Table 4. Evolutionary conservation information for rs61742029 and L59P

Mutation	Species	Match	Gene	AA	Alignment
L59P	Human	—	ENST00000380393	59	K T S N P T S S L T S *L S V A P T F S P N I T L
	Mutant	Not conserved	—	59	K T S N P T S S L T S *P S V A P T F S P N I T
	P. Troglodytes	All identical	ENSPTRG00000033879	59	K T S N P T S S L T S *L S V A P T F S P N I T
	M. Mulatta	All identical	ENSMMUG00000005878	59	K T S N P T S S L T S *L S V A P T F S P N I T
	F. Catus	All identical	ENSFCAG00000019232	59	K T S S P A S S V T S *L S V A P T F S P N L T
	M. Musculus	All identical	ENSMUSG00000027303	59	K T S N S T S S V I S *L S V A P T F S P N L T
	G. Gallus	All identical	ENSGALG00000015995	56	*L N V S - - - S P M T T
	T. Rubripes	All identical	ENSTRUG00000014770	99	P T P S P A S D G T L *L Q A D P N A T G R V L
	D.rerio	Not conserved	ENSDARG00000001769	101	P P V V P P P A V P I *P T V V L P V P P T P T
	D. Melanogaster	No homologue	—	N/A	
	C. Elegans	No alignment	C09D8.1	N/A	
	X. Tropicalis	All conserved	ENSXETG00000017982	71	T T A PF T T T R T A *V I L A P N V T D S I F
rs61742029	Human			664	L K K E E E C E S Y T *V R D L L V T N T R E N
	mutated	all conserved		664	S Y T . R D L L V T N T R E N
	Ptroglodytes	all identical	ENSPTRG00000033879	673	L K K E E E C E S Y T *V R D L L V T N T R E N
	Mmulatta	all identical	ENSMMUG00000005878	673	L K K E E E C E S Y T *V R D L L V T N T R E N
	Fcatus	all identical	ENSFCAG00000019232	674	L K K E E E C E S Y T *V R D L L V T N T R E N
	Mmusculus	all identical	ENSMUSG00000027303	700	L K K E E E C E S Y T *V R D L L V T N T R E N
	Ggallus	all identical	ENSGALG00000015995	680	L K K E E E C E S Y T *V R D L L V T N T R E N
	Trubripes	all identical	ENSTRUG00000014770	710	Y T *V R D L L V T N N R E N

*Marks the position of the amino acid change due to mutation.
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Table 5. In silico functional effect prediction for rs61742029 and L59P.

Mutation	Prediction Tool		
	PolyPhen-2	Pmut	SIFT
rs61742029	Probably damaging	Neutral	Tolerated
L59P	Benign	Neutral	Tolerated

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areas, were not sequenced (the rare intronic mutations we detected close to the exons can be viewed in Table S1).

Conclusion

In conclusion, our study did not detect any rare missense mutations within the *PTPRA* gene in our samples that showed statistical association with SCZ or ASD. Nonetheless, some potentially interesting variants were identified that might increase the susceptibility of their carriers to the disorders. Also, our results may help provide genetic clues for the involvement of the *PTPRA* gene in the pathogenesis of psychiatric disorders.

Supporting Information

Table S1 Rare intronic mutations identified during the resequencing stage. [a]: Based on NCBI build 37.1. [b]: Based on NCBI Reference Sequence NC_000020.10. All mutations are heterozygous.
(DOCX)

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Author Contributions

Conceived and designed the experiments: JX BA MI NI NO. Performed the experiments: JX CW HK YT. Analyzed the data: JX SK AY YN TK IK BA NO. Contributed reagents/materials/analysis tools: JX YU TO BA MI NI NO. Wrote the paper: JX SK AY YN TK MB IK YU TO BA NO.

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Novel rare variants in F-box protein 45 (*FBXO45*) in schizophrenia

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ABSTRACT

The ubiquitin ligase F-box protein 45 (FBXO45) is critical for synaptogenesis, neuronal migration, and synaptic transmission. *FBXO45* is included in the 3q29 microdeletion region that confers a significant risk for schizophrenia, as shown by rare structural variant studies. Thus, *FBXO45* is considered a prominent candidate for mediating schizophrenia pathogenesis. Here, we investigated rare, deleterious single nucleotide variants (SNVs) as well as small insertions and deletions (INDELs) in *FBXO45* that may contribute to schizophrenia susceptibility.

Using Sanger sequencing, we performed mutation screening in *FBXO45* exon regions in 337 schizophrenia patients. Novel missense or nonsense variants were followed up with a genetic association study in an independent sample set of 601 schizophrenia patients and 916 controls, a case report for assessing the clinical consequence of the mutations, a pedigree study for measuring mutation inheritance in the proband's family, bioinformatics analyses for evaluating mutation effect on protein structure and function, and mRNA expression analysis for examining mutation transcriptional influence on *FBXO45* expression.

One heterozygous, novel, and rare missense mutation (R108C) was identified in a single schizophrenia patient and in his healthy mother. At age 20, this patient was diagnosed with paranoid schizophrenia and carried some clinical features of 3q29 deletion phenotypes, including premorbid IQ decline. With follow-up genotyping, this mutation was not found in either the schizophrenia group (0/601) or the healthy control group (0/916). Bioinformatics analyses predicted that R108C probably pathologically impacted the structure and function of the FBXO45 protein. The relative expression of *FBXO45* in SCZ case with R108C mutation was relatively low when compared to 50 schizophrenia patients and 52 healthy controls.

The R108C mutation in *FBXO45* is a rare variant with a modest effect on schizophrenia risk that may disrupt the structure and function of the FBXO45 protein. Our findings also suggest that *FBXO45* may be a new attractive candidate gene for schizophrenia.

1. Introduction

Schizophrenia (SCZ) is a severe psychiatric disorder with a lifetime prevalence of around 1% (Lewis and Lieberman, 2000) and a heritability of 64% (Lichtenstein et al., 2009). Despite high heritability, the genetic basis of SCZ remains largely unknown despite many years of researches. The genetic architecture of SCZ has been explored through genome-wide association studies (GWAS), rare structural variant studies, and next-generation sequencing (NGS). GWAS have identified common variants with extremely small effects on risk and have clarified that heritability of SCZ cannot be explained only by such common variants (Purcell et al., 2009; Shi et al., 2009; Stefansson et al., 2009). The rare variant model is supported by rare structural variant studies in which individual rare copy number variants (CNVs) with large effects increase susceptibility to SCZ (Consortium, 2008; Stefansson et al., 2008; Walsh et al., 2008). NGS analysis suggested that some SCZ cases are caused by highly penetrant de novo variants (Girard et al., 2011; Xu et al., 2011; Need et al., 2012; Xu et al., 2012).

Several lines of evidence from rare structural variant studies have demonstrated that a 0.8- to 1.6-Mb deletion spanning 3q29 confers a significant risk for SCZ (Consortium, 2008; Walsh et al., 2008; Mulle et al., 2010; Levinson et al., 2011; Vacic et al., 2011). The clinical phenotype of the 3q29 deletion often includes mild to moderate mental retardation, autistic features, symptoms of SCZ, microcephaly, and dysmorphology (chest wall deformity, high nasal bridge, cleft palate,

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horseshoe kidney, etc.) (Willatt et al., 2005; Ballif et al., 2008). Additionally, genome-wide linkage analyses suggest a significant linkage between SCZ and bipolar disorder and the chromosome 3q29 telomere region (Bailer et al., 2002; Devlin et al., 2002; Schosser et al., 2004; Schosser et al., 2007). The commonly deleted 3q29 microdeletion region spans about 20 genes, one of which is *F-box protein 45* (*FBXO45*), which encodes an ubiquitin ligase.

Ubiquitylation is a rapid, local, and reversible post-translational modification of proteins that is related to regulation of synaptic processes (DiAntonio and Hicke, 2004; Kawabe and Brose, 2011). The correlation between SCZ and dysregulation of ubiquitin proteasome system (UPS) has been implicated by a variety of gene expression analyses in post-mortem brain tissue (Vawter et al., 2001; Middleton et al., 2002; Vawter et al., 2002; Altar et al., 2005) and peripheral blood (Bousman et al., 2010a; Bousman et al., 2010b). Protein ubiquitylation is catalyzed by a cascade of enzyme reactions that includes three classes of enzymes: E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin-protein ligases). The specificity of ubiquitylation is mainly determined by the E3 ligases, which transfer ubiquitin to substrate proteins (Kawabe and Brose, 2011). F-box proteins, a type of E3 ligase, are a large and diverse family of proteins present in all eukaryotes. Their activity is crucial for selecting proteins that will be targeted by E3 ligase (Bai et al., 1996). FBXO45 is a member of the F-box protein family and is required for normal synaptogenesis, axon navigation, and neuronal migration in developing central and peripheral neurons through UPS (Saiga et al., 2009). FBXO45 also negatively regulates neurotransmission in mature hippocampal neurons through ubiquitylation (Tada et al., 2010). Two proteins, FSN-1 and Fsn, which are the invertebrate homologues of FBXO45, were reported to regulate presynaptic differentiation (Liao et al., 2004) and terminal synaptic growth (Wu et al., 2007) through ubiquitylation proteolysis.

