control for confounding in all analyses. Because it is desirable to adjust for each subject's global GM volume,[11] adjustment was performed by entering the global GM values as a covariate. Nonsphericity estimation was used. These analyses yielded statistical parametric maps (SPM (*t*)) based on a voxel-level height threshold of $P < .001$ (uncorrected for multiple comparisons). Only the clusters of more than 100 contiguous voxels were considered in the analyses. Additionally, small volume correction (SVC) was applied in order to protect against type I error using family wise error (FWE). The significance level was set $P < 0.05$ (FWE corrected) after SVC, spheres with radius 10 mm around the peak.

In second stage of the analysis, we extracted a sphere of 10 mm volume of interest (VOI)-radius on left superior temporal gyrus and left middle temporal gyrus to compare regions of the genotype effects. Anatomic localization was according to both MNI coordinates and Talairach coordinates, obtained from M. Brett's transformations (http://www.mrccbu.cam.ac.uk/Imaging/Common/mnispace.shtml) and presented as Talairach coordinates.

Statistical analyses were performed using PASW Statistics 18.0 software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2 tests for categorical variables and the Mann-Whitney *U* test for continuous variables. We extracted the "*y*" values from the left superior temporal gyrus and left middle temporal gyrus maxima voxel and used these values in the VOI analysis using PASW. The effects of the variant in *VAV3* on extracted VOI were tested using the 2-way ANOVA without covariates because the extraction of VOI was performed after confounding factors, including age, sex, education years, and total GM volumes, were included in the whole-brain search analyses. Statistical significance was defined as $P < .05$.

Mutation Screening

For the purpose of mutation screening, we have designed a custom resequencing microarray, based on NCBI36 build (Affymetrix, Santa Clara, CA), NAGOYA_DESIGN, which primarily focuses on the genes selected, based on the JPN_GWAS findings. The sequences tiled on the microarray included the sequences of all *VAV3* exons totaling 4933 bps (consensus CDS transcripts ENST00000370056 and ENST00000343258). Because the principle of the resequencing microarray is based on sequencing by hybridization, it was crucially important to avoid cross-hybridization to increase the accuracy of resequencing. For this purpose, we conducted an in-silico screening to compare the tiled sequences with a sliding 25-nucleotide window to detect the sequences with an identity exceeding 22 bases in the tiled sequences and optimized the design of the microarrays and polymerase chain reaction (PCR) primers. Initially, the arrays were run according to the manufacturer's protocol. Briefly, long-range PCR conditions for the LA TaKaRa Polymerase (Takara, Japan) were: TaKaRa LA Taq 0.05 U/μl, 1X LA PCR Buffer II, 400 μM (each) deoxynucleotide triphosphate, 0.3 μM (each) primers, 4 ng/μl genomic DNA in a 25 μl reaction volume. Modifications using standard approaches to PCR optimization were made for some difficult reactions. All PCR assays were quantified using PicoGreen (Molecular Probes, Eugene, OR) and then pooled in equimolar amounts. The PCR products were then purified, fragmented, labeled, and hybridized to the array. Finally, affymetrix GSEQ 4.0 Software (default settings) was used to process raw data and analyze the nucleotide sequences. SeqC Ver. 3.2.1.5 (JSI-medisys, www.jsi-medisys.de) was used to reanalyze the acquired datasets and assign annotation (based on NCBI 36 build). Novel variants with frequency of less than 5% were validated by cycle sequencing on ABI 3130xl DNA Analyzer (Applied Biosystems) according to standard manufacturer protocol. Allelic discrimination was performed using Taqman (Applied Biosystems) custom probes (details about DNA sequences, and PCR conditions are available upon request). Each 384 microtiter plate contained at least 3 nontemplate controls and the sample(s) in which novel variant was observed. Analysis was performed on HT7500 instrument (Applied Biosystems) according to the standard protocol. In-silico analysis of deleterious effect of amino acid substitution was done by algorithms implemented in LRT,[13] PMUT,[14] and PANTHER.[15] All these tools operate using approximately the same principles, that is, they are all supervised and employ features based on protein sequence, sequence conservation, and/or protein structure. The interpretation of the results was done based on score of the likelihood that missense variants, which cause a single amino acid substitution within a protein sequence, may or may not lead to altered protein function.

As basis for a more detailed functional interpretation of the novel rare variants, we performed ab-initio structure predictions based on I-TASSER algorithm.[16] This automated pipeline predicts secondary and tertiary protein structure based on sequence homology between protein/domain of interest and the proteins/domains with experimentally determined structures. The output of I-TASSER is analyzed and visualized using UCSF Chimera.[17]

All allele-wise association analyses were carried out by calculating the *P* values for each SNP using Fisher's exact test. In meta-analysis (JPN_GWAS and Rep_JPN sample), *P* values were generated by Cochran-Mantel-Haenszel stratified analysis. Two-tailed *P* values of less than 0.05 were considered significant. Calculations were done using Plink v1.07.[18]

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Table 1. Demographic Information for Patients With Schizophrenia and Healthy Controls Included in the VBM Analysis

	Schizophrenia (N = 100)					Control (N = 264)					Group Difference
	A/A		G-carrier			A/A		G-carrier			
	(N = 52)		(N = 48)			(N = 131)		(N = 133)			
Variables[a]	Mean	SD	Mean	SD	P values (z)[b]	Mean	SD	Mean	SD	P values (z)[b]	P values (z)[b]
Age (years)	37.7	12.5	39	13.5	0.91 (−0.12)	36.2	11.9	37.2	11.3	0.35 (−0.93)	0.37 (−0.89)
Sex (male/female)	25/27		28/20		0.30 (1.05)	51/80		69/64		**0.035 (4.46)**	0.20 (1.66)
Education (years)	14.1	2.5	13.7	2.2	0.38 (−0.87)	14.9	2.1	15.3	2.4	0.21 (−1.26)	**<0.001 (−4.13)**
Estimated premorbid IQ	102.7	10.8	99.9	9.2	0.23 (−1.20)	107.4	8.6	106.9	7.6	0.58 (−0.55)	**<0.001 (−5.07)**
Gray matter volume (mm^3)	666.2	73.1	688	82.6	0.23 (−1.20)	702.4	71.7	706.2	81.3	0.79 (−0.26)	**0.003 (−2.96)**
CPZ-eq (mg/day)[c]	584.4	510.2	639.5	610.7	0.75 (−0.32)	—	—	—	—	—	—
Age at onset (years)	25.5	10.6	25.1	10.9	0.91 (−0.11)	—	—	—	—	—	—
Duration of illness (years)	12.2	10	13.9	10.7	0.37 (−0.90)	—	—	—	—	—	—
PANSS positive symptoms	20.1	5.7	17.1	5.9	**0.009 (−2.61)**	—	—	—	—	—	—
PANSS negative symptoms	19.8	6.4	18.3	6.2	0.13 (−1.50)	—	—	—	—	—	—

Note: PANSS, Positive and Negative Syndrome Scale.
[a]Some demographic information was obtained in part of subjects (estimated premorbid IQ and PANSS in patients: A/A, N = 49; G-carriers, N = 46, Estimated premorbid IQ in controls: A/A, N = 130).
[b]Significant results are bolded and underlined.
[c]CPZ-eq: chlorpromazine equivalent of total antipsychotics.

Results

Voxel-Based Morphometry

We investigated effects of diagnosis, genotype, and their interaction on GM volumes in the whole brain analyses. There was no difference in demographic variables between *VAV3* genotype groups, except for scores of positive symptom between the genotype groups in patients with schizophrenia and sex between the genotype groups in healthy controls (table 1). Patients with schizophrenia showed reduced GM volumes compared with controls mainly in the frontal lobe and temporal lobe (data not shown), consistent with previous studies.[19,20] Genotype effects on GM volume in several brain regions were found in all subjects (uncorrected $P < .001$, table 2). Individuals with A/A genotype had reduced GM volumes of left superior and middle temporal gyri than G-carriers, while individuals with A/A genotype had larger GM volumes of cerebellum anterior lobe (culmen) and right medial frontal gyrus than G-carriers (uncorrected $P < .001$, table 2). Additionally, we found significant diagnosis-genotype interaction of GM volume in the right medial frontal

Table 2. Effects of the VAV3 Genotype on Brain Morphology in All Subjects

						Talairach Coordinates		
Brain regions	R/L	BA	Cluster Size	T_{356}	P values[a]	x	y	z
A/A < G-carriers								
Superior temporal gyrus	L	13	194	3.8	**0.011**	−50	−48	18
Middle temporal gyrus	L	22	124	3.8	**0.013**	−53	−42	0
A/A > G-carriers								
Cerebellum anterior lobe (culmen)	R	NA	1533	4.1	**0.004**	7	−40	−12
Medial frontal gyrus	R	25	286	3.8	**0.012**	6	6	−17
VAV3 genotype × diagnosis interaction								
Medial frontal gyrus	R	25	271	3.9	**0.009**	6	7	−18

Note: R, right; L, left; BA, brodmann area.
[a]Significant results ($P < .05$ [FWE corrected]) are indicated with bold and underline.

