Molecular Psychiatry (2012), 1–2
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www.nature.com/mp

LETTER TO THE EDITOR

Genetic evidence for association between *NOTCH4* and schizophrenia supported by a GWAS follow-up study in a Japanese population

Molecular Psychiatry advance online publication, 29 May 2012; doi:10.1038/mp.2012.74

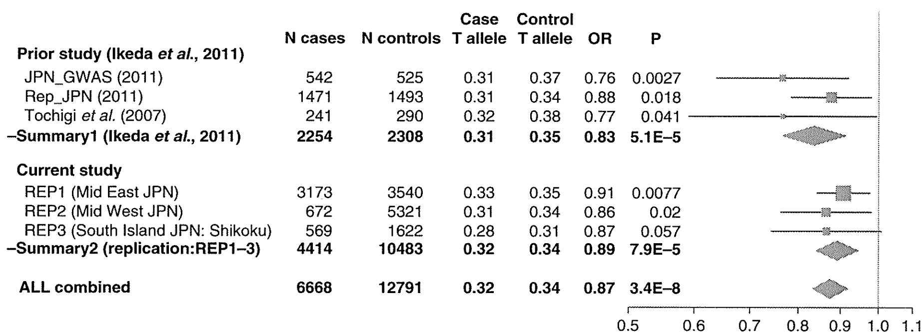
Using stringent criteria, we followed-up a genome-wide association study (GWAS) finding[1] to examine the involvement of the developmental regulatory gene *NOTCH4* in schizophrenia. This is the first study in a Japanese population that satisfies genome-wide significance between a particular single-nucleotide polymorphism (SNP) and schizophrenia.

The past 5 years have seen significant genetic discoveries made through GWASs. Although in schizophrenia the effect sizes have been relatively small (odds ratio (OR) of <1.2), these studies collectively provide strong evidence for association of a number of susceptibility genes. The major loci identified for schizophrenia risk are located in the major histocompatibility complex region in 6p21.3–p22.1. The Psychiatric GWAS Consortium, a sample consisting mainly of Caucasian subjects, reported 136 SNPs with genome-wide significance (5×10^{-8}), and among them 129 SNPs were located from 25 to 33 Mb at chromosome 6.[2] In addition, a recent Asian GWAS from a Chinese population showed a stringent level of significance at *ZKSCAN4* (chr6: 28.32 Mb),[3] even though another larger Chinese population GWAS[4] and our prior Japanese population-based GWAS (total sample size: 1108)[1] did not detect any genome-wide significance in this region. In our previous study,[1] however, only one signal within these loci maximized at a SNP (rs2071287: chr6: 32 170 433 bp on hg19) in *NOTCH4* (chr6: 32.16 to 32.19 Mb). To provide a more complete analysis, in the current study, we expanded the sample size to verify whether this SNP (rs2071287: C>T) in *NOTCH4* shows strong support for genetic association.

Six case–control data sets from Japanese population, including our screening GWAS (JPN_GWAS), replication set (Rep_JPN) and the sample of Tochigi *et al.*,[1] were evaluated. The other three new data sets consist of REP1, REP2 and REP3, the sample sets being divided by the collection regions in Japan: REP1 (Mid-east Japan: case = 3173, control = 3540), REP2 (Mid-west Japan: case = 672, control = 5321), and REP3 (South island of Japan (Shikoku): case = 569, control = 1622) (Supplementary Method). The TaqMan assay (Applied Biosystems, Foster City, CA, USA) was used to determine the genotypic distribution of rs2071287. A meta-analysis was performed with a fixed model by 'rmeta', an R package, as we did not apply a principal component analysis, which is the most common approach to adjust the population structure. Instead, we conducted a stratified analysis according to the sample collection site in order to rule out possible population stratification. A test for heterogeneity did not show significant deviation for combined replication data sets (REP1–REP3, $\chi^2 = 0.63$, df = 2, $P = 0.73$) and all data sets, including samples from our prior study ($\chi^2 = 4.2$, df = 5, $P = 0.52$).

Previously, we reported an association based upon three data sets with P_{meta} at 5.1×10^{-5} and OR of 0.83 (reference: T allele: minor allele, 95% confidence intervals: CIs: 0.77–0.91: 'Summary1' in Figure 1).[1] In the current study, in two out of three replication sets, we detected statistical evidence ($P<0.05$, two-tailed) for a consistent association that was similar in magnitude and direction (T allele in control is more frequent than in case: Figure 1). Further meta-analysis of the combined samples for the new data sets (REP1–REP3: case = 4414, control = 10 483) suggests strong support for the association of this SNP ($P = 7.9 \times 10^{-5}$: OR = 0.89, 95% CI = 0.84–0.94, 'Summary2' in Figure 1). Finally, genome-wide significance was detected in the meta-analysis combining all of the data sets (6668 case and 12 791 controls) in the Japanese population ($P = 3.4 \times 10^{-8}$: OR = 0.87, 95% CI = 0.83–0.92, 'ALL combined' in Figure 1).

Our results provide stringent support for previous genetic findings reporting the association of an intronic SNP (rs3131296, chr6: 32 172 993 bp on hg19) in *NOTCH4* in a European cohort.[5] However, the pattern of linkage disequilibrium (LD) between the current (rs2071287) and previous (rs3131296) SNPs, in addition to the minor allele frequencies (MAFs) of these SNPs vary across populations (Supplementary Figures 1 and 2): (1) a greater level of LD is observed in the Caucasian (HapMap CEU, release28, $D' = 1$, $r^2 = 0.13$) compared with the Japanese population (HapMap JPT, release28, $D' = 0.49$, $r^2 = 0$); and (2) rs3131296, the SGENE-plus study[5] reported significant association, shows substantially higher



	N cases	N controls	Case T allele	Control T allele	OR	P
Prior study (Ikeda *et al.*, 2011)						
JPN_GWAS (2011)	542	525	0.31	0.37	0.76	0.0027
Rep_JPN (2011)	1471	1493	0.31	0.34	0.88	0.018
Tochigi *et al.* (2007)	241	290	0.32	0.38	0.77	0.041
–Summary1 (Ikeda *et al.*, 2011)	**2254**	**2308**	**0.31**	**0.35**	**0.83**	**5.1E–5**
Current study						
REP1 (Mid East JPN)	3173	3540	0.33	0.35	0.91	0.0077
REP2 (Mid West JPN)	672	5321	0.31	0.34	0.86	0.02
REP3 (South Island JPN: Shikoku)	569	1622	0.28	0.31	0.87	0.057
–Summary2 (replication:REP1–3)	**4414**	**10483**	**0.32**	**0.34**	**0.89**	**7.9E–5**
ALL combined	**6668**	**12791**	**0.32**	**0.34**	**0.87**	**3.4E–8**

Figure 1. Association of rs2071287 with schizophrenia.

