TBX1* (GI_18104949-I) of 28 genes located in the 22q11DS region were expressed at lower levels in schizophrenic brains than in the control brains in the Australian samples ($P < .05$) (Supplementary Table S2). However, the signals of these transcripts were low, and reliable confidence was not obtained from any subject. Expression of *CDC45L* (GI_34335230-S) tended to be lower in schizophrenic brains than in control brains ($P = .07$). Data of *GNB1L* were not available in this platform (Supplementary Table S2).

We used real-time PCR experiments to evaluate expression of the 3 genes that were potentially underexpressed in schizophrenia prefrontal cortex by microarray and *GNB1L*, which was not assessed by the microarray in the Australian and Japanese brain samples. The difference in gene expression between the schizophrenia and control groups was not confirmed for *CDC45L*. In addition, because the reliability of *HIRA* and *SEPT5* was not sufficient due to weakly expressed sequences in the array screening, we reexamined expression levels of these genes by real-time PCR method and did not find significant differences in gene expression between the schizophrenia and control groups. Expressions of *TBX1* and *GSCL* were too low to obtain reliable signals with the TaqMan gene expression assay (Hs00271949_m1 and Hs00232019_m1, respectively). Relative expression of *GNB1L* was significantly lower in Australian schizophrenic prefrontal brains than in Australian control brains (average ratio = 0.57, $P < .001$) and in Japanese patients with schizophrenia than in control subjects (average ratio = 0.53, $P < .0001$) (figure 1A). No difference in *GNB1L* expression was observed between the Japanese and Australian schizophrenic patient groups (data not shown). *GNB1L* expression was not significantly correlated with pH of the brain tissue samples overall (figure 2), neither with gender ($P = .62$) nor PMI ($F = 0.61$, $P = .44$). Western blotting analysis also demonstrated the lower levels of GNB1L protein in brains of the schizophrenia sample than in those of the control sample from each ethnic group (approximate average ratio = 0.75, $P = .027$ in Australian sample and approximate average ratio = 0.69, $P = .033$ in Japanese sample) (figure 1B). There is a significant correlation between gene and protein expression observed in our samples ($F = 4.7$, $P = .037$).

There were no significant associations of tag SNPs at the *GNB1L* gene studied in the present study with schizophrenia in our Japanese case-control sample (table 1). Also no significant differences were found in distributions of homozygotes and heterozygotes between schizophrenics and controls (table 1). Williams et al[26] reported male-specific associations of rs5746832 and rs2269726 with schizophrenia and correlation between those markers and the gene expression. However, such male-specific associations of rs5746832 and rs2269726 were not observed in our sample (table 1).

There was a nominally significant correlation between rs5748832 and *GNB1L* expressions in whole subjects ($P = .014$) and in Japanese ($P = .028$), but not in Australian ($P = .66$) (table 2). An allele of rs5748832 is correlated with high *GNB1L* expression in this study, while the previous study showed the opposite direction of correlation.[26]

Significant deviation from HWE in the genotypic distributions was observed at rs4819523 in the control group. Lower proportions of heterozygotes than those expected by HWE seemed to cause these deviations. Although genotype errors, chance findings, or actual