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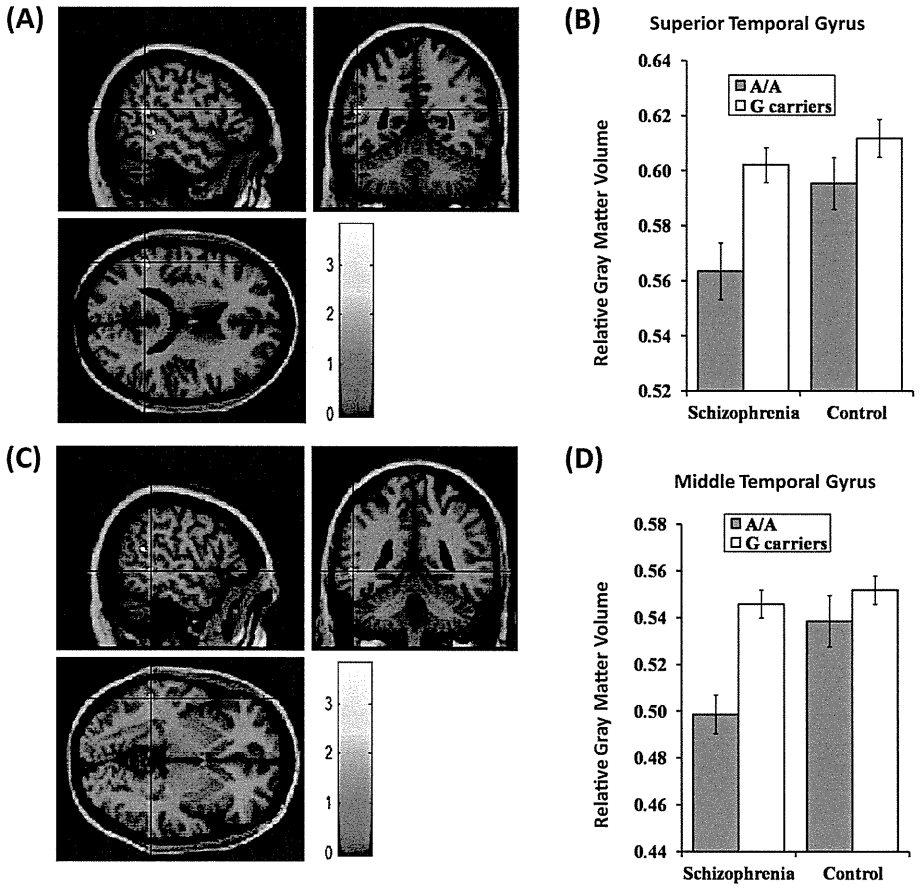

Fig. 1. Voxel-based morphometry. Impacts of VAV3 genotype on gray matter (GM) volumes of left superior temporal gyrus and left middle temporal gyrus. (A and C) Anatomical localizations were displayed on coronal, sagittal, and axial sections of a normal magnetic resonance imaging spatially normalized into the Montreal Neurological Institute template (uncorrected $P < .001$, cluster size >100). Significant clusters of genotype effect were in the left superior temporal gyrus (Talairach cordinate; −50, −48, 18) (A) and in the left middle temporal gyrus (−53, −42, 0) (C). These regions were showed as cross-hairline. The color bars showed *t* values corresponding to color in the figure. (B and D) Each column showed relative GM volumes extracted from left superior temporal gyrus (−50, −48, 18) (B) and left middle temporal gyrus (−53, −42, 0) (D). Data represent means ± SEM.

gyrus (uncorrected $P < .001$, table 2). These results remained positive after SVC for multiple tests (FWE corrected $P < .05$ after SVC).

Systematic searches for VBM studies have reported that smaller volumes of the left temporal gyrus were found in patients with schizophrenia than those in healthy subjects. Based on the JPN_GWAS data, individuals with A/A genotype of the rs1410403 were enriched in patients with schizophrenia. Therefore, we focused on the individuals with A/A genotype of the rs1410403 in *VAV3* as they may have a reduction of GM in the left superior temporal and left middle temporal gyri. Two-way ANOVA revealed significant effects of diagnosis ($F_{1,360} = 5.77$, $\eta^2 = 0.016$, $P = .017$) and genotype ($F_{1,360} = 10.04$, $\eta^2 = 0.027$, $P = .0017$) in the extracted region centering the left superior temporal gyrus (−50, −48, 18) (figure 1A and B). No interaction was found in the left superior temporal gyrus ($F_{1,360} = 1.65$, $\eta^2 = 0.0046$, $P = .20$). Individuals with homozygous A had smaller GM volumes of the left superior temporal gyrus than G-carriers. We also found significant effects of diagnosis ($F_{1,360} = 8.03$, $\eta^2 = 0.022$, $P = .0049$) and genotype ($F_{1,360} = 14.04$, $\eta^2 = 0.038$, $P < .001$) and their interaction ($F_{1,360} = 4.40$, $\eta^2 = 0.012$, $P = .037$) in the extracted region centering the left middle temporal gyrus (−53, −42, 0) (figure 1C and D). As the genotype-diagnosis interaction was found in the left middle temporal gyrus, we analyzed the effects of genotype on the region in patients and controls separately. Patients with schizophrenia showed that patients with A/A genotype had smaller GM volumes of the region than G-carriers ($F_{1,98} = 12.00$, $\eta^2 = 0.11$, $P < .001$). In contrast, controls showed no genotype effect on GM volumes of the region ($F_{1,262} = 2.46$, $\eta^2 = 0.0093$, $P = .12$).

Mutation Screening

We detected 4 novel nonsynonymous heterozygous variants within the JMut sample (321 schizophrenic

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patients). Protein homology analysis showed that VAV3 is highly conserved between species (~95% identical amino acids between human and mouse), accordingly the identified point mutations affected conserved residues (see online supplementary table 2). All the variants detected were located in the C-terminal region of VAV3 (see online supplementary figure 1).

The identified novel variants were reconfirmed by cycle sequencing and followed-up in 2 large independent schizophrenia case-control samples (Rep_JPN and JPN_GWAS sample). Only one rare variant (Glu741Gly) showed statistical evidence for association in meta-analysis (P_{CMH} = .020, OR = 0.58), while the others were observed at similar frequencies both in case and control samples (table 3). In-silico analysis of the missense variants applying 3 different algorithms predicted Glu741Gly as variant of functional relevance (table 4). Detailed 3-dimensional structural analysis of SH2 domain (wild type—figure 2a) indicated specific interaction (hydrogen bond) between Glu741 (side chain) and Lys735 (main chain). Point mutation at position 741 (Glu→Gly) would abolish hydrogen bonding because glycine does not contain a side chain (figure 2b). Moreover, beta strand extending into Lys735 is lost in the model of VAV3 mutant. The functional consequence of the associated point mutation is disappearance of casein kinase 2 phosphorylation site (SXXE)[21] as shown on figure 2c and d.

Discussion

Our study reports the systematic genetic evaluation of *VAV3*, as a candidate gene for schizophrenia based on our genome wide screening.[4] Specifically, meta-analysis of the JPN_GWAS and follow-up sample provided genetic evidence for the involvement of *VAV3* locus in schizophrenia in the Japanese population. Mutation screening of all *VAV3* coding exons did not reveal evidence for the existence of a common (minor allele frequency >5%) nonsynonymous variant that explains the association signal in JPN_GWAS.

The VBM analysis showed that the associated common SNP (rs1410403) might affect the volume of the left superior and middle temporal gyri, which were reduced in patients with schizophrenia compared with healthy subjects. VBM analysis suggests that *VAV3*'s influence is focal to the aforementioned regions. Furthermore, the Allen Brain Atlas (http://human.brain-map.org) records relatively high levels of expression of the human *VAV3* gene and lower expression levels of other VAV family GEFs (*VAV1* or *VAV2*) in left superior and middle temporal gyri. Considering VAV3 biological function (developmental processes in particular), we speculate that our macroscopic observation using VBM approach is result of neuronal distribution and differential activity of VAV3 protein associated with rs1410403 genotypes.

Table 3. Resequencing Results

Chr	Variant	Physical Position[a]	Protein Domain	M	JMut (Minor Allele Count)	m	JPN_GWAS (MAF)			Rep_JPN (MAF)			Meta-Analysis	
							Cases	Control	P_{allele}	OR	Cases	Control	P_{allele}	OR	P_{CMH}	OR
1	p.Asp623Val	107.986.810	N-SH3	A	2	T	0.0006964	0.0008993	0.8561	0.7742	0.0003344	0	0.3171	NA	0.6649	1.662
1	p.Glu685Lys	107.947.271	SH2	G	1	A	0.0006974	0.001821	0.4151	0.3824	0.0003336	0	0.3168	NA	0.8415	0.8246
1	p.Glu705Lys	107.947.211	SH2	G	3	A	0.0007022	0.0009074	0.8557	0.7737	0.0006658	0.0003311	0.5605	2.011	0.7354	1.355
1	P.Glu741Gly	107.940.485	SH2	A	7	G	0.004972	0.01087	0.09038	0.4547	0.0074480	0.0117400	0.0897	0.6314	0.02065	0.5821

Note: M, major allele; m, minor allele; MAF, minor allele frequency; OR, odds ratio; P_{CMH}, Cochran-Mantel-Hentzel test.
[a]NCBI 36 build.

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Table 4. In-Silico Analysis

Variant	Protein Domain	Genomic Data: Physical Position[a]	Strand	Alleles M/m	Impact on Protein Structure/Function: PMUT (Prediction Score)[b]	Panther (subPSEC Score)[c]	LRT (P value)[d]
p.Asp623Val	N-SH3	Chr1: 107986810	−1	A/T	Yes (0.97)	Yes (−3.99)	Yes (4.29×10^{-8})
p.Glu685Lys	SH2	Chr1: 107947271	−1	G/A	Yes (0.93)	No (−2.04)	Yes (7.08×10^{-10})
p.Glu705Lys	SH2	Chr1: 107947211	−1	G/A	Yes (0.75)	No (−1.74)	Yes (1.83×10^{-7})
p.Glu741Gly	SH2	Chr1: 107940485	−1	A/G	Yes (0.89)	Yes (−3.24)	Yes (1.64×10^{-6})

Note: M/m, major/minor allele.
[a]NCBI36 build.
[b] >0.5 is interpreted as nonneutral substitution.
[c]Less than −3 is interpreted as nonneutral substitution.
[d]<0.001 interpreted as nonneutral substitution.