Considering that FBXO45 is included in the 3q29 microdeletion region and that the FBXO45 protein plays various roles in synaptic development and transmission via UPS, FBXO45 may be a novel candidate gene for SCZ. No common variants associated with SCZ were detected with the Japanese GWAS of SCZ (JPN_GWAS) in the region of FBXO45 (Fig. S1) (Ikeda et al., 2011) or by other SCZ GWAS (Purcell et al., 2009; Shi et al., 2009; Stefansson et al., 2009). To investigate rare variants in FBXO45 that may contribute to susceptibility to SCZ, we conducted mutation screening in the exon regions of FBXO45 and performed follow-up analyses.

2. Materials and methods

2.1. Participants

Three sample groups were used in this study. The first group (resequencing sample set), comprising 337 SCZ patients (mean age 49.3 ± 14.6 years, male/female = 200/137), was used for mutation screening. The second group (genotyping sample set), included 601 SCZ patients (mean age 52.2 ± 15.0 years, male/female = 355/246) and 916 healthy comparison individuals (mean age 38.9 ± 15.5 years, male/female = 386/530), was used for a genetic association study. The third group (mRNA expression sample set), comprised 50 SCZ patients (mean age 42.5 ± 11.0 years, male/female = 24/26), 52 healthy controls (mean age 41.7 ± 11.5 years old, male/female = 25/27), one SCZ patient with a rare missense mutation (R108C; 50 years old, male) detected with resequencing analysis and his mother with same mutation (77 years old female). It was a smaller but representative (matched in age, and gender) sample set for assessment of genetic expression. The mutation screening, genetic association and mRNA expression samples were collected independently at each university hospital. The Ethics Committees of the Nagoya University Graduate School of Medicine and associated institutes and hospitals approved this study. Written informed consent was obtained from all participants. In addition, the patients' capacity to consent was confirmed by a family member when needed. Individuals with a legal measure of reduced capacity were excluded. Patients were included in the study if they (1) met DSM-IV-TR criteria for SCZ and (2) were physically healthy. A general characterization and psychiatric assessment of the participants is available elsewhere (Ikeda et al., 2011). Controls were selected from the general population and had no personal or family history of psychiatric disorders (first-degree relatives only based on the subject's interview). The selection was based on questionnaire responses from the controls themselves during the sample inclusion step and based on an unstructured diagnostic interview done by an experienced psychiatrist during the blood collection step.

2.2. Resequencing analysis

Human *FBXO45* spans approximately 20 kb on chromosome 3q29 (chr3: 196,295,559-196,315,930; human reference sequence GRCh37). Genomic DNA was extracted from whole blood or saliva using a QIAamp DNA blood kit or tissue kit (QIAGEN Ltd., Hilden, Germany). Optimal polymerase chain reaction (PCR) primer sequences were generated with FastPCR (PrimerDigital Ltd., Helsinki, Finland) (Kalendar et al., 2011) and validated with PerlPrimer (Marshall, 2004). To target *FBXO45*, we designed three amplicons to cover the coding exons. After PCR amplification, aliquots of PCR products were purified using Illustra Exonuclease I and Alkaline Phosphatase (GE Healthcare & Life Science, Little Chalfont, United Kingdom). These were then sequenced using the Sanger method and a 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Mutation Surveyor® (Softgenetics, State College, PA, USA) was used for mutation detection analysis in all *FBXO45* exons. The genetic variants were verified by re-amplifying and resequencing the fragments. Considering that one of the limitations of Sanger sequencing is that it cannot discover large structural variations, we screened for deletion or duplication within *FBXO45* or 3q29 region in our resequencing sample set using TaqMan copy number assays (detailed information are provided in supplementary method section).

2.3. Follow-up analyses

2.3.1. Prioritizing steps of genetic variants for follow-up analyses

Two prioritizing step genetic variants were conducted as follows: (1) we included only novel genetic variants. "Novel" was defined in our study as variants not registered in either dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) or the Exome Variant Server (http://evs.gs.washington.edu/EVS/) or 1000 Genomes (http://www.1000genomes.org/home), and (2) we included only nonsense or missense (functionally relevant) SNVs, splicing variants, and small (<900 base pairs) INDELs.

2.3.2. Genetic association study

First, to verify the frequency of variants detected in the resequencing procedure in SCZ cases and controls, we conducted a genetic association study with the genotyping sample set. Variants were designated as 'rare' if their minor allele frequency (MAF) in combined cases and controls was <1% (Schork et al., 2009). Genotyping was conducted with Custom TaqMan SNP genotyping assays (Applied Biosystems) and a Real-Time PCR System (7900HT Fast Real-Time PCR System, Applied Biosystems). Differences in the allele and genotype frequencies of SNPs between SCZ patients and controls were evaluated using Fisher's exact test (one-tail). The threshold of significance was set as $P < 0.05$.

2.3.3. Bioinformatics analyses

The genetic position and sequence were obtained from the Ensembl Genome Browser (Ensembl 70, Jan 2013). The potential structural and functional consequences of the missense mutation were evaluated using the following tools: (1) localization of the protein domain with the Human Protein Reference Database (http://www.hprd.org/index_html), (2) prediction and comparison of secondary and tertiary protein

structure changes with the I-TASSER algorithm (Roy et al., 2010) and UCSF Chimera (Pettersen et al., 2004), (3) prediction of qualitatively functional effects, i.e., benign/possibly damaging/probably damaging with Polyphen-2 and PMut software (Ferrer-Costa et al., 2005; Adzhubei et al., 2013), (4) sequence alignment of F-box proteins with BLAST (http://blast.ncbi.nlm.nih.gov/), and (5) evolutionary conservation with the HomoloGene database (http://www.ncbi.nlm.nih.gov/homologene/).

2.3.4. Analysis of mRNA levels by gene expression profiling

To investigate the transcriptional impact of the rare missense mutation R108C in *FBXO45*, we performed gene expression profiling of lymphoblastoid cell lines (LCLs) from the expression sample set. LCLs were established by Epstein–Barr virus transformation of lymphocytes and cultured in RPMI-1460 medium containing 20% fetal bovine serum, penicillin, and streptomycin. Total RNA was extracted from LCLs using a RNAqueous Kit (Invitrogen, Carlsbad, CA, USA), treated with DNase using a TURBO DNA-free™ Kit (Invitrogen), and reverse transcribed to cDNA with a High capacity RNA-to-cDNA Kit (Invitrogen). Two housekeeping genes, beta-2-microglobulin (*B2M*) and glucuronidase-beta (*GUSB*), were selected as internal control genes to normalize the PCR. Real-Time quantitative PCR was performed with the probes in the predesigned TaqMan Gene Expression Assay (Hs00397889_m1 for *FBXO45*, Hs99999907_ml for *B2M*, and Hs99999908_ml for *GUSB*; Applied Biosystems) using Applied Biosystems 7900HT. The expression probe for *FBXO45* was designed to bind the region which is not harboring mutation detected in the mutation screening analysis. Measurement of the cycle threshold was performed in duplicate. Data of relative expression level were analyzed with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The Mann–Whitney *U* test was used to compare expression levels of *FBXO45* between SCZ patients and controls because this test is robust in the case of deviation from normal distribution. $P < 0.05$ was considered significant.

3. Results

3.1. Mutations detected with resequencing analysis

We detected one missense variant (R108C) in exon 2, one synonymous (A11A) variant in exon 1, and two intronic variants (ss709035634 and ss79085393). These were all single-base heterozygous substitutions. One of the intronic variants was a known SNP (rs79085393), and the other three mutations were novel. The location of these variants in *FBXO45* is illustrated in Fig. 1a and b and summarized in Table 1. No deletions or duplications within *FBXO45* and 3q29 region were detected in our resequencing sample set using TaqMan copy number assays (supplement). Because the purpose of our research was to identify novel, functional mutations (missense and nonsense mutations), we excluded the known SNPs and the intronic and synonymous variants from our subsequent analyses. R108C, a heterozygous nucleotide substitution from cytidine to thymidine that was exclusively identified in a single patient out of 337, was studied with follow-up analyses (case study, pedigree study, genetic association study, bioinformatics analyses, and mRNA expression analysis).