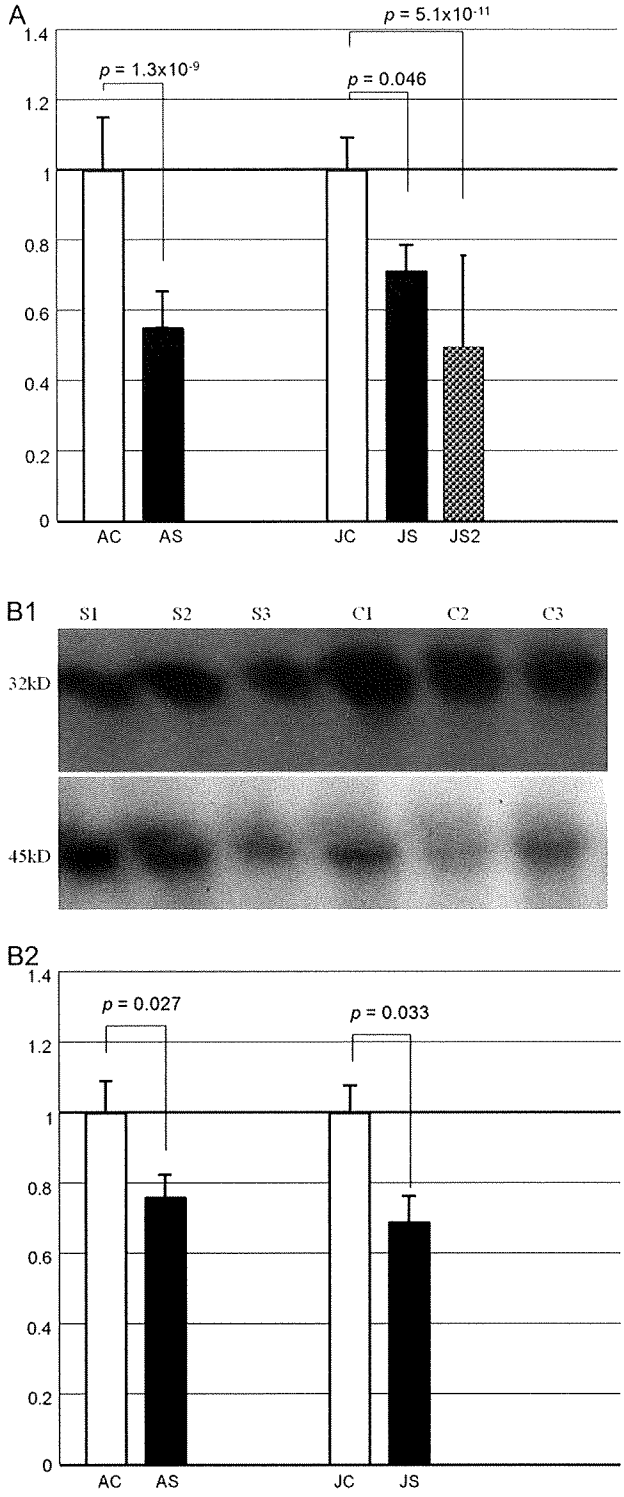

Fig. 1. GNB1L expression in schizophrenic brain (A). Relative expression of the *GNB1L* gene in prefrontal cortex from Australian control subjects (AC, n = 10), Australian schizophrenics (AS, n = 10), Japanese controls (JC, n = 11), Japanese schizophrenics (JS, n = 6), and additional Japanese schizophrenics (JS2, n = 37). The vertical scores show average of relative expression and ±1 SD in comparison with control subjects in each ethnic population, respectively. (B-1) A partial result of Western blotting was shown. Upper: GNB1L (Although expected size would be 35 kD, bands are expressed at 32 kD according to the antibody protocol) Lower: beta actin (45 kD). Samples S1–S3 are from schizophrenic patients and C1–C3 are from controls. (B-2) Relative expression of the GNB1L protein in prefrontal cortex from Australian control subjects (AC, n = 10), Australian schizophrenics (AS, n = 10), Japanese controls (JC, n = 11), and Japanese schizophrenics (JS, n = 6). The vertical scores show average of relative expression and ±1 SD in comparison with control subjects in each ethnic population, respectively.

structural variations in some subjects might have potentially caused these deviations, we could not determine which was most likely to cause these HWE deviations.

Gnb1l expression in mice was examined to exclude the possibility that reduced *GNB1L* expression was the effects of chronic treatment with antipsychotic drugs. The patients whose brains were examined in the present study had received long-term medication of typical antipsychotic drugs; therefore, we chose haloperidol as a representative antipsychotic drug. As a result, while *Gnb1l* gene expression in prefrontal cortex of mice treated with haloperidol for 4 weeks was not changed, the expression was higher in those treated with haloperidol for 50 weeks than in those with saline injected (P = .02) as shown in figure 3.

Discussion

In the present study, we hypothesized that haploinsufficiency of some genes in the 22q11DS region might increase the susceptibility to schizophrenia not only in patients with 22q11DS but also in the those without 22q11DS and that such genes would be expressed at lower levels in the brains of schizophrenic patients than in control subjects. *GNB1L* appears to meet this hypothesis. Reduced *GNB1L* gene expression was detected in both mRNA and protein levels in Australian and Japanese subjects, suggesting that lower *GNB1L* gene expression produces lower GNB1L protein levels which underlie schizophrenia across ethnicities. Treatment of mice with haloperidol indicated that the reduction of *GNB1L* expression is not likely a consequence of antipsychotic medication treatment, though the possibility of reduction of *GNB1L* expression by other antipsychotic drugs remains. The present study did not provide evidence of whether *TBX1* expression is altered significantly in schizophrenic brains because the signals detected by Illumina's Sentrix® Human-6 Expression BeadChip or TaqMan assay were very weak. Paylor et al[9] mapped PPI deficits in a panel of mouse mutants and found that PPI was impaired by either haploinsufficiency of *Tbx1* or *Gnb1l*. The present study of human brains confirms that *GNB1L* is an important candidate for susceptibility to schizophrenia.

There is little information about the function of GNB1L. *GNB1L* expression is relatively low in adult brain but is high in fetal brain. *GNB1L* encodes a guanine