Systematic searches for VBM studies have reported that reduced volumes of the left temporal gyrus were found in patients with schizophrenia.[19] Furthermore, the data from previous study of the superior temporal gyrus[22] and a study of the middle temporal gyrus in which patients with schizophrenia who were predisposed to auditory hallucinations showed reduced activation of the left middle temporal gyrus when imagining sentences in another person's voice.[23] Several other functional magnetic resonance imaging studies have reported decreased left and increased right middle temporal gyrus activation in schizophrenia during auditory verbal hallucinations.[24,25] In our exploratory analysis (see online supplementary material), positive symptoms scores of Positive and Negative Syndrome Scale (PANSS) were not able to predict the VOIs in left superior and left middle temporal gyri ($P > .05$). It is of note that VBM sample size in the current study might not be sufficient to detect the effect of positive symptoms score of PANSS on VOIs in left superior and left middle temporal gyri because positive symptoms score of PANSS is derived from symptomatology that is characterized by excess or distortion

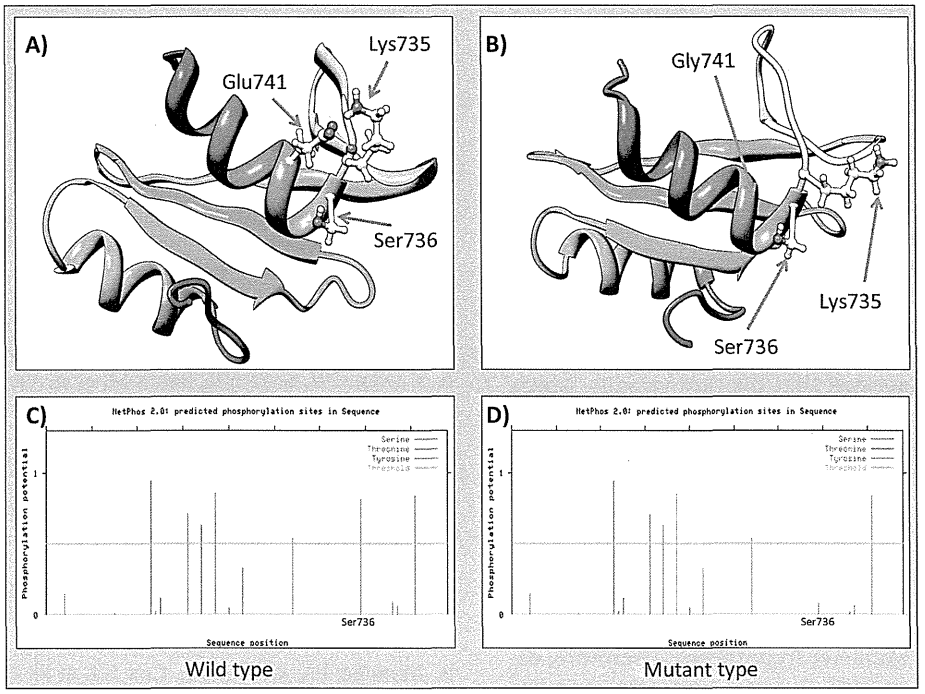

Fig. 2. Ab-initio 3D modeling of SH2 domain (VAV3). (a) Wild type, note hydrogen bond between Glu741 and Lys735 (blue line); (b) Mutant type, hydrogen bond between Gly741 and Lys735 cannot be formed; (c) and (d) posphorylation potential of serine (blue), threonine (green), and tyrosine (red). Threshold is marked by horizontal gray line (phosphorylation analysis performed by NetPhos 2.0).

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of the individual's normal functioning and therefore may not reflect only auditory hallucinations.

We detected several rare variants close to original association signal and in case of one rare variant (Glu741Gly), we observed nonsignificant association trend. When the evidence was combined across the 2 samples (JPN_GWAS and Rep_JPN sample), we found that the *P* values had strengthen. VAV3 is composed of 8 domains: calponin homology (CH), Acidic (Ac), Dbl homology (DH), pleckstrin homology (PH), zinc finger (ZF), Src homology 3 (SH3), Src homology 2 (SH2), and a second SH3 (see online supplementary figure 1).[26] Interestingly, the associated common variant (rs1410403) is located in LD block that encompass coding exons of SH2 domain of vav3 (see online supplementary figure 2), and the associated rare variant (Glu741Gly) is located within the exon that is translated into the SH2 domain of vav3 (see online supplementary figure 1).

VAV family GEFs have been implicated as regulators of Eph receptor endocytosis, the event which is required for efficient cell detachment.[27] This can provide a complex and dynamic set of cues that either repel or attract axons toward their synaptic targets, converting initially adhesive interaction into a repulsive force. Several studies had shown that SH2 domain of the VAV3 binds to phosphorylated tyrosine residue(s) on the EphA receptors,[6,28] which triggers endocytosis of EphA receptors and growth cone collapse.[27] Although the mechanisms by which Glu741Gly contributes to schizophrenia pathogenesis remain to be explored, we note that 3 different bioinformatics algorithms had predicted functional effect, and Glu741Gly have stronger protective effects on schizophrenia risk (OR = 0.58) than does the associated common SNP (OR = 0.81). Moreover, analysis of phosphorylation sites showed that point mutation at position 741 (Glu→Gly) would abolish phosphorylation of Ser736 by casein kinase II. The substitution of glutamic acid by Glycine at position 741 might have as a consequence alteration of the biological function of VAV3 because a substrate with *n* phosphorylation sites has an exponential number (2^n) of phosphoforms and each phosphoform may have distinct properties.[29] Our genetic association results suggested that the rare variant, which is predicted to alter function of the VAV3, would decrease the risk of schizophrenia, whereas normal function is associated with schizophrenia. Same protective allelic effect was observed for common variant identified by JPN_GWAS.

As the conclusion, our results showed that in case of schizophrenia, the "rare high-risk variant" vs the "common variant with low effect" hypotheses should not be viewed as 2 mutually exclusive hypotheses. Therefore, direct resequencing of candidate genes and copy number variants on the one side and GWAS analyses on the other side could be viewed as complementary approaches to analyze the genetic susceptibilities to schizophrenia.

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Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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Resequencing and Association Analysis of the *KALRN* and *EPHB1* Genes And Their Contribution to Schizophrenia Susceptibility

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Background: **Our genome-wide association study of schizophrenia found association signals at the Kalirin gene (*KALRN*) and EPH receptor B1 gene (*EPHB1*) in a Japanese population. The importance of these synaptogenic pathway genes in schizophrenia is gaining independent supports. Although there has been growing interest in rare (<1%) missense mutations as potential contributors to the unexplained heritability of schizophrenia, there are no population-based studies targeting rare (<1%) coding mutations with a larger effect size (eg, OR >1.5) in *KALRN* or *EPHB1*. *Methods and Results:*** **The present study design consisted of 3 phases. At the discovery phase, we conducted resequencing analyses for all exon regions of *KALRN* and *EPHB1* using a DNA microarray–based method. Seventeen rare (<1%) missense mutations were discovered in the first sample set (320 schizophrenic patients). After the prioritization phase based on frequencies in the second sample set (729 cases and 562 controls), we performed association analyses for each selected mutation using the third sample set (1511 cases and 1517 controls), along with a combined association analysis across all selected mutations. In *KALRN*, we detected a significant association between schizophrenia and P2255T (OR = 2.09, corrected P = .048, 1 tailed); this was supported in the combined association analysis (OR = 2.07, corrected P = .006, 1 tailed). We found no evidence of association of *EPHB1* with schizophrenia. *In silico* analysis indicated the functional relevance of these rare missense mutations.** ***Conclusion:*** **We provide evidence that multiple rare (<1%) missense mutations in *KALRN* may be genetic risk factors for schizophrenia.**

Key words: synaptogenic pathway/rare missense mutations/GWAS/Japanese population

Introduction

Schizophrenia is a genetically heterogeneous disorder with heritability estimated at up to 80%.[1] According to a recent simulation based on genome-wide association study (GWAS) datasets, a highly polygenic model involving a number of common variants of very small effect may explain more than one-third of the total variation in risk of schizophrenia.[2] On the other hand, interest has been growing in rare variants as potential contributors to the unexplained heritability of schizophrenia.[3] This is partly triggered by recent studies establishing an important role for rare genomic copy number variants (CNVs) in the etiology of schizophrenia.[4] Another potential genetic variation to explain the remaining heritability is rare missense mutations. Kryukov et al[5] reported that ~20% of new (de novo) missense mutations in humans result in a loss of function, whereas ~53% have mildly deleterious effects and ~27% are effectively neutral with respect to phenotype by a combined analysis of mutations causing human Mendelian diseases, mutations driving human-chimpanzee sequence divergence, and systematic data on human genetic variation. Their results were supported by an independent study.[6] Because the pressure of purifying selection acting on the mildly deleterious mutations is weak, their cumulative high frequency in the human population is being maintained

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by "mutation-selection balance." This provides support to a speculation that the accumulation of mildly deleterious missense mutations in individual human genomes can be a genetic basis for complex diseases.[5] The importance of rare missense mutations in schizophrenia is demonstrated by a study of the *ABCA13* gene in which multiple rare (<1%) coding variants were associated with schizophrenia.[7]

We recently performed a GWAS for schizophrenia in a Japanese population.[8] Although single locus analysis did not reveal genome-wide support for any locus, a shared polygenic risk of schizophrenia between the Japanese and the Caucasian samples was confirmed. In our GWAS, association signals were detected at the regions of the Kalirin gene (*KALRN*) on 3q21.2 and the EPH receptor B1 gene (*EPHB1*) on 3q21-q23, both of which are in the same synaptogenic pathway[9] (supplementary figure S1). Associations of each gene with schizophrenia have recently received support from independent GWASs in different populations.[10,11] Furthermore, a rare de novo CNV overlapping with the *EPHB1* gene locus was detected in a patient with schizophrenia.[12]

KALRN is a large neuronal dual Rho guanine nucleotide exchange factor (GEF) that activates small guanosine triphosphate–binding proteins of the Rho family, including Rac1.[13] This activation enables KALRN to regulate neurite initiation, axonal growth, dendritic morphogenesis, and spine morphogenesis. Consistent with its biological function, KALRN is a key factor responsible for reduced densities of dendritic spines on pyramidal neurons in the dorsolateral prefrontal cortex (DLPFC)[14] observed in postmortem brains from schizophrenic patients. The messenger RNA expression level of *KALRN* is significantly reduced in DLPFC of patients with schizophrenia and strongly correlated with spine density.[15] In addition, *KALRN*-knockout mice not only exhibit spine loss and reduced glutamatergic transmission in the frontal cortex but also schizophrenia-like phenotypes including robust deficits in working memory, sociability, prepulse inhibition, and locomotor hyperactivity reversible by clozapine, an atypical antipsychotic.[16] These synaptic and behavioral dysfunctions are apparent during young adulthood in mice (12 weeks old), which coincides with the onset of schizophrenia in patients. Notably, Disrupted-in-Schizophrenia 1, a prominent schizophrenia risk factor, was shown to be involved in the maintenance of spine morphology and function by regulating access of KALRN to Rac1.[17] *EPHB1* belongs to a receptor tyrosine kinase family and controls multiple aspects of neuronal development, including synapse formation and maturation, as well as synaptic structural and functional plasticity. In neurons, activation of EphB receptors by its ligand B-type ephrins induces the rapid formation and enlargement of dendritic spines, as well as rapid synapse maturation. One of the downstream effectors of ephrinB/EphB signaling is KALRN. In young hippocampal neurons, KALRN is reported to play an important role in the maturation of synapses induced by trans-synaptic ephrinB/EphB signaling.[18]

According to the above-mentioned study,[5] most missense mutations with a frequency of <1% are mildly deleterious, indicating that a low frequency of missense mutation per se can serve as a strong predictor of a deleterious effect of variants. Therefore, the working hypothesis of the present study is that rare (<1%) missense or nonsense mutations with a larger effect size (eg, OR >1.5) in *KALRN* and *EPHB1* may be genetic risk factors for schizophrenia. Recently, a DNA microarray–based resequencing method has been developed to enable accurate and rapid resequencing analysis of candidate genes.[19] Using this system, we conducted resequencing analyses for all exon regions of *KALRN* and *EPHB1* in 320 schizophrenic patients and found evidence that rare (<1%) missense mutations in *KALRN* are significantly associated with schizophrenia using the 3-phase study design.