MAF in Caucasian (8.5% in HapMap CEU, 13% in SGENE-plus) than in Japanese (1.9% in HapMap JPT). Considering that the major histocompatibility complex region consists of large blocks with a very high LD,[6] it would be difficult to ascribe the difference to particular SNP(s). Nevertheless, given the evidence for the association of *NOTCH4* with schizophrenia based upon current and previous genetic and biological support,[7] we suggest that *NOTCH4* remains one of the strongest candidate susceptibility genes for schizophrenia, and we propose this SNP is a key to the identification of causal variant(s).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; Ministry of Health, Labor and Welfare of Japan; Academic Frontier Project for Private Universities, Comparative Cognitive Science Institutes; Core Research for Evolutional Science and Technology; Uehara Memorial Foundation; SENSHIN Medical Research Foundation; Takeda Science Foundation; Novaltis Foundation, Japan; Strategic Research Program for Brain Sciences of the MEXT of Japan.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)

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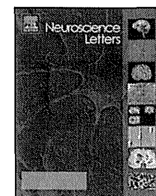
Neuroscience Letters 529 (2012) 66–69

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet



An evaluation of polymorphisms in casein kinase 1 delta and epsilon genes in major psychiatric disorders

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HIGHLIGHTS

- We report the candidate gene analysis of *CSNK1D*/*CSNK1E* with psychiatric disorders.
- Two-stage analysis was carried out using large sample size.
- No association was detected in bipolar disorder, schizophrenia and depression.
- These genes may not play a major role in the risk of major psychiatric disorders.

ARTICLE INFO

Article history:
Received 9 July 2012
Received in revised form 31 July 2012
Accepted 8 August 2012

Keywords:
Schizophrenia
Bipolar disorder
Major depressive disorder
Single nucleotide polymorphism

ABSTRACT

Disturbances of the circadian rhythm are involved in the pathophysiology of bipolar disorder (BD), schizophrenia (SCZ) and major depressive disorder (MDD). Specifically, because clock gene dysfunction is good candidate for enhancing the susceptibility to these psychiatric disorders, we selected two circadian rhythm-related genes (*CSNK1D* and *CSNK1E*) and investigated genetic associations of the genes with these three disorders.

None of the SNPs showed a significant association with MDD, but a SNP (rs2075984) in *CSNK1E* and SNP (rs6502097) in *CSNK1D* were associated with SCZ ($P = 0.0091$, uncorrected) and BD ($P = 0.030$, uncorrected), respectively. To confirm these findings, we analyzed an independent dataset (maximum $N = 3815$) but found a lack of association ($P = 0.63$ for rs2075984 and $P = 0.61$ for rs6502097). The final meta-analysis showed no association between these SNPs with SCZ ($P = 0.21$) and BD ($P = 0.53$). These results do not support that genetic variation in *CSNK1D* and *CSNK1E* is a susceptibility factor for major psychiatric disorders in the Japanese population.

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1. Introduction

As patients with psychiatric disorders often show various rhythm disturbances, including sleep–wake disruption, abnormalities in the circadian system have been hypothesized to be involved in the pathophysiology of these disorders [4,10]. Typically, in mood disorders such as bipolar disorder (BD) and major depressive disorder (MDD), many patients demonstrate sleep disturbances, social rhythm maladaptation and appetite and hormonal rhythm changes

Abbreviations: MDD, major depressive disorder; BD, bipolar disorder; SCZ, schizophrenia; *CSNK1D*, casein kinase 1 delta; *CSNK1E*, casein kinase 1 epsilon; SNP, single nucleotide polymorphism; OR, odds ratio.

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http://dx.doi.org/10.1016/j.neulet.2012.08.070

[5]. Therefore, chronobiological interventions (e.g., sleep deprivation and light therapy) have been shown to be promising treatment options for mood disorders [19]. Potent mood stabilizers, such as lithium and valproic acid, are also known to modulate circadian rhythms via the circadian rhythm-related genes [2,24]. For schizophrenia (SCZ), a recent study reported disruptions in sleep and circadian rhythm disturbances in patients with SCZ, despite a lack of changes in melatonin production [22]. The anatomical center of the circadian rhythm is in the hypothalamic suprachiasmatic nucleus, which is regulated by the transcription of genes related to the circadian rhythm pathway (GO: 7623) [21], i.e., the so-called clock genes.

Casein kinase 1 delta (*CSNK1D*) and epsilon (*CSNK1E*) are members of the clock gene family and act as serine/threonine protein kinases that phosphorylate period (*PER*), a core member of the circadian clock gene group. Several lines of evidence suggest that homologs of human *CSNK1D* and *CSNK1E* have essential roles in circadian rhythm generation in mammals [11]. In a human study, for example, a point mutation in *CSNK1D* has causality in familial advanced sleep phase syndrome [23]. Another study also reported that *CSNK1E* is associated with circadian rhythm sleep disorders [17]. These findings indicate that the two genes have potential to affect human phenotypes relating to circadian rhythm regulation. In addition, Casein kinase 1 indirectly modulates the dopaminergic neurotransmitter system, which is well known to be involved in the pathophysiology of SCZ and mood disorders. First, casein kinase 1 interacts with Darpp-32 (Dopamine-And-cAMP-Regulated-Phosphoprotein-32 kDa) [3], which has a key role in dopaminergic neurotransmission. Second, recent animal studies suggest that casein kinase 1 is associated with altered locomotor activities by regulating dopaminergic signaling [1,25]. Finally, a human genetic study reported that a single nucleotide polymorphism (SNP: rs135745) in *CSNK1E* was associated with the subjective response to D-amphetamine [20].

Based on these data collectively, we hypothesized that *CSNK1D* and *CSNK1E* may have a possible role in a shared pathophysiology of a broad range of psychiatric disorders. More recently, a genetic case–control association analysis indicated that the same SNP (rs135745) in *CSNK1E* was significantly associated with SCZ-susceptibility in the Chinese population [7], although several reports did not detect the consistent evidence for the association between SNPs in these two genes and psychiatric disorders [8,9,15,16]; these indicate that another study with larger sample size and robust method is needed. In this study, we conducted a two-stage genetic association study in which significantly associated SNPs detected in the first-set screening sample were followed up in independent replication sample.

2. Materials and methods

2.1. Subjects

Two independent sample sets were used in this study. In the first-set screening analysis, we used 1004 patients with BD (492 males and 512 females; mean age ± standard deviation; 50.0 ± 14.4 years, BDI = 710, BDII = 291, schizoaffective disorder = 3), 808 patients with SCZ (443 males and 365 females; 46.1 ± 15.1 years), 452 patients with MDD (209 males and 243 females; 48.5 ± 16.1 years) and 1712 healthy controls (922 males and 790 females; 44.7 ± 15.2 years).

For rs2075984 and rs6502097, which showed a significant association with SCZ ($P = 0.0091$) and BD ($P = 0.030$), respectively, we used independent datasets consisting of 1380 patients with SCZ (718 males and 662 females; 49.0 ± 15.0 years), 324 patients with BD (163 males and 161 females; 47.6 ± 14.0 years, BDI = 118, BDII = 206) and 1502 healthy controls (782 males and 720 females: 46.6 ± 15.2 years). Specifically for rs2075984, 933 unscreened controls from the public database of Japanese Single Nucleotide Polymorphisms (JSNP) were used to increase the sample size. A detailed description of the general characterization and psychiatric assessment of our subjects is available in the Supplementary methods section. Written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, University of Occupational and Environmental Health, and institutes participating in the Collaborative Study of Mood Disorder (COSMO).

