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ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Necessity of normative data on the Japanese version of the Wechsler Memory Scale-Revised Logical Memory subtest for old-old people

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Aim: Episodic memory is vulnerable to deterioration in people suffering from Alzheimer's disease. Currently, the Logical Memory (LM) subtest of the Wechsler Memory Scale-Revised (WMS-R) is used internationally as an operational definition to identify people with mild cognitive impairment (MCI). However, the Japanese version of the LM has not been adequately normalized for old-old people. Therefore, norms of the LM for people aged 75 years and over are required, and the effects of sex, age and education on performance were evaluated.

Methods: A total of 50 (27 female and 23 male) participants without a history of dementia and symptomatic stroke events recruited from the community and hospital populations were investigated using the Mini-Mental State Examination, the LM and some interference tasks.

Results: The mean scores (standard deviations) of the sample were 15.5 (5.4) on LM-I and 9.9 (6.6) on LM-II. The distributions of the LM-I and -II scores satisfied the normality assumption. The LM-I and LM-II scores correlated with age and the LM-I score correlated with educational background.

Conclusions: For the Japanese version of the LM, the means, standard deviations and distribution features of the old-old sample are presented. Although the normal sample was chosen to closely match the demographic profile of the Japanese population, the present sample might have had a higher educational background than the age-matched population, especially the males. Further study is required to standardize the Japanese version of the LM subtest for each 5-year interval for latter-stage elderly people. **Geriatr Gerontol Int 2013; 13: 726–730.**

Keywords: episodic memory, Logical Memory, mild cognitive impairment, normative data, old-old people.

Introduction

A mild cognitive impairment (MCI) as a result of Alzheimer's disease (AD) is seen as memory impairment, and this symptom is the key early marker in the prodromal stages of AD.[1] Although the memory deficits in individuals with MCI are clinically discernible, in order to make a diagnosis of MCI, amnesia that does not interfere notably with activities of daily life (ADL) must be identified. It is operationally defined as performance 1.0–1.5 standard deviations (SD) below age- and education-adjusted norms on an episodic memory measure of delayed verbal recall.[2]

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The Logical Memory (LM) subtest of the Wechsler Memory Scale (WMS), which includes immediate (LM-I) and 30-min delayed (LM-II) trials of prose recall, is a large contributor to discriminating between healthy older adults and individuals with very mild AD. Guillozet *et al.* reported that AD pathology is more numerous in medial temporal lobe regions associated with the LM scores of the revised version of WMS (WMS-R),[3] and shows a relationship with LM performance on the WMS-R in individuals in the non-demented stage.[4] Although the relative ability of memory tests to discriminate between the AD convertor type of MCI and normal aging has not been well characterized, a previous study reported that the LM-II was one of the best predictors for detecting progression from MCI to AD over a 4-year period.[1] The LM of the WMS-R is one of the standard memory criteria for MCI clinical and research; for example, in the Alzheimer's Disease Neuroimaging Initiative study.[5]

Various factors have been associated with LM test score differences. Abikoff *et al.* have already reported that age and education norms are generated for immediate, 30-min delayed and 24-h delayed recall in the LM of the WMS, and performance is more closely related to educational background.[6] Their sample ranged in age from 18 to 81 years, with a mean educational level of 13.96 years (range 6–18 years). The LM performance increase is somewhat more common with higher levels of education.[6,7] However, Abikoff *et al.* noted that "Although education was more highly related to scores than was age, small but significant relationships between age and verbal recall remained over and above the influence of education.[6]" The impact of age is most obvious in 24-h delayed recall, and drop-off in performance occurs over the age of 60 years. Therefore, the latest version of the WMS has paid attention to elderly participants in the form of advancing an elderly battery.

However, the WMS-R version is the only LM task that has been standardized for Japanese people, and the normative sample has been limited to the ages of between 16 and 74 years. The incidences of AD, combined dementia and other types of dementia rise with increasing age, particularly after the age of 85 years.[8] Although not only for young-old people, but also for old-old or oldest-old people, an amnestic state examination of high accuracy is required, because the Japanese versions of the WMS-R, LM-I and LM-II have not been adequately normalized for latter-stage elderly people. In the current study, normative data for the LM in Japanese elderly people aged 75 years and older were gathered.

Methods

Participants

A total of 50 (27 female and 23 male) participants without a history of dementia and symptomatic stroke events were recruited from the community and hospital populations living in two urban areas. All participants could attend the trial sites alone. The sample size determination was based on the original version of the WMS-R and the general recommendation on statistics in psychology and education, taking a sample of 50 and over per age group interval.[9] A total of 30 participants (60%) had no history of psychiatric problem as assessed by the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disoriders, Fourth Edition revised (SCID),[10] and they did not report clinical evidence of amnesia and ADL impairment. There is nothing to suggest that participants did not hear something at the time of auditory stimulus presentation in the study. They ranged in age between 75 and 87 years (mean ± SD: 79.3 ± 3.6 years), and in educational background between 6 and 18 years (mean ± SD: 11.7 ± 3.1 years).

The ethics committee of the Tokyo Metropolitan Institute of Gerontology and the Nagoya University School of Medicine approved the present study, and each participant signed a consent form after being sufficiently informed about the outline of the study by the principal investigator.

Tasks

Logical Memory (LM)-I and -II from the Japanese version of the WMS-R[11] were carried out. In the LM-I, participants were asked to immediately recall from the number of prose units twice: the first trial presented story A verbally, and the second trial presented story B verbally. In the LM-II, participants were asked to recall words from the two stories 30 min later. During the time delay, participants were asked to carry out the Mini-Mental State Examination (MMSE)[12] and some interference tasks.