Methods and Materials

Subjects

Three sample sets were used in this study. The first sample set, comprising 320 schizophrenic patients (mean age, 54.2 ± 14.1 years, 49.1% male), with long-term hospitalization for severe symptoms, was used to search for rare missense or nonsense mutations. We used the first sample set for mutation screenings because patients with extreme phenotypes (severe symptoms) can be expected to carry more deleterious mutations.[20] The second sample set, including 729 cases (45.4 ± 15.1 years, 52.2% male) and 562 controls (44.0 ± 14.4 years, 49.8% male), was used to prioritize detected functional variants for subsequent association analyses. The third sample set, including 1511 cases (45.9 ± 14.0 years, 49.6% male) and 1517 controls (46.0 ± 14.6 years, 49.6% male), was used for association analyses. Age and gender were matched in the second and third sample sets, respectively. All patients were diagnosed according to *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*, criteria, and controls were evaluated using unstructured interviews to exclude individuals with history of mental disorders. Detailed information regarding diagnostic procedures is available elsewhere.[21] All subjects were ethnically Japanese and provided written informed consent. This study was approved by the ethics committees at each participating university.

Array Design for Resequencing Analyses

We used the Affymetrix GeneChip CustomSeq Resequencing Array (Affymetrix, Santa Clara, California) for exon sequencing in the first sample set. These arrays rely on allele-specific hybridization for determining DNA

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sequence.[19] Each individual nucleotide of both the sense and the antisense DNA strands is interrogated with four 25-mer probes that differ only with respect to the central position (A, C, G, and T). According to Affymetrix's Custom-Seq Array Design Guide, we designed arrays covering all exon regions of *KALRN* and *EPHB1* (Ensembl release 52 [Human CCDS set]; Transcript: ENST00000360013, ENST00000240874, and ENST00000291478 for *KALRN*; ENST00000398015 for *EPHB1*). Because the principle of the resequencing arrays is based on hybridization, it is necessary to avoid cross-hybridization for accurate resequencing. For this purpose, we removed repetitive elements and highly homologous sequences from the array design.

Array-Based Resequencing

The experiments were conducted according to the manufacturer's instructions (supplementary figure S2). Genomic DNA was extracted from peripheral blood using standard methods. To generate enough target-enriched subject material for hybridization to the arrays, we generated 47 and 14 amplicons per sample for *KALRN* and *EPHB1*, respectively, using long-range polymerase chain reaction (PCR). The PCR conditions were as follows: 94°C for 2 minutes followed by 30 cycles consisting of 94°C for 15 seconds, 68°C for 3 minutes, followed by a final extension of 68°C for 8 minutes, using TaKaRa LA Taq™ (Takara Bio, Otsu, Shiga, Japan). Each PCR product was quantified using PicoGreen (Molecular Probes, Eugene, Oregon), pooled in an equimolar fashion. The PCR products were then purified, fragmented, labeled, and hybridized to the arrays, following the protocol. Finally, the arrays were washed and stained using the GeneChip Fluidics Station 450 (Affymetrix) and scanned using the GeneChip Scanner 3000 (Affymetrix). The data were analyzed using the GeneChip Operating Software (GCOS; Affymetrix), the GeneChip Sequence Analysis Software (GSEQ; Affymetrix), and SeqC (JSI Medical Systems, Kippenheim, Germany; http://www.jsi-medisys.de/html/products/SeqC/SeqC.htm) to automate the generation of sequence and genotype calls from the intensity data. In this study, around 17 kb was sequenced per sample, meaning that more than 5.4 Mb was sequenced in total. All missense mutations presented in this study were confirmed using both Sanger sequencing and Custom TaqMan SNP genotyping assays (Applied Biosystems, Foster City, California).

Association Analysis of Each Missense Mutation

Although the rare (<1%) missense mutations were originally discovered among 320 schizophrenic patients, it was possible that a portion of them might have neutral or protective effects.[5] In addition, it was necessary to reduce the number of statistical tests for multiple comparison problems. To accomplish this, we prioritized rare (<1%) deleterious variants for subsequent association analyses based on the frequencies in the second case-control sample set because rare deleterious variants relevant to schizophrenia can be assumed to have higher frequency in cases than in controls. The criteria for prioritization were as follows: (1) frequencies of mutations were <1% in controls and (2) frequencies of mutations were higher in cases (ie, OR > 1). Mutations not detected in the second sample set were not followed up in this analysis. The frequencies of such mutations can be so low (<0.0005) that the results of association analyses are unlikely to be statistically significant in our sample size. For mutations meeting the above criteria, we conducted association analyses with schizophrenia using the third sample set. Genotyping was conducted by Custom TaqMan SNP genotyping assays (Applied Biosystems). For quality control, samples with missing call rates of 10% or higher were excluded from the analyses.

Combined Association Analyses

In general, it is difficult to establish an association of a rare mutation with a phenotype because statistical power is limited by low population frequency and because the number of rare variants requires a strict multiple test correction. Therefore, we conducted combined association analyses across rare mutations observed in each gene in the third sample set, comparing the number of mutations in cases with the number in controls. The criteria for mutations included in these analyses were same as the above criteria with 1 exception: Mutations not detected in the second sample set were included in the combined association analyses.

In Silico Analysis

The potential influence of missense mutations was evaluated using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and[22] PMut (http://mmb2.pcb.ub.es:8080/PMut/)[23] softwares. PolyPhen-2 uses 8 sequence-based and 3 structure-based predictive features and compares a property of the wild-type allele and the corresponding property of the mutant allele. PolyPhen-2 trained on HumDiv datasets is reported to achieve true positive prediction rates of 92% with a false-positive rate of 20%.[22] A mutation is appraised qualitatively as benign, possibly damaging, or probably damaging based on naive Bayes posterior probability that a given mutation is damaging. PMut also allows the fast and accurate prediction (~80% success rate in humans) of the pathological character of missense mutations based on the use of neural networks. The final output is a pathogenicity index ranging from 0 to 1 (indexes >0.5 signal pathological mutations).

We also examined evolutionary conservation of the mutated residues and surrounding amino acids. Multiple sequence alignment of human *KALRN* or *EPHB1* with 6 orthologs was performed for this purpose.

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Power Calculation

Power calculation was performed with a power calculator called CaTS (http://www.sph.umich.edu/csg/abecasis/CaTS/).[24] Power was estimated under the following parameter assumptions with respect to association test statistics: genetic relative risk = 2, prevalence of disease = 0.01, risk allele frequency = the values frequency observed in controls, and $\alpha = .05$; a multiplicative model was used.

Statistical Analysis

For the association analysis of each variant, Fisher exact test was used to examine whether rare deleterious variants were significantly overrepresented in the patient group rather than the control group.

A combined association test was performed following a previous study.[7] In brief, to account for variable sample size, sample size was adjusted to $N=n/(\sum(1/Ni))$, where Ni is the sample size at the ith variant, and n is the number of variants. The number of observed variants was adjusted as $\sum(pi)\times N$, where pi is the frequency of the ith variant. Fisher exact test was used in this test as well to examine an overrepresentation of rare deleterious missense mutations in the patient group rather than control group.

All statistical tests were 1 tailed, and a P value less than 0.05 was considered significant. Bonferroni correction was used for solving multiple testing problems.

Results

Discovery of Mutations

We detected 12 and 6 missense mutations with a frequency of <5% in *KALRN* and *EPHB1*, respectively, among 320 cases in the first sample set (table 1). All but 2 mutations (N2973S in *KALRN* and T981M in *EPHB1*) were novel. All mutations were validated by both Sanger sequencing and Custom TaqMan SNP genotyping assays. In the first sample set, 2 patients were compound heterozygotes for rare missense mutations in the 2 genes. One patient had R410H in *KALRN* and R905C in *EPHB1*. The other had A2382V in *KALRN* and D375N in *EPHB1*. There were no clinical characteristics shared between these patients. No nonsense mutations were identified in this study.

Association Analysis of Each Missense Mutation

In the prioritization phase using the second sample set, T1207M and P2255T in *KALRN* and R637C and R905C in *EPHB1* showed a higher frequency in cases than in controls (table 1). Seven missense mutations (R410H, Q770K, and A2382V in *KALRN* and F151S, D375N, D577N, and T981M in *EPHB1*) were not detected. The frequency of P1695Q was more than 4% both in cases and in controls. Based on our criteria, we selected 4 missense mutations (T1207M and P2255T in *KALRN* and R637C and R905C in *EPHB1*) for subsequent association analyses using the third sample set.

In the third phase, P2255T showed a nominally significant association with schizophrenia (OR = 2.09, P = .012) in the third sample set (table 2). This remained significant after correction for multiple testing of 4 variants (corrected $P = .048$). T1207M in *KALRN* and R637C and R905C in *EPHB1* were also more frequent in cases, although differences were not significant.

We excluded mutations not detected in the second sample set from this analysis. This was supported by a power analysis showing that the third sample set had only 10% power in analysis of very rare mutations.

