

Failure to confirm an association between *Epsin 4* and schizophrenia in a Japanese population

Misty Richards · Yoshimi Iijima · Tomoko Shizuno · Yoko Kamegaya · Hiroaki Hori · Mayu Omori · Kunimasa Arima · Osamu Saitoh · Hiroshi Kunugi

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Abstract Previous studies suggested that genetic variations in the 5' region of *Epsin 4*, a gene encoding enthoprotin on chromosome 5q33, are associated with schizophrenia. However, conflicting results have also been reported. We examined the possible association in a Japanese sample of 354 patients and 365 controls. Seventeen polymorphisms of *Epsin 4* [3 microsatellites and 14 single nucleotide polymorphisms (SNPs)] were selected. A microsatellite marker (D5S1403) demonstrated a significant difference in the allele frequency between patients and controls (uncorrected $P = 0.04$). However, there was no significant difference in the genotype or allele frequency between the two groups for the other microsatellites or SNPs. Haplotype-based analysis provided no evidence for an association. The positive result at D5S1403 no longer reached statistical significance when multiple testing was taken into consideration. Our results suggest that the examined region of *Epsin 4* does not have a major influence on susceptibility to schizophrenia in Japanese.

Keywords Association study · *Epsin 4* · Enthoprotin · Schizophrenia · Polymorphism

M. Richards · Y. Iijima · T. Shizuno · Y. Kamegaya · H. Hori · H. Kunugi (✉)
Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
e-mail: hkunugi@ncnp.go.jp

M. Richards
Albany Medical College, Albany, NY 12208, USA

M. Omori · K. Arima · O. Saitoh
Department of Psychiatry, National Center of Neurology and Psychiatry Hospital, Tokyo 187-8502, Japan

Introduction

Schizophrenia is a debilitating psychiatric disorder that affects approximately 1% of the world's population (Fenton et al. 2003; Hyman 2000). Patients may suffer from delusions, hallucinations, disorganized speech and behavior, as well as impairment in short-term verbal and non-verbal memory. The complete etiology of the disease remains unknown, though twin and adoption studies have demonstrated that schizophrenia is highly heritable (estimated heritability of >80%) (Cardno et al. 1999). While the contributing genes and pathophysiological mechanisms remain elusive, identifying the susceptibility genes is essential in discovering the true pathogenesis of schizophrenia. The mode of schizophrenia transmission is complex and is thought to be polygenic (Owen et al. 2004). Thus far, linkage and association studies have been successful approaches in searching for complex disease genes, discovering such candidate genes as neuregulin 1, dysbindin, G72, and D-amino acid oxidase (DAAO). Through such analysis, several chromosomal regions have been identified and investigated as potential sources for schizophrenia susceptibility genes (Chumakov et al. 2002; Stefansson et al. 2002; Straub et al. 2002a). As a result, the long arm of chromosome 5q has been identified as a putative chromosomal region of interest and subsequently investigated for susceptibility loci (Lewis et al. 2003; Straub et al. 2002b).

An association study of English, Irish, Welsh, and Scottish populations found *Epsin 4* on chromosome 5q33 as a strong candidate for schizophrenia susceptibility (Pimm et al. 2005). Four associated polymorphisms were discovered at the 5' end of *Epsin 4*, a gene encoding the clathrin-associated protein enthoprotin. These included two microsatellite markers, D5S1403 and AAAT11, and

two single-nucleotide-polymorphism (SNP) markers, rs10046055 and rs254664. Entropin plays a critical role in the formation, transport and stability of clathrin-coated vesicles (CCVs) and is therefore thought to regulate the transport and storage of neurotransmitters in the brain (McPherson and Ritter 2005; Wasiaik et al. 2002). Neuronal CCVs are critical in the insertion and recycling of neurotransmitter receptors at the postsynaptic membrane and have been implicated as a regulatory mechanism for synaptic plasticity (Blanpied et al. 2002; Wang and Linden 2000). Specifically, CCVs facilitate AMPA receptor trafficking on the postsynaptic membrane, therefore affecting overall glutamatergic neurotransmission (Malinow and Malenka 2002; Man et al. 2002). Dysfunction of entropin could, therefore, stimulate disturbances in glutamatergic brain signaling as well as synaptic plasticity, both postulated to be integral components of schizophrenia pathophysiology (Christison et al. 1989; Carter et al. 2006). Moreover, it has been demonstrated that entropin interacts with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex found in hippocampal pyramidal cells (Antonin et al. 2000; Chidambaram et al. 2004). This suggests a possible role for entropin in calcium-mediated vesicle fusion of excitatory neurotransmitters released from the hippocampus. If the SNARE complex does not function properly in this way, abnormal neural connectivity may result, which is another characteristic of schizophrenia (Honer et al. 2002).