3.2. Follow-up analysis of the rare missense variant R108C

3.2.1. Case report of the patient with the FBXO45 R108C mutation

The patient with the *FBXO45* R108C mutation was a male diagnosed with paranoid SCZ. The premorbid IQ score of the patient was estimated

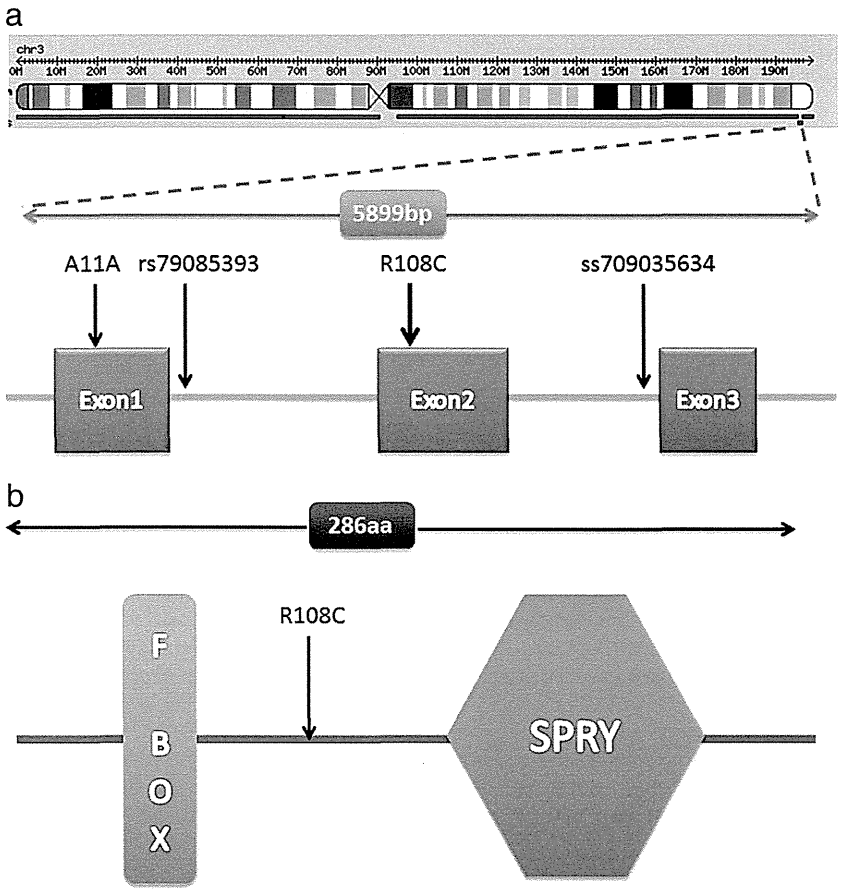

Fig. 1. Schematic genomic structure and domains of *FBXO45* and the locations of the variants studied. Legend: (a) gene structure of *FBXO45* and the locations of all the variants we detected; (b) domains of FBXO45 including the N-terminal F-BOX motif, the SPRY motif, and the location of the R108C mutation that we analyzed further.

Table 1
FBXO45 variants identified in mutation screening and association study of the missense mutation.

Chr	Genomic position[a]	Nucleotide change	dbSNP reference	Novel[b]	AA change[c]	Mutation screening[d]	Association study[d]		Combined association study[d]	
						SCZ	SCZ	CONT	SCZ	CONT
3	196295888	c.33C>T	ss709035633	Yes	A11A	0/14/323				
3	196296182	G>C	rs79085393	No	–	0/10/327				
3	196304327	c.322C>T	ss709035628	Yes	R108C	0/1/336	0/0/601	0/0/916	0/1/937	0/0/916
3	196310954	A>G	ss709035634	Yes	–	0/1/336				

[a] Genomic position based on NCBI build 37.1.
[b] No registration in either dbSNP and/or Exome Variant Server was considered as "novel".
[c] Amino acid change based on NCBI reference sequence NP_001099043.1.
[d] Genotype count: homozygote of minor allele/heterozygote/homozygote of major allele.

at 88 with the Japanese Adult Reading Test (JART), suggesting a decline of approximately 1 S.D. from the mean premorbid IQ of SCZ patients (102.2 ± 11.6) (Hori et al., 2008). We examined the cognitive performance and symptomatology of the patient with the variant (R108C) using the Positive and Negative Symptom Scale (PANSS), Brief Assessment of Cognition in Schizophrenia, Japanese Version (BACS-J) (Kaneda et al., 2007), and Continuous Performance Test, Identical Pairs version (CPT-IP) (Koide et al., 2012), the results of which are described in the supplements (Tables S1 and S2, Figs. S2 and S3). The other phenotypes of 3q29 microdeletion including autism, microcephaly, cleft palate, pectus excavatum, and horseshoe kidney were not found in this patient (Ballif et al., 2008).

3.3. Pedigree study

The mother of the proband who carried the R108C variant had no history of medical or mental illness, but the father suffered from Alzheimer's disease. The proband had no brothers, sisters, or children. DNA was obtained from the mother in whom the R108C variant was detected. The father's DNA could not be obtained due to his physical condition.

3.4. Genetic association study

The R108C variant was then searched for in the genotyping sample set and was not detected in either the 601 SCZ patients or the 916 healthy controls. Using the combined resequencing and genotyping samples, the R108C mutation was not statistically overrepresented in SCZ patients compared to controls (Table 1).

3.5. Bioinformatics analyses

Prediction of the structural effect of the R108C mutation was presented as follows. Judging the parallel between the wild-type and mutant FBXO45 protein structure predicted by the I-TASSER server, a neutral charged amino acid (cysteine) was substituted for an amino acid with a positive side chain (arginine) at codon 108, resulting in a reversed hydrophobic distribution in the alpha helix next to the SPRY domain (Fig. 2).

Prediction of the functional effect of the R108C mutation was presented as follows, two different kinds of algorithms (Polyphen-2 and PMut) both predicted that the R108C mutation will have a damaging impact on the function of the FBXO45 protein.

Conservation analysis in species was performed, and the protein and DNA sequences of *FBXO45* in different species are highly conserved from *Caenorhabditis elegans* to mammals (over 90% identical amino acids between human and mouse). R108C was located in an evolutionarily conserved region (Table 2).

Conservation analysis of F-box proteins was performed. We aligned the FBXO45 protein sequence with other F-box proteins (FBXW7, SKP2) and found that Arg108 was conserved among these F-box proteins (Table 2).

3.6. Analysis of mRNA expression

To investigate the effect of the R108C mutation on *FBXO45* mRNA expression, we separately compared the relative expression of R108C carriers with 50 SCZ patients and 52 controls. As seen with the box plot depicting data of the relative expression of *FBXO45* normalized to the two housekeeping genes, *B2M* and *GUSB*, the relative expression of *FBXO45* in the R108C case appeared to deviate markedly from the 50 SCZ patients and the 52 controls (Fig. 3). Interestingly, patient's mother (who is not suffering from schizophrenia) is R108C mutation carrier; however, her *FBXO45* expression level was not reduced (Fig. 3). The relative expression level of *FBXO45* did not show a nominally significant difference among the 50 SCZ patients and 52 controls ($P = 0.36$, directional test, with the Mann–Whitney *U* test).

4. Discussion

4.1. Main findings

To our knowledge, this is the first study that systematically screened all *FBXO45* coding regions to search for rare mutations in SCZ patients and assessed the association of an identified mutation with SCZ.

We found one heterozygous, novel, and rare missense mutation (R108C) in a single patient among 337 SCZ patients (resequencing sample set). Follow-up genotyping in an independent sample of 601 cases and 916 controls (genotyping sample set) revealed no carrier of R108C. Using the combined association study including the resequencing and genotyping sample set, no significant association between SCZ and R108C in *FBXO45* was detected ($P > 0.05$). Because the R108C mutation was a very rare variant in our sample set, we could not determine its significant association with SCZ or estimate its odds ratio. A further study with a larger sample size is needed to precisely verify the odds ratio of the R108C mutation in SCZ, and reconsider an association between mutation and disease.

We followed up the rare missense mutation R108C using a case report for assessing the clinical consequence of the mutation, a pedigree study for measuring the inheritance of the mutation in the proband's family, bioinformatics analyses for evaluating the impact of the mutation on the structure and function of the protein, and analysis of mRNA expression for examining the influence of transcription. We tried to analyze protein expression of FBXO45 in LCLs using western blotting. However, due to low enrichment of FBXO45 protein in LCLs, we were not able to detect FBXO45 in schizophrenia patients and healthy controls (supplementary information).

The patient with the *FBXO45* R108C mutation was a male who was diagnosed with the paranoid type of SCZ. Of all the symptoms, physical signs, and examination results of this patient, only the lower premorbid IQ (score = 88 with JART) appeared to us to be important. SCZ has been consistently associated with a range of early neurodevelopmental abnormalities (Murray and Lewis, 1987; Weinberger, 1987; Seidman, 1990). One measure that may reflect early neurodevelopmental