In the present study, not all of the participants carried out every task item, other than LM-I, II and MMSE. As the purpose of the present study was to provide normative data for LM-I and -II, the sample size was kept the same; hence, missing data were not substituted. The results are based on the eight task scales.

Statistical analysis

All statistical analyses were carried out using SPSS 17.0 J. for Windows (SPSS, Chicago. IL, USA). Normative data are provided in the form of means and standard deviations (SD) broken down by sex, age and educational background. Correlation analyses between LM scores and various factors were carried out using the Pearson product-moment correlation coefficient. A *P*-value of less than 0.05 was considered significant. The percentile rank of each LM-I or -II score was calculated, after the Shapiro–Wilk test was carried out to check the normality of the sampling distribution.

Results

Sample characteristics

In the present sample, the mean ± SD score of MMSE (27.3 ± 2.2) reflected the expected distribution of general cognitive status for aged groups. The normative sample was confirmed to match closely the demographic profile of this population as reported in a recent census. Table 1 shows the percentiles of the normalization sample by age, sex and educational background compared with these population averages in Japan (Statistics Bureau 2010: Ministry of Internal Affairs and Communications). The results showed that the sample might have had a higher educational background than Japan's age-matched population.

Table 1 Percentiles of the normalization sample by age, sex and educational background

Age (years)	Sex	Education 0–11 (years) Sample	Population (Japan)	12 (years) Sample	Population (Japan)	>13 (years) Sample	Population (Japan)
>75	Male	25.9	45.6	18.5	37.6	40.7	16.8
	Female	63.0	53.3	18.5	41.3	18.5	5.4
	Total	48.0	50.3	20.0	39.9	32.0	9.8

The estimated population was calculated excluding active students and unknown individuals of education backgrounds from total number.

Table 2 Performance of the sample aged 75 years and older

		Mean SD (min–max)
LM-I		15.5 ± 5.4 (5–8)
	Story A	8.3 ± 3.2 (3–16)
	Story B	7.4 ± 2.8 (2–14)
LM-II		9.9 ± 6.6 (0–25)
	Story A	5.0 ± 3.8 (0–13)
	Story B	4.9 ± 3.2 (0–12)

I, immediate recall; II, delayed recall; LM, Wechsler Memory Scale-Revised Logical Memory subtest.

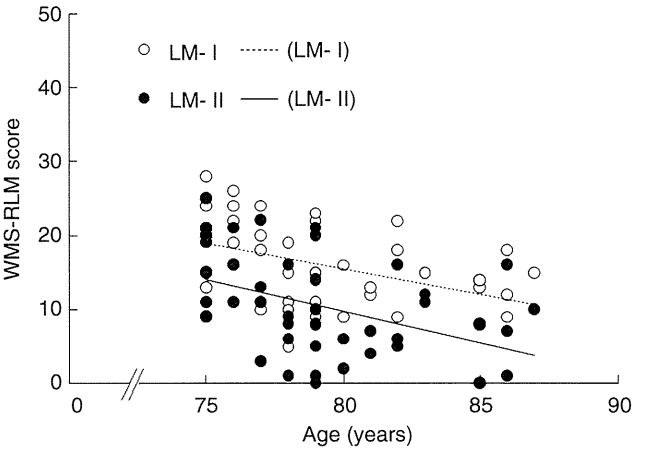

Figure 1 The scatter plot of Wechsler Memory Scale-Revised Logical Memory (WMS-RLM) subtest scores (y-axis) and age in years (x-axis). I, immediate recall; II, delayed recall; LM, Logical Memory subtest.

Reference data of the normal group

Mean scores (SD) of the sample were 15.5 (5.4) on LM-I and 9.9 (6.6) on LM-II. Table 2 summarizes the performance of the sample. To check the normality of the sampling distribution, coefficients of skewness and kurtosis were calculated for each trial. In the LM-I, the skewness value was 0.19 and the kurtosis value was −0.89; in the LM-II, the skewness value was 0.32 and the kurtosis value was −0.82. The distributions of the LM-I and -II scores satisfied the normality assumption using the Shapiro–Wilk test ($P > 0.05$).

Characteristics and performances

To examine the effect of sex on performance, unpaired t-tests comparing the LM-I and -II scores in male and female participants were carried out. In both the LM-I and -II, no significant difference was found. The mean scores (SD) of the male group were 15.6 (5.5) on the LM-I and 10.8 (6.4) on the LM-II, compared with 16.3 (5.5) on the LM-I and 10.0 (6.8) on the LM-II in the female group.

To examine associations between age (years) or educational background (years) and LM scores, correlation analyses were carried out. The LM-I and LM-II scores were moderately correlated with age ($r = -0.44$, $P < 0.01$; $r = -0.45$, $P < 0.01$), and the LM-I score was moderately correlated with educational background ($r = 0.36$, $P < 0.05$). There was no significant correlation between the LM-II score and educational background ($r = 0.23$, not significant.). The figures show scatter plots of the WMS-R LM scores and age in years (Fig. 1), or years of education (Fig. 2). Considering that the sample had a moderate to high education, partial correlation analyses between age (years) and LM scores were carried out. The LM-I and LM-II scores were moderately correlated with age ($r = -0.36$, $P < 0.05$; $r = -0.40$, $P < 0.01$).