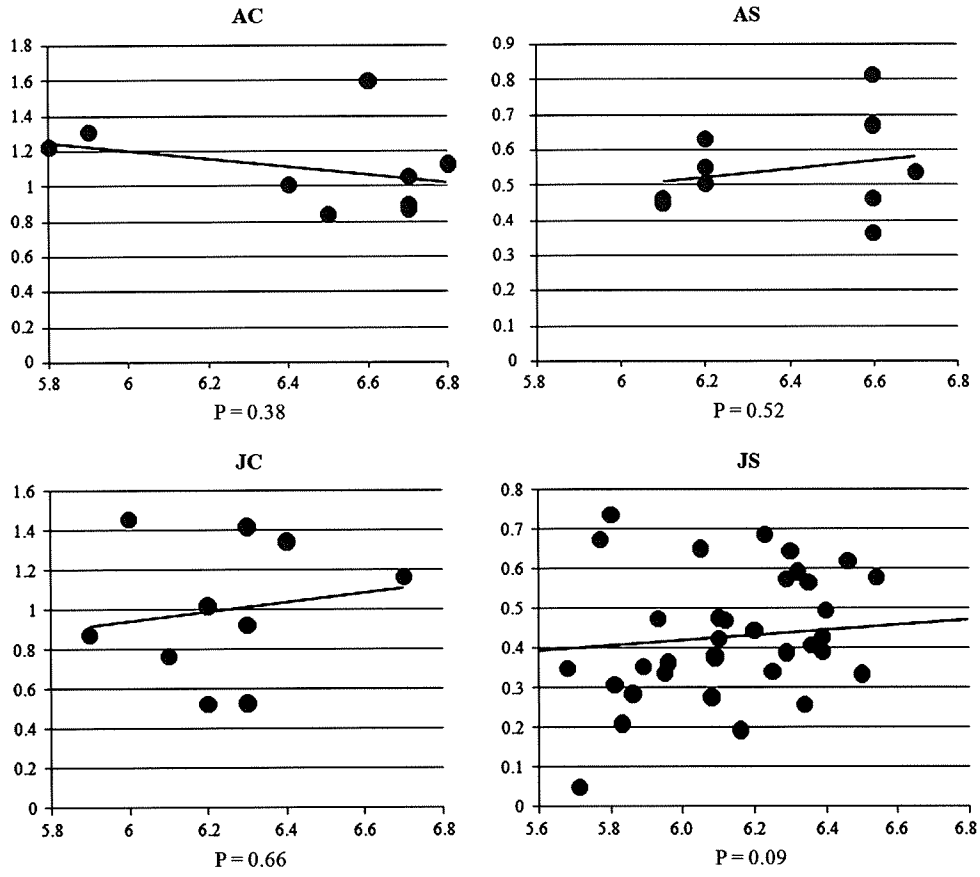

Fig. 2. *GNB1L* expression and pH in human postmortem brain Correlation of *GNB1L* expression and pH of the human postmortem brain subjects used in the same experiments shown in figure 1; AC, Australian controls; AS, Australian schizophrenics; JC, Japanese controls; JS, Japanese schizophrenics; JS2, additional Japanese schizophrenic samples. The vertical scale shows relative *GNB1L* expression and horizontal scale shows pH. Statistical *P* values are calculated below each graph.

nucleotide–binding protein (G protein), beta polypeptide 1–like, which is a member of the WD repeat protein family. WD repeats are minimally conserved regions of approximately 40 amino acids typically bracketed by Gly-His and Trp-Asp (GH-WD) that may facilitate formation of heterotrimeric or multiprotein complexes. Members of this family are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation. GNB1L contains 6 WD repeats.[32] GNB1L shows homology to the human guanine nucleotide–binding protein β subunit (GNB1). GNB1 functions in G-protein–coupled receptor protein signaling pathways and intracellular signaling cascade.

Williams et al[26] reported excess homozygosity at rs5746832 and rs2269726 in male schizophrenia subjects and that the markers associated with male schizophrenia were related with cis-acting changes in *GNB1L* expression. Firstly in the present study, we failed to confirm the association in our Japanese case-control population. Secondly, we found a nominally significant correlation between rs5746832 and *GNB1L* expression in the Japanese brain samples, but failed to find it in our limited number of the Australian samples. Further, the association between allele and gene expression in our Japanese samples was in the opposite direction from that reported in the Caucasian samples. It might be due to possible differences in LD block between haplotype phases across rs5746832 and harboring potential cis-acting variations of the gene between 2 ethnic populations. Even if such cis-acting variations are present, diagnosis has tremendous effect on the gene expression, in comparison to that of the SNP. The power of the present study to replicate the findings of excess homozygosity in male subjects is greater than 90% assuming the odd ratio of greater than 1.5 found in UK populations by Williams et al.[26] However, if the odd ratio assumes 1.3 observed in a German population by them, the power drops to 0.65. Although the gene frequencies of rs5746832 and rs2269726 were significantly different between Caucasian and Japanese populations, the frequencies of homozygotes were almost the same between 2 populations. Because of small sample size, we did not attempt allele-specific expression analysis in our brain sample. Therefore, we could not conclude whether lower GNB1L gene/protein expression in schizophrenia was

Table 1. . Analysis of Tag Single-Nucleotide Polymorphisms at the *GNB1L* Gene in the Japanese Case-Control Population