Thus far, two replication studies have been conducted in the Han Chinese population (Liou et al. 2006; Tang et al. 2006). Both studies found no association between the four markers previously reported (D5S1403, AAAT11, rs10046055, or rs254664) and schizophrenia. Tang et al. (2006), however, did detect haplotypes near the 5' end of *Epsin 4* (252T consisted of AAAT11 and rs10046055, global $P = 0.0021$; T/T of rs1145603 and rs254664, $P = 0.0033$) showing an association with schizophrenia in Han Chinese family trios. Liou et al. (2006) analyzed nine SNPs on *Epsin 4* in a case-control design and found a significant difference in the allele frequency of a SNP on the 5' upstream region of *Epsin 4* (rs1186922); however, this difference was not significant after multiple testing. These conflicting results prompted us to examine *Epsin 4* for an association with schizophrenia in a Japanese sample.

Materials and methods

Subjects

Subjects were 354 patients with schizophrenia [212 males, mean age of 44.0 years (SD 13.7)] and 365 healthy controls

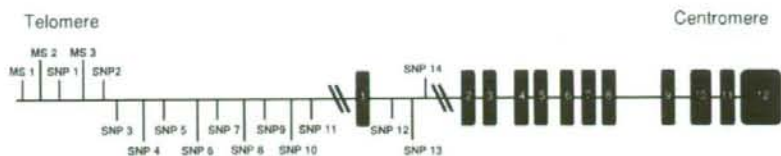
[113 males, mean age of 39.7 years (SD 14.1)]. All subjects were biologically unrelated Japanese and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association 1994) on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers recruited from hospital staffs and their associates. Control individuals were interviewed and those with a current or past history of psychiatric treatment or regular use of psychotropic agents were not enrolled in the study. Participants were excluded from both the patient and control groups if they had prior medical histories of central nervous system disease or severe head injury, or if they met criteria for alcohol/drug dependence or mental retardation. The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject.

Genotyping

Venous blood was drawn from the subjects and Genomic DNA was extracted from peripheral leukocytes using the Wizard Genomic DNA Purification System kit (Promega, Madison, WI, USA) based on the solution-based method according to the manufacturer's instructions. Fourteen SNPs (rs1186922, rs10046055, rs1894962, rs6556290, rs7735412, rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032, rs254664) and three microsatellites (D5S1400, D5S1403, AAAT11) were genotyped. Five SNPs (rs1186922, rs10046055, rs1186930, rs1145603, rs254664) were selected based on previous studies conducted by Liou et al. (2006), Tang et al. (2006), and Pimm et al. (2005). The three microsatellites demonstrated to be significantly associated with schizophrenia by Pimm et al. (2005) and Tang et al. (2006) were selected in order to replicate their positive findings. The other nine SNPs (rs1894962, rs6556290, rs7735412, rs1145585, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032) were selected from the international HapMap project (<http://hapmap.org/index.html.en>) using Paul de Bakker's Tagger algorithm in the Haploview V 3.32 program. The 17 studied polymorphisms cover approximately 193 kb of *Epsin 4* from the 5' upstream region to intron 1 (Fig. 1).

The SNPs were genotyped using the TaqMan 5'-exonuclease allelic discrimination assay. TaqMan probes of the assay on demand (rs1186922, rs10046055, rs6556290, rs7735412, rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032, rs254664)

Fig. 1 The genetic structure of *Epsin 4* and location of studied markers. Upper six markers were studied in the previous reports. Lower 11 markers were additionally selected for Tag SNPs in this area



and assay by design (rs1894962) with Universal PCR Master Mix were obtained from Applied Biosystems (Foster City, CA, USA). Thermal cycling conditions for polymerase chain reaction (PCR) were one cycle at 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. After amplification, the allelic specific fluorescence was measured on ABI PRISM 7900 Sequence Detector Systems (Applied Biosystems, Foster City, CA, USA). Genotype data were read blind to the case-control status. Ambiguous genotype data were not included in the analysis.

Primer sequences for PCR amplification of the microsatellite markers D5S1400 and D5S1403 were obtained from the UniSTS database in NCBI (<http://www.ncbi.nlm.nih.gov/>), while the primer sequence of microsatellite marker AAAT11 was obtained from Pimm et al. (2005). PCR amplification of microsatellite markers was performed using primers fluorescently labeled with Beckman dyes. PCR products were subject to electrophoresis on Beckman CEQ 8000 (Beckman Coulter, Fullerton, CA, USA). As the PCR fragments of microsatellites were not the same length as those in the original work, we adjusted the sizes of our PCR fragments to Pimm et al. (2005).