Combined Association Analysis

In addition to 4 mutations (T1207M and P2255T in *KALRN* and R637C and R905C in *EPHB1*), 7 very rare mutations (R410H, Q770K, and A2382V in *KALRN* and F151S, D375N, D577N, and T981M in *EPHB1*), which were not detected in the second samples set, were included in the combined association analysis. A global comparison of the frequencies of 5 selected mutations in *KALRN* between cases and controls in the third sample set showed a significant increase in frequency in schizophrenic patients (OR = 2.07, P = .003) (table 3). This remained significant after correction for multiple testing (corrected $P = .006$). On the other hand, a global comparison of the frequencies of 6 selected mutations in *EPHB1* did not show a significant difference (OR = 1.09, $P = .438$).

In Silico Analysis

Results of *in silico* analysis are shown in table 4. All missense mutations but A2382V in *KALRN* were predicted to have functional relevance by PolyPhen-2 or PMut software.

A multiple alignment of the region of *KALRN* or *EPHB1* containing rare missense mutations with 6 orthologs is shown in table 4. Most of the rare missense mutations showed a high degree of amino acid conservation in different species.

Discussion

In this study, we conducted resequencing analyses for the 2 synaptogenic pathway genes (*KALRN* and *EPHB1*) in schizophrenia using a DNA microarray–based method. After resequencing more than 5.4 Mb, we discovered 17 rare (<1%) missense mutations in *KALRN* or *EPHB1* and detected a significant association between schizophrenia and P2255T in *KALRN*, as well as in the combined association analysis for *KALRN*. These findings are consistent with an estimation that most rare (<1%) missense mutations are mildly deleterious and are associated with a heterozygous fitness loss.[5]

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Table 1. *KALRN* And *EPHB1* Missense Mutations Identified in The First Sample Set And Their Frequencies in The Second Sample Set

Gene	Genomic Position	Base Change	dbSNP Reference	AA Change	First Sample Set		Second Sample Set				
							Genotype Counts		Mutation Frequency		
					Homo	Hetero	SCZ	CONT	SCZ	CONT	OR >1
KALRN	125527659	G → A	ss250607852	R410H	0	1	0/0/701	0/0/541	0	0	
KALRN	125531474	T → A	ss250607853	L452Q	0	1	0/1/709	0/2/541	0.0007	0.0018	
KALRN	125600376	C → A	ss250607854	Q770K	0	1	0/0/706	0/0/544	0	0	
KALRN	125656787	C → T	ss250607855	T1207M	0	1	0/2/705	0/1/542	0.0014	0.0009	+
KALRN	125764534	C → A	ss250607856	P1695Q	0	1	0/59/636	1/44/492	0.0425	0.0428	
KALRN	125764599	A → T	ss250607857	M1717L	0	1	0/0/705	0/1/540	0	0.0009	
KALRN	125860927	G → A	ss250607858	R2049K	0	1	0/1/696	0/1/540	0.0007	0.0009	
KALRN	125873259	C → A	ss250607859	P2255T	0	7	1/14/684	0/7/536	0.0114	0.0064	+
KALRN	125873289	C → T	ss250607860	P2265S	1	0	0/6/701	0/7/533	0.0042	0.0065	
KALRN	125873382	G → T	ss250607861	G2296C	0	1	0/1/703	0/1/542	0.0007	0.0009	
KALRN	125876103	C → T	ss250607862	A2382V	0	1	0/0/697	0/0/540	0	0	
KALRN	125920964	A → G	rs16835896	N2973S	0	3	0/3/698	0/6/538	0.0021	0.0055	
EPHB1	136153231	T → C	ss252863894	F151S	0	1	0/0/710	0/0/543	0	0	
EPHB1	136334407	G → A	ss252863895	D375N	0	1	0/0/708	0/0/544	0	0	
EPHB1	136368508	G → A	ss252863896	D577N	0	1	0/0/707	0/0/544	0	0	
EPHB1	136394134	C → T	ss252863897	R637C	0	2	1/1/707	0/2/541	0.0021	0.0018	+
EPHB1	136450890	C → T	ss252863898	R905C	0	3	0/9/695	0/1/543	0.0064	0.0009	+
EPHB1	136460639	C → T	rs56186270	T981M	0	2	0/0/706	0/0/541	0	0	

Note: Genomic position based on NCBI build 36, chromosome 3. Amino acid changes based on NCBI Reference Sequence NP_001019831.2 (2986 aa) for KALRN and NP_004432.1 (984 aa) for EPHB1. All but N2973S (rs16835896) and T981M (rs56186270) are novel. AA change, amino acid change; dbSNP, Single Nucleotide Polymorphism Database; Homo, homozygote; Hetero, heterozygote; SCZ, schizophrenia; CONT, control; NCBI, National Center for Biotechnology Information.

Schizophrenia is a genetically heterogeneous disorder, with both very rare variants with a high effect size (eg, CNVs in 1q21.1, 15q13.3) and common variants with a low effect size (eg, rs1344706 in *ZNF804A*) involved in its genetic architecture. In this frequency-effect size spectrum, P2255T (OR: ~2, risk allele frequency in controls: ~0.005) is located between the CNV in 1q21.1 (OR: ~10, frequency in controls: ~0.0001)[25] and rs1344706[T] in *ZNF804A* (OR: ~1.1, risk allele frequency in controls: ~0.6),[26] both of which have been recently associated with schizophrenia. The relatively modest effect size of P2255T compared with that of the above CNVs can be attributable to the difference in the effect of each variant on gene(s): Although CNVs strongly influence the expression of multiple genes, missense mutations in *KALRN* are presumed to have limited effects on KALRN function. P2255T is located in the evolutionally conserved proline-rich region between the C-terminal GEF and SH3 domains[27] and is surrounded by 2 nearby phosphorylation sites (S2237 and S2262), according to Human Protein Reference Database (http://www.hprd.org/index_html)[28] (figure 1). *In silico* analysis with PhosphoMotif Finder[29] shows that T2255 itself can be recognized and phosphorylated by many kinases, suggesting functional implications of P2255T (figure 1). In addition, *in silico* analysis predicts that phosphorylation of T2255 will induce that of nearby S2253. Thus, P2255T may greatly change the phosphorylation status in a narrow

Table 2. Association Analyses of Each Missense Mutation in the Third Sample Set

	AA Change	Third Sample Set					
		Genotype Counts		Mutation Frequency			
		SCZ	CONT	SCZ	CONT	OR	*P* Value
KALRN	T1207M	0/7/1477	0/3/1482	0.0024	0.0010	2.34	.171
KALRN	P2255T	0/31/1448	0/15/1473	0.0105	0.0050	2.09	.012
EPHB1	R637C	0/4/1477	0/4/1478	0.0014	0.0014	1.00	.636
EPHB1	R905C	0/15/1458	0/12/1466	0.0051	0.0041	1.26	.347

Note: Abbreviations are explained in the first footnote to table 1. *P* values were calculated by Fisher exact test (1 tailed).

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Table 3. Combined Association Analysis in The Third Sample Set

Gene	AA Change	Third Sample Set				Combined Analysis	
		Genotype Counts		Mutation Frequency		Gene Based	
		SCZ	CONT	SCZ	CONT	OR	*P* value
KALRN	R410H	0/0/1481	0/0/1484	0	0	2.07	.003
KALRN	Q770K	0/0/1486	0/0/1490	0	0		
KALRN	T1207M	0/7/1477	0/3/1482	0.0024	0.0010		
KALRN	P2255T	0/31/1448	0/15/1473	0.0105	0.0050		
KALRN	A2382V	0/7/1473	0/4/1480	0.0024	0.0013		
EPHB1	F151S	0/0/1478	0/0/1484	0	0	1.09	.438
EPHB1	D375N	0/0/1483	0/0/1490	0	0		
EPHB1	D577N	0/0/1486	0/2/1483	0	0.000673		
EPHB1	R637C	0/4/1477	0/4/1478	0.0014	0.0014		
EPHB1	R905C	0/15/1458	0/12/1466	0.0051	0.0041		
EPHB1	T981M	0/5/1481	0/4/1484	0.0017	0.0013		

Note: Abbreviations are explained in the first footnote to table 1. *P* values were calculated by Fisher exact test (1 tailed).

region between the C-terminal GEF and SH3 domain. A protein with multiple phosphorylated sites like KALRN can be assumed to have an exponential number of phospho-forms, and individual phospho-forms may have distinct biological effects. The diffuse distribution of these phospho-forms at steady state enables the phosphoproteome to encode information and flexibly respond to varying demands.[30] Thus, it is conceivable that P2255T may influence such plasticity in KALRN by changing the number of phosphorylated sites. Interestingly, detailed examination of clinical information from the first sample set, which was uniquely available to us, revealed that congenital or early-onset vascular disease was observed in 5 of 7 cases with P2255T (supplementary table S1). Because *KALRN* may represent a candidate gene for vascular diseases,[31,32] it is tempting to speculate that P2255T may be a potential risk factor for vascular disease.

In addition to P2255T, we detected multiple rare (<1%) missense mutations in *KALRN* or *EPHB1*. Such variants are not sufficiently frequent to be covered by GWAS nor do they have sufficiently large effect sizes to be detected by linkage analysis in family studies. For modest effect sizes, it is suggested that association testing may require composite tests of overall mutational load,

Table 4. Results of *In Silico*/Conservation Analysis

KALRN							
Analysis		R410H	Q770K	T1207M	P2255T	A2382V	
PolyPhen-2		Probably damaging	Probably damaging	Probably damaging	Benign	Benign	
PMut		Pathological	Neutral	Pathological	Pathological	Neutral	
Conservation analysis	Human (NP_001019831.2)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**ARL	SILAPLT	
	Chimpanzee (XP_516703.2)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**ARL	SILAPLT	
	Dog (XP_535768.2)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**SRV	SVLAPLT	
	Cattle (XP_001790302.1)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**ARV	SILTPLT	
	Mouse (XP_001481079.1)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**PRV	SILAPLA	
	Rat (NP_114451.1)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**PRV	SILAPLT	
EPHB1							
Analysis		F151S	D375N	D577N	R637C	R905C	T981M
PolyPhen-2		Benign	Probably damaging	Possibly damaging	Probably damaging	Probably damaging	Probably damaging
PMut		Pathological	Neutral	Neutral	Pathological	Pathological	Pathological
Conservation analysis	Human (NP_004432.1)	QVDFGGR	RCD**D**NVE	VYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSPTAMA
	Chimpanzee (XP_001150963.1)	QVDFGGR	RCD**D**NVE	LLVEQWQ	YKG**R**LKL	LLD**R**SIP	QSPTAMA
	Dog (XP_542791.2)	QVDFGGR	RCD**D**NVE	VYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSPTTMA
	Cattle (XP_614602.4)	QVDFGGR	RCD**D**NVE	VYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSPTAMA
	Mouse (NP_775623.2)	QVDFGGR	RCD**D**NVE	AYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSPSVMA
	Rat (XP_217250.1)	QVDFGGR	RCD**D**NVE	VYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSPSVMA

Note: The bold are the mutated amino acids.