Discussion

In the current study, LM normal performances of healthy Japanese people aged 75 years and older were surveyed, and the effects of sex, age and education on performance were identified. The means, SD and distribution features of the LM-I and -II of the WMS-R are presented for Japanese old-old people.

The sample had mean (SD) scores of 15.5 (3.2) on the LM-I and 9.9 (6.6) on the LM-II. According to

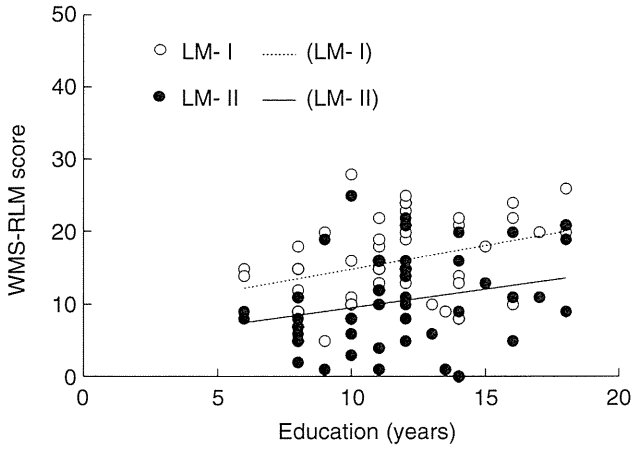

Figure 2 The scatter plot of Wechsler Memory Scale-Revised Logical Memory (WMS-RLM) scores (*y*-axis) and years of education (*x*-axis). I, immediate recall; II, delayed recall; LM, Logical Memory subtest.

Sugishita,[11] people in each age group (16–17 years ($n = 50$), 20–24 years ($n = 54$), 35–44 years ($n = 56$), 55–64 years ($n = 50$), 65–69 years ($n = 52$) and 70–74 years ($n = 54$)) had the following scores: on the LM-I, 27.7 (7.2), 26.6 (6.4), 25.1 (7.5), 22.0 (7.1), 19.5 (6.8) and 18.5 (7.5), respectively; and on the LM-II, 24.9 (7.7), 22.8 (6.7), 20.7 (7.6), 16.8 (7.0), 15.3 (7.0) and 13.2 (6.8), respectively. These results are consistent with previous data, and indicate an age-related decrease in LM task performance.[6,13] Considering the Flynn effect, the present data should not be compared directly with the previous data by Sugishita.[11] It is recommended that a larger study for the normalization of LM in older people be carried out.

Furthermore, the study showed that the LM-I and -II scores were moderately negatively correlated with age in a healthy sample aged 75 years and older. In particular, the LM-II score reflected the individual difference associated with age, independent of educational background. The result also confirms the age-related changes in memory functions. This finding, that the LM-II was *not* correlated with education leaves room for interpretation. Although the present sample from among community-dwelling older adults had generally better health and education, high-risk MCI persons might have been present in definite proportions, or the normal population might have individuals who, despite educational levels, may have been less able in cognitive abilities throughout their life.[14,15] According to the Mayo clinic's team, the LM-II data were *not* correlated with education in a community-based healthy sample.[15] They noted that the education-WMS performance association in the restricted age range of their older sample did not reflect true underlying relationships between the intelligence quotient (IQ) and task performance, and they recommended that WMS norms be stratified by IQ. The education-LM performance association might reflect these confounding factors.

Although the present sample was chosen to closely match the demographic profile of the Japanese population, the sample might have had a higher educational background than the age-matched population, especially among males. Community-based surveys in rural areas should also be carried out at the same time as surveys in urban areas. Thus, the sample bias is inappropriate for determining the "range of normal" memory functioning in an older population.[15] Norms stratified to be representative of the general population have great diagnostic value. However, the present result showed that the aging-related memory decline was observed in highly educated people, who had a greater likelihood of preserving cognitive function than people with low educational achievement. The result suggests that normalization of LM must be carried out for latter-stage elderly Japanese people. To establish the norms for the Japanese version of the LM, a further community-based study using the Intelligence Scale in parallel will be necessary. In addition, it will be necessary to compare between the LM norms based on the separately-carried out condition and that based on the completely-carried out WMS-R condition, and to normalize the latest version of WMS in Japanese people, because the latest version has a short battery for ages 65–90 years (the Older Adult Battery), including the new LM composed of the 14-paragraph-story (story A) and the 25-paragraph-story (story B).

The present estimated values based on LM scores of people aged 75 years and older, which are currently based on the population aged less than 75 years, show that current percentile ranks underestimate the memory ability of people aged 75 years and older. Furthermore, the present study obviously showed that the LM-I and LM-II scores were correlated with age. These results suggest the necessity of normative data on the Japanese version of the WMS-R LM subtest for each 5-year interval for the population aged 75 years and older, like the original version. In the future, for old-old people, it will be necessary to carry out a survey to establish norms of the WMS-R LM for each 5-year interval.

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Disclosure statement

No potential conflicts of interest were disclosed.