2.2. SNP selection and quality control

Four tagging SNPs within *CSNK1D* and seven tagging SNPs within *CSNK1E* were selected based upon HapMap information (note: although rs6502097 is an intronic SNP in another gene [*SECTM1*], we included this SNP to cover the 5′ region of *CSNK1D*). All of the SNPs were genotyped using a TaqMan assay (Supplementary text).

Quality control for genotyping was applied based upon the missing rate per person (excluded if more than 4 of 11 SNPs failed) and per SNP (included if less than 10%). A total of 128 subjects (7 BD, 40 MDD, 13 SCZ and 68 controls) were removed from the following association analysis. None of the SNPs were removed from the analysis.

2.3. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium and allelic associations were evaluated using the chi-square test. For the significant SNPs in the screening SCZ or BD datasets (rs2075984 and rs6502097) and rs135745 that was associated with SCZ in the Chinese population [7], we conducted a meta-analysis combining screening and replication datasets (rs2075984 and rs6502097) or results from our screening sample and Huang's data (rs135745) using a random-effect model because the I^2 heterogeneity index was relatively high ($I^2 = 67.4$ for rs2075984, 62.5 for rs6502097 and 72.5 for rs135745). All of the statistical procedures were calculated using PLINK ver1.07 [14].

The significance level for all statistical tests was 0.05. To correct for multiple testing, we used two-stage analysis, which provides conservative results if a significant association is detected in the screening sample.

3. Results

We observed a significant association between rs2075984 in *CSNK1E* with SCZ ($P = 0.0091$, odds ratio (OR) = 0.85) and a nominal association between rs6502097 in *CSNK1D* with BD ($P = 0.030$, OR = 1.19). To exclude the possibility of genotyping errors, we genotyped randomly selected samples from the first-set screening dataset using direct sequencing using ABI 3100 (case = 83, control = 94). We found 100% concordance between genotypes (data not shown), indicating that the observed statistical significance was not derived from mistakenly assigned genotype calls.

Rs135745, which was associated with SCZ in the Chinese population [7], did not show a significant association in our screening dataset ($P = 0.12$). We next conducted a meta-analysis of this SNP in SCZ, combining our screening data and that from Huang et al. [7]. Although the direction of the effect size was same, we found non-significant trend for association ($P = 0.095$, OR = 1.26). In the MDD samples, none of the SNPs showed a significant association between the tagging SNPs in these genes.

In the following replication analysis using an independent case–control dataset (SCZ = 1380, control = 2435 for rs2075984 and BD = 324, control = 1502 for rs6502097), no association was

Table 1
Association analysis of SNPs in *CSNK1D* and *CSNK1E* with major psychiatric disorders.

SNP								Screening			Replication			Meta analysis	
Gene	CHR	SNP	BP[a]	type	A1[b]	A2[c]		MAF	*P* value	OR	MAF	*P* value	OR	*P* value	OR
CSNK1D	17	rs9901910	77791546	3′ region	G	C	Control	0.27							
							BP	0.25	0.26	0.93					
							SCZ	0.26	0.48	0.95					
							MDD	0.29	0.14	1.14					
	17	rs3829773	77814724	intron	A	G	Control	0.08							
							BP	0.07	0.44	0.91					
							SCZ	0.09	0.26	1.14					
							MDD	0.07	0.39	0.87					
	17	rs4789846	77819084	intron	T	C	Control	0.26							
							BP	0.25	0.57	0.96					
							SCZ	0.25	0.65	0.97					
							MDD	0.29	0.068	1.17					
	17	rs6502097	77879718	5′ region	C	G	Control	0.15			0.17				
				intron			BP	0.17	0.030	1.19	0.16	0.61	0.94	0.53	1.11
				(*SECTM1*)			SCZ	0.16	0.15	1.13					
							MDD	0.14	0.48	0.92					
CSNK1E	22	rs135745	37013833	3′ region	C	G	Control	0.24							
							BP	0.25	0.39	1.06					
							SCZ	0.26	0.12	1.12				0.095[d]	1.26[d]
							MDD	0.21	0.08	0.85					
	22	rs2075984	37021085	intron	C	A	Control	0.47			0.45				
							BP	0.47	0.81	0.99					
							SCZ	0.43	0.0091	0.85	0.45	0.63	0.98	0.21	0.92
							MDD	0.45	0.36	0.93					
	22	rs3890379	37022576	intron	G	A	Control	0.14							
							BP	0.16	0.052	1.17					
							SCZ	0.15	0.49	1.06					
							MDD	0.14	0.69	0.96					
	22	rs6001093	37031589	intron	C	T	Control	0.09							
							BP	0.10	0.12	1.16					
							SCZ	0.08	0.18	0.86					
							MDD	0.09	0.92	0.99					
	22	rs135757	37033849	intron	A	G	Control	0.23							
							BP	0.22	0.74	0.98					
							SCZ	0.22	0.67	0.97					
							MDD	0.22	0.57	0.95					
	22	rs135764	37040608	intron	A	G	Control	0.16							
							BP	0.16	0.77	0.98					
							SCZ	0.15	0.48	0.94					
							MDD	0.17	0.48	1.08					
	22	rs1997644	37045418	5′ region	A	G	Control	0.38							
							BP	0.36	0.051	0.89					
							SCZ	0.38	0.89	0.99					
							MDD	0.37	0.49	0.95					

MAF = minor allele frequency, OR = odds ratio, BP = bipolar disorder, SCZ = schizophrenia, MDD = major depressive disorder.
[a] Based upon hg19.
[b] A1: minor allele based upon whole sample.
[c] A2: major allele based upon whole sample.
[d] Results from our screening sample and Huang's data [7].

observed (Table 1). Finally, we conducted a meta-analysis using the screening and replication samples. However, no associations were detected (P = 0.21 and 0.53 for rs2075984 and rs6502097, respectively), thus providing no evidence that supports the association between the common SNPs in *CSNK1E* with SCZ or *CSNK1D* with BD (Table 1 and Supplementary Table 1).

4. Discussion

In this study, our results could not support evidence for an association between two casein 1 genes (*CSNK1D* and *CSNK1E*) and major psychiatric disorders (BD, SCZ and MDD). Four and seven tagging SNPs in *CSNK1D* and *CSNK1E*, respectively, were included in our study based upon linkage disequilibrium. In addition, an imputation analysis was conducted to achieve full-range coverage, but none of the analyzed SNPs showed more significant *P* values than the signals observed at rs2075984 for SCZ or rs6502097 for BD, which are both SNPs that did not reach statistical significance in the final meta-analysis for SCZ and BD (Supplementary Figs. 3 and 4). We also could not replicate the previous finding of significant association of SNP with SCZ (rs135745) in our dataset. One possible explanation is that the discrepancy might be derived from the population difference between Japanese and Chinese population.

Recent genome-wide association studies (GWASs) suggest that the effect size for psychiatric disorders is low; for example, the effect sizes for SNPs with genome-wide significance range from 1.13 (0.88) to 1.14 for BD [12] and 1.09 to 1.22 for SCZ [18]. Although our approach was based upon candidate genes and assumes a

higher prior probability for association, a larger sample for replication samples is required. Our sample size for screening the dataset was relatively large, and the power analysis suggested that the samples had 99.9% power for BD, 99.7% for MDD and 99.9% for SCZ to detect a significant association (assuming a relaxed OR of 1.5) of risk with 15% minor allele frequency under an additive model (type I error rate = 0.05). However, our replication datasets, especially for rs6502097 in BD, had only 35% power, if the same OR detected in the screening analysis and the same conditions are assumed [13], indicated that a more conclusive result will be reported with a larger sample.