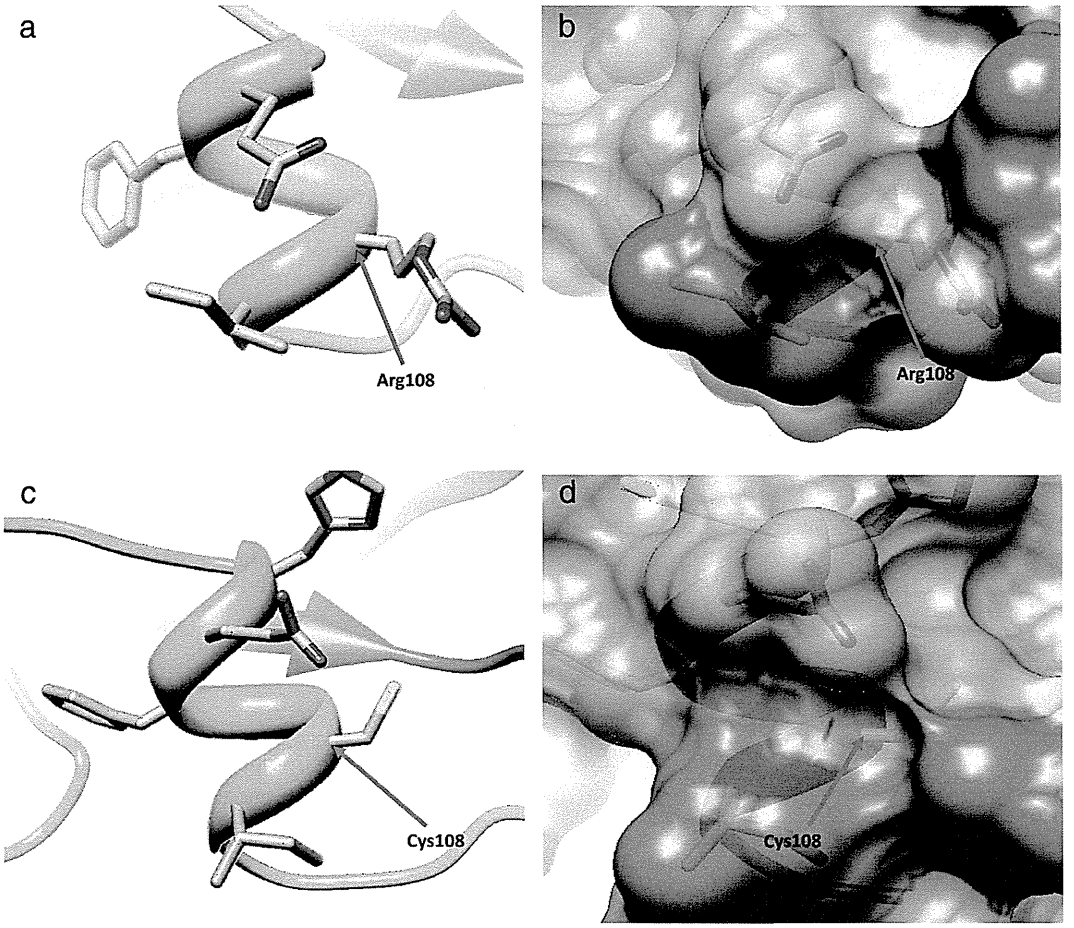

Fig. 2. 3D protein modeling of the alpha helix adjacent to the SPRY domain (FBXO45). Legend: (a) Wild type; note Arg108 on the hydrophilic chain (blue line); (b) wild type surface; note Arg108 on the hydrophilic surface (transparent blue); (c) mutant; note Cys108 on the hydrophobic chain (yellow line); (d) mutant surface; note Cys108 on the hydrophobic surface (transparent orange).

abnormalities and that has received considerable attention is premorbid intelligence (premorbid IQ) (Woodberry et al., 2008; Khandaker et al., 2011). The premorbid IQ was estimated to be 88 with JART, which was a decrease of more than 1 S.D. from the mean premorbid IQ of SCZ patients (102 ± 11.6) and controls (102.3 ± 7.4) (Hori et al., 2008). The patient's score on the Token motor test from BACS-J was

Table 2
Multiple alignments of FBXO45's homologous proteins and F-box proteins.

Homologue	Start																															End
NP_498046.1 C. elegans	146	L	G	S	A	K	K	K	L	R	A	W	Y	F	A	W	N	T	S	D	I	S	R	N	N	Y	I	R	T	N	G	F	T	V	H	179
NP_956889.1 zebrafish	105	L	A	S	Y	K	G	K	L	K	S	F	Q	H	A	L	S	S	H	D	C	S	R	N	V	Y	I	K	K	N	G	F	T	L	H	138
XP_001232841.2 chicken	118	V	P	T	Y	K	G	K	V	R	A	F	H	H	A	F	S	T	N	D	C	S	R	N	V	Y	I	K	K	N	G	F	T	L	H	151
XP_851899.2 dog	97	L	P	S	Y	K	A	K	V	R	A	F	Q	H	A	F	S	T	N	D	C	S	R	N	V	Y	I	K	K	N	G	F	T	L	H	130
NP_775615.2 mouse	100	L	P	S	Y	K	A	K	V	R	A	F	Q	H	A	F	S	T	N	D	C	S	R	N	V	Y	I	K	K	N	G	F	T	L	H	133
NP_001193687.1 cow	95	L	P	S	Y	K	A	K	I	R	A	F	Q	H	A	F	S	T	N	D	C	S	R	N	V	Y	I	K	K	N	G	F	T	L	H	128
NP_001099043.1 human	100	L	P	S	Y	K	A	K	I	R	A	F	Q	H	A	F	S	T	N	D	C	S	R	N	V	Y	I	K	K	N	G	F	T	L	H	133
XP_002803137.1 rhesus monkey	100	L	P	S	Y	K	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	104
NP_610849.1 Drosophila	69	V	S	T	Y	K	T	K	L	R	A	Y	F	H	A	W	S	P	N	D	C	S	R	N	V	Y	I	K	P	N	G	F	T	L	H	102
XP_309899.3 A. gambie	72	V	P	T	Y	K	A	K	L	R	A	Y	F	H	A	W	N	P	F	D	C	S	R	H	V	Y	I	K	P	N	G	F	T	L	H	105

F-box protein	F-box														Linker																	
FBXO45	K	H	W	Y	R	C	L	H	G	D	E	N	S	E	V	W	R	S	L	C	A	R	S	L	A	E	E	A	L	–	–	–	R	T
Fbxw7	R	Y	W	–	R	I	L	A	–	–	E	D	N	L	L	W	R	E	K	C	K	E	E	G	I	D	E	P	L	H	I	K	R	R
Skp2	K	R	W	–	Y	R	L	A	–	–	S	D	E	S	L	W	Q	T	L	–	–	–	–	D	–	–	–	–	L	T	G	K	N	

	Linker																															
FBXO45	D	I	L	–	–	–	–	C	N	L	P	S	Y	K	A	K	I	R	A	F	–	Q	H	A	F	S	T	N	D	C	S	R	N	V
Fbxw7	K	V	I	K	P	G	F	I	H	S	P	W	K	S	A	Y	I	R	–	–	–	Q	H	R	I	D	T	N	W	R	R	G	E	–
Skp2		L								H	P	D	V		T		G	R	L	L	S	Q	G	V	I			A	F	R	C	P	R	S

Arginine

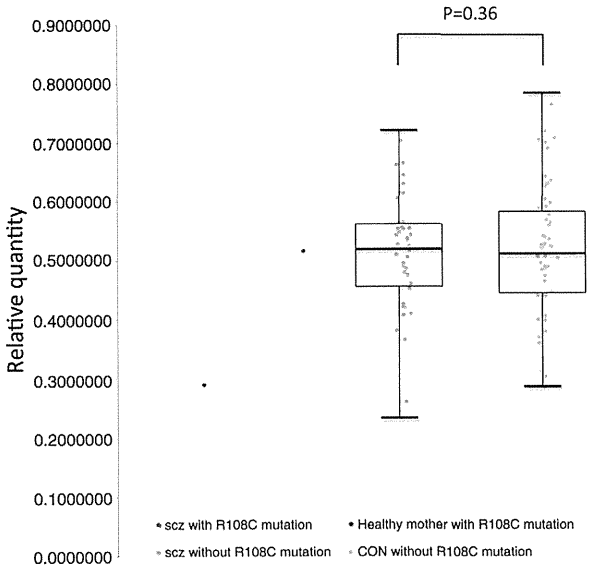

Fig. 3. Relative expression of FBXO45. Legend: Box plot: the box represents the middle 50% of observations. The middle bold line represents the median gene expression. Whiskers represent the minimum and maximum observations. Each dot represents the relative expression of each sample, which was calculated with the $2^{-\Delta\Delta C_T}$ method. The relative expression of SCZ patient with R108C mutation was an outlier of the entire sample set, while his healthy mother with the same mutation had no deviation of gene expression. The relative expression of FBXO45 was not significantly different between the 50 SCZ patients and the 52 controls (P = 0.36, directional test, with the Mann–Whitney *U* test).

less than 1 S.D. away from the mean score of SCZ patients, meaning that the patient may have a severe deficiency in motor speed (Table S1 and Fig. S2) (Kaneda et al., 2007). The CPT-IP score in this patient was less than 1 S.D. away from the mean score of SCZ patients, indicating that he may suffer from serious attention/vigilance deficits (Table S2 and Fig. S3) (Koide et al., 2012).

As a result of the pedigree study, we found that the mother of the proband was a heterozygous carrier of the R108C mutation; the DNA of the father was not available. The R108C mutation of the proband may have been inherited from the unaffected healthy mother because frequency of the mutation in cases and controls was very rare (MAF < 0.001). In other words, it is much less likely that the father was a carrier of the R108C mutation who transmitted it to the proband. If the R108C mutation was inherited from both parents, this pedigree case showed incomplete penetrance of this mutation regarding the SCZ phenotype.