Statistical analysis

Deviations of genotype distributions from Hardy-Weinberg equilibrium were assessed with the χ^2 test for goodness of fit. Genotype and allele distributions of each SNP were compared between patients and controls by using the χ^2 test for independence. These tests were performed with the SPSS software ver. 11 (SPSS Japan, Tokyo, Japan). The allelic association of microsatellite markers with schizophrenia was examined by use of the CLUMP program (Sham and Curtis 1995), which assesses the significance of departure between the observed and expected values in a $2 \times N$ contingency table using a Monte Carlo approach. The standardized measure of linkage disequilibrium (LD), (D') and r^2 , were estimated using the online software SHEsis (<http://202.120.7.14/analysis/myAnalysis.php>). Haplotype-based association analyses were examined with the COCAPHASE software ver. 2.4 (<http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/>) (Dudbridge et al. 2000). The expectation-maximization (EM) and "droprare" options were

used. Haplotypes with frequencies less than 3% were considered to be rare.

Results

All investigated SNPs and microsatellite markers for case and control groups resulted in distributions that did not significantly deviate from Hardy-Weinberg Equilibrium. LD estimates of pairwise markers, expressed in D' and r^2 , are presented in Table 1. Pairs in LD are represented as gray-shaded values, with estimates of $D' > 0.8$ and $r^2 > 0.8$. Pairwise LD analysis and haplotype block determination demonstrated that there were two blocks across the studied genomic region (Fig. 2). Measurement of pairwise LD showed that the middle seven SNPs (rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, and rs1186998) were in strong LD with each other and were located in the sample block (haplotype block 2). The remaining block, haplotype block 1, consisted of three SNPs (rs1186922, rs10046055, and rs1894962) in the 5' upstream region. In the haplotype-based analysis, the haplotypes in the two blocks were analyzed separately.

In the single-marker analysis, the microsatellite 2 marker (D5S1403) demonstrated a significant difference in allele frequency between case and control groups (Table 2: Allele 203, $\chi^2 = 4.26$, $df = 1$, $P = 0.04$ from the "T3" analysis of CLUMP). The remaining microsatellite markers and SNPs did not show any significant allelic association with schizophrenia (Table 2, all $P > 0.05$). The obtained evidence for an association at microsatellite 2 with schizophrenia was weak and non-significant when multiple testing was taken into consideration.

Results of the haplotype-based analysis are also presented in Table 2. None of the haplotypes showed a statistically significant difference between case and control groups. Even when haplotypes were examined according to the groupings established by Pimm et al. (2005), no evidence for an association was obtained. Tang et al. (2006) reported a highly significant transmission of haplotypes consisting of AAAT11 and rs10046055 ($P = 0.0048$) in addition to rs254664 and rs1145603 ($P = 0.0047$) to affected offspring in Han Chinese trios. Our results, again, failed to provide evidence to support this finding.