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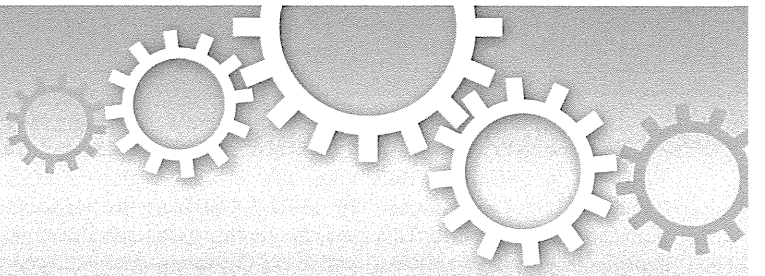

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Definition and refinement of the 7q36.3 duplication region associated with schizophrenia

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Using a very high-resolution oligonucleotide array for copy number variant (CNV) screening of samples comprising schizophrenic patients, we detected a novel CNV within the critical region (NCBI36/hg18, Chr7: 158,630,410–158,719,410) previously shown to be associated with schizophrenia. We investigated the association between the novel CNV identified in the current study and schizophrenia. Three independent samples were used: (1) *Screening set*, 300 Japanese schizophrenic patients (53.28 ± 14.66 years); (2) *Confirmation set*, 531 schizophrenic patients (46.03 ± 12.15 years); and (3) 711 healthy controls (47.12 ± 11.03 years). All subjects enrolled in the study were Japanese. Chromosomal position was determined using fluorescence in situ hybridization. We identified a novel duplication within the region associated with schizophrenia identified on 7q36.3 that is adjacent to *VIPR2* and is not associated with schizophrenia. In the Japanese population, the 35-kb region that harbors the common, novel CNV should be excluded from the region associated with schizophrenia on 7q36.3.

Schizophrenia is a chronic, debilitating illness characterized by impairments in cognition, affect and behaviour[1]. The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM IV-TR)[2] defines the essential features of schizophrenia as a mixture of characteristic signs and symptoms (both positive and negative) that have been present for a significant portion of time during a 1-month period (or for a shorter time if successfully treated), with some signs of the disorder persisting for at least 6 months. In this regard, positive refers to the presence of active symptoms including delusions and hallucinations. Negative symptoms refer to a loss, typically of emotions, speech, or motivation. Schizophrenic disorders exist on a continuum from mild to severe. The DSM IV-TR[2] recognizes a number of different types, which include disorganized, catatonic, paranoid, schizophreniform, residual, schizoaffective, undifferentiated and not otherwise specified[2]. Schizophrenia is a relatively common disorder, with a lifetime prevalence of about 1%[1]. Although the overall sex ratio is almost equal, males tend to have an earlier onset than females, a finding accounted for by the later age of onset in those females who lack a family history of the disease[3]. Family history is the most important risk factor for schizophrenia, consistent with a genetic contribution to its etiology[4]. However, as with most mental disorders, the origins and mechanisms of schizophrenia are not fully understood.

Genetic factors influence human disorders by determining disease susceptibility or resistance[5]. Therefore, genetic studies can help pinpoint the exact molecular mechanism of a disease. Recent successes in the genetic mapping and molecular mechanism of the Mendelian traits have been remarkable, owning to the development of genome wide screening techniques[6]. As such, attention has been gradually shifting towards more complex, common, genetic disorders and traits that involve multiple genes and environmental effects, such as celiac disease[7], diabetes[8], rheumatoid arthritis[9] and psychiatric disorders[10]. In this context, recurrent microdeletions at 1q21.1[11], 15q13.3[12], and 15q11.2[12], microduplications at 16p11.2[13], and copy number variations (CNVs) at other genomic loci[14] have been shown to be associated with schizophrenia in large cohorts examined by CNV analyses and other molecular studies. Furthermore, duplication at chromosome 7q36.3, encompassing *VIPR2*, was implicated in schizophrenia for the first time in a recent report[15]. In a specific genome-wide association study of 8,290 patients with schizophrenia performed by Vacic et al.[15], the authors found that 0.35% of these patients carry rare CNVs in the chromosomal locus 7q36.3. In contrast, these microduplications were much less frequent

(0.03%) among the 7,431 healthy controls. All variants overlap with *VIPR2* or lie within the noncoding subtelomeric region, <89 kb (NCBI36/hg18, Chr7: 158,630,410–158,719,410) from the transcriptional start site of *VIPR2*. This gene encodes the vasoactive intestinal peptide (VIP) receptor VPAC2, which is a G-protein-coupled receptor that is expressed in the suprachiasmatic nucleus, hippocampus, amygdala and hypothalamus[16]. VPAC2 binds VIP, activates cyclic AMP (cAMP)-signalling and PKA, regulates synaptic transmission in the hippocampus, and promotes the proliferation of neural progenitor cells in the dentate gyrus[17]. Moreover, it has been shown that alteration in synaptic plasticity of hippocampal neurons may contribute to the symptoms observed in schizophrenic patients[18]. The aforementioned lines of evidence provide support for the role of *VIPR2* as a candidate gene for schizophrenia from a biological point of view.

In the present study, by using a very high-resolution oligonucleotide array for CNV screening of samples from schizophrenic patients, we were able to detect CNV within the critical region (NCBI36/hg18, Chr7: 158,630,410–158,719,410) on 7q36.3 that was shown to be associated with schizophrenia by the Vacic et al. study[15]. Thus, the goal of the present study was to follow-up on the novel CNV that was previously detected in schizophrenic patients and further investigate any association between this CNV and schizophrenia.

Results

In the present study, we detected a smaller (35 kb) duplication (NCBI36/hg18, Chr7: 158,658,128–158,693,128) within the critical region identified by Vacic et al.[15] (Figure 1). The observed frequency of the CNVs was ~2% and we did not detect any statistically significant difference between the patients and controls (Table 1). There was a 100% concordance rate between the custom NimbleGen 12 × 135,000 CGH arrays and the NimbleGen 3 × 720,000 CGH arrays or custom TaqMan copy number assay for the detection of the smaller (35 kb) duplication (NCBI36/hg18, Chr7: 158,658,128–158,693,128) within the critical region (NCBI36/hg18, Chr7: 158,630,410–158,719,410) previously identified by Vacic et al.[15].