In addition, following limitation should be considered. First, we did not evaluate gene–gene interaction: Because the clock genes have been shown to form complicated pathways centering on the core negative feedback loop, such interaction among clock genes is presumably an important factor for developing psychiatric disorders. Following this concept, a genetic study found significant association between three circadian genes including *CSNK1E* and BD demonstrating a multi-locus interaction [15]. Therefore, epistasis test [15] or pathway-based analysis [6] concerning the pathogenesis of the whole circadian rhythm system should be conducted. Second, our sample sets were collected from multiple areas of Japan, thus likely to have genetic heterogeneity, which could skew the results in the first-set screening samples.

In conclusion, these results could not support the genetic variants in *CSNK1D* and *CSNK1E* as a susceptibility factor for major psychiatric disorders in the Japanese population. Although our sample has moderate power to detect association, further sample size and sophisticated and robust analytic strategies will be required for conclusive result.

Acknowledgements

We thank Ms. M. Miyata and Ms. M. Aizawa for their technical support. This work was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; Ministry of Health, Labor and Welfare of Japan; Academic Frontier Project for Private Universities, Comparative Cognitive Science Institutes; Core Research for Evolutional Science and Technology; Uehara Memorial Foundation; SENSHIN Medical Research Foundation; Takeda Science Foundation; Novartis Foundation, Japan; and Strategic Research Program for Brain Sciences of the MEXT of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2012.08.070.

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Schizophrenia Bulletin vol. 38 no. 3 pp. 552–560, 2012
doi:10.1093/schbul/sbq118
Advance Access publication on November 1, 2010

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

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 $N{=}n/\left(\sum(1/Ni)\right)$, 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]

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 Phospho-Motif 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	SIL**A**PLT
	Chimpanzee (XP_516703.2)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**ARL	SIL**A**PLT
	Dog (XP_535768.2)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**SRV	SVL**A**PLT
	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	SIL**A**PLA
	Rat (NP_114451.1)	LDE**R**STI	IFL**Q**LRI	IHA**T**EIR	RSQ**P**PRV	SIL**A**PLT

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)	QVD**F**GGR	RCD**D**NVE	VYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSP**T**AMA
	Chimpanzee (XP_001150963.1)	QVD**F**GGR	RCD**D**NVE	LLVEQWQ	YKG**R**LKL	LLD**R**SIP	QSP**T**AMA
	Dog (XP_542791.2)	QVD**F**GGR	RCD**D**NVE	VYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSP**T**TMA
	Cattle (XP_614602.4)	QVD**F**GGR	RCD**D**NVE	VYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSP**T**AMA
	Mouse (NP_775623.2)	QVD**F**GGR	RCD**D**NVE	AYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSPSVMA
	Rat (XP_217250.1)	QVD**F**GGR	RCD**D**NVE	VYS**D**KLQ	YKG**R**LKL	LLD**R**SIP	QSPSVMA

Note: The bold are the mutated amino acids.

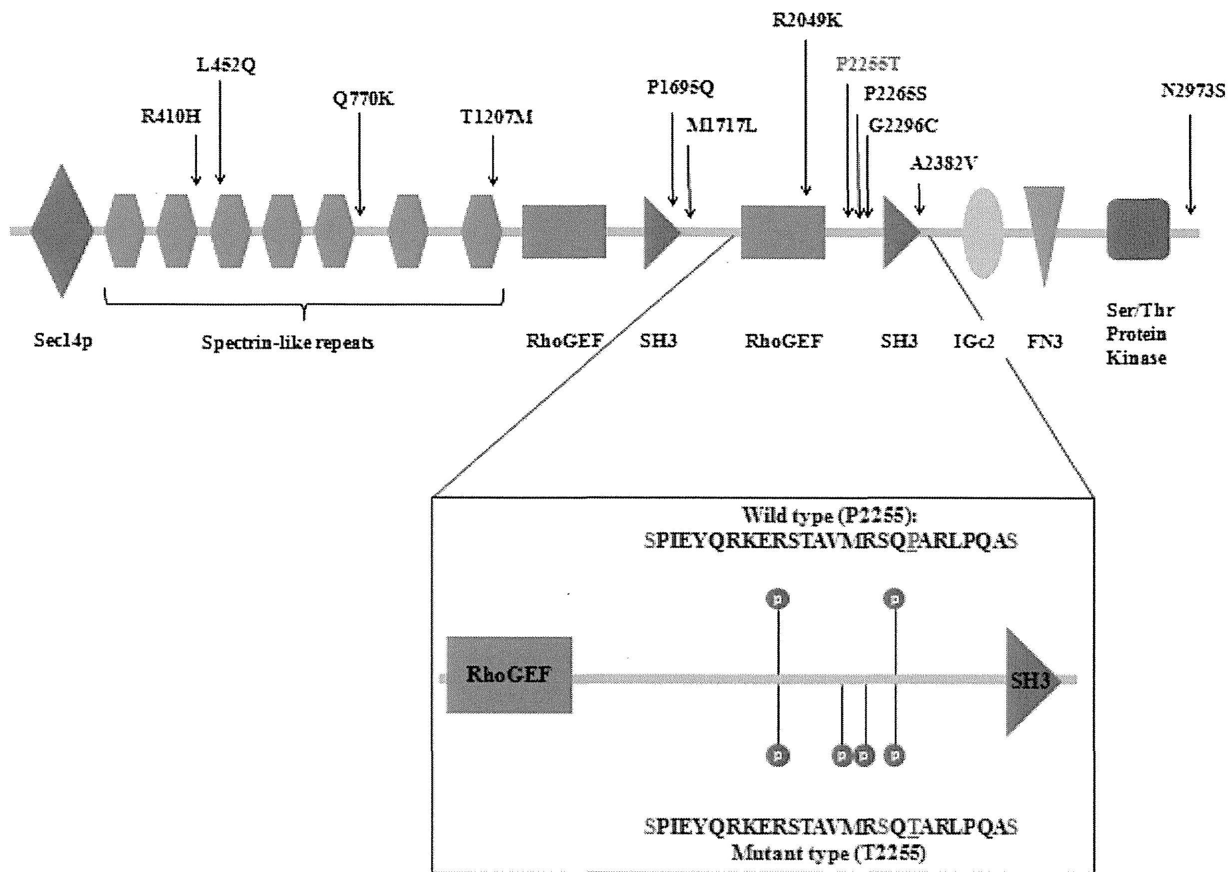

Fig. 1. Rare Missense Mutations in *KALRN* and Change in Phosphorylation Status by P2255T.

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.

Funding

This work was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Ministry of Health, Labor and Welfare of Japan; Grant-in-Aid for Scientific Research on Pathomechanisms of Brain Disorders from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Academic Frontier Project for Private Universities, Comparative Cognitive Science Institutes; Core Research for Evolutional Science and Technology.

Supplementary Material

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

Acknowledgments

We sincerely thank the patients and healthy volunteers for their participation in this study. We also thank Ryoko Ishihara and Junko Tsuda for technical assistance.
The authors report no biomedical financial interests or potential conflicts of interest.