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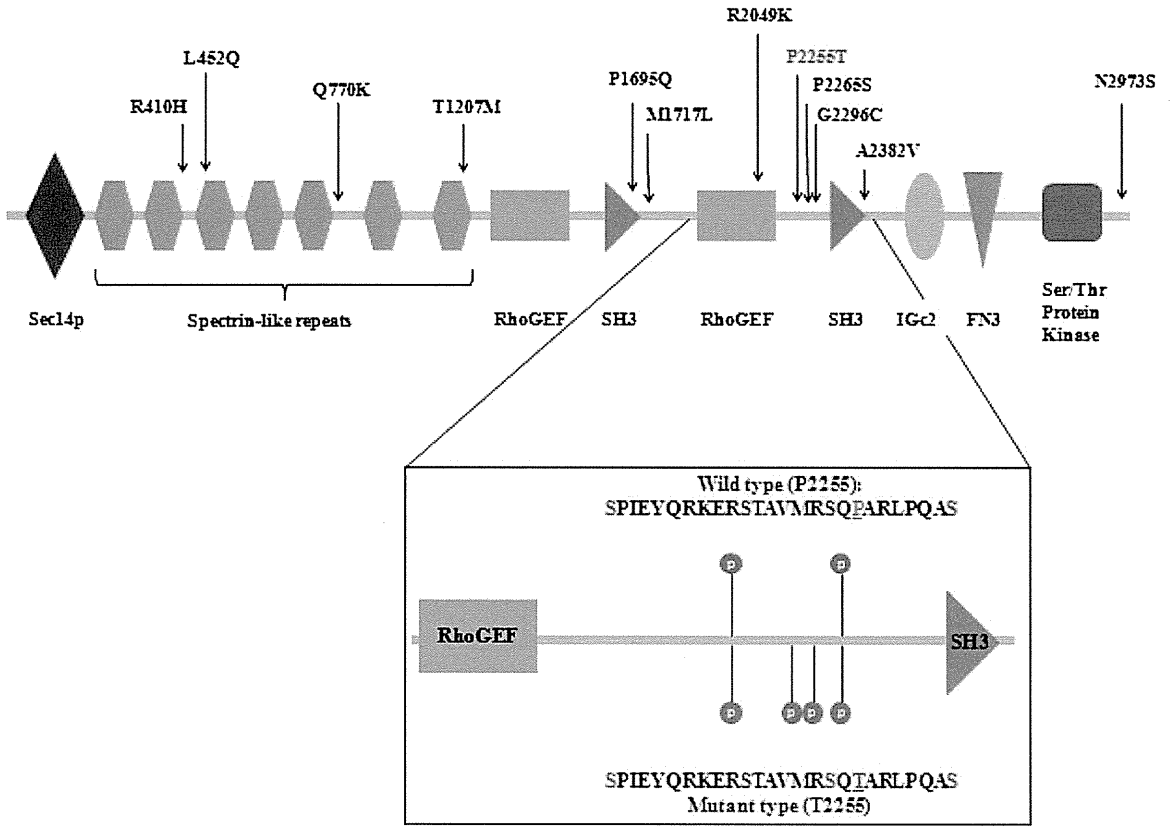

Fig. 1. Rare Missense Mutations in *KALRN* and Change in Phosphorylation Status by P2255T.

comparing frequencies of mutations of potentially similar functional effect in cases and controls. Thus, we also performed combined association analyses for *KALRN* or *EPHB1* and found evidence that multiple rare (<1%) missense mutations in *KALRN* as a whole are associated with schizophrenia. This finding is supported by *in silico* analyses showing that most of the mutations are predicted as being of functional relevance and that they are located in evolutionally conserved regions. In contrast, there were no significant differences in the cumulative frequencies of rare missense mutations in *EPHB1*. This might be due to a type II error. The cumulative frequency of rare mutations of *EPHB1* in controls is almost same as the one of *KALRN* in controls (0.0075 vs 0.0073), indicating that cumulative effect size of rare missense mutations in *EPHB1* may be smaller than the one in *KALRN*. In the mammalian genome, there are 5 different EphB receptors (EphB1, EphB2, EphB3, EphB4, and EphB6), with a high similarity at the amino acid level. Analysis of double and triple knockout mice lacking EphB1, EphB2, and EphB3 in different combinations revealed that EphBs have functional redundancy even though all these EphBs are responsible for spine morphogenesis and synapse formation to varying degrees.[33] This is in contrast with the drastic phenotypes observed in *KALRN*-knockout mice.[16] Therefore, biological effects of rare missense mutations in *EPHB1* may be compensated for by other intact *EPHBs*. This might lower the ORs of rare missense mutations in *EPHB1*. Given that all the mutations detected in *EPHB1* were predicted to have pathogenicity by PolyPhen-2 or PMut, a larger-scale case-control study with sufficient power may provide a significant result in a combined analysis for *EPHB1*.

One important aspect of the present study is that we found rare mutations associated with schizophrenia in the *KALRN* gene, in which GWASs detected association signals for schizophrenia. Several studies have recently reported the 1 gene may harbor both rare and common variants associated with the same diseases, including schizophrenia,[34] type 2 diabetes,[35] and hypertriglyceridemia.[36] Given that the cost of whole-genome sequencing is still high to search for rare mutations, resequencing analyses for genes with support from GWAS might be a better strategy for detection of rare mutations with larger effect size.

There are several limitations to this study. First, we could not conduct segregation analyses for mutations due to limited access to family members. Furthermore, given the modest risk (OR ~2), these mutations would show incomplete penetrance. In fact, it is reported that penetrance estimates of CNVs at 1q21.1 and 15q13.3,

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both of which show higher ORs, are 0.061 and 0.074, respectively.[25] Therefore, a population-based study is a better choice to evaluate genetic associations for missense mutations with modest risk.[37] The second limitation is population stratification. Although a Japanese population is considered relatively homogenous, small population stratifications may have influenced our findings.[38] However, we believe that the recruitment of subjects in local regions minimized this concern. Third, we did not conduct functional analyses for detected missense mutations. The detailed effects of these mutations on the pathophysiology of schizophrenia need to be examined in a future study. Fourth, our resequencing analyses were not comprehensive in terms of the kind of variants and the number of genes. In other words, the present study did not cover indels or CNVs because of the methodological limitation of the DNA microarray–based method. Because these classes of variants could have a more profound effect on protein function, their genetic contribution to schizophrenia might be revealed in future studies. Also, as shown in *EPHB1*, it is assumed that a variety of molecules or pathways have a role in spine formation or synapse plasticity, which are impaired in patients with schizophrenia, to compensate for each other. A combined analysis of a large number of genes relevant for synaptic function might provide more robust evidence that rare missense mutations as a whole contribute to pathomechanisms of schizophrenia.

In conclusion, we provide the first evidence that multiple rare (<1%) missense mutations in *KALRN* may be genetic risk factors for schizophrenia. Further studies will be needed to examine the pathogenicity of these mutations from a biologic point of view.

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Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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META-ANALYSIS

Plasma L-Tryptophan Concentration in Major Depressive Disorder: New Data and Meta-Analysis

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ABSTRACT

Objective: Tryptophan, an essential amino acid, is the precursor to serotonin and is metabolized mainly by the kynurenine pathway. Both serotonin and kynurenine have been implicated in the pathophysiology of major depressive disorder (MDD). However, plasma tryptophan concentration in patients with MDD has not unequivocally been reported to be decreased, which prompted us to perform a meta-analysis on previous studies and our own data.

Data Sources: We searched the PubMed database for case-control studies published until August 31, 2013, using the search terms *plasma* AND *tryptophan* AND synonyms for MDD. An additional search was performed for the term *amino acid* instead of *tryptophan*. We obtained our own data in 66 patients with MDD (*DSM-IV*) and 82 controls who were recruited from March 2011 to July 2012. The majority of the patients were medicated (N = 53). Total plasma tryptophan concentrations were measured by the liquid chromatography/mass spectrometry method.

Study Selection: We scrutinized 160 studies for eligibility. Original articles that were written in English and documented plasma tryptophan values in patients and controls were selected.

Data Extraction: We included 24 studies from the literature and our own data in the meta-analysis, which involved a total of 744 patients and 793 controls. Data on unmedicated patients (N = 156) and their comparison subjects (N = 203) were also extracted. To see the possible correlation between tryptophan concentrations and depression severity, meta-regression analysis was performed for 10 studies with the Hamilton Depression Rating Scale 17-item version score.

Results: In our case-control study, mean (SD) plasma tryptophan level was significantly decreased in the MDD patients versus the controls (53.9 [10.9] vs 57.2 [11.3] μmol/L; $P = .03$). The meta-analysis after adjusting for publication bias showed a significant decrease in patients with MDD with a modest effect size (Hedges *g*, −0.45). However, analysis on unmedicated subjects yielded a large effect (Hedges *g*, −0.84; $P = .00015$). We found a weak association with depression severity in the meta-regression analysis ($P = .049$).

Conclusions: This meta-analysis provides convincing evidence for reduced plasma tryptophan levels in patients with MDD, particularly in unmedicated patients.