The R108C mutation changed the hydrophobic distribution of the amino acids in the FBXO45 protein as predicted by structural analysis (Fig. 2). The R108C mutation was located in an evolutionarily conserved region (Table 2) between the F-box domain and the SPRY domain (Fig. 1b). The R108C mutation was predicted to be a damaging mutation for FBXO45 protein function by two kinds of algorithms (PMut and Polyphen-2). Because our sequence alignment of F-box protein family members and structural analysis of FBXO45 suggested that the mutation was located in the flexible linker region, we speculate that it may be relevant to our understanding of how the function of FBXO45 is affected by the R108C mutation (supplements). Hence, R108C is likely to play a role in a conformational change that prevents FBXO45 from correctly forming the ubiquitin ligase complex, further impacting ubiquitylation proteolysis and disrupting synaptic function.

Because genetic variation affects disease susceptibility in two ways (affecting the structure of the encoded protein and expression of the gene), thereby changing the amount or distribution of the protein (Harrison and Weinberger, 2005), we further conducted gene expression analysis of LCLs. Interestingly, the patient in whom we detected the R108C mutation had much lower *FBXO45* expression compared to the 50 SCZ patients and the 52 healthy controls, while in the case of his healthy mother who is also R108C mutation carrier, *FBXO45* expression was not reduced (Fig. 3). This observation implies that R108C mutation might not be a causal variant affecting gene expression of *FBXO45*. However, we cannot rule out the possibility that down-regulation of *FBXO45* in this patient with the R108C mutation might be relevant to the R108C mutation along with other joint factors, such as the effect of epistasis (Lappalainen et al., 2013), long-term antipsychotic medication (Hashimoto et al., 2004). Because of only two samples with R108C mutation, an estimation of R108C transcriptional influence using mRNA expression analysis seemed to be a coin-tossing situation. It would be useful for further estimation to increase genotype sample set in order to discover more R108C carriers.

In addition, we also identified one synonymous and two intronic variants. They were excluded from our follow-up analyses because our study design focused on putatively functional variants in exons. However, it may be premature to conclude that "non-functional" variants have no effect on protein function. A growing amount of evidence suggests that synonymous mutations may not always be silent; they may influence the abundance and function of proteins by activating cryptic splicing sites, affecting the stability of the mRNA, or altering the protein-folding pathway (Plotkin and Kudla, 2011; Sauna and Kimchi-Sarfaty, 2011).

4.2. Limitations

This study has several limitations. First, the sample size of our study was relatively small and lacked the statistical power to detect an association between the very rare variant R108C in *FBXO45* and SCZ. Second, several potentially valuable regions were not sequenced, including the promoter and the 5′- and 3′-UTR ends. Third, we did not validate the pathological effect of the R108C mutation with a biological experiment. Fourth, *FBXO45* gene expression profiling was evaluated using LCLs from a small sample size, and neuronal tissues were not examined. Because of controversy involving the use of non-neuronal tissues for detecting gene expression differences associated with predisposition to SCZ (Matigian et al., 2008), *FBXO45* expression should be examined in the central nervous system, and a larger sample size should be examined. In addition it would be interesting to investigate polygenic risk burden of the individuals selected for resequencing. If the case with the candidate rare variant has a low polygenic risk score, it might suggest that this variant is more likely to be causative.

5. Conclusion

The novel, heterozygous, rare, and missense mutation R108C was discovered using our mutation screening. This mutation may have a potentially pathogenic effect on FBXO45 protein structure and function, and thus, the variant may have a modest effect on SCZ risk. In addition, our findings suggest that *FBXO45* may be a new and interesting candidate gene for SCZ.

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Contributors

Conceived and designed the experiments: CW TK BA. Performed the experiments: CW HK YN AM AY. Analyzed the data: CW TK AY MB BA. Contributed reagents/materials/analysis tools: HK TK AY YN NK YT JX BA MI TO Tli Tin NI. Wrote the paper: CW TK SK IK BA NO.

Conflict of interest

The authors declare that they have no competing financial or other interests that might be perceived to influence the results and discussion reported in this paper.

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Plasma dehydroepiandrosterone sulfate levels in patients with major depressive disorder correlate with remission during treatment with antidepressants

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Objective We attempted to investigate whether dehydroepiandrosterone sulfate (DHEA-S) levels are associated with remission of major depressive disorder by assessing scores on the 17-Item Structured Interview Guide for the Hamilton Depression before and after antidepressant treatment.
Methods Plasma DHEA-S levels in 24 patients diagnosed with major depressive disorder on the basis of Diagnostic and Statistical Manual of Mental Disorders, fourth edition (text revision) before and after antidepressant treatment, and 24 healthy, gender-matched, and age-matched controls were measured using a radioimmunoassay kit.
Results Plasma DHEA-S levels in patients were significantly higher than those in healthy controls. In patients who achieved remission after antidepressant treatment, plasma DHEA-S levels significantly declined compared with the levels before treatment. A significant correlation was observed between changes in DHEA-S levels and Absence of Depressive and Anxious Mood scores, which are calculated from the 2-Item Structured Interview Guide for the Hamilton Depression rating as follows: severity of depressive mood and anxiety in patients before and after antidepressant treatment.
Conclusions These findings suggest that plasma DHEA-S levels can be used as a putative indicator of the state of remission in patients with major depressive disorder. Copyright © 2014 John Wiley & Sons, Ltd.

KEY WORDS—dehydroepiandrosterone sulfate (DHEA-S); major depressive disorder; remission; Absence of Depressive and Anxious Mood (ADAM) score

INTRODUCTION

Dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) are neurosteroids synthesized in the body either from cholesterol or from steroid hormone precursors, which are abundant in the brain (Baulieu, 1981, 1998; Baulieu and Robel, 1996). DHEA and DHEA-S regulate neuronal activity through receptors on the cell membrane of neurons. Although all of the target receptors and functional mechanisms of neurosteroids have not yet been elucidated (Webb *et al.*, 2006), DHEA and DHEA-S are $GABA_A$ receptor antagonists (Majewska *et al.*, 1990; Rupprecht, 1997, 2003; Imamura and Prasad, 1998) and sigma-1 receptor agonists (Monnet *et al.*, 1995; Bergeron *et al.*, 1996; Maurice *et al.*, 1996, 1999, 2006; Skuza, 2003; Bermack and Debonnel, 2005; Dhir and Kulkarni, 2008). In addition, DHEA and DHEA-S have been suggested to have antidepressive and anxiolytic effects. The antidepressive effects of DHEA have been clinically reported. For example, the Hamilton Depression Rating Scale scores is significantly decreased in cases in which oral administration of DHEA alleviates the symptoms of major depressive disorder (Wolkowitz *et al.*, 1997, 1999; Bloch *et al.*, 1999; Schmidt *et al.*, 2005).

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In previous studies, it was unclear whether DHEA-S levels were decreased because of the direct pharmacological effect of imipramine (Tollefson *et al.*, 1990) or clomipramine (Takebayashi *et al.*, 1998) or because of the remission state mediated by these drugs. Subsequent research has shown that a decrease in DHEA-S levels after treatment with venlafaxine is dependent on remission; however, the assessment of DHEA-S levels was not conducted in unremitted patients (Hsiao, 2006a). Investigation of mirtazapine treatment has also shown a decrease in DHEA-S levels; however, healthy controls were not included in this evaluation (Schüle *et al.*, 2009). Paslakis *et al.* (2010) recently reported that DHEA-S levels in remitted, but not in unremitted depressive patients, were significantly decreased after treatment with both venlafaxine and mirtazapine. These findings suggest that changes in DHEA-S levels are associated with the state of remission after antidepressant treatment (Paslakis *et al.*, 2010). Nevertheless, further investigation including more clinically used antidepressant treatments is needed to confirm the association of the change in DHEA-S levels with remission state of major depressive disorder.

A preliminary pilot study has indicated that morning plasma DHEA-S levels positively correlate with the Hospital Anxiety and Depression Scale anxiety subscale score (which assesses depressive mood and severity of anxiety) in medication-free outpatients experiencing major depressive disorder (Silverstone *et al.*, 2002; Hsiao, 2006b). Depressive mood and anxiety are essential psychological symptoms not always associated with somatic conditions such as change in sleep, nutrition, appetite, or weight. They have been used for evaluation of the effect of antidepressants. However, this pilot data have not yet been assessed in a follow-up study. In the present study, DHEA-S, which is more stable than DHEA in blood, was investigated in detail, particularly focusing on the relationship between changes in plasma DHEA-S levels and depressive symptoms before and after antidepressant treatment.

SUBJECTS AND METHODS

This study was approved by the Ethical Review Board of Fujita Health University. All patients and controls of the study at Fujita Health University Hospital consented for participation in this research after reviewing documents detailing the study, written with the guidance of the Ethical Review Board. Written informed consent was obtained from each subject.

Subjects

Study subjects included 24 patients diagnosed with major depressive disorder [15 men and 9 women; average age, 40.1 ± 2.5 years (mean ± SEM)] and 24 healthy, gender-matched, and age-matched controls (healthy controls; 40.2 ± 2.5 years of age). The criteria for selecting patients were as follows: (i) no comorbidities from Axes I-III of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (text revision) (DSM-IV-TR); (ii) single or repeated major depressive episodes according to DSM-IV-TR; (iii) no substance dependence or habitual use of drugs or alcohol; and (iv) no treatment with psychotropic drugs for at least 2 weeks before the initiation of this study. Details of the patient's characteristics are provided in Table 1.