Population		Genotype count (frequency)			HWE *P*	*P*	Allele count (frequency)		*P*	Homozygote	Heterozygote	*P*
rs5746832		AA	AG	GG			A	G				
Affected	*n* = 1889	501 (0.27)	958 (0.51)	430 (0.23)	.49		1960 (0.52)	1818 (0.48)		931 (0.49)	958 (0.51)	
Male only	*n* = 1008	256 (0.25)	531 (0.53)	221 (0.22)	.08					477 (0.47)	531 (0.53)	
Controls	*n* = 1876	484 (0.26)	916 (0.49)	476 (0.25)	.31	.17	1884 (0.50)	1868 (0.50)	.14	960 (0.51)	916 (0.49)	.24
Male only	*n* = 1047	260 (0.25)	524 (0.50)	263 (0.25)	.97	.23				523 (0.50)	524 (0.50)	.23
rs5746834		GG	GT	TT			G	T				
Affected	*n* = 1898	1652 (0.87)	234 (0.12)	12 (0.01)	.24		3538 (0.93)	258 (0.07)		1664 (0.88)	234 (0.12)	
Controls	*n* = 1893	1653 (0.87)	228 (0.12)	12 (0.01)	.18	.96	3534 (0.93)	252 (0.07)	.81	1665 (0.88)	228 (0.12)	.77
rs2269726		TT	TC	CC			T	C				
Affected	*n* = 1905	338 (0.18)	896 (0.47)	671 (0.35)	.20		1572 (0.41)	2238 (0.59)		1009 (0.53)	896 (0.47)	
Male only	*n* = 1050	176 (0.17)	51 (0.49)	363 (0.35)	.87					539 (0.51)	511 (0.49)	
Controls	*n* = 1906	309 (0.16)	911 (0.48)	686 (0.36)	.82	.45	1529 (0.40)	2283 (0.60)	.31	995 (0.52)	911 (0.48)	.65
Male only	*n* = 1042	174 (0.17)	49 (0.48)	370 (0.36)	.77	.90				544 (0.52)	498 (0.48)	.69
rs748806		TT	TC	CC			T	C				
Affected	*n* = 1888	537 (0.28)	919 (0.49)	432 (0.23)	.31		1993 (0.53)	1783 (0.47)		969 (0.52)	919 (0.48)	
Controls	*n* = 1895	526 (0.28)	911 (0.48)	458 (0.24)	.10	.64	1963 (0.52)	1827 (0.48)	.39	984 (0.51)	911 (0.49)	.72
rs29807124		CC	CT	TT			A	G				
Affected	*n* = 1872	1688 (0.90)	177 (0.09)	7 (0.00)	.31		3553 (0.95)	191 (0.05)		1695 (0.91)	177 (0.09)	
Controls	*n* = 1873	1693 (0.90)	174 (0.09)	6 (0.00)	.50	.95	3560 (0.95)	186 (0.05)	.79	1699 (0.90)	174 (0.10)	.82
rs5993835		AA	AG	GG			A	G				
Affected	*n* = 1881	1474 (0.78)	374 (0.20)	33 (0.02)	.10		3322 (0.88)	440 (0.12)		1507 (0.80)	374 (0.20)	
Controls	*n* = 1871	1484 (0.79)	365 (0.20)	22 (0.01)	.93	.31	3333 (0.89)	409 (0.11)	.30	1506 (0.80)	365 (0.20)	.77
rs13057609		AA	AG	GG			A	G				
Affected	*n* = 1888	13 (0.01)	271 (0.14)	1604 (0.85)	.68		297 (0.08)	3479 (0.92)		1617 (0.86)	271 (0.14)	
Controls	*n* = 1883	10 (0.01)	272 (0.14)	1601 (0.85)	.67	.82	292 (0.08)	3474 (0.92)	.86	1611 (0.86)	272 (0.14)	.93
rs4819523		GG	GC	CC			G	C				
Affected	*n* = 1886	536 (0.28)	932 (0.49)	418 (0.22)	.74		2004 (0.53)	1768 (0.47)		954 (0.51)	932 (0.49)	
Controls	*n* = 1896	562 (0.30)	887 (0.47)	447 (0.24)	.008	.26	2011 (0.53)	1781 (0.47)	.90	1009 (0.53)	887 (0.47)	.10
rs2073765		CC	CT	TT			C	T				
Affected	*n* = 1887	90 (0.05)	586 (0.31)	1211 (0.64)	.08		766 (0.20)	3008 (0.80)		1301 (0.69)	586 (0.31)	
Controls	*n* = 1895	76 (0.04)	590 (0.31)	1229 (0.65)	.62	.52	742 (0.20)	3048 (0.80)	.47	1305 (0.69)	590 (0.31)	.97
rs7286924		AA	AT	TT			A	T				
Affected	*n* = 1892	808 (0.43)	848 (0.45)	236 (0.12)	.56		2464 (0.65)	1320 (0.35)		1044 (0.55)	848 (0.45)	
Controls	*n* = 1898	800 (0.42)	872 (0.46)	226 (0.12)	.62	.75	2472 (0.65)	1324 (0.35)	.98	1026 (0.54)	872 (0.46)	.47
rs10372		AA	AG	GG			A	G				
Affected	*n* = 1902	10 (0.01)	244 (0.13)	1648 (0.87)	.77		264 (0.07)	3540 (0.93)		1658 (0.87)	244 (0.13)	
Controls	*n* = 1904	6 (0.00)	244 (0.13)	1654 (0.87)	.34	.60	256 (0.07)	3552 (0.93)	.65	1660 (0.87)	244 (0.13)	.96
rs3788304		CC	CG	GG			C	G				
Affected	*n* = 1907	1056 (0.55)	720 (0.38)	131 (0.07)	.58		2832 (0.74)	982 (0.26)		1187 (0.62)	720 (0.38)	
Controls	*n* = 1906	1058 (0.56)	720 (0.38)	128 (0.07)	.71	.98	2836 (0.74)	976 (0.26)	.81	1186 (0.62)	720 (0.38)	.97
rs11704083		AA	AG	GG			A	G				
Affected	*n* = 1909	660 (0.35)	915 (0.48)	334 (0.17)	.58		2235 (0.59)	1583 (0.41)		994 (0.52)	915 (0.48)	
Controls	*n* = 1897	646 (0.34)	931 (0.49)	320 (0.17)	.62	.76	2223 (0.59)	1571 (0.41)	.92	966 (0.51)	931 (0.49)	.50

Table 2. Correlation Between Genotype and *GNBIL* Gene Expression in Brain

SNP	Genotype	*n*	Expression	Genotype	*n*	Expression	Genotype	*n*	Expression	*P* value
rs5746832	AA	19	0.82	AG	16	0.63	GG	21	0.56	.014
Australian	AA	8	0.90	AG	7	0.75	GG	4	0.84	.660
Japanese	AA	11	0.77	AG	9	0.54	GG	17	0.50	.028
rs5746834	GG	46	0.68	GT	11	0.52	TT	2	0.71	.391
rs2269726	TT	22	0.58	TC	20	0.72	CC	15	0.72	.224
rs748806	TT	15	0.84	TC	15	0.55	CC	32	0.63	.105
rs29807124	CC	47	0.68	CT	7	0.85	TT	3	0.29	.063
rs5993835	AA	53	0.61	AG	6	0.62	GG	0	NA	.794
rs 13057609	AA	0	NA	AG	7	0.57	GG	54	0.67	.479
rs4819523	GG	14	0.66	GC	26	0.61	CC	19	0.72	.601
rs2073765	CC	4	0.89	CT	17	0.48	TT	38	0.70	.520
rs7286924	AA	33	0.64	AT	21	0.77	TT	9	0.49	.112
rslO372	AA	0	NA	AG	8	0.67	GG	52	0.52	.261
rs3788304	CC	37	0.65	CG	21	0.64	GG	5	0.73	.731
rs11704083	AA	22	0.75	AG	23	0.56	GG	17	0.67	.412

due to cis-acting differences by genetic polymorphisms in this locus or not in this study.

The present study showed that reduced expression of *GNB1L* may be involved in the pathophysiology of schizophrenia; however, it does not exclude the possibility that other genes in the 22q11DS region contribute to the susceptibility to schizophrenia. The array used in the present study did not examine all isoforms of the genes in the 22q11DS region. In addition, the reliability of weakly expressed sequences in the array screening is not sufficient. Therefore, we reexamined expression levels of the genes, which reliable data (greater than 0.96 confidence) was produced by the array in no subjects, by real-time PCR method. The study is also limited by the areas and ages of the brains examined. We examined only adult postmortem prefrontal cortex. Differential gene expression in other brain regions or during other developmental stages may also influence the susceptibility to schizophrenia.