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Schizophrenia Research 134 (2012) 137–142

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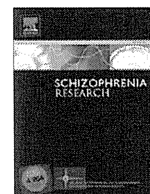

Effects of aging on the morphologies of Heschl's gyrus and the superior temporal gyrus in schizophrenia: A postmortem study

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ARTICLE INFO

Article history:
Received 16 May 2011
Received in revised form 9 October 2011
Accepted 29 October 2011
Available online 23 November 2011

Keywords:
Schizophrenia
Postmortem study
Aging
Heschl's gyrus
Superior temporal gyrus
Neuropathology

ABSTRACT

The etiology of schizophrenia has been proposed to be neurodevelopmental based on neuroimaging and molecular biological studies. If there is neuronal vulnerability based on neurodevelopment failures in schizophrenic brains, then the impact of aging may have a greater effect on schizophrenic brains than on normal brains. To determine the impact of aging on schizophrenic brains, we investigated the age-related morphological changes of the cross-sectional area of the gray matter (GM) in the left Heschl's gyrus (HG) and the left superior gyrus (STG) in 22 schizophrenic and 24 age- and sex-matched normal control postmortem brains two-dimensionally. The subject groups were divided into younger groups (30–54 years of age) and older groups (65–84 years of age) on the basis of age at death. Both in schizophrenic and control subjects, the GM area in HG and the STG was significantly smaller in the older group than in the younger group, however, no significant differences were observed between the schizophrenic and control subjects. In the STG, the cross-sectional area of the white matter (WM) was also measured. In the older group, the ratio of the GM area to the WM area in the STG was significantly larger in schizophrenic subjects than controls, although there was no significant difference between the schizophrenic and control subjects in the younger group. These findings indicate that the impact of aging has a greater effect on the WM in the STG in schizophrenic subjects than in normal individuals, although the pathological basis is still unclear.

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1. Introduction

A decreased cortical volume in schizophrenic brains has been repeatedly reported in cross-sectional imaging studies (Honea et al., 2005; Steen et al., 2006) and postmortem studies (Harrison, 1999; Iritani, 2007). It is understood that this morphological change occurs during the very early period of the formation of the central nervous system, because no gliosis has been found in the postmortem brains of schizophrenic patients (Arnold and Trojanowski, 1996). On the other hand, recent longitudinal imaging studies have also reproducibly reported a greater progressive loss of the cortical volume after disease onset (DeLisi et al., 1997; Kasai et al., 2003b; Nakamura et al., 2007). It is assumed that these phenomena depend on not only neuronal development (Thompson et al., 2001; Vidal et al., 2006) but also continued neuronal degeneration, the long-term use of antipsychotic drugs (Lieberman et al., 2005; Thompson et al., 2009) and unknown interactions between aging and schizophrenia (Harrison, 1999). However, the basis of the volumetric change after disease onset still remains unclear.

To investigate the impact of aging on schizophrenia, it is necessary to consider physiological atrophy and the concomitant presence of neurodegenerative disorders (e.g. Alzheimer-type dementia [ATD], frontotemporal lobar degeneration, etc.) separately. Most neuropathological studies have reported the frequency of Alzheimer-type neuropathological changes in schizophrenic brains to be equal to that in the general population (Powchik et al., 1998; Purohit et al., 1998; Jellinger and Gabriel, 1999), but it remains unclear how the physiological changes in the brain volume of schizophrenic patients occurs, compared to that of the normal brain. To investigate the impact of physiological atrophy, it is necessary to select elderly cases without significant neuropathological changes as study subjects. It is

Abbreviations: ATD, Alzheimer-type dementia; BA, Brodmann area; CPZ, chlorpromazine; CSI, circular sulcus of insula; DTI, diffusion tensor imaging; FTS, first transverse sulcus; GAF, Global Assessment of Functioning; GM, gray matter; HG, Heschl's gyrus; HS, Heschl's sulcus; PMI, postmortem interval; SI, sulcus intermedius; STG, superior temporal gyrus; STS, superior temporal sulcus; WM, white matter.

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doi:10.1016/j.schres.2011.10.024

impossible to detect neuropathological changes with the subtle and subclinical concomitant presence of neurodegenerative disorders or other organic factors, such as cerebrovascular changes, by neuroimaging investigations. A postmortem analysis has advantages in that it can determine subtle or subclinical neurodegenerative and organic changes which cannot be detected during the neuroimaging analysis of living subjects. In some cases, early onset frontotemporal dementia, organic psychotic disorders and so on are misdiagnosed as schizophrenia (Fujii et al., 2004; Velakoulis et al., 2009). Generally, schizophrenia is diagnosed based on the operational diagnostic criteria, such as the DSM IV-TR based on clinical symptoms, because so far no definite biological diagnostic tools have yet been established. In addition, neuropathological investigations of the schizophrenic brain are hardly ever performed posthumously in usual clinical settings. Therefore, it is important to perform postmortem neuropathological evaluations, even if the subjects are relatively young at the time of death.

Heschl's gyrus (HG) and its posterior region, the planum temporal (PT), are located on the dorsal surface of the superior temporal gyrus (STG). HG forms part of the primary auditory cortex (Brodmann area [BA] 41/42) and plays a crucial role in auditory perception, while the anterior portion of the PT, which surrounds HG, forms part of the unimodal auditory association cortex (part of BA22) and plays a critical role in language processing (Shapleske et al., 1999). In structural imaging studies, the gray matter (GM) in the STG and its sub-regions, such as HG or the PT, has been found to be smaller (McCarley et al., 2002; Takahashi et al., 2006) and even to decrease over time in schizophrenic patients (Kasai et al., 2003b; Salisbury et al., 2007). Moreover, their reduced size has been correlated with the degree of thought disorder (Shenton et al., 1992; Anderson et al., 2002) and auditory hallucinations (Barta et al., 1990; Onitsuka et al., 2004), especially when the difference is noted on the left side (Sun et al., 2009).

In this study, to determine whether aging has a greater impact on schizophrenic brains than on normal brains, we investigated the age-related changes in the cross-sectional GM area in the left HG and the STG using post-mortem neuropathological slide specimens without significant neuropathological changes. In addition, we also investigated the age-related changes of the cross-sectional area of the white matter (WM) and the ratio of the GM area to the WM area in the left STG to identify differences due to aging in schizophrenic patients and normal subjects.

2. Experimental/Materials and methods

2.1. Subjects

Brain specimens obtained from 22 schizophrenic patients and 24 age- and sex-matched normal control brain specimens were obtained from autopsy cases at Tokyo Metropolitan Matsuzawa Hospital based on the following criterion: age at death ≧30 years and ≦54 years for the younger groups (11 schizophrenic patients, 11 control subjects), and ≧65 years and ≦84 years for the older groups (11 schizophrenic patients, 13 control subjects). The demographic details of the subjects are summarized in Table 1.

We confirmed the diagnosis by reviewing the clinical records to verify that the cases satisfied the DSM-IV-TR criteria for schizophrenia, and that the control subjects had no evidence of psychiatric or neurological disorders.