Table 1 Pair-wise linkage disequilibrium in *Epsin 4*

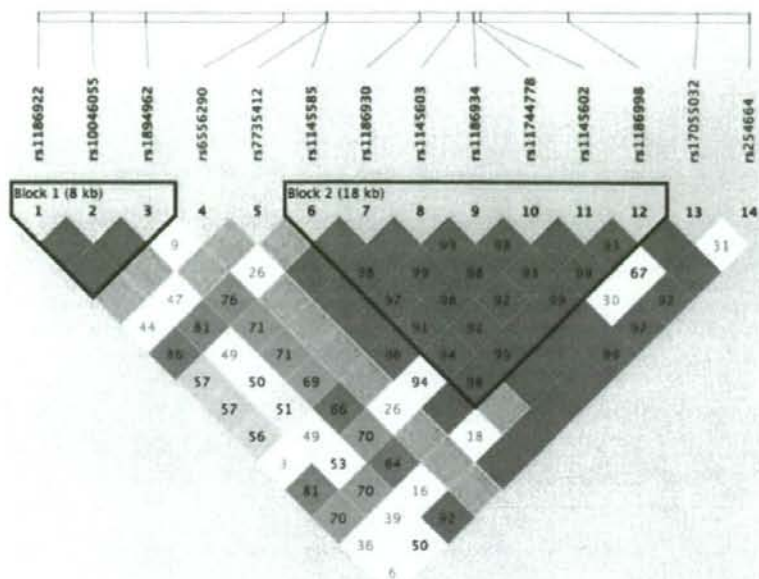
| | MS1 DSS1400 | MS2 DSS1403 | MS3 AAAT11 | SNP1 rs1186922 | SNP2 rs10046055 | SNP3 rs1894962 | SNP4 rs6556290 | SNP5 rs7735412 | SNP6 rs1145585 | SNP7 rs1186930 | SNP8 rs145603 | SNP9 rs186934 | SNP10 rs11744778 | SNP11 rs1145602 | SNP12 rs1186998 | SNP13 rs17055032 | SNP14 rs254664 |
|-------|----------------|----------------|---------------|-------------------|--------------------|-------------------|-------------------|-------------------|-------------------|-------------------|------------------|------------------|---------------------|--------------------|--------------------|---------------------|-------------------|
| MS1 | | | | | | | | | | | | | | | | | |
| MS2 | 0.001 | | | | | | | | | | | | | | | | |
| SNP1 | 0.005 | 0.051 | | | | | | | | | | | | | | | |
| MS3 | 0.011 | 0.014 | | | | | | | | | | | | | | | |
| SNP2 | 0.008 | 0.013 | 0.063 | 0.775 | | | | | | | | | | | | | |
| SNP3 | 0.007 | 0.004 | 0.425 | 0.008 | 0.001 | | | | | | | | | | | | |
| SNP4 | 0.002 | 0.004 | 0.002 | 0.013 | 0.002 | 0.003 | | | | | | | | | | | |
| SNP5 | 0.000 | 0.002 | 0.415 | 0.015 | 0.035 | 0.455 | 0.001 | | | | | | | | | | |
| SNP6 | 0.000 | 0.041 | 0.251 | 0.065 | 0.092 | 0.165 | 0.006 | 0.416 | | | | | | | | | |
| SNP7 | 0.000 | 0.043 | 0.067 | 0.068 | 0.101 | 0.159 | 0.006 | 0.046 | 0.406 | | | | | | | | |
| SNP8 | 0.000 | 0.042 | 0.243 | 0.068 | 0.101 | 0.159 | 0.006 | 0.044 | 0.399 | 0.560 | | | | | | | |
| SNP9 | 0.009 | 0.035 | 0.000 | 0.185 | 0.249 | 0.020 | 0.000 | 0.110 | 0.029 | 0.372 | 0.375 | | | | | | |
| SNP10 | 0.000 | 0.003 | 0.368 | 0.014 | 0.012 | 0.395 | 0.001 | 0.003 | 0.883 | 0.363 | 0.365 | 0.374 | | | | | |
| SNP11 | 0.003 | 0.050 | 0.295 | 0.111 | 0.150 | 0.179 | 0.005 | 0.036 | 0.309 | 0.759 | 0.766 | 0.755 | 0.292 | | | | |
| SNP12 | 0.003 | 0.000 | 0.022 | 0.034 | 0.057 | 0.008 | 0.000 | 0.001 | 0.012 | 0.030 | 0.029 | 0.029 | 0.003 | 0.284 | | | |
| SNP13 | 0.003 | 0.000 | 0.034 | 0.034 | 0.057 | 0.008 | 0.000 | 0.001 | 0.012 | 0.030 | 0.029 | 0.029 | 0.003 | 0.284 | 0.105 | | |
| SNP14 | 0.010 | 0.034 | 0.000 | 0.184 | 0.262 | 0.023 | 0.000 | 0.116 | 0.035 | 0.384 | 0.386 | 0.381 | 0.957 | 0.029 | 0.297 | 0.003 | |

Upper diagonal figures are D' and lower diagonal figures are r^2

Pairwise LD measurement using the online software SHEsis

Pairs in LD ($D' > 0.8$ or $r^2 > 0.8$) are shown in italics

Fig. 2 Linkage disequilibrium (LD) block structure estimated from 14 SNPs by using Haploview



Discussion

The purpose of this investigation was to replicate Pimm et al.'s (2005) study of *Epsin 4* and its association with schizophrenia in a Japanese population. Pimm et al. (2005) originally reported that two SNPs, rs254664 (intron 1) and rs10046055 (5' upstream region), in addition to two microsatellite markers D5S1403 (5' flanking region) and AAAT11 (5' upstream region), may be involved in the susceptibility to schizophrenia in the English, Irish, Welsh, and Scottish populations. In the present study, we examined a total of 17 polymorphisms and obtained weak evidence for an association at microsatellite 2 (D5S1403). However, when multiple testing was taken into consideration, the finding no longer reached significance. In addition, the risk-conferring allele was inconsistent between the two studies; we found an enrichment of the 203rd allele of microsatellite 2 in schizophrenics, whereas Pimm et al. (2005) found an enrichment of the same allele in controls. Furthermore, in the haplotype-based analysis, we did not find any evidence for an association.