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Depression imposes a great burden on afflicted individuals and society, while its pathophysiology remains elusive. Since the serotonin hypothesis was proposed by Coppen,[1] it has long been a major hypothesis in the pathophysiology of major depressive disorder (MDD). Early evidence supporting the serotonin hypothesis was decreased plasma levels of tryptophan, the amino acid precursor to serotonin.[2] Subsequently, growing evidence has suggested that tryptophan may play an important role in MDD through the kynurenine pathway as well.[3–5] In the inflammation-associated depression, for example, proinflammatory cytokines activate indoleamine 2,3-dioxygenase (IDO), the first and rate-limiting enzyme that degrades tryptophan along the kynurenine pathway, which would result in decreased plasma tryptophan. However, in recent studies, plasma tryptophan concentration in patients with MDD has not unequivocally been reported to be lower when compared with that of controls. Some studies found significantly decreased plasma tryptophan levels in MDD patients compared with healthy controls,[6–9] while others obtained contradictive negative results.[10–12] These inconsistent results require further investigations and warrant performing meta-analysis. To our knowledge, there is no study of meta-analysis on plasma tryptophan levels in MDD. In addition, most previous studies were conducted in white subjects. To our knowledge, there have been only 2 studies from Asian populations. Xu et al[9] reported reduced tryptophan levels in Han Chinese patients with MDD compared with controls, while Myint et al[10] reported no significant difference in plasma tryptophan levels between Korean patients with MDD and controls, although "tryptophan breakdown index" was found to be increased in the patients.

The aims of the present study were 2-fold: to examine whether plasma tryptophan concentration is different between Japanese patients with MDD and controls, and to perform meta-analysis on previous studies, including ours, to determine whether plasma tryptophan concentration is lowered in MDD patients.

DATA SOURCES

Our Case-Control Study

Subjects. Subjects were 66 patients with MDD and 82 healthy controls; they were also included in this meta-analysis. Participants were recruited at the outpatient clinic of the National Center of Neurology and Psychiatry (NCNP) Hospital, Tokyo, Japan, or through advertisements in free local magazines and our website announcement. Diagnosis

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Clinical Points

- Patients with major depressive disorder (MDD) have decreased plasma tryptophan levels compared with healthy controls.
- Decrease in plasma tryptophan levels may be more marked for unmedicated than medicated patients.
- There may be a weak correlation between severity of MDD and plasma tryptophan level.

of MDD according to the *Diagnostic and Statistical Manual of Mental Disorders,* Fourth Edition,[13] was made based on a structured interview, medical charts, and information from the psychiatrist in charge of the patients. All patients were interviewed by using either the Mini-International Neuropsychiatric Interview (M.I.N.I.)[14] (N = 54) or the Structured Clinical Interview for *DSM-IV* Axis I disorders[15] (N = 12) by a research psychiatrist. Depression severity was assessed by the Hamilton Depression Rating Scale 17-item version (HDRS-17).[16] Remitted patients (HDRS-17 score < 8) and those patients with comorbid other Axis I disorders were not enrolled. Additional exclusion criteria for study participation were as follows: having a prior medical history of central nervous system disease or severe head injury; having a history of substance abuse/dependence; taking corticosteroids, antihypertensives, or oral contraceptives; and being on hormone replacement therapy.

Of the 66 MDD patients, there were 13 patients who did not take any psychotropic drugs at the time of study. The remaining 53 patients were medicated with psychotropic drugs such as antidepressants, antipsychotics, and benzodiazepine derivatives. There were 37 patients on antidepressant medication (mean imipramine equivalent dose: 114.9 ± 76.2 mg/d), 22 on antipsychotic medication (mean chlorpromazine equivalent dose: 178.8 ± 184.1 mg/d), and 38 on benzodiazepines (mean diazepam equivalent dose: 7.4 ± 4.8 mg/d). There were 19 patients who had a history of admission to the psychiatric ward and 7 who had a history of attempted suicide.

The present study was conducted in accordance with the Declaration of Helsinki. After the nature of the study procedures had been fully explained, written informed consent was obtained from every subject. This study was approved by the ethics committee of the NCNP (No. A2010-007).

Blood Collection and Measurement of the Plasma Tryptophan Concentration

Measurement of plasma tryptophan concentration was done in the "real world" setting. Without fasting, venous blood was drawn between 9:30 AM and 4:30 PM to an ethylenediaminetetraacetic acid disodium (EDTA-2Na)–containing Vacutainer tube (Terumo, Tokyo, Japan), immediately centrifuged at 3000 rpm for 15 min at 4°C (H-103HR, Kokusan, Tokyo, Japan), and supernatant was collected into a polyethylene tube and stored at −20°C until analysis. Plasma tryptophan concentration was measured using the liquid chromatography/mass spectrometry (LC-MS) method at SRL Co, Inc (Hachioji, Tokyo, Japan). In plasma, tryptophan takes 2 forms (ie, free and loosely albumin-bound forms). We obtained plasma total (free + albumin-bound forms) tryptophan levels.

STUDY SELECTION

Relevant studies published in English were identified from systematic searches of the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed?otool=ijpncnplib) through all publications available up to August 31, 2013. The following search terms were used: *plasma* AND *tryptophan* AND (*depress**[title] OR *mood disorder*[title] OR *mood disorders*[title] OR *affective disorder*[title] OR *affective disorders*[title]) AND (*normal* OR *healthy* OR *comparison* OR *control* OR *controls*).

This search strategy obtained a total of 159 records. In addition, the study of Pinto et al[11] was found by using another search term *amino acid* instead of *tryptophan* to extend our search in the PubMed database. We then scrutinized eligibility of articles for meta-analysis. Studies on minor depression and seasonal affective disorder (MDD with seasonal pattern) were excluded. The study of Maes et al[6] was excluded from analysis because the participants seemed to overlap in another study of Maes and Rief[7] The study selection flow is shown in Figure 1 in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (http://www.prisma-statement.org), which outlines the preferred way to report meta-analysis studies.[17]

Finally, 24 articles were selected. Study quality of each article was assessed using the checklist of the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) statement (http://www.strobe-statement.org), which describes the preferred way to report observational studies.[18] Following a previous report,[19] studies were assigned a low, medium, or high possibility of reporting bias depending on how many items were checked (cutoff points were set at 33% and 66%). No study was classified as having high possibility of reporting bias. We included our case-control data in the meta-analysis.

DATA EXTRACTION

Our Case-Control Study

For our subjects, averages are reported as mean (SD). Ratios of categorical variables and means of continuous variables with normal distribution were compared using χ^2 test and *t* test, respectively. Analysis of covariance (ANCOVA) was performed to examine the effect of diagnosis on plasma tryptophan levels controlling for age, sex, and body mass index (BMI). The possible association between HDRS-17 score and tryptophan level was examined by multiple regression analysis within the patient group, controlling for age, sex, and BMI. Statistical significance was set at 2-tailed $P < .05$. Analyses were performed using the Statistical Package for Social Science (SPSS) Japanese edition version 11.0 (SPSS Japan, Tokyo).

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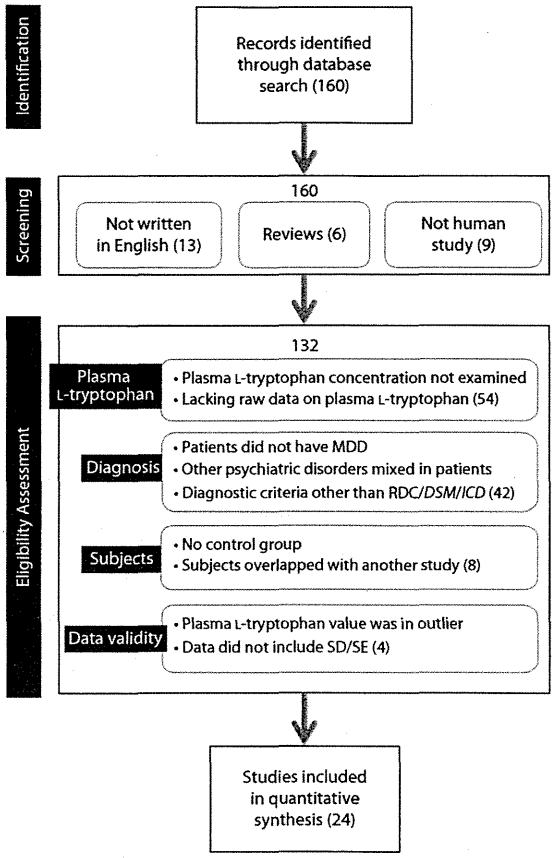
Figure 1. Flowchart of the Literature Search and Eligibility Assessment



Abbreviations: *DSM = Diagnostic and Statistical Manual of Mental Disorders, ICD = International Classification of Diseases*, MDD = major depressive disorder, RDC = Research Diagnostic Criteria.

Meta-Analytic Method

Data on means and SDs for plasma tryptophan concentration in MDD patients and controls were drawn from each study. We used the Comprehensive Meta-Analysis software (version 2.2.04; Biostat, Englewood, New Jersey). Tryptophan concentration data expressed as a unit of measure other than μmol/L (eg, μg/mL) were converted to data using the μmol/L unit. We chose Hedges g[20] for expressing the effect size of meta-analysis. To evaluate the effect size, we used the method of interpretation from Cohen convention.[21–23] Four studies[24–27] reported means and SDs of plasma tryptophan concentration only for subgroups (ie, MDD with and without melancholia; young and elderly groups). In these studies, we recalculated means and SDs for the total MDD patients.

Furthermore, we extracted data on unmedicated patients and their comparison subjects from 8 studies and from our own data and performed meta-analysis similarly.

Meta-regression analysis was also performed on the Comprehensive Meta-Analysis software. We chose only studies that used the HDRS-17 for estimating severity because this scale was most commonly used in the 24 studies.

Table 1. Demographic and Clinical Data of Our Case-Control Study[a]

Variable[b]	Patients With MDD	Healthy Controls	Differences
Patients, N (n female/n male)	66 (31/35)	82 (54/28)	$\chi^2_1 = 5.3$, $P = .021$
Age (y)	44.0 ± 12.9	43.9 ± 13.9	$t_{146} = -0.06$, $P = .95$
BMI	22.9 ± 4.8	22.1 ± 3.5	$t_{146} = -1.19$, $P = .24$
HDRS-17 score	14.3 ± 5.0	NA	NA
Tryptophan (μmol/L)	53.9 ± 10.9	57.2 ± 11.3	$F_1 = 4.83$, $P = .030$[a]

[a]Based on analysis of covariance (ANCOVA) controlling for age, sex, and BMI. Significant P values are denoted in bold text.
[b]Variables shown as mean (SD) except for N/n values included in the first row.
Abbreviations: BMI = body mass index, HDRS-17 = Hamilton Depression Rating Scale 17-item version, MDD = major depressive disorder, NA = not applicable.