We also excluded cases with significant neuropathological changes, such as neurodegenerative disorders (e.g. ATD, frontotemporal lobar degeneration, etc.), cerebrovascular diseases, brain invasion of tumors, severe malnutrition, metabolic encephalopathies, inflammatory or traumatic processes, and so on, based on the records of clinico-pathological conferences with several expert neuropathologists. We selected cases without any history of alcohol or substance abuse, convulsions or mental retardation based on the patient clinical records. We confirmed that all cases had sufficient social function before onset and had completed compulsory education.

Table 1
The basic demographic characteristics of the subjects.

	Control		Schizophrenia	
	Younger group	Older group	Younger group	Older group
Number	11	13	11	11
Sex (M/F)	6/5	8/5	7/4	5/6
Age at death (years)	45.0 ± 8.1	72.7 ± 4.4	44.4 ± 6.8	70.9 ± 5.1
range (years)	(31–54)	(66–81)	(33–54)	(65–83)
Age at onset (years)	–	–	27.7 ± 13.6	29.7 ± 8.6
Duration of illness (years)	–	–	16.6 ± 10.7	41.2 ± 8.9
Subtype (Paranoid/ Disorganized/Catatonic/ Undifferentiated)	–	–	7/3/1/0	5/4/1/1
GAF scores	–	–	28 ± 4.3	24.3 ± 5.8
Mean daily antipsychotic dosage[d] (mg/day)	–	–	561.3 ± 216.0[a]	562.5 ± 459.6[a]
Lifetime daily antipsychotic dosage[d] (g)	–	–	3343.9 ± 1921.5[a]	8479.4 ± 6139.9[a]
Cause of death (Cardiac/ Respiratory/Other)	3/3/5	2/6/5	1/5/5	1/5/5
Cerebrum weight (kg)	1332.5 ± 165.9[b]	1310.8 ± 206.9	1360.0 ± 171.1	1265.9 ± 149.6
PMI (hours)	6.1 ± 5.4[c]	6.8 ± 4.9[c]	8.3 ± 13.6	10.9 ± 8.6[c]

GAF: Global Assessment of Functioning, PMI: postmortem interval.
There were no significant differences, except for in the age at death and duration of illness, between the younger and older groups ($p < 0.01$, Mann–Whitney U test).
[a] Not known for 1 younger subject and 3 older subjects.
[b] Not known for 1 control subject.
[c] Not known for 2 younger control, 2 older control and 2 older schizophrenic subjects.
[d] Chlorpromazine milligram or gram equivalents.

We assessed the clinical severity in the predominant state using the Global Assessment of Functioning (GAF) scale (American Psychiatric Association, 2000), because this scale was easy to pick up retrospectively from the clinical records. It was impossible to evaluate the detailed degrees of symptoms in life retrospectively using the Brief Psychiatric Rating Scale or Positive and Negative Symptom Scale. We measured the sum of antipsychotic dosage taken throughout their lifetime (lifetime antipsychotic dosage) and the mean daily antipsychotic dosage (chlorpromazine [CPZ] milligram equivalents per day) by reviewing the clinical records.

This study was approved by the Nagoya University School of Medicine Ethical Review Board.

2.2. Brain tissue processing

In all cases, the cadavers were kept at 4° C before autopsies to prevent autolysis and tissue degeneration. All brains were extracted and fixed in 10% formalin within 48 h after death. After fixation, the brains were sectioned in the coronal plane. The brains were embedded in paraffin and cut at a thickness of about 10 μm. The sections were stained with hematoxylin-eosin and/or Klüver-Barrera.

2.3. Morphometric analysis

A morphometric analysis was performed by one of the coauthors blind to the diagnosis. In each subject, we used a neuropathological specimen slide from each left coronal slice at the level where HG, the hippocampus and the subthalamic nucleus appeared. Each slice was scanned into a computer as digital data (TIFF file) using an image scanner (CanoScan LiDE 500F; Canon Inc., Japan) at 600 dpi. The tracing and measurements of regions of interest were performed manually using the Adobe Photoshop 6.0 (Adobe Systems Inc., CA,

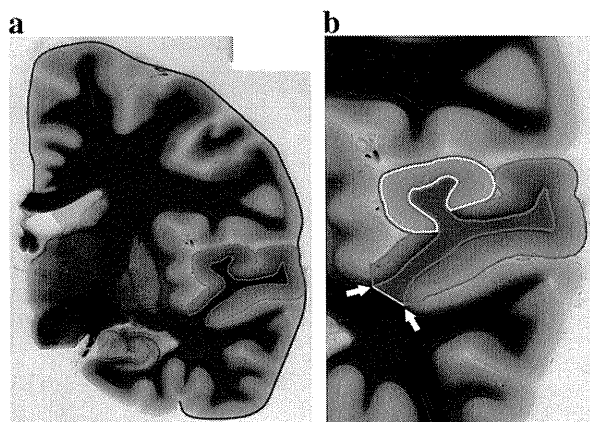

Fig. 1. Delineation of Heschl's gyrus and the superior temporal gyrus. (a) The gray matter (GM) in the left superior temporal gyrus (STG) is shown in red. The length of the external surface of the hemisphere from the callosal sulcus to the occipitotemporal sulcus is shown in blue. (b) The GM in the left STG is shown in red. The GM in the left Heschl's gyrus is shown in yellow. In the STG, after delineating the GM, the boundary of the white matter (WM) was delineated by connecting a straight line between the two open ends of the area of the GM (arrows). The WM in the left STG is shown in green.

USA) and ImageJ 1.43u (free software presented by NIH: http://rsb.info.nih.gov/ij/) software programs on the computer.

We identified the circular sulcus of insula (CSI), Heschl's sulcus (HS), the sulcus intermedius (SI), the first transverse sulcus (FTS) and the superior temporal sulcus (STS). Then, the area of GM bounded by the HS or SI laterally and the FTS medially was delineated and measured as the absolute GM area in HG based on the guidelines for the parcellation of the temporal lobe (Kim et al., 2000) (Fig. 1b). The area of the GM bounded by the STS laterally and the CSI medially was considered the absolute GM area in the STG (Fig. 1a). The length of the external surface of the hemisphere from the callosal sulcus to the occipitotemporal sulcus was measured as the hemisphere circumference (Fig. 1a). The relative GM area in HG and the STG ([absolute GM area of region of interest]/[hemisphere circumference]) was calculated to correct for individual differences in each brain size.

In the STG, after delineating the GM, the boundary of the WM was delineated by connecting a straight line between the two open ends of the area of the GM based on a previous report (Lee et al., 2009). In this way, the absolute WM area was also measured (Fig. 1b). The relative WM area ([absolute WM area of region of interest]/[hemisphere circumference]) and the ratio of the GM area to the WM area was calculated.

2.4. Statistical analysis

For the statistical analyses, nonparametric tests were chosen, because our relatively small sample size was insufficient to evaluate the distribution. The Kruskal–Wallis test was used to assess the differences in the cerebrum weights and postmortem interval (PMI) among the four groups (the younger schizophrenia group, the older schizophrenia group, the younger control group and the older control group). Fisher's exact test was used to assess the differences in gender and causes of death among the four groups, and the differences in subtype between the younger and older schizophrenia groups. The Mann–Whitney *U* test was used to verify the differences in the age at death between the controls and schizophrenic patients in each age group, and the differences in the age at onset, GAF scores or mean daily antipsychotic dosage between the younger and older schizophrenic patients. To assess of impact of aging on the cross-sectional area in regions of interest, the Mann–Whitney *U* test was used between age groups in each diagnostic group and between diagnostic groups in each age group. Spearman's rank correlation was calculated to assess the association between the hemisphere circumference and the cerebrum weight. Spearman's rank correlation was used to assess the association between the cross-sectional area in regions of interest and the PMI, GAF scores or lifetime antipsychotic dosage. The data were expressed as the means ± standard deviation. A value of $p<0.05$ was considered to be statistically significant. All statistical analyses were performed using the SPSS Statistics 17.0 software program (SPSS Inc., IL, USA).