Methods

Measurement of dehydroepiandrosterone sulfate levels. Venous blood was collected once from the healthy controls and twice from the patients, once before and once, when the patients were tested, after the average of more than 8 weeks since the initiation of antidepressant treatment. Blood collection was performed only between 10AM and 2PM to minimize the effects of diurnal variation of DHEA-S levels.

After collection in heparinized vacuum blood tubes (Venoject, Terumo Corporation, Tokyo, Japan), the blood samples were immediately centrifuged at 2900 rpm for 10 min. Plasma was separated and stored at −70°C until DHEA-S levels were measured in duplicate using a radioimmunoassay kit (Diagnostic Product Corporation, Los Angeles, CA, USA).

Treatment with antidepressants. A suitable antidepressant and dosage were selected based upon the condition of each patient. The antidepressants used in the current study were imipramine, clomipramine, amoxapine (tricyclic antidepressant), maprotiline (tetracyclic antidepressant), trazodone (serotonin antagonist and reuptake inhibitor), and sulpiride (benzamide antipsychotic used as an antidepressant at a low dose). The new class antidepressants including selective serotonin reuptake inhibitors were not used in this study, because almost all have not been approved and have not yet been used commonly to treat depression in Japan. Depending on the depressive state, a second antidepressant was administered to patients, which included mood stabilizers, carbamazepine, sodium valproate, lithium carbonate, and second generation antipsychotics. The typical antidepressant administration period was

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Table 1. Characteristic of patients in this study

		Healthy controls			Depressive patients	
Number	Total	24			24 (18[#], 12[##]) ([#]*Remitter-1*, [##]*Remitter-2*)	
	Gender	Male	Female		Male	Female
		15	9		15 (11, 7)	9 (7, 5)
Mean age (aged range)	Total	40.2 ± 2.5			40.1 ± 2.5	
	Gender	42.8 ± 3.1	35.9 ± 4.5		42.7 ± 3.1	35.8 ± 4.5
	(20–29)	2	4		2 (2, 1)	4 (4, 2)
	(30–39)	3	3		3 (3, 2)	3 (2, 2)
	(40–49)	7	0		7 (3, 2)	
	(<50)	3	2		3 (3, 2)	2 (1, 1)
Treatment	Period	—	—		174 ± 36	
Used drug[†]	Single	—	—	IMP		1 (1, 0)
				CMI	3 (2, 1)	1 (0, 0)
				AMX	1 (1, 0)	3 (2, 2)
				MAP	1 (1, 0)	3 (3, 2)
				TRZ	1 (0, 0)	
				SLP	3 (3, 3)	
	≧2 drugs	—	—		5 (3, 2)	
	None	15	9		1 (1, 1)	1 (1, 1)

IMP, imipramine; CMI, clomipramine; AMX, amoxapine; MAP, maplotiline; TRZ, trazodone; SLP, sulpiride.
[#]Remitter-1: 17-Item Structured Interview Guide for the Hamilton Depression (SIGH-D 17) scores were decreased less than 7.
[##]Remitter-2: Absence of Depressive and Anxious Mood (ADAM) scores were equal zero. Used drug.
[†]Drugs taking at the end-point.

174 days (minimum: 20 days, maximum: 307 days) or about 25 weeks on average.

Rating of the depressive state. The depressive state of the 24 patients was evaluated before and after the average of more than 8 weeks since the initiation of antidepressant treatment by four psychiatrists using 17-Item Structured Interview Guide for the Hamilton Depression (SIGH-D 17). The analysis of variance (ANOVA) intraclass correlation coefficient was 0.96. The remission of major depressive disorder was defined as a SIGH-D 17 score of ≤7. Patients in remission were designated as *remitter-1*. Patients with a zero Absence of Depressive and Anxious Mood (ADAM) score were referred to as *remitter-2*. ADAM is a subscale calculated by adding up scores of item 1 (depression) and item 10 (anxiety) of SIGH-D 17.

Statistical analysis. Statistical significance of differences between the two groups was assessed using Student's *t*-test (plasma DHEA-S levels) and Mann–Whitney *U*-test (SIGH-D 17 and ADAM scores). Statistical differences among subjects, genders, and treatments were determined using three-way ANOVA, followed by Fisher's protected least significant difference test for comparison of multiple factors. Correlations between the percentage of change in plasma DHEA-S levels and a decrease in SIGH-D 17 or ADAM scores were analyzed using Pearson's correlation coefficient test. $P < 0.05$ was assumed to indicate statistically significant differences.

RESULTS

(1) Plasma DHEA-S levels in healthy controls and patients before and after antidepressant treatment

The average plasma DHEA-S level in patients before antidepressant treatment was 1825.7 ± 294.9 ng/ml (mean ± SEM), which was significantly higher than that in healthy controls (1204.7 ± 193.2 ng/ml). After antidepressant treatment, the average plasma DHEA-S level in patients significantly decreased to approximately the same level (1328.6 ± 234.3 ng/ml) as that in healthy controls (Figure 1(a)).When analyzed by gender, a similar phenomenon was observed in female (before treatment: 1065.6 ± 183.8 ng/ml, after treatment: 683.0 ± 150.0 ng/ml) and male patients (before treatment: 2281.8 ± 421.2 ng/ml, after treatment: 1716.0 ± 240.8 ng/ml; Figure 1(b)). A three-way ANOVA revealed a significant effect of gender [$F_{(1, 52)} = 16.21$, $P < 0.01$] and subject [$F_{(14, 52)} = 1.92$, $P < 0.05$], but there were no significant differences among treatments [$F_{(2, 52)} = 2.03$, $P = 0.14$]. None of the previous interactions was significant. *Post hoc* comparisons revealed a significant effect of DHEA-S levels between healthy controls and the patients before but not after the treatment (Fisher's protected least significant difference test: $P < 0.05$; Figure 1(b)).

(2) SIGH-D 17 and ADAM scores of patients before and after antidepressant treatment

The average SIGH-D 17 score in patients before treatment was 23.8 ± 1.5, and it decreased to 4.8 ± 1.1 after

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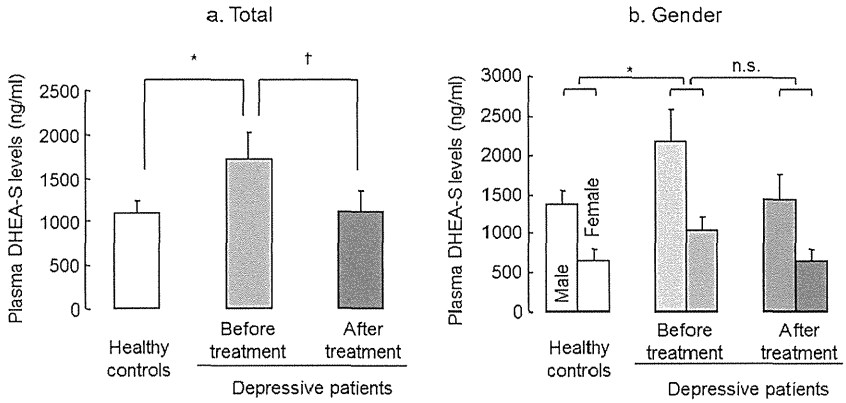

Figure 1. Plasma dehydroepiandrosterone sulfate (DHEA-S) levels in healthy controls and depressive patients before and after treatment. (a) The data are shown as mean ± SEM ($n = 24$). $^{*}P < 0.05$ compared with healthy controls (Student's unpaired t-test), $^{\dagger}P < 0.05$ compared with depressive patients before treatment (Student's paired t-test). (b) The data are shown as mean ± SEM (men: $n = 15$, women: $n = 9$). Three-way analysis of variance: gender $F_{(1, 52)} = 16.21$, $P < 0.01$; treatment $F_{(2, 52)} = 2.03$, $P = 0.14$; subject $F_{(14, 52)} = 1.92$, $P < 0.05$. *Post hoc* comparison revealed a significant effect of DHEA-S levels between healthy controls and patients before treatment (Fisher's protected least significant difference test: $P < 0.05$); n.s., not significant

treatment. The average SIGH-D 17 score in *remitter-1* patients ($n = 18$, 11 male and 7 female patients) decreased from 22.9 ± 3.7 before treatment to 2.3 ± 0.9 after treatment. In contrast, the average SIGH-D 17 score in *non-remitter*-1 patients ($n = 6$, 4 male and 2 female patients) failed to show a clinically significant decline (disease to remission) in spite of a statistically significant change (before treatment: 26.2 ± 1.6, after treatment: 12.5 ± 1.3). There were 12 (seven male and five female patients) *remitter-2* patients with a zero ADAM score and 12 (eight male and four female patients) *non-remitter-2* patients (Figure 2).