Studies that were unclear as to which version (HDRS-17 or -21) was used were excluded.

RESULTS

Our Case-Control Study

Demographic and clinical data, including plasma tryptophan levels, are shown in Table 1. There was no significant difference in mean age or BMI between the patients with MDD and controls; however, there was a significant difference in sex distribution ($P = .021$). ANCOVA analysis controlling for age, sex, and BMI showed a significant main effect of diagnosis ($F_1 = 4.83$, $P = .030$) on tryptophan concentration, indicating that plasma tryptophan concentration was lower in the patients than in the controls (53.9 [10.9] vs 57.2 [11.3] μmol/L). In multiple regression analysis within the patient group, there was no evidence for an association between HDRS score and tryptophan levels ($t_{4,61} = -0.24$, $P = .81$).

Meta-Analysis

Details of the 24 articles selected for meta-analysis[7–12,24–41] are described in Table 2. Data from the 24 articles and our study yielded 25 comparisons (Figure 2A). The total numbers of patients with MDD and controls were 744 and 793, respectively. Since we detected significant heterogeneity across the studies ($P < .001$), we employed the random effects model. In the combined sample, there was a highly significant difference in standardized mean tryptophan concentration between patients and controls (Hedges g, −0.63; 95% CI, −0.82 to −0.44; $P < .00000001$; fail-safe number, 687). Funnel plot and Egger's regression analysis indicated a publication bias (intercept, −2.19; 95% CI, −3.81 to −0.58; $df = 23$; $P = .0098$). We then used "trim-and-fill method"[42] to adjust for the bias (Figure 2B). After the adjustment, the Hedges g showed a modest effect (Hedges g, −0.45; 95% CI, −0.66 to −0.23), although the statistical significance remained high ($P = .00006$) (Figure 2B).

We also performed meta-analysis in patients who did not take psychotropic drugs, which yielded 9 comparisons. The total numbers of patients with MDD and controls were 156

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Table 2. Characteristics of Studies Included in Meta-Analysis on Comparison With Plasma L-Tryptophan Concentration Between Patients With MDD and Healthy Controls

Study	Subjects' Country (race)	Case Group Name (criteria)	Case N (n female/ n male)	Mean (SD) Plasma L-Tryptophan Concentration (μmol/L)	Control N (n female/ n male)	Mean (SD) Plasma L-Tryptophan Concentration (μmol/L)	Evaluation of Depressive State (mean [SD] score)	Drug Free in Patients[a] (period)
DeMyer et al, 1981[41]	USA	MDD (RDC)	18 (13/5)	42 (11)	10 (7/3)	56.9 (12)	HDRS-17 (22.4 [8.9])	Yes (3 wk)
Menna-Perper et al, 1983[40]	USA	MDD with melancholia (*DSM-III*)	9 (3/6)	42.8 (8.2)	6 (3/3)	46.3 (4.7)	HDRS, BDI (NA)	No
Joseph et al, 1984[39]	USA	MDD (*DSM-III*)	16 (10/6)	31.3 (7.2)	8 (5/3)	43.8 (16)	HDRS, BDI (NA)	Yes (1 wk)
Anderson et al, 1990[38]	UK	MDD (*DSM-III*)	31 (15/16)	38.2 (8.9)	31 (15/16)	45.4 (11.1)	HDRS-17 (22.8 [NA])	No
Chiaroni et al, 1990[37]	France, Switzerland	MRD (*DSM-III*)	25 (19/6)	48.8 (13.3)	33 (19/14)	59.5 (12.7)	AMDP (NA)	No
Russ et al, 1990[36]	USA	MDD (*DSM-III-R*)	16 (10/6)	59 (11)	9 (7/2)	52 (14)	HDRS-21 (31 [7])	No
Maes et al, 1990[24]	Belgium	MDD – melancholia (*DSM-III*)	22 (12/10)	56.6 (10.5)	16 (8/8)	60.6 (4.8)	HDRS (NA)	No
		MDD + melancholia (*DSM-III*)	13 (7/6)	50 (12.1)	16 (8/8)	60.6 (4.8)	HDRS (NA)	
Price et al, 1991[35]	USA	MDD (*DSM-III-R*)	109 (78/31)	35.3 (8.3)	58 (41/17)	36.7 (8.3)	HDRS-25 (34 [11])	No
Quintana, 1992[33]	Spain	MDD (RDC)	25 (15/10)	42.5 (8.3)	25 (NA)	47.6 (11.3)	HDRS (NA)	No
Lucca et al, 1992[34]	Italy	MDD (*DSM-III-R*)	19 (12/7)	52 (20)	29 (14/15)	74 (12)	HDRS-21 (24.7 [4.1])	No
Maes et al, 1993[25]	Belgium	MDD – melancholia (*DSM-III-R*)	7 (NA)	56 (14)	8 (NA)	79 (12)	HDRS (NA)	No
		MDD + melancholia (*DSM-III-R*)	10 (NA)	55 (15)	8 (NA)	79 (12)	HDRS (NA)	
Ortiz et al, 1993[26]	Spain	MDD adults (*DSM-III-R*)	10 (8/2)	69.0 (9.8)	10 (NA)	70.0 (14.7)	MADRS (26.8 [2.0])	No
		MDD elderly (*DSM-III-R*)	7 (5/2)	64.1 (8.8)	10 (NA)	70.0 (14.7)	MADRS (28.3 [1.4])	
Moller, 1993[32]	Denmark	All depressives (*DSM-III*)	26 (18/8)	36 (6)	55 (39/16)	39 (8)	HDRS-17 (24 [5])	No
Maes et al, 1995[27]	Belgium	MDD – melancholia (*DSM-III*)	47 (35/12)	61 (12)	50 (24/26)	66 (12)	HDRS-17 (21.3 [2.9])	No
		MDD + melancholia (*DSM-III*)	35 (21/14)	57 (12)	50 (24/26)	66 (12)	HDRS-17 (26.7 [3.2])	
Mauri et al, 1998[31]	Italy	MDD (*DSM-IV*)	29 (14/15)	33.3 (27.3)	28 (12/16)	56.7 (79.9)	HDRS (NA)	Yes (4 wk)
Song et al, 1998[30]	Belgium	MDD (*DSM-IV*)	6 (4/2)	69 (11)	14 (6/8)	73 (19)	(NA)	Yes (10 d)
Hoekstra et al, 2001[29]	Netherlands	MDD (*DSM-IV*)	20 (13/7)	35.5 (9)	29 (13/16)	45.6 (6.1)	HDRS-17 (31 [NA])	No
Mauri et al, 2001[28]	Italy	MDD (*DSM-IV*)	16 (11/5)	28.6 (34.1)	11 (2/9)	45.6 (13.0)	HDRS-17 (22.4 [5.6])	No
Myint et al, 2007[10]	Korea	MDD (*DSM-IV*)	58 (32/26)	65.8 (15.6)	189 (86/103)	69.7 (13.7)	HDRS-17 (27.2 [7.3])	No
Manjarrez-Gutierrez et al, 2009[8]	Mexico	MDD (*DSM-IV*)	8 (4/4)	48.1 (1.2)	9 (5/4)	57.7 (3.3)	(NA)	Yes (NA)
Sublette et al, 2011[12]	USA (white/ nonwhite)	MDD (*DSM-IV*)	30 (14/16)	59.2 (10.4)	31 (21/10)	60.2 (7.7)	HDRS-17 (20.1 [3.4]) BDI (25.9 [8.2])	No
Maes and Rief, 2012[7]	Germany	MDD (*DSM-IV*)	35 (22/13)	69.8 (14.4)	22 (8/14)	82.9 (15.9)	BDI (27.1 [8.3])	Yes (NA)
Pinto et al, 2012[11]	Brazil	MDD (*DSM-IV*)	5 (NA)	35 (6)	5 (NA)	36 (2)	HDRS (22 [2])	Yes (NA)
Xu et al, 2012[9]	China (Han Chinese)	MDD (*DSM-IV*)	26 (19/7)	42.9 (6.4)	25 (16/9)	49.8 (7.2)	HDRS-17 (24.2 [4.5])	Yes (NA)

[a]*Drug free* means being completely excepted from the administration of psychotropic drugs, not only antidepressants but also benzodiazepines and antipsychotics.

Abbreviations: AMDP = The Association for Methodology and Documentation in Psychiatry, BDI = Beck Depression Inventory, *DSM* = *Diagnostic and Statistical Manual of Mental Disorders*, HDRS = Hamilton Depression Rating Scale, MADRS = Montgomery-Asberg Depression Rating Scale, MDD = major depressive disorder, MRD = major recurrent depression, NA = not mentioned in the article, RDC = Research Diagnostic Criteria, TRP = plasma L-tryptophan.

and 203, respectively (Figure 2C). There was a significant heterogeneity ($P = .002$), and the random effects model was applied. As a result, there was a highly significant difference in standardized mean tryptophan concentration between the 2 groups (Hedges g, −0.84; 95% CI, −1.27 to −0.40; $P = .00015$; fail-safe number, 93). Funnel plot and Egger regression analysis did not indicate publication bias (intercept, −2.73; 95% CI, −6.92 to 1.47; $df = 7$; $P = .17$) (Figure 2D).

To elucidate the relationship between plasma tryptophan concentration and depression severity, we performed meta-regression analysis in 11 comparisons (Figure 3A). When we set the HDRS-17 score as an outcome variable and Hedges g value as an explanatory variable, we found that Hedges g value had a significant, albeit weak, effect on HDRS-17 score by adopting the fixed effects model as the estimation method ($\tau^2 = 0.068$, slope, −0.029; 95% CI, −0.057 to −0.00012; $P = .049$) (Figure 3B).

DISCUSSION

In our case-control study, the ANCOVA analysis controlling for age, sex, and BMI showed that the patients with MDD had significantly lower plasma tryptophan

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