3. Results

The mean values of the cross-sectional area and the hemisphere circumference in each region are reported in Table 2.

3.1. Basic demographic data

There were no significant differences in the gender ($p=0.91$), cause of death ($p=0.90$), cerebrum weight ($p=0.44$) or PMI ($p=0.76$) among the various groups. There were also no significant differences in the age at death between the schizophrenic and control subjects in both the younger and older groups ($p=0.74$, $p=0.26$). There were no significant differences in the age at onset of disease ($p=0.21$), disease subtype ($p=0.82$), GAF scores ($p=0.11$) and mean daily antipsychotic dosage ($p=0.86$) between the younger and older schizophrenic patients.

The PMI and lifetime antipsychotic dosage were not correlated with the cross-sectional area in the regions of interest (the relative GM area in HG; $p=0.53$, $p=0.17$, the relative GM area in the STG; $p=0.67$, $p=0.71$, the relative WM area in the STG; $p=0.51$, $p=0.25$) or the ratio of the GM area to the WM area in the STG ($p=0.91$, $p=0.10$).

3.2. The association between hemisphere circumference and cerebrum weight

The hemisphere circumference was positively correlated with the cerebrum weight in all subjects (Spearman rank correlation, $r=0.44$, $p=0.0034$). As the hemisphere circumference reflected the brain size, it was thought that using the hemisphere circumference was adequate for standardizing individual differences in brain size.

Table 2
The mean values of the cross-sectional area and hemisphere circumference.

	Control		Schizophrenia	
	Younger group	Older group	Younger group	Older group
Hemisphere circumference (mm)	187.2 ± 17.9	178.7 ± 10.2	193.0 ± 12.6	180.2 ± 12.7
HG				
Absolute GM area (mm^2)	58.0 ± 17.0	37.8 ± 17.7	55.6 ± 16.9	39.4 ± 16.6
Relative GM area (mm^2/mm)	0.31 ± 0.10	0.21 ± 0.09	0.29 ± 0.08	0.22 ± 0.10
STG				
Absolute GM area (mm^2)	221.1 ± 46.1	166.9 ± 36.1	212.2 ± 44.6	160.3 ± 31.3
Relative GM area (mm^2/mm)	1.18 ± 0.18	0.93 ± 0.18	1.10 ± 0.21	0.89 ± 0.19
Absolute WM area (mm^2)	84.1 ± 27.4	69.4 ± 22.5	80.7 ± 33.9	54.6 ± 13.4
Relative WM area (mm^2/mm)	0.45 ± 0.12	0.39 ± 0.11	0.42 ± 0.16	0.30 ± 0.07
GM area/WM area (mm^2/mm^2)	2.73 ± 0.49	2.53 ± 0.54	2.82 ± 0.58	3.00 ± 0.48

HG: Heschl's gyrus, STG: superior temporal gyrus, GM: gray matter, WM: white matter.

3.3. The cross-sectional GM area in HG and the STG

The relative GM area in HG was significantly smaller in the older group than in the younger group, in both schizophrenic and control subjects ($p = 0.045$, $p = 0.02$) (Fig. 2a). However, in both the younger and older groups, there were no significant differences in the relative GM area in HG between the brains from the schizophrenic and control subjects ($p = 0.49$, $p = 0.58$) (Fig. 2a).

In the STG, the relative GM area was significantly smaller in the older group than in the younger group, in both controls ($p = 0.008$) and schizophrenic patients ($p = 0.03$) (Fig. 2b). In both the younger and older groups, there were no significant differences in the relative GM area in the STG between the brains of the schizophrenic and control subjects ($p = 0.58$, $p = 0.58$) (Fig. 2b).

3.4. The cross-sectional WM area in the STG

The relative WM area in the STG was smaller in the older group than the younger group in schizophrenic patients, although this difference was not statistically significant ($p = 0.053$), while there was very little age-related reduction in the relative WM area in controls ($p = 0.34$) (Fig. 2c). In the younger subjects, there were no significant differences in the relative WM area in the STG between schizophrenic patients and controls ($p = 0.49$). In the older groups, the relative WM area in the STG was significantly smaller in the schizophrenic patients than in controls ($p = 0.04$) (Fig. 2c). In addition, in the older subjects, the ratio of the GM area to the WM area in the STG was significantly larger in schizophrenic patients than controls ($p = 0.04$). However, in the younger groups, there was no significant difference between the ratios in the schizophrenic and control subjects ($p = 0.62$) (Fig. 2d).

3.5. The effects of the severity of illness

The GAF scores were positively correlated with the relative GM area in the HG in schizophrenic patients (Spearman's rank correlation, $r = 0.48$, $p = 0.02$). This correlation was observed especially in the younger schizophrenic patients, although it was barely significant ($r = 0.60$, $p = 0.050$). On the other hand, in older schizophrenic patients, no correlation was observed ($r = 0.10$, $p = 0.767$). The GAF scores were not correlated with the relative GM area, WM area or the ratio of the GM area to the WM area in the STG ($p = 0.24$, $p = 0.23$, $p = 0.28$).

4. Discussion

4.1. The GM cross-sectional area and volume reduction in schizophrenic and normal subjects

The first key finding from this study is that there were no significant differences in the cross-sectional GM area loss with age in the STG and its sub-region, HG, between the schizophrenic and normal postmortem brains. We could select elderly subjects without any significant neuropathological changes for the older group, who were older than those in most imaging studies, based on the results of clinico-pathological discussions with several expert neuropathologists and based on physical evidence in the brains themselves observed upon autopsy. It is assumed that the older the subjects are, the stronger the impact of aging would be, however, even the older age of our current subjects did not show any significant impact on the acceleration of GM area reduction, or physiological atrophy, in the schizophrenic brains. Longitudinal imaging studies of the STG and HG have demonstrated the progressive reduction of the GM volume to be larger in the early stage of illness (Jacobsen et al., 1998; Kasai et al., 2003a, 2003b; Salisbury et al., 2007), while the GM volume thereafter stabilizes during the chronic stage of illness (DeLisi and Hoff, 2005; Yoshida et al., 2009). Taking these findings from longitudinal imaging studies into consideration, our results support the assumption that the progression of the GM volume reduction in the STG and its sub-regions in schizophrenic patients does not reflect a greater impact of aging on schizophrenic brains, but such progression is instead due to the neurodevelopmental or neurodegenerative pathogenetic core at the initial stage of schizophrenia.