Thus, we failed to replicate the widespread and highly significant associations with schizophrenia across 200,000 bp of *Epsin 4* reported by Pimm et al. (2005). Though we examined this region thoroughly by using 17 markers, we obtained no evidence for an association. The discrepancy found between Pimm et al.'s (2005) study and ours could result from a combination of factors. The first

possibility may be due to the putative difference in allele frequencies between the English, Irish, Welsh, and Scottish population versus the Japanese population. It has been suggested that variations between ethnicities and populations in allele frequencies can dramatically affect the power to detect marginal differences (Marchini et al. 2005). According to the data bank of the International HapMap project (<http://www.hapmap.org/index.html.en/>), the allele frequency variation for rs10046055 and rs254664 is noticeable. Therefore, ethnic diversity of allele frequency of the SNPs may be a reason for negative findings in our present study. It is possible that there is a potential risk locus that is in strong LD with the investigated genetic variants in Pimm et al.'s (2005) population that is in weak or no LD with the variants in our population. However, to address this issue, we analyzed ten additional SNPs to thoroughly cover the entire genomic region of interest according to the HapMap database for the Japanese population; therefore, such a possibility is unlikely. Another possibility is that our negative results may be a type II error due to the potentially inadequate sample size. Our sample size (354 patients and 365 controls) had a power of 90% to detect an odds ratio of 1.9 or more if the T/A haplotype of HapB was assumed to be a risk (see Table 3). Incidentally, this haplotype gave rise to the most significant result ($P = 0.0005$) in the study of Pimm et al. (2005). This relatively weak power is due to the low frequency (0.06) of the T/A haplotype in our Japanese controls. If the A allele

Table 2 Allelic and haplotypic association analyses between *Epsin 4* and schizophrenia

| dbSNP ID | Position ^a | Inter-SNP distance (bp) | Allele frequency | | | χ^2 | P^c | P^d | Haplotype P (global) ^e | | | | |
|-------------|-----------------------|-------------------------|---------------------|------------|---------------|------------|-------|-------|-------------------------------------|---------|---------|---------|--|
| | | | Allele ^b | Control | Schizophrenia | | | | 2 Locus | 3 Locus | 4 Locus | 5 Locus | |
| MS1 D5S1400 | 157439237 | - | A | 368 | 104 (0.16) | 115 (0.18) | 1.03 | 0.31 | | | | | |
| | | | T | 371 | 304 (0.47) | 292 (0.46) | 0.07 | 0.80 | | | | | |
| | | | C | 377 | 124 (0.19) | 101 (0.16) | 2.20 | 0.14 | | | | | |
| | | | G | 380 | 55 (0.08) | 70 (0.11) | 2.43 | 0.12 | (T3) | | | | |
| | | | A | 383 | 45 (0.07) | 45 (0.07) | 0.01 | 0.90 | 0.12 | | | | |
| MS2 D5S1403 | 157346617 | 92,620 | A | 203 | 417 (0.66) | 444 (0.71) | 4.26 | 0.04 | | | | | |
| | | | T | 207 | 114 (0.18) | 99 (0.16) | 1.00 | 0.32 | | | | | |
| | | | C | 211 | 18 (0.03) | 15 (0.02) | 0.23 | 0.63 | (T3) | | | | |
| | | | G | 215 | 67 (0.11) | 56 (0.09) | 0.91 | 0.34 | 0.04 | 0.64 | | | |
| SNP1 | 157302083 | 44,534 | A | 179 (0.25) | 189 (0.27) | | | | | | | | |
| rs1186922 | | | T | 547 (0.75) | 511 (0.73) | 1.02 | 0.31 | | 0.23 | 0.43 | | | |
| MS3 AAAT11 | 157301697 | 386 | A | 248 | 567 (0.84) | 562 (0.87) | 1.48 | 0.22 | (T1) | | | | |
| | | | T | 252 | 100 (0.15) | 86 (0.13) | 0.71 | 0.40 | 0.13 | 0.47 | 0.58 | 0.34 | |
| SNP2 | 157297912 | 3,785 | A | 120 (0.17) | 99 (0.14) | | | | | | | | |
| rs10046055 | | | T | 606 (0.83) | 607 (0.86) | 1.74 | 0.19 | | 0.23 | 0.43 | 0.77 | 0.31 | |
| SNP3 | UTR | 4,191 | C | 86 (0.12) | 99 (0.14) | | | | | | | | |
| rs1894962 | 157293721 | | T | 642 (0.88) | 605 (0.86) | 1.61 | 0.21 | | 0.23 | 0.26 | 0.47 | 0.77 | |
| SNP4 | UTR | 10,737 | C | 2 (0.00) | 2 (0.00) | | | | | | | | |
| rs6556290 | 157282984 | | T | 726 (1.00) | 706 (1.00) | 0.00 | 0.98 | | 0.22 | 0.23 | 0.27 | 0.47 | |
| SNP5 | UTR | 3,395 | C | 14 (0.02) | 16 (0.02) | | | | | | | | |
| rs7735412 | 157279589 | | T | 712 (0.98) | 690 (0.98) | 0.20 | 0.66 | | 1.00 | 0.21 | 0.23 | 0.26 | |
| SNP6 | UTR | 42 | C | 608 (0.84) | 589 (0.83) | | | | | | | | |
| rs1145585 | 157279547 | | T | 116 (0.16) | 117 (0.17) | 0.08 | 0.78 | | 0.76 | 0.84 | 0.43 | 0.38 | |
| SNP7 | UTR | 7,239 | T | 494 (0.68) | 482 (0.68) | | | | | | | | |
| rs1186930 | 157272308 | | C | 232 (0.32) | 222 (0.32) | 0.03 | 0.86 | | 0.78 | 0.67 | 0.67 | 0.52 | |
| SNP8 | UTR | 2,984 | C | 496 (0.68) | 484 (0.69) | | | | | | | | |
| rs1145603 | 157269324 | | T | 230 (0.32) | 222 (0.31) | 0.01 | 0.92 | | 0.92 | 0.74 | 0.64 | 0.63 | |
| SNP9 | UTR | 1,215 | A | 229 (0.32) | 217 (0.31) | | | | | | | | |
| rs1186934 | 157268109 | | G | 491 (0.68) | 479 (0.69) | 0.06 | 0.80 | | 0.89 | 0.89 | 0.68 | 0.57 | |
| SNP10 | UTR | 42 | A | 114 (0.16) | 102 (0.14) | | | | | | | | |
| rs11744778 | 157268067 | | G | 608 (0.84) | 604 (0.86) | 0.50 | 0.48 | | 0.78 | 0.75 | 0.75 | 0.72 | |
| SNP11 | UTR | 517 | A | 117 (0.16) | 115 (0.16) | | | | | | | | |
| rs1145602 | 157267550 | | G | 609 (0.84) | 587 (0.84) | 0.02 | 0.89 | | 0.78 | 0.58 | 0.56 | 0.56 | |
| SNP12 | Intron 1 | 6,895 | C | 279 (0.38) | 258 (0.37) | | | | | | | | |
| rs1186998 | 157260655 | | G | 447 (0.62) | 448 (0.63) | 0.54 | 0.46 | | 0.23 | 0.30 | 0.42 | 0.54 | |
| SNP13 | Intron 1 | 10,039 | A | 49 (0.07) | 37 (0.05) | | | | | | | | |
| rs17055032 | 157250616 | | C | 679 (0.93) | 671 (0.95) | 1.44 | 0.23 | | 0.39 | 0.36 | 0.54 | 0.51 | |
| SNP14 | Intron 1 | 4,036 | C | 611 (0.84) | 603 (0.85) | | | | | | | | |
| rs254664 | 157246580 | | T | 113 (0.16) | 103 (0.15) | 0.29 | 0.59 | | 0.47 | 0.51 | 0.48 | 0.51 | |