(3) Plasma DHEA-S levels in remitted and non-remitted patients before and after antidepressant treatment

The average plasma DHEA-S level in *remitter-1* patients before treatment (1829.7 ± 113.4 ng/ml) significantly decreased after treatment (1281.5 ± 216.7 ng/ml). Nevertheless, the average plasma DHEA-S level in *non-remitter-1* patients did not decrease significantly after treatment (before treatment: 1814.0 ± 324.8 ng/ml, after treatment: 1470.1 ± 729.7 ng/ml). In *remitter-2* and *non-remitter-2* patients, a similar phenomenon was observed. The average plasma DHEA-S level in *remitter-2*

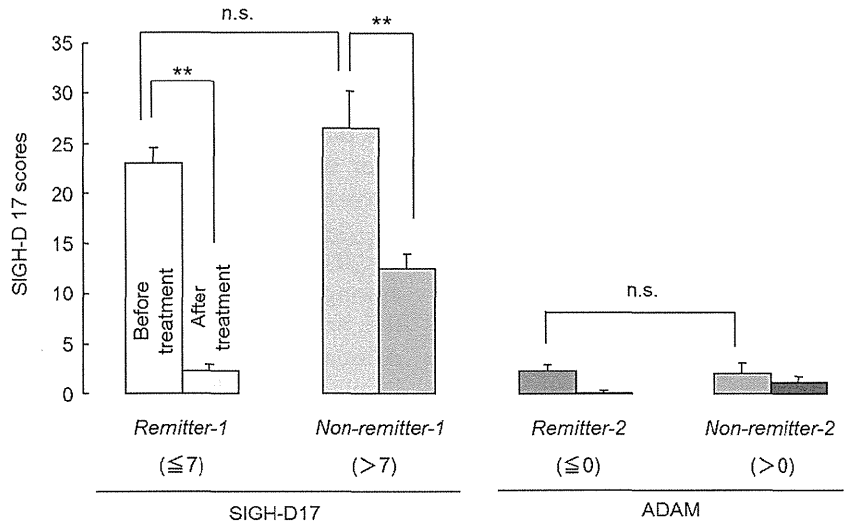

Figure 2. The 17-Item Structured Interview Guide for the Hamilton Depression (SIGH-D 17) and Absence of Depressive and Anxious Mood (ADAM) scores (a subscale of SIGH-D-17) before and after treatment. *Remitter-1*: SIGH-D 17 scores decreased to less than 7. *Remitter-2*: ADAM scores equaled zero. The data are shown as mean ± SEM (*remitter-1*: $n = 18$, *non-remitter-1*: $n = 6$, *remitter-2*: $n = 12$, and *non-remitter-2*: $n = 12$). $^{**}P < 0.01$ compared with before treatment (Mann–Whitney U-test); n.s., not significant

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patients before treatment (1929.7 ± 510.1 ng/ml) significantly decreased after treatment (1255.8 ± 285.4 ng/ml). Nonetheless, the average plasma DHEA-S level in *non-remitter-2* patients did not decrease significantly after treatment (before treatment: 1721.8 ± 324.8 ng/ml, after treatment: 1470.1 ± 729.7 ng/ml; Figure 3).

(4) Correlation between the change in plasma DHEA-S levels and the decrease in SIGH-D 17 and ADAM scores after antidepressant treatment

No significant correlation was observed between the percentage of change in plasma DHEA-S levels and the decrease in SIGH-D 17 scores after antidepressant treatment ($r = 0.229$, $P > 0.05$). However, a strong correlation was identified between the percentage of change in plasma DHEA-S levels and the decrease in ADAM scores after antidepressant treatment ($r = 0.607$, $P < 0.01$; Figure 4).

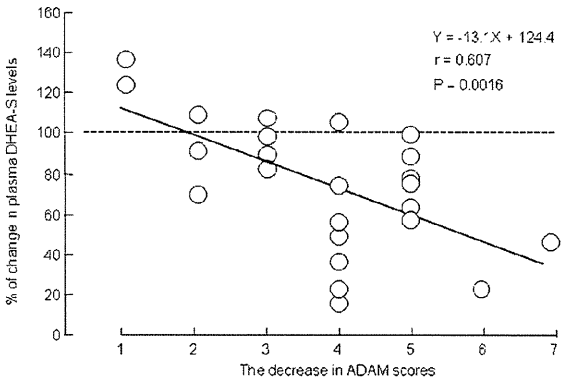

Figure 4. Correlation of the percentage of change in plasma dehydroepiandrosterone sulfate (DHEA-S) levels with a decrease in Absence of Depressive and Anxious Mood (ADAM) scores (a subscale of 17-Item Structured Interview Guide for the Hamilton Depression). $Y = -13.1X + 124.4$, $r = 0.607$, $P = 0.0016$

DISCUSSION

In this study, plasma DHEA-S levels in patients with major depressive disorder were found to be higher than those in healthy controls. It is believed that psychosocial stressors are among the causes of mood disorders such as major depressive disorder and affect the levels of DHEA-S. Izawa *et al.* (2008) found that acute psychosocial stress significantly increases salivary DHEA levels in male subjects. They pointed out that this result may be partly explained by the hypothalamic–pituitary–adrenal (HPA) axis activity because of the known correlation between DHEA and cortisol secretion. Under stressful situations, cortisol secretion is induced by increased adrenocorticotrophic hormone secretion. A significant increase in plasma DHEA-S levels during a graded adrenocorticotrophic hormone infusion has been reported in normal young and elderly human subjects (Ohashi *et al.*, 1986). Therefore, the higher DHEA-S levels in medication-free patients than in healthy controls in the present study could be explained by the HPA axis activity in response to stressors associated with major depressive disorder.

Overall, despite of the differences in types and doses of antidepressants, plasma DHEA-S levels decreased in remitted patients but not in non-remitted patients in our study, and this result is consistent with previous findings (Fabian *et al.*, 2001; Hsiao, 2006a; Schüle *et al.*, 2009; Paslakis *et al.*, 2010). Because of the relationship of the decline in DHEA-S levels with remission, the HPA axis activity may be attenuated after remission caused by antidepressant treatment, and DHEA-S levels would then decrease. Therefore, the change in plasma DHEA-S levels could be an accurate biomarker of remission of depression.

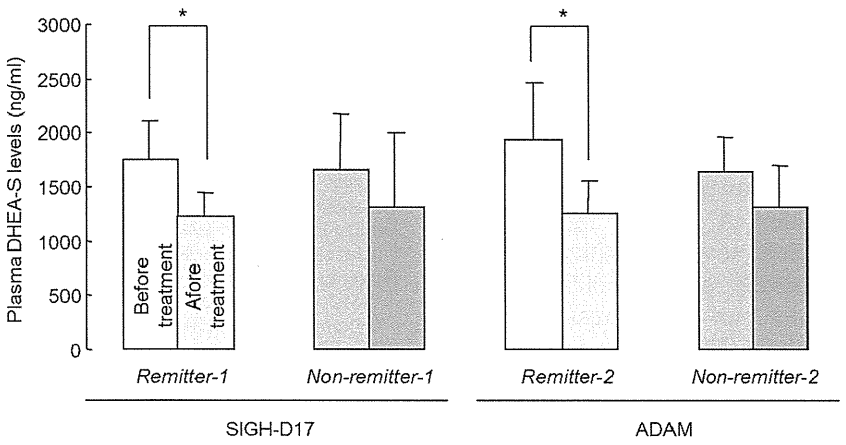

Figure 3. Plasma dehydroepiandrosterone sulfate (DHEA-S) levels in patients who achieved remission and in those who did not. *Remitter-1*: 17-Item Structured Interview Guide for the Hamilton Depression (SIGH-D 17) scores decreased to less than 7. *Remitter-2*: Absence of Depressive and Anxious Mood (ADAM) scores equaled zero. The data are shown as mean ± SEM (*remitter-1*: $n = 18$, *non-remitter-1*: $n = 6$, *remitter-2*: $n = 12$, and *non-remitter-2*: $n = 12$). $^*P < 0.05$ compared with before treatment (Mann–Whitney *U*-test)

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Regarding the gender effect observed in this study, plasma DHEA-S levels in female patients were lower than in male patients, and a similar trend was observed in female and male patients before and after antidepressant treatment. Plasma DHEA-S levels in remitted patients were decreased significantly. Consequently, no gender effect was found in the relationship between the decrease in plasma DHEA-S levels and remission. In addition, in this study, we quantified plasma progesterone, a sex steroid hormone. As a result, no correlation was observed between the change in plasma progesterone levels before/after antidepressant treatment and remission (data not shown). Our results show that the changes in DHEA-S but not progesterone levels reflect the success of treatment in major depressive disorder. Reduced levels of allopregnanolone, a 3α-reduced metabolite of progesterone, are associated with major depressive disorder and other psychiatric disorder (Schüle *et al.*, 2014). Further studies will be needed to clarify the relationship between gender and various steroid hormones with the success of treatment in major depressive disorder.

Here, we found a significant and strong correlation between the decrease in ADAM scores and the rate of change in plasma DHEA-S levels, whereas no such correlation was identified between the percentage of change in plasma DHEA-S levels and the decrease in SIGH-D 17 scores. This result demonstrates that a change in plasma DHEA-S levels is involved in the pathophysiology of the main symptoms of major depressive disorder, severity of anxiety, and depressive mood, thus, warrants for further research.