In metaphase cells, all duplication-specific FISH signals localize to the subtelomeric region of 7q, confirming that the duplications lie adjacent to each other in the 7q36.3 region (Figure 2). In addition, NS102 exhibited two signals, one of which had a higher intensity compared to that of the other. This suggests that there is unilocus duplication in the *VIPR2* promoter region. During the orientation analysis, an amplicon was detected by electrophoresis only in samples with duplication, which indicates that there is a head-to-tail orientation of the repeated DNA fragment (Supplementary Figure 1). Additionally, sequence analysis of the repeat junction revealed that all samples with duplication shared exactly the same sequence within the junction region (Supplementary Figure 2). Based on the

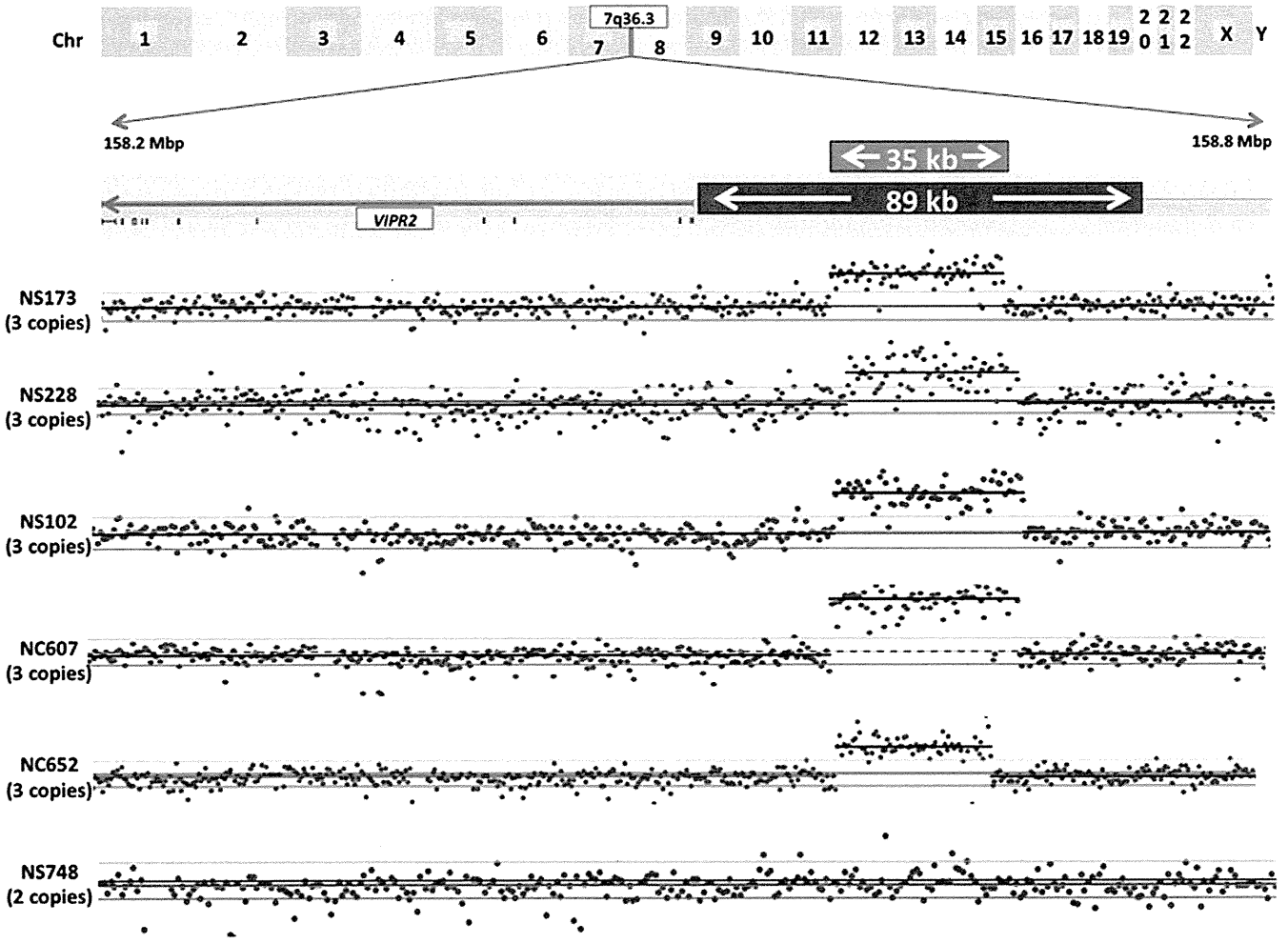

Figure 1 | High-resolution aCGH data. Probe intensity ratios. The orange box (NCBI36/hg18, Chr7: 158,658,128–158,693,128) represents CNVs detected in the present study and the purple box (NCBI36/hg18, Chr7: 158,630,410–158,719,410) represents the region revealed by Vacic et al. to show an association peak in a schizophrenia group. Coordinates are based on the NCBI36 build.

Table 1 | Frequency distribution (confirmation set)

		Number of copies										Aggregated data		
		0		1		2		3		4		>2 copies		P-Value
Set	Sample size	N	%	N	%	N	%	N	%	N	%	N	%	(Fisher exact test)
Cases	531	0	0.0%	0	0.0%	520	97.9%	10	1.9%	1	0.2%	11	2.1%	0.96
Controls	711	0	0.0%	0	0.0%	696	97.9%	13	1.8%	2	0.3%	15	2.1%	

panel of 8 SNPs in the CNV region that were detected in the current study, we did not observe the existence of any common haplotype (Supplementary Table 2).