^a Chromosome position was obtained from the HapMap database^b Microsatellites alleles presented by PCR fragment size (bp) with frequencies >0.03 in controls in either study^c P from $2 \times 2 \chi^2$, with one df ^d P from CLUMP Monte Carlo^e P from COCAPHASE-EM, -drop rare 0.03 options

Table 3 Replication study of haplotype-based analysis between *Epsin 4* and schizophrenia

| Group | Markers and haplotype | | | Case freq. | Cont freq. | P value ^a | |
|-------|-----------------------|------------|--------|------------|------------|----------------------|--------|
| | | | | | | Individual | Global |
| HapA | rs254664 | rs10046055 | | | | | |
| | C | A | | 0.05 | 0.08 | 0.098 | |
| | T | A | | 0.09 | 0.09 | 0.743 | |
| | C | T | | 0.80 | 0.77 | 0.151 | |
| | T | T | | 0.06 | 0.07 | 0.664 | 0.341 |
| HapB | rs1186930 | rs10046055 | | | | | |
| | C | A | | 0.10 | 0.10 | 0.563 | |
| | T | A | | 0.04 | 0.06 | 0.133 | |
| | C | T | | 0.22 | 0.22 | 0.835 | |
| | T | T | | 0.64 | 0.62 | 0.418 | 0.471 |
| HapC | rs10046055 | AAAT11 | | | | | |
| | T | 248 | | 0.85 | 0.82 | 0.189 | |
| | A | 252 | | 0.12 | 0.14 | 0.249 | 0.234 |
| HapD | rs254664 | rs10046055 | AAAT11 | | | | |
| | G | T | 248 | 0.80 | 0.76 | 0.10 | |
| | G | T | 248 | 0.06 | 0.07 | 0.42 | |
| | T | A | 252 | 0.05 | 0.06 | 0.25 | |
| | T | A | 252 | 0.07 | 0.08 | 0.58 | 0.429 |
| HapE | rs1145603 | rs10046055 | AAAT11 | | | | |
| | C | T | 248 | 0.64 | 0.61 | 0.292 | |
| | T | T | 248 | 0.21 | 0.22 | 0.969 | |
| | C | A | 252 | 0.04 | 0.06 | 0.131 | |
| | T | A | 252 | 0.08 | 0.08 | 0.698 | 0.440 |
| HapF | rs1145603 | rs254664 | | | | | |
| | C | C | | 0.69 | 0.68 | 0.940 | |
| | T | C | | 0.17 | 0.16 | 0.671 | |
| | T | T | | 0.15 | 0.16 | 0.591 | 0.819 |