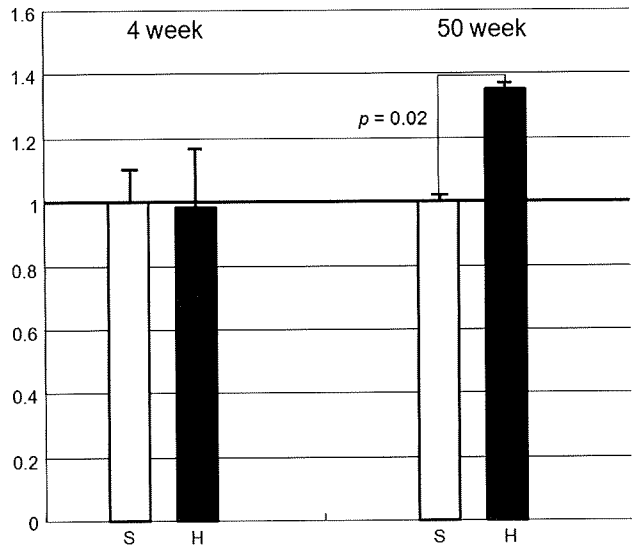

Fig. 3. Effect of haloperidol treatment on *Gnb1l* expression. Relative expression of the *Gnb1l* gene in mouse prefrontal cortex in saline treated (S) or haloperidol treated (H) mice during 4 or 50 weeks. The vertical scale shows relative *Gnb1l* expression compared with that in saline-treated mice, with bars for ±1 SD calculated in each group, respectively.

The consortium data of the Stanley Medical Research Institute showed no significant differences ($P > .05$) in the following gene expression levels in postmortem prefrontal cortex between patients with schizophrenia and controls: *DGCR6, PRODH, DGCR2, STK22B, DGCR14, CLTCL1, CLTCL1, HIRA, UFD1L, CDC45L, CLDN5, TBX1, FLJ21125, TXNRD2, COMT, ARVCF, DKFZp761P1121, DGCR8, HTF9C, RANBP1*, and *ZDHHC8*. The expression of *RTN4R* might be potentially reduced ($P = .02$). No data were available for *GSCL, MRPL40, SEPT5, GP1BB*, and *GNB1L* (http://www.stanleyresearch.org/brain/menu.asp).

A trans-acting effect on expression of the disease gene may also be expected to modulate disease susceptibility. Large-scale studies in humans have indicated that a significant proportion of the heritable variance in gene expression is attributable to trans-acting polymorphism.[33,34] As one of the examples, recent study reported that microRNAs regulate gene expression posttranscriptionally.[35] Even for schizophrenia, Bray et al[36] indicated that the reduction in *DTNBP1* expression in schizophrenia is likely to result in part from trans-acting risk factors. Such

trans-acting factors that regulate *GNB1L* gene expression, however, have not been identified.

In conclusion, the present study further supports the role of *GNB1L* in the pathophysiology of schizophrenia.

Supplementary Material

Supplementary tables are available at http://schizophreniabulletin.oxfordjournals.org/.

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Original article

Study of *HOXD* genes in autism particularly regarding the ratio of second to fourth digit length

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Abstract

Multiple genes are involved in the pathogenesis of autism. To study the causative gene, the relationship between autism endophenotypes and their closely related genes has been analyzed. There is a subgroup of autism spectrum disorder (ASD) in which the ratio of second digit length to fourth digit length (2D/4D) is low (short digit group, SDG). We studied the relationship between ASD and *HOXD* genes, which are located in the candidate locus for ASD and are associated with digit morphogenesis, with a particular focus on SDG. We analyzed 25 SNPs of *HOXD11*, *HOXD12*, and *HOXD13* in the subject of 98 ASD, 89 healthy controls, and 16 non-autistic patients (non-ASD). There was no significant difference in the genotype frequencies between the ASD and the healthy controls. However, the G-112T heterozygote in the promoter region of *HOXD11* was observed in only four patients with ASD and in none of the healthy controls or non-ASD subjects. Moreover, this *HOXD11* G-112T was observed in three of 11 SDG with ASD but in none of the 15 non-SDG patients with ASD. There were eight SDG patients among the non-ASD ones, but this polymorphism was observed in none of them. Considering the above results, it is expected that candidate genes will be further identified, using *HOXD11* G-112T polymorphism as a marker, by analyzing genes located near 2q in a larger number of ASD subjects with clinical signs of SDG.

Keywords: Autism; *HOXD*; 2D/4D; Endophenotype; Genetic polymorphism

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1. Introduction

Autism is basically characterized by severely impaired social interaction and communication, and a limited range of activities and interests. As the diagnosis of autism is made on the basis of patients' behavioral characteristics, the disorder is not caused by only one factor. It is considered that various genetic and environmental factors are involved in the occurrence of autism, and their interactions are complex. In 1998, the International Molecular Genetic Study of Autism Consortium (IMGSC) reported their genome-wide linkage analysis of families in which there was more than one member with idiopathic autism [1]. On the basis of the results of a subsequent large-scale genome-wide scan, candidate