Discussion

Using samples from only Japanese subjects, we identified a smaller 35-kb (NCBI36/hg18, Chr7: 158,658,128–158,693,128) common (>1%) duplication within the region that Vacic et al.[15] previously showed was associated with schizophrenia (NCBI36/hg18, Chr7: 158,630,410–158,719,410). However, the common duplication that we detected in the present study was not found to be associated with schizophrenia. It is of note that these results may be specific to Japanese subjects, and therefore further studies involving other population groups will need to be undertaken. We have experimentally confirmed that the common CNV detected in the current study was located adjacent to *VIPR2*. Our analysis of the breakpoint junctions at the sequence level showed there was no difference among the CNV carriers. On the basis of the 2-bp microhomology found at this junction (Supplementary Figure 2), we speculate that duplication formation occurs by the FoSTeS (fork stalling and template switching)/MMBIR (microhomology mediated break-induced replication) mechanism previously proposed by Lupski's research group[23]. In addition, we did not find any sequence motif that was characteristic for the breakpoint of recurrent rearrangements at the junction region. Although the junction sequence was exactly the same in all of the subjects in whom the 35-kb CNV was detected, we could not confirm that these subjects shared any common haplotype. Differences in the haplotype among the duplication carriers were likely due to the high recombination rate that occurs at the subtelomeric region[24]. Regarding the origin of the CNV that was identified in the current study, our observation of the same breakpoint junction sequence that was observed in the CNV carriers is highly suggestive of a common ancestral founder.

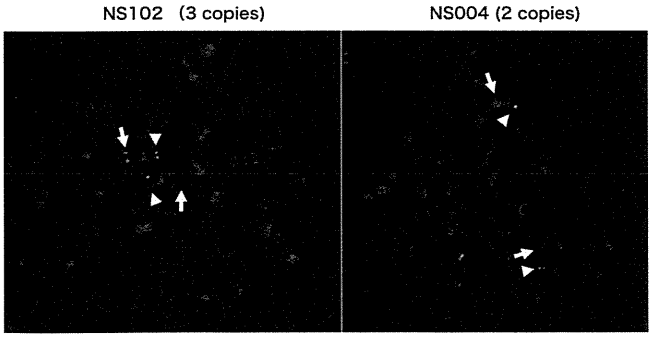

Figure 2 | Tandem duplications of 7q36.3 confirmed in two patients by fluorescence in situ hybridization (FISH). 7p-green (arrowheads) and 7q-red (arrows, CNV specific). Left NS102 (3 copies); Right NS004 (2 copies). Cytogenetic confirmation was obtained for two samples with and without duplication of VIPR2. Probes for duplicated region were produced by long range PCR. The subtelomeric probe, 7p-green (Abbott Molecular), was used as a reference. Hybridizations were performed according to the manufacturer's protocols.

The main limitation of the current study was that we examined a much smaller number of samples as compared to the Vacic et al. study[15]. It is of note that the frequency of the common 35-kb CNV detected in the current study was 2%, and thus with our current sample size of 300 schizophrenic patients, it was large enough to capture the variation. Regarding the individual with 4 copies, we do not have any data indicating whether the individual is a homozygote of duplication or is a carrier of triplication. This point should be considered as another limitation of the current study. The results of the current study do suggest that in case of a duplication event on 7q36.3, the relevant region is not the *VIPR2* promoter (as has been suggested by Vacic et al.[15]), but rather suggest that it is the *VIPR2* gene region. In addition, the 2-bp microhomology in the promoter region of VIPR2 may be associated with the relative meiotic instability of the region harboring the common CNV that is adjacent to the *VIPR2* gene[25]. This in turn may give rise to the larger *VIPR2* duplications that were shown to be associated with schizophrenia with an odds ratio of 4.0[14].

It is interesting that our findings demonstrated that CNV was detected in our study in contrast to the previous reports by both Vacic et al.[15] and Beri et al.[26]. Moreover, CNV has not been listed in the database of genomic variants[27]. Although the CNV detected by our group may be specific to Japanese populations, further studies should be undertaken to ensure comprehensive characterization of the region surrounding the *VIPR2* gene. In addition, to address the question regarding the origin of the CNV detected in the current study, it is necessary to perform family analysis of the carriers and determine whether CNV is a *de novo* event, or if it is transmitted from the parents. In conclusion, the 35-kb region that harbors the common CNV in the Japanese population should be excluded from the region of the association peak in the schizophrenia group reported in the Vacic et al. study[15].