^a P from COCAPHASE-EM, -drop rare 0.03 options

of rs10046055, which gave rise to the second significant result ($P = 0.002$) by Pimm et al. (2005), was assumed to be a risk, our sample size had a power of 90% to detect an odds ratio of 1.6 or more. Thus our sample size may have been limited by the inadequacy to detect a potentially weak effect of *Epsin 4* in Japanese. It is also possible that there may be a differential effect of *Epsin 4* on the development of schizophrenia between ethnic groups due to unknown factors. Alternatively, the positive association between schizophrenia and *Epsin 4* reported by Pimm et al. (2005) may have arisen by chance.

Subsequent to Pimm et al.'s (2005) investigation, two replication studies have been reported. Tang et al. (2006) reported a significant association of schizophrenia with some haplotypes of *Epsin 4* (252/T composed of AAAT11 and rs10046055, global $P = 0.0021$; T/T of rs1145603 and rs254664, $P = 0.0033$) in Han Chinese family trios, providing evidence for an association between schizophrenia and *Epsin 4*. The other replication study of Liou et al. (2006) found some evidence for an association, discovering

a significant difference in allele frequency at rs1186922 ($P = 0.038$) and a significant difference in the frequency of a haplotype composed of two markers (rs1186922 and rs10046055). Liou et al. (2006) conservatively concluded that these findings occurred by multiple testing and chance and that their data did not support an association between schizophrenia and *Epsin 4*. However, Gurling et al. (2007) argued that the data of Liou et al. (2006) should be interpreted as supportive evidence for association. Since these two studies and ours were performed in Asian populations, if the association really exists, the obtained results should be similar. However, we obtained no evidence supporting the positive findings of Tang et al. (2006) or Liou et al. (2006).

In conclusion, we failed to identify a significant association between genetic variations of *Epsin 4* and schizophrenia in a Japanese population, suggesting that the examined region (5' side) of *Epsin 4* does not have a major influence on susceptibility to schizophrenia in Japanese. Given the inconsistent findings across studies, more studies

should be conducted within different ethnic populations to draw more concrete conclusions. A meta-analysis of such studies and a multicenter study with a very large sample size will eventually be required. Since we examined only the 5' side of the gene, the possibility remains that other unknown polymorphisms linked to the 3' region of the *Epsin 4* gene may be associated with schizophrenia.

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Microarray comparative genomic hybridization analysis of 59 patients with schizophrenia

Takeshi Mizuguchi · Ryota Hashimoto · Masanari Itokawa · Akira Sano · Osamu Shimokawa · Yukiko Yoshimura · Naoki Harada · Noriko Miyake · Akira Nishimura · Hiroto Saito · Nadiya Sosonkina · Norio Niikawa · Hiroshi Kunugi · Naomichi Matsumoto

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Abstract Schizophrenia is a common psychiatric disorder with a strong genetic contribution. Disease-associated chromosomal abnormalities in this condition may provide important clues, such as *DISC1*. In this study, 59 schizophrenia patients were analyzed by microarray comparative genomic hybridization (CGH) using custom bacterial artificial chromosome (BAC) microarray (4,219 BACs with 0.7-Mb resolution). Chromosomal abnormalities were found in six patients (10%): 46,XY,der(13)t(12;13)(p12.1;p11).ish del(5)(p11p12); 46,XY, ish del(17)(p12p12); 46,XX.ish dup(11)(p13p13); and 46,X,idi(Y)(q11.2); and in two cases, mos 45,X/46XX. Autosomal abnormalities in three cases are likely to be pathogenic, and sex chromosome abnormalities in three follow previous findings. It is noteworthy that 10% of patients with schizophrenia have (sub)microscopic chromosomal abnormalities, indicating

that genome-wide copy number survey should be considered in genetic studies of schizophrenia.