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gene loci, including 7q21.2–q36.2, 16p12.1–p13.3, 6q14.3–q23.2, 2q24.1–q33.1, 17q11.1–q21.2, 1q21–q44, and 3q21.3–q29, were identified [2]. In an attempt to increase the linkage, a nearly homogeneous group was selected among patients with autism of heterogeneous causes. Autism patients were classified into subgroups or subsets in accordance with the phenotype of autism [3], such as through a quantitative trait locus (QTL) analysis of the constituent elements of endophenotypes in autism [4], and an ordered-subset analysis [5] was carried out. The ratio of second digit (2D) length to fourth digit (4D) length (2D/4D) is very low in some autism patients [6,7]. The *homeo box D (HOXD)* gene family is involved in skeletal morphogenesis, and correlations between digit length and the expression levels of *HOXD11*, *HOXD12*, and *HOXD13* have been observed [8,9]. In addition, *HOXD* genes form a cluster at 2q24.1–q33.1, which has been found to be a candidate locus by a genome-wide scan [3]. Therefore, we considered that digit length is one of the small physical signs of autism. Hence, we investigated the relationships between autism and polymorphism of *HOXD11*, *HOXD12*, and *HOXD13*. Moreover, we classified autism patients into two categories: patients with a low 2D/4D formed the short digit group (SDG), while the remaining patients formed the non-short-digit group (non-SDG). We also examined the genetic polymorphism of these three genes between SDG and non-SDG with autism and also between SDG with and without autism. No analysis of autism focusing on these relationships has been reported to date.

2. Subjects and methods

Seven patients with autism in the SDG were screened for the presence or absence of gene mutations in the exon and intron of *HOXD11*, *HOXD12*, and *HOXD13*, and for gene polymorphisms. The genotypic frequencies of the detected polymorphisms and the polymorphisms already listed in the GenBank were compared between the autism patients and the controls. Finally, the genotypes of the above polymorphisms of the autism patients in SDG were investigated.

2.1. Subjects

The subjects examined by genetic analysis in this study were 98 patients who visited the Department of Pediatrics, Hamamatsu University School of Medicine and Hamamatsu City Medical Center for Developmental Medicine, and who were diagnosed as having autism, PDD-NOS, and Asperger syndrome on the basis of the criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV [10]). Patients with clear underlying diseases such as chromosomal abnormalities, tuberous sclerosis, and Fragile X syndrome were excluded from the study. The patients were of 82 males and 16 females with ages ranging from 5 years and 2 months to 31 years and 10 months (mean age: 12 years and 7 months). In terms of ethnicity, 95 patients had Japanese parents, 2 had Japanese fathers and Filipino mothers, and 1 had Bangladeshi parents. Eighty-nine subjects without any neurological abnormality served as healthy controls for gene analysis; all of them were Japanese and their sex and age were not determined. Thirty patients were also examined as disease controls, including 16 non-autistic patients, 14 mentally retarded patients, and 2 AD/HD patients, all of whom were Japanese.

2.2. Measurement of second and fourth digit lengths

A digital camera providing three-megapixel images was used for the measurement of the 2D and 4D lengths. Each subject's right hand was placed palm-up on a flat desk, and was photographed with the camera 20 cm above the hand. Three pediatric neurologists separately measured the 2D and 4D lengths from the line of the base to the tip of the digits three times using the image analyzing software Scion Image (NIH). The mean ratio of 2D length to 4D length (2D/4D) was calculated. In this study, patients with lower than the mean 2D/4D of the autism patients reported by Osawa et al., that is, a 2D/4D of 0.94 or lower, were classified as SDG [7].

2.3. Gene analysis

Seven patients with autism (6 males and 1 female) in the SDG were screened for the presence or absence of gene mutations and gene polymorphisms by the direct sequencing method. *HOXD11*, *HOXD12*, and *HOXD13* – each consisting of two exons and one intron – were searched for in a region from approximately 500 bp upstream, including a promoter, to approximately 500 bp downstream of the gene. Genomic DNA extracted from lymphocytes using a DNA extraction kit (Takara Co., Shiga, Japan) was used. DNA was amplified by PCR using a Taq PCR Core kit (QIAGEN Co., CA, USA), and the base sequence was obtained by the direct sequencing method. Genotypes were determined for single nucleotide polymorphism (SNP) in five loci that were newly found by this method in this study and for SNP in 20 loci that are listed in the online database GenBank (NCBL dbSNP). Genotypes in some loci were also determined by real-time PCR analysis using a TaqMan allelic discrimination assay (Applied Biosystems).

2.4. Statistical analysis

Genotypic frequency and allelic frequency of the autism patients were compared to those of the healthy con-

trol group using a χ^2 test or Fisher's exact test with SPSS 12.0J for a Windows-based System. A statistical significance level of $p \leqq 0.05$ was set.

3. Results

2D/4D was determined in 28 patients (24 males and 4 females) out of the 98 autism patients. Eleven patients (9 males and 2 females) of these 28 patients were classified as SDG. The clinical features of these patients, including sex, age, and the severity of mental retardation, are shown in Table 1. A high percentage of patients with severe mental retardation were observed in SDG with autism, whereas no patients with severe mental retardation were observed in non-SDG with autism. We also measured 2D/4D in 16 non-autistic patients in the disease control group, and 8 patients were classified as SDG and 8 patients as non-SDG. The results of the 2D/4D values of the 28 ASD and 30 non-ASD patients are shown in Fig. 1.