Methods

Three independent samples were used in the current study: (1) *Screening set*, 300 Japanese patients suffering from schizophrenia (53.28 ± 14.66 years); (2) *Confirmation set*, 531 patients suffering from schizophrenia (46.03 ± 12.15 years); and (3) 711 healthy control subjects (47.12 ± 11.03 years). All schizophrenic patients met the current DSM IV-TR criteria[2], which was reflected by consensual diagnosis of two experienced psychiatrists. Prior to inclusion in the control set, subjects were screened on the basis of a brief diagnostic interview. Detailed characterization and psychiatric assessment of the subjects is available elsewhere[19]. All subjects enrolled in the study were Japanese and provided written informed consent prior to the study. Venous blood was drawn from each subject and genomic DNA was extracted according to the standard phenol/chloroform method. Comparative genomic hybridization of DNA was performed using the high-resolution NimbleGen (Roche NimbleGen, Inc., U.S.) CGH array (3 × 720,000 or 12 × 135,000). Labeling and hybridization of patient (test) and sex-matched commercial (Promega Corporation, U.S.) reference DNA was performed according to the manufacturer's protocols. Test and reference DNA were labeled by Cy3- and Cy5-labeled random primers, respectively, and were combined and hybridized to the array for 40–72 h. Arrays were washed in four steps, as indicated in the protocol. Two-color scanning was performed using a NimbleGen MS 200 microarray scanner. Acquisition of the microarray images was performed with NimbleGen MS 200 software. Data extraction, analysis and visualization were done using NimbleScan version 2.4 software. CNV calling was performed using NEXUS software. The FASST2 Segmentation Algorithm, a Hidden Markov Model (HMM) based approach, was used to make copy number calls. The FASST2 algorithm, unlike other common HMM methods for copy number estimation, does not aim to estimate the copy number state at each probe, but uses many states to cover more possibilities, such as mosaic events. These state values are then used to make calls based on a log ratio threshold. The significance threshold for

segmentation was set at 10^{-6} and also required a minimum of three probes per segment. The log ratio thresholds for single copy gain and single copy loss were set at 0.3 and −0.3, respectively. The log ratio thresholds for the gain of two or more copies and homozygous loss were set at 0.9 and −0.9 respectively.

Custom TaqMan copy number assay was specifically designed to interrogate a duplication region (NCBI36/hg18, Chr7: 158,630,410–158,719,410) without interspersed repeats, low complexity or a homologous DNA sequence. A TaqMan copy number assay for RNase P was used as a reference. Experiments were carried out on four technical replicates according to the manufacturer's protocol. CNV typing of the screening sample was performed using Roche NimbleGen, Inc. CGH array 3 × 720,000, while confirmation of the sample was performed using the TaqMan copy number assay. Sixteen randomly selected duplication events (both in the screening and confirmation samples) were validated using custom NimbleGen 12 × 135,000 CGH arrays (Roche NimbleGen, Inc., U.S.) covering the region (NCBI36/hg18, Chr7: 158,630,410–158,719,410) implicated in the Vacic et al. study[15], with an average of one probe per 500 bp. P values derived from association analysis were based on Fisher's exact test.

We performed PCR based analysis to determine the orientation of the detected duplications. We designed forward and reversed primers to align with the region of the duplication junction (F: 5′-TGTGGATTCCCTTCAGAGGCGAC-3′, R: 5′-CATTCTTCAGCCCATGGAGTCATC-3′) (Supplementary Figure 1). Cytogenetic confirmation was obtained for two samples with and without duplication of VIPR2. Probes for duplicated region were produced by long range PCR (NCBI36/hg18, Chr7: 158,658,128–158,693,128). Subtelomeric probe, 7p-green (Abbott Molecular, U.S.), was used as a reference. Hybridizations were performed according to the manufacturer's protocols.

Haplotypes were estimated using the statistical software package PHASE version 3.4.1 (http://www.stat.washington.edu/stephens/)[20–22]. This program is based on a Bayesian statistical method using coalescent-based models that infers phases at loci from unphased genotype data for a sample of unrelated individuals[20]. The algorithm uses a flexible model for the decay of linkage disequilibrium with distance and explicitly incorporates an assumption about the recombination rate variation. PHASE uses Gibbs sampling, a Markov-Chain Monte Carlo algorithm for the estimation of the posterior distribution. Hence, the individual haplotype can be estimated from the posterior distribution by choosing the most likely haplotype reconstruction for each individual. Using the extension for unrelated individuals, we used the default settings to infer the haplotypes from the genotype data[19] of the 8 SNPs (Supplementary Table 1) surrounding the duplication in sample that comprised 517 subjects (7 with a structural variant detected in the current study). Estimates of the sample haplotype frequencies together with their standard deviation, a list of the most likely pairs of haplotypes for each individual together with their probability, and the estimates of recombination parameters in the region, were calculated using the same software.

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Author contributions

B.A., I.K., T.O. and N.O. designed the study and wrote the protocol. B.A., I.K., T.O., N.I., H.K. and N.O. performed the literature review. B.A., I.K. and T.O. made and managed the sample database. B.A., I.K., T.O., M.I., S.K., Y.N., A.Y., T.K., S.I., H.K., N.I. and N.O. collected and managed the genome samples. B.A., I.K., T.O., M.I., S.K., Y.N., A.Y., T.K. and S.I. conducted the statistical analysis. B.A., I.K., T.O., M.I., S.K., Y.N., A.Y., T.K., S.I., H.K., N.I. and N.O. interpreted and discussed the results. B.A., I.K., T.O., M.I., H.K., N.I. and N.O. wrote the manuscript and edited the final manuscript.

Additional information

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

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

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

Introduction

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

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

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

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

Methods

Sample

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

Voxel-Based Morphometry

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

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

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]

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.