Keywords Schizophrenia · Chromosomal abnormality · Array comparative genomic hybridization · Copy number variation

Introduction

Schizophrenia is a common psychiatric disorder involving approximately 1% of the population worldwide. Family, twin, and adoption studies suggest genetic factors contribute to this illness (Lang et al. 2007; McGuffin et al. 1995). Meta-analysis including 18 genome scans revealed strong evidence at chromosomal regions 22q, 8p, and 13q

T. Mizuguchi · Y. Yoshimura · A. Nishimura · H. Saito · N. Matsumoto (✉)
Department of Human Genetics, Yokohama City University Graduate School of Medicine, Fukuura 3-9, Kanazawa-ku, Yokohama 236-0004, Japan
e-mail: naomat@yokohama-cu.ac.jp

R. Hashimoto · H. Kunugi
Department of Mental Disorder Research,
National Center of Neurology and Psychiatry,
Kodaira, Japan

R. Hashimoto
Department of Psychiatry,
Osaka University Graduate School of Medicine,
Suita, Japan

M. Itokawa
Schizophrenia Research Team,
Schizophrenia Project,
Tokyo Institute of Psychiatry,
Tokyo, Japan

A. Sano
Department of Psychiatry,
Kagoshima University Graduate School of Medical
and Dental Sciences, Kagoshima, Japan

O. Shimokawa · N. Harada
Department of Molecular Cytogenetics,
Kyushu Medical Science, Inc., Nagasaki, Japan

N. Miyake
Department of Medicine (Genetics),
Children's Hospital Boston, Boston, MA, USA

N. Miyake
Department of Neurology, Harvard Medical School,
Boston, MA, USA

N. Sosonkina · N. Niikawa
The Research Institute of Personalized Health Sciences,
Health Sciences University of Hokkaido,
Tobetsu-cho, Ishikari-gun, Japan

as the susceptibility loci (Badner and Gershon 2002), and another meta-analysis of 20 genome-wide scans suggested regions of chromosomes 2q, 5q, 3p, 11q, 6p, 1q, 22q, 8p, 20q, and 14p as the significant loci (Lewis et al. 2003). Chromosomal abnormalities in patients with schizophrenia may provide useful information regarding the susceptible loci (Bassett et al. 2000). Disrupted in schizophrenia 1 (*DISC1*) gene isolated from a large Scottish family with t(1;11)(q42.1;q14.3) and high risk of schizophrenia in velo-cardio-facial syndrome (VCFS) with a 22q11 deletion are good examples (Arinami 2006; Millar et al. 2000; Murphy 2002). Some linkage and association studies support that schizophrenia could be associated with *DISC1* and genes at 22q11 (Chubb et al. 2008; Liu et al. 2002; O'Donovan et al. 2003; Shifman et al. 2002).

Microarray technologies have now become practical tools for detection of submicroscopic copy number changes. Using custom bacterial artificial chromosome (BAC) microarray (4,219 BACs at 0.7-Mb resolution), we analyzed 59 patients with schizophrenia. Chromosomal abnormalities found in this study are presented.

Materials and methods

Subjects

A total of 59 subjects (31 men and 28 women) with schizophrenia were recruited in this study. Forty-one had family history. Diagnosis was made for each patient according to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV) criteria on the basis of unstructured interviews and information from medical records. Participants were excluded if they had organic brain diseases, including head injury and infection, or if they met criteria for alcohol/drug dependence. After written informed consent, genomic deoxyribonucleic acid (DNA) from lymphoblastoid cell line (LCL) of all patients was isolated using DNA isolation systems [Quick Gene-800 (Fujifilm, Tokyo, Japan) and/or NA-3000 (Kurabo, Osaka, Japan)]. Microarray comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) analysis were performed using materials from LCL. Peripheral blood lymphocytes were reevaluated in ID394, MZ102, and MZ127, but could not be obtained for reexamination in ID67, ID345, or ID391. Only parents of ID345 subjects were available for familial analysis. Other parents or sibs could not be evaluated. Experimental protocols were approved by the Committee for Ethical Issues at Yokohama City University School of Medicine.

Microarray CGH analysis

Comparative genomic hybridization analysis was performed using our custom BAC microarray containing 4,219 BAC clones, as previously described (Saito et al. 2008). In brief, after complete digestion using *DpnII*, subject's DNA was labeled with Cy-5 dCTP (Amersham Biosciences, Piscataway, NJ), and reference DNA was labeled with Cy-3 deoxycytidine triphosphate (dCTP) (Amersham Biosciences) using the DNA random primer Kit (Invitrogen). Prehybridization, probe hybridization, washing, and drying steps for arrays were performed on a Tecan hybridization station HS400 (Tecan Japan, Kawasaki, Japan). Arrays were scanned by GenePix 4000B (Axon Instruments, Union City, CA, USA) and analyzed using GenePix Pro 6.0 (Axon Instruments). The signal intensity ratio between patient and control DNA was calculated from the data of the single-slide experiment using the ratio of means formula ($F635 \text{ mean} - B635 \text{ median}/F532 \text{ mean} - B532 \text{ median}$) according to GenePix Pro. 6.0. The standard deviation was calculated from the data of all clones. We regarded the signal ratio as abnormal if it ranged out of ± 3 standard deviations (SD). Clones showing abnormal copy number were checked to see whether they were in the position of previously registered copy number variations using the Human Genome Variation Database (<http://www.hgvbase.org/>) (Iafate