The results of the polymorphism analysis are shown in Table 2. No significant difference in polymorphism was observed between the autism patients and the healthy control group. However, with regarding to SNP in the promoter region of *HOXD11* G-112T, heterozygosity was observed in 4 autism patients, but not in the healthy or disease control group. The SNP in the promoter region of *HOXD12* -C226A and the SNP in

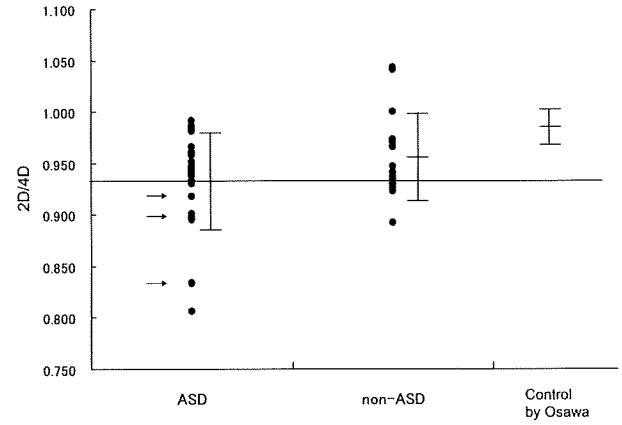

Fig. 1. 2D/4D values for the ASD28 cases and the non-ASD30 cases. Mean ± SD was presented. The M-ASD line is the average for the ASD cases; at or below this line is the SDG. As a reference, we showed the mean ± SD for normally healthy children as calculated by Osawa et al. [7]. The arrow indicates cases with *HOXD11* heterogeneity.

Table 1
Clinical features of patients.

	All the autistic disorder patients	Patients with 2D/4D determined		
		Total	SDG	NSDG
Number of patients	98	28	11	17
Sex				
Males:females	82:16 (5.1:1)	24:4 (5.5:1)	9:2 (4.5:1)	15:2 (7.5:1)
Age	5 y 2 m–31 y 10 m	5 y 4 m–31 y 10 m	8 y 1 m–31 y 10 m	5 y 4 m–16 y 7 m
Median	11 y 6 m	12 y 0 m	14 y 4 m	9 y 2 m
Mean	12 y 7 m	12 y 11 m	16 y 6 m	10 y 4 m
Family history: (3 generations)				
With[a]	22 (22.4%)	10 (35.7%)	3 (30.0%)	7 (41.2%)
Those with autism	7 (7.3%)	5 (17.9%)	2 (20.0%)	3 (17.4%)
Without	69 (70.4%)	16 (57.1%)	7 (70.0%)	7 (41.2%)
Mental retardation				
Without	10 (10.3%)	7 (25.0%)	2 (18.2%)	5 (29.4%)
Minor	21 (21.6%)	6 (21.4%)	2 (18.2%)	4 (23.5%)
Moderate	44 (45.4%)	10 (35.7%)	2 (18.2%)	8 (47.1%)
Severe	22 (22.7%)	5 (17.9%)	5 (45.5%)	0
Age at walk alone	9–48 m (91 cases)	9–48 m (26)	11–48 m (10)	9–18 m (16)
Median	13 m	12 m	12 m	12 m
Mean	13.9 m	14.3 m	18 m	12.9 m
Age at first word	10 m–6 y 10 m (80 cases)	11 m–6 y 10 m (25)	11 m–6 y 10 m (10)	1 y 3 m–3 y 5 m (15)
Median	1 y 6 m	1 y 6 m	1 y 6 m, 1 y 11 m	1 y 10 m
Mean	1 y 9 m	2 y 1 m	2 y 4 m	1 y 11 m
No. of patients 2 y or over	28	10	4	6
Age at first phrase	1 y 6 m–5 y 0 m (31 cases)	1 y 7 m–5 y 0 m (13)	2 y 6 m–5 y 0 m (5)	1 y 7 m–4 y 0 m (8)
Median	2 y 11 m	2 y 11 m	3 y 0 m	2 y
Mean	2 y 10 m	2 y 9 m	3 y 2 m	2 y 5 m
No. of patients 3 y or over	16	5	3	2

[a] Family history with psychiatric disorders including major depression, autism etc.

Table 2
Results of analysis of gene polymorphisms.

Gene	Location in gene	dtSNP ID	Allele	Frequency		Genotype	Frequency	
				Autism	Control		Autism	Control
HOXD11	Promoter		G	0.979	1	GG	0.959	1
			T	0.021	0	GT	0.042	0
						TT	0	0
	Intron	rs84746	A	0.711	0.721	AA	0.571	0.561
			C	0.289	0.288	AC	0.230	0.371
						CC	0.133	0.067
	Exon 2	rs863678	G	0.541	0.567	GG	0.316	0.292
			T	0.459	0.443	GT	0.449	0.551
						TT	0.235	0.157
	Exon2	rs6745764	A	0.214	0.18	AA	0.031	0.011
			G	0.786	0.82	AG	0.367	0.337
						GG	0.602	0.652
HOXD12	Promoter		A	0.041	0.028	AA	0	0
			C	0.959	0.972	AC	0.082	0.056
						CC	0.918	0.944
	Promoter		G	0.929	0.955	GG	0.929	0.955
			T	0.071	0.045	GT	0.071	0.045
						TT	0	0
	Exon 1	rs847151	A	0.041	0.028	AA	0	0
			G	0.959	0.972	AG	0.082	0.056
						GG	0.918	0.944
HOXD13	Promoter	rs847196	C	0.893	0.938	CC	0.786	0.876
			G	0.107	0.061	CG	0.214	0.124
						GG	0	0
	Promoter		A	0.082	0.107	AA	0	0
			T	0.918	0.893	AT	0.163	0.213
						TT	0.837	0.787
	Exon 1		C	0.985	0.989	CC	0.969	0.978
			T	0.015	0.011	CT	0.031	0.022
						TT	0	0
	Exon 1	rs2518053	A	0.408	0.455	AA	0.173	0.235
			G	0.592	0.545	AG	0.469	0.438
						GG	0.357	0.326
	Intron	rs847194	A	0.684	0.657	AA	0.459	0.404
			C	0.316	0.343	AC	0.449	0.506
						CC	0.092	0.09

SNP with no polymorphism detected in the present cases analyzed among the SNPs listed at the GenBank

HOXD11	Promoter	rs2736846	*HOXD13*	Exon 1	rs847195
	Intron	rs2736847		Exon 1	rs13392701
	Exon 2	rs12995279		Intron	rs847193
	Exon2	rs12995280		Intron	rs847192
HOXD12	Exon 1	rs2551807		Exon 2	rs28928892
	Exon2	rs2553776		Exon 2	rs28933082
				Exon 2	rs28928891

exon 1 of *HOXD12* (rs847151, G364A) showed a nearly complete linkage disequilibrium. Heterozygosity for both *HOXD12* -C226A and *HOXD12* G364A was observed in five healthy controls and eight autism patients. Furthermore, all of the five controls heterozygous for *HOXD12* -C226A and *HOXD12* G364A were homozygous for *HOXD11* -G112G. On the other hand, of the eight autism patients heterozygous for both *HOXD12* -C226A and *HOXD12* G364A, four were homozygous and four were heterozygous for *HOXD11* G-112T. Taken together, heterozygosity in all the three loci *HOXD11* G-112T, *HOXD12* -C226A, and *HOXD12* G364A was found in four autism patients but not in the healthy controls. Table 3 shows the relationships between the polymorphisms in these three loci for two cases: SDG and non-SDG with autism and SDG and non-SDG without autism. Of the four patients heterozygous for *HOXD11* G-112T, three in whom digit length was measured were classified into SDG with autism and the rest was unknown. The clinical type of ASD of the patients with having *HOXD11* heterogeneity was classified as autistic disorder in all cases. No patients

Table 3
Frequency of *HOXD* gene polymorphisms between SDG and NSDG patients with or without autism.

Gene	dbSNP ID	Genotype	Autistic patients			Normal	Non-autistic	
			Total	SDG	NSDG	Control	SDG	NSDG
HOXD11		GG	94	8	17	89	8	8
		GT	4	3	0	0	0	0
HOXD12		CC	90	8	17	84	8	8
		AC	8	3	0	5	0	0
HOXD12	rs847151	GG	90	8	17	84	8	8
		AG	8	3	0	5	0	0

heterozygous for *HOXD11* G-112T were observed among the 16 non-autistic disease controls including the eight patients with SDG.

4. Discussion

In genetic research for autism, some studies have been conducted that focused mainly on language development skills (e.g., age at first word, age at first phrase, onset of first phrase >36 months, and nonverbal communication) skill. Other studies have focused on the establishment of motor language development, bladder and bowel control milestones, developmental regression, repetitive/stereotyped behavior, restricted behavior, interest, and activity [2–4,11–13].

Manning et al. [6] reported that 2D/4D is low in autism and Asperger syndrome. In Japan, Osawa et al. [7] reported a higher incidence of low 2D/4D in autism patients than in healthy children. From their report, we assumed that it is possible to consider a low 2D/4D as a specific feature in some autism patients. Such patients formed part of a group of subjects (SDG) for investigation in our study. It was assumed that SDG in autism may express one of the common features; hence, 2D/4D may be associated with one of the etiological genes of autism. Manning et al. [14] reported the findings of their 2D/4D measurement as follows: (1) there is a gender difference in 2D/4D measurements (2D/4D is lower in males than in females); (2) a low 2D/4D is observed across races and countries; (3) 2D/4D is closely related to fetal growth, sperm count, family size, myocardial infarction, and breast cancer; and (4) 2D/4D is related to sexual differentiation, the production of sex hormones in the fetal stage, and disease programming in the fetal stage. In addition, there is an inverse correlation between 2D/4D and testosterone concentration at the fetal stage, and 2D/4D correlates with the CAG repeat number in the androgen receptor gene [15].

A study of female twins conducted by Paul et al. [16] showed that the concordance rate of 2D/4D is higher in monozygotic twins than in dizygotic twins, that the heritability of 2D/4D is approximately 66%, and that the genetic contribution to 2D/4D in females may be more influential than the effects of prenatal environmental factors. Although it is uncertain whether these findings differ significantly between males and females in the absence of any report for males, it seems possible that 2D/4D is affected by both hereditary and secondary perinatal environmental factors.

One study showed that the mean 2D/4D did not change with gestational age from the 9th week to the 40th week [17]. In addition, there was a small increase in 2D/4D with age, which was lowest in the right hand [18]. This study indicates that 2D/4D is probably established in the uterus and that this ratio remains almost constant until adult life.

Because 2D/4D, an easily measurable physical feature, is already determined in utero and remains constant until adult life, it can be used regardless of age differences among subjects and is universal; moreover, its measurement is noninvasive. Therefore, 2D/4D is an excellent parameter for evaluating a group of autistic patients.

In genomic scans of families having more than one member with autism, the susceptibility loci for autism were investigated, and identified; these included 2q21–q33 [3,4]. In the candidate genes located here, the NRP2 gene is reported as one of the genes related to autism [19]. In addition, specific polymorphism has been found in distal-less 2 (DLX2) and cAMP guanine nucleotide exchange factor II (cAMP-GEFII) in a few cases of autism [20]. On the other hand, no significant correlation has been reported between autism and distal-less 1 (DLX1) [20,21] and DLX2 [20]. With regard to *HOXD* genes, Bacchlli et al. reported that there is no relationship between *HOXD1* and autism [20]. There has been no report on *HOXD11*, *HOXD12* or *HOXD13* to date.

It seems that these genes may be found to be significant in the development of autism when cases as a study subject have been carefully chosen and classified by the specific characteristics of presenting behavior or phenotypic clinical presentations.

The present study has limitations because it is a case-control study, rather than a family study, with a small number of subjects enrolled. However, in this study,

HOXD11 SNP -112G/-112T heterozygosity was specifically observed in autism patients with low 2D/4D. On the basis of this result, we expect that the relationships between autism and the *HOXD* genes or other candidate genes located in 2q will be clarified by studying a larger population with low 2D/4D, that is, by studying patients heterozygous for -112G/-112T in the *HOXD11* promoter.

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