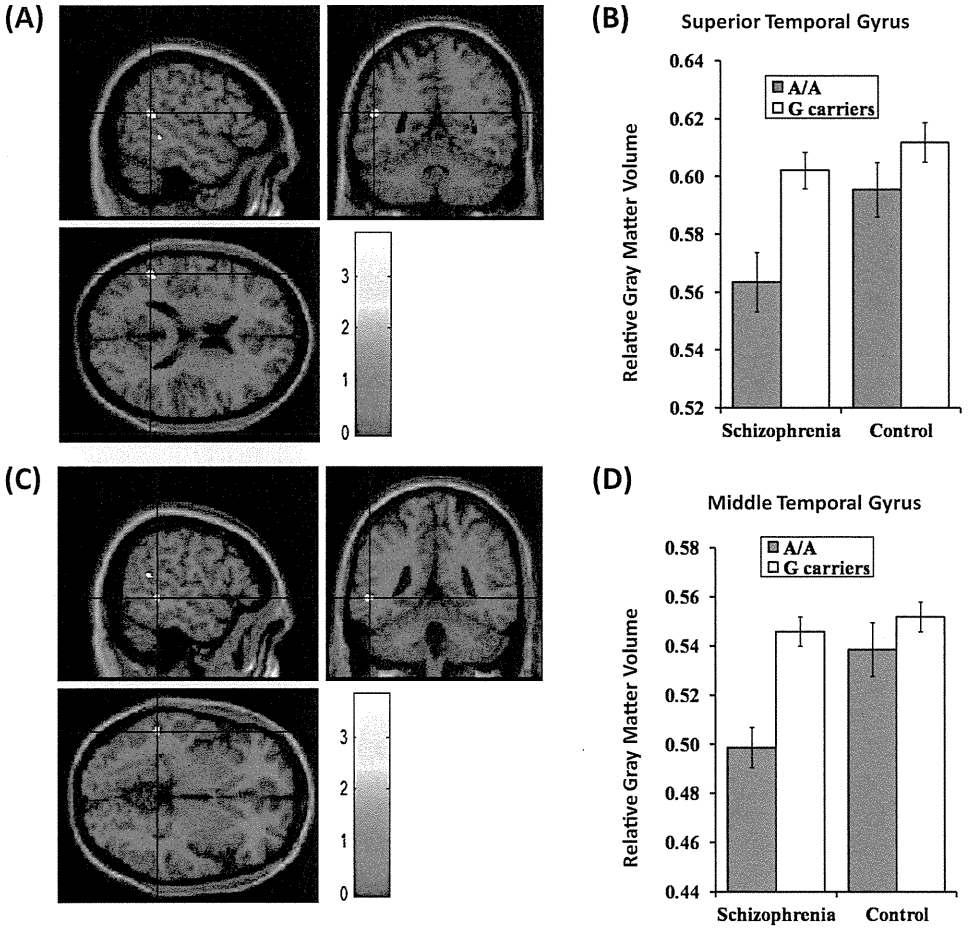

Fig. 1. Voxel-based morphometry. Impacts of VAV3 genotype on gray matter (GM) volumes of left superior temporal gyrus and left middle temporal gyrus. (A and C) Anatomical localizations were displayed on coronal, sagittal, and axial sections of a normal magnetic resonance imaging spatially normalized 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

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.

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

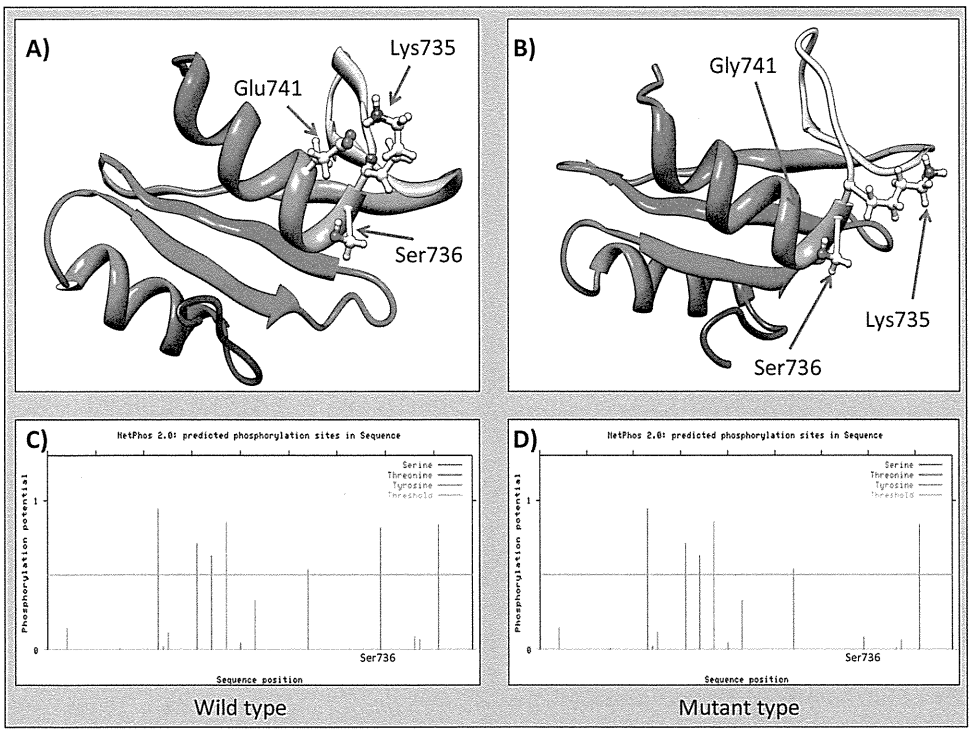

Fig. 2. Ab-initio 3D modeling of SH2 domain (VAV3). (a) Wild type, note hydrogen bond between Glu741 and Lys735 (blue line); (b) Mutant type, hydrogen bond between Gly741 and Lys735 cannot be formed; (c) and (d) posphorylation potential of serine (blue), threonine (green), and tyrosine (red). Threshold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

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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