After analysis of antidepressant efficacy in this study, we found that a patient treated with trazodone alone did not experience remission, whereas another patient achieved remission after treatment with the same dose of trazodone plus imipramine. A patient treated with imipramine alone also remitted. One possible explanation for this discrepancy is different affinity of antidepressants for sigma-1 receptor. Higher affinity of imipramine (compared with trazodone) for sigma-1 receptor has been shown in previous studies that compared the affinities of various ligands for sigma-1 receptor, including several antidepressants (Narita *et al.*, 1996; Garrone *et al.*, 2000; Cobos *et al.*, 2008). Amitriptyline, an antidepressant with low sigma-1 receptor affinity (Werling *et al.*, 2007), also failed to produce remission in the present study. These results are consistent with Hashimoto's suggestion (2009) that the differences in affinity for sigma-1 receptor among various antidepressants may be responsible for the varied efficacy of antidepressants (Volz and Stoll, 2004; Hashimoto, 2009; Maurice and Su, 2009). Medium affinity of DHEA-S has been reported, which is lower than that of imipramine and fluvoxamine but the same as that of fluoxetine (Cobos *et al.*, 2008). Therefore, the decline in plasma DHEA-S levels observed during remission may be related to the affinity of antidepressants for sigma-1 receptor and the resulting changes in the association of DHEA-S with sigma-1 receptor. On the other hand, not all of the functional mechanisms of antidepressants in our study can be explained by the sigma-1 receptor connection.

According to the results of our study, DHEA-S is likely to be involved in the pathophysiology of major depressive disorder; therefore, plasma DHEA-S may be an accurate biomarker of remission.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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Analysis of the *VAV3* as Candidate Gene for Schizophrenia: Evidences From Voxel-Based Morphometry and Mutation Screening

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In recently completed Japanese genome-wide association studies (GWAS) of schizophrenia (JPN_GWAS) one of the top association signals was detected in the region of *VAV3*, a gene that maps to the chromosome 1p13.3. In order to complement JPN_GWAS findings, we tested the association of rs1410403 with brain structure in healthy individuals and schizophrenic patients and performed exon resequencing of *VAV3*. We performed voxel-based morphometry (VBM) and mutation screening of *VAV3*. Four independent samples were used in the present study: (1) for VBM analysis, we used case-control sample comprising 100 patients with schizophrenia and 264 healthy controls, (2) mutation analysis was performed on a total of 321 patients suffering from schizophrenia, and 2 case-control samples (3) 729 unrelated patients with schizophrenia and 564 healthy comparison subjects, and (4) sample comprising 1511 cases and 1517 healthy comparison subjects and were used for genetic association analysis of novel coding variants with schizophrenia. The VBM analysis suggests that rs1410403 might affect the volume of the left superior and middle temporal gyri ($P = .011$ and $P = .013$, respectively), which were reduced in patients with schizophrenia compared with healthy subjects. Moreover, 4 rare novel missense variants were detected. The mutations were followed-up in large independent sample, and one of the novel variants (Glu741Gly) was associated with schizophrenia ($P = .02$). These findings demonstrate that *VAV3* can be seen as novel candidate gene for schizophrenia in which both rare and common variants may be related to increased genetic risk for schizophrenia in Japanese population.

Key words: resequencing/MRI/Japanese population/axon guidance/rare variant/GWAS

Introduction

Schizophrenia is a severe mental disorder with a lifetime risk of about 1%, characterized by hallucinations, delusions, and cognitive deficits, with heritability estimated at up to 80%. Recently, there have been a few major advances in identifying common variants associated with schizophrenia by genome-wide association studies (GWAS). The GWAS approach has both highlighted genes previously identified by the candidate gene approach studies or by basic biological investigation and illuminated novel genomic loci clearly associated with schizophrenia that were previously unsuspected.[1–3]

In recently completed Japanese GWAS of schizophrenia (JPN_GWAS),[4] one of the top association signals based on the meta-analysis for Japanese sample (rs1410403, $P_{CMH} = 9.3 \times 10^{-4}$, OR = 0.86) was detected in the region of VAV3 (see online supplementary table 1). VAV3 is closely related to the axon guidance pathways, which are implicated in etiology of schizophrenia.[5] During axon guidance, ephrin binding to Ephs triggers VAV-dependent endocytosis of the ligand-receptor

complex, converting an initially adhesive interaction into a repulsive event. In the absence of VAVs, ephrin-Eph endocytosis is blocked, leading to defects in growth cone collapse in vitro and significant defects in the ipsilateral retinogeniculate projections in vivo.[6] Therefore, VAV family guanine nucleotide exchange factors (GEFs) may play an important role as regulators of ligand-receptor endocytosis and determinants of repulsive signaling during axon guidance. The additional findings implicating the relevance of this locus for pathogenesis of schizophrenia came from genomewide linkage analysis of 236 Japanese families.[7] Specifically, they located the strongest evidence of linkage at rs2048839 (LOD [logarithm (base 10) of odds] = 3.39) and 95% CI comprises *VAV3* locus (Chr1: 102.0–111.9 Mbp, based on NCBI36 annotation).

Based on genetic evidence from the JPN_GWAS, the meta-analysis for Japanese sample, biological studies, and linkage evidence *VAV3* can be seen as novel candidate gene for schizophrenia. Therefore, to follow-up JPN_GWAS findings, we tested the association of rs1410403 with brain structure in healthy individuals and schizophrenic patients. Because biological phenotypes (eg, brain structure and function) are thought to more closely reflect the effects of genetic variation as compared with manifest psychiatric illness, endophenotype studies have proven to be more robust and require vastly smaller sample sizes than purely diagnosis-based studies.[8] Furthermore, statistical genetic association studies can provide a link between genes and complex polygenetic constructs like schizophrenia, but this approach does not illuminate the possible underlying pathophysiology impacted or the mechanisms of association. Here, we used imaging approach to examine the impact of variation in *VAV3* on risk for schizophrenia and function and structure in human brain of neural circuitries implicated in the pathophysiology of schizophrenia. Furthermore, in terms of genetic architecture, liability to schizophrenia is related to the number of loci involved and the effect size of each risk variant, and on the population level, these 2 factors combine to form an "allelic spectrum" which is bounded by "common disease/common variant" and "multiple rare variant" models.[9] Based on the results of recent schizophrenia GWAS, it was suggested that common variants can explain at least one-third of the total variation in liability, and genetic transmission patterns in schizophrenia may be a complex hybrid of common, low-penetrant alleles and rare, highly penetrant variants.[1] Therefore, in order to complement JPN_GWAS findings and search for novel rare variants with larger effect, we performed exon resequencing of *VAV3*.

Methods

Sample

Four independent samples were used in the present study: (1) for the voxel-based morphometry (VBM) analysis, we used case-control sample comprising 100 patients with schizophrenia (38.3 ± 13.0 y) and 264 healthy comparison subjects (36.7 ± 11.9 y), (2) mutation analysis was performed on a total of 321 patients suffering from schizophrenia (54.3 ± 14.1 y) (JMut sample) (3) JPN_GWAS comprised of 729 unrelated patients with schizophrenia (45.4 ± 15.1 y) and 564 healthy comparison subjects (44.0 ± 14.4 y) and (4) Rep_JPN comprising 1511 cases (45.9 ± 14.0 y) and 1517 healthy comparison subjects (46.0 ± 14.6 y). JPN_GWAS and Rep_JPN were used for genetic association analysis of novel coding variants with schizophrenia. The individuals with personal or family history of psychiatric disorders (first-degree relatives only based on the subject's interview) were not included in the healthy comparison group. After complete description of the study to the subjects, written informed consent was obtained. A general characterization and psychiatric assessment of subjects are available elsewhere.[10]

Voxel-Based Morphometry

All magnetic resonance (MR) studies were performed on a 1.5T GE Sigma EXCITE system. A 3-dimensional volumetric acquisition of a T1-weighted gradient echo sequence produced a gapless series of 124 sagittal sections using a spoiled gradient recalled acquisition in the steady state sequence (TE [Eecho time]/TR [repetition time], 4.2/12.6 ms; flip angle, 15°; acquisition matrix, 256 × 256; 1NEX [number of excitations], FOV [Field of view], 24 × 24 cm; and slice thickness, 1.4 mm). Statistical analyses were performed with Statistical Parametric Mapping 5 (SPM5) software (http//www.fil.ion.ucl.ac.uk/spm) running on MATLAB R2007a (MathWorks, Natick, MA). MR images were processed using optimized VBM in SPM5 according to VBM5.1-Manual (http://dbm.neuro.uni-jena.de/vbm/vbm5-for-spm5/manual/) as described in detail previously.[11,12] Each image was confirmed to eliminate images with artifacts and then anterior commissure-posterior commissure line was adjusted. The normalized segmented images were modulated by multiplication with Jacobian determinants of the spatial normalization function to encode the deformation field for each subject as tissue density changes in the normal space. Finally, images were smoothed with a 12-mm full-width half-maximum of isotropic Gaussian kernel.

In first stage of the analysis, we performed whole brain search to explore the effects of diagnosis, genotype, and their interaction on gray matter (GM) volume in patients with schizophrenia and controls. These effects on GM volume were assessed statistically using the full factorial model for a 2 × 2 ANOVA in SPM5. We contrasted GM volumes between the genotype groups (individuals with A/A genotype and G-carriers), the diagnosis groups (smaller volume region in patients with schizophrenia relative to controls), and the diagnosis-genotype interaction. Age, sex, and education years were included to