In our study, no significant differences were seen in the GM area between the schizophrenic and control subjects in the younger groups. A few imaging studies reported results consistent with our findings (Kulynych et al., 1996; Barta et al., 1997). Nevertheless, GM or total volume reduction in the STG in patients with schizophrenia

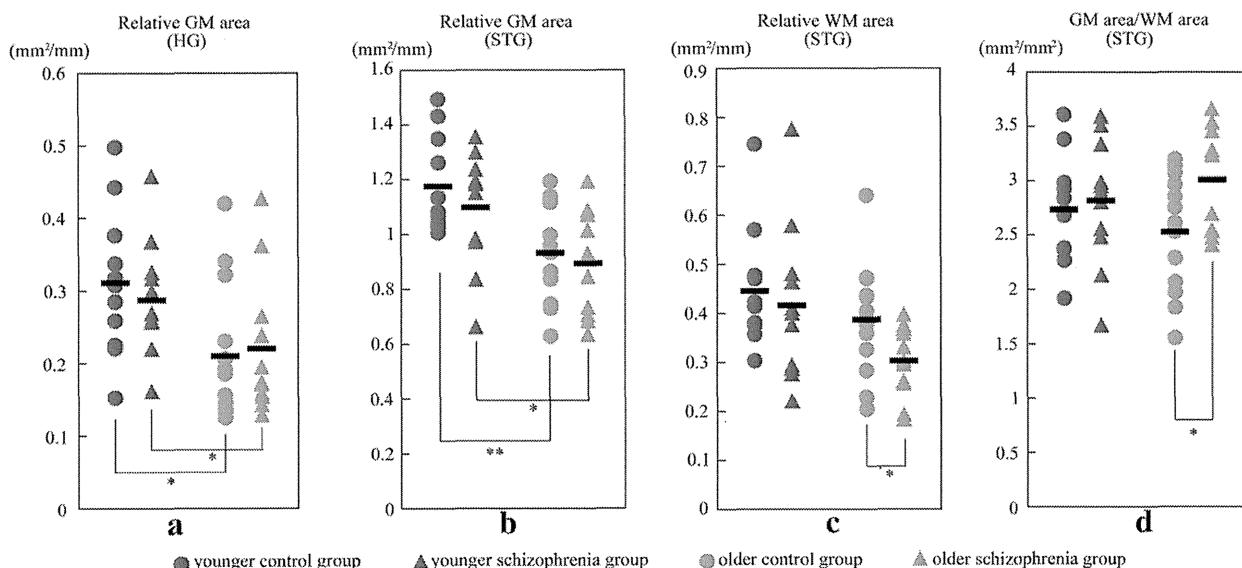

Fig. 2. Effects of aging on Heschl's gyrus and the superior temporal gyrus in the schizophrenic and control subjects. (a) The relative gray matter (GM) area in Heschl's gyrus (HG) in the schizophrenic and control subjects. (b) The relative GM area in the superior temporal gyrus (STG) in the subjects with schizophrenia and the controls. (c) The relative white matter (WM) area in the STG in the schizophrenic and control subjects. (d) The ratio of the GM area to the WM area in the STG in the subjects with schizophrenia and the controls. The horizontal black lines indicate the means. **: $p<0.01$, *: $p<0.05$.

has also been frequently reported in some imaging studies where the mean age is in the 40s, which is equal to that of our younger group (Anderson et al., 2002; Onitsuka et al., 2004). In comparison to the findings of imaging studies, this discrepancy may be explained by the older mean age at onset and the shorter illness duration of our younger group (Matsumoto et al., 2001; Crespo-Facorro et al., 2004; DeLisi and Hoff, 2005), in addition to the methodological differences.

4.2. WM cross-sectional area or volume reduction in schizophrenia

Our second key finding is that the WM loss increased with age in the STG in the schizophrenic patients, but not in the controls. In younger schizophrenic patients, some cross-sectional imaging studies have reported an absence of volumetric differences in the WM in the STG (Gur et al., 2000; Matsumoto et al., 2001; Buchanan et al., 2004), which is in agreement with our finding, although other studies have reported volumetric differences (Spalletta et al., 2003; O'Daly et al., 2007). To the best of our knowledge, there have been no previous reports about volumetric changes in the WM in the STG in elderly schizophrenic patients. With regard to other brain regions, there have been a few reports about volumetric changes of the WM with age. Our finding is in agreement with the studies which have noted an increase in the WM loss with age in schizophrenic patients in the frontal lobes (Ho et al., 2003) and total brain (Bose et al., 2009) and is in contrast to a study that reported no difference in the WM loss with age in the total brain between schizophrenic and control subjects (Hulshoff Pol et al., 2002). Therefore, controversy remains in regard to whether there is a WM volume change as a result of schizophrenia.

Recent diffusion tensor imaging (DTI) studies have repeatedly reported WM abnormalities in schizophrenic patients based on decreases in anisotropy indices in the WM tracts and structures (Hubl et al., 2004; Wang et al., 2004). It is thought that the WM changes noted in DTI studies could reflect a loss of organization in WM fiber tracts, such as loss of myelin, axonal fibers, and/or increased extracellular space in this region. However, further examinations will be required to determine what volumetric change in the WM is reflected in our studies, because there were differences in the methodologies between the various studies.

In postmortem studies, in addition to reduced glial cell density (Beasley et al., 2009) in the WM in the STG in schizophrenic patients, it has been demonstrated that there is decreased expression of myelination-related genes (e.g. CNP, MAG, PLP1, ERBB3, etc.) (Tkachev et al., 2003; Peirce et al., 2006) and decreased intracortical myelin markers, such as myelin basic protein, have been reported in other brain regions (Flynn et al., 2003; Chambers and Perrone-Bizzozero, 2004). Therefore, further studies are required to investigate whether there are microscopic and/or genetic findings which could correlate with the age-related macroscopic findings identified in our studies.

4.3. The effects of antipsychotics and the severity of illness

In our study, the lifetime antipsychotic dosage did not correlate with the cross-sectional GM and WM area in the STG and HG. Another postmortem study reported that the long-term use of antipsychotic medication by schizophrenic patients does not promote the development of ATD pathology in the brain (Niizato and Ikeda, 1996). These findings may indicate that antipsychotics do not affect the age-related changes of the brain tissue.

In our study, the severity of illness was more likely to affect the brain volume in the younger patients, not in older patients. This finding may be due to many factors related to aging, such as physical disease. It is assumed that the effects of schizophrenia itself may be obscured by many of these factors.

4.4. Future studies

There are a few limitations to this study. First, we measured the morphological changes two-dimensionally in glass slide specimens. It would therefore be better to measure the actual volume of HG and the STG three-dimensionally, but it is difficult to measure the volume when evaluating two-dimensional glass slide specimens. Second, our data cannot be directly compared with the data of neuroimaging studies, because the brain tissue would likely constrict during the course of fixation.

Ultimately, a histopathological or neuropathological investigation of the WM of schizophrenic patients, focusing on the formation of the neuronal fibers, including neurofilament or myelin protein, is thus called for to elucidate the pathological basis of our age-related findings.

Role of funding source

This research was supported by Grants-in-Aid for Scientific Research (No. 20591400) from the Japan Society for the Promotion of Science (2008–2010).

Contributors

The study was conceptualized by SI, TA and KI. The sample was controlled by YT, SI, TA, KI and HA. The analyses were designed and conducted by YT, HS, CH and NO. The manuscript was written by YT, SI, HS, CH, MH and NO. All authors have approved the final manuscript.

Conflict of interest

None.

Acknowledgments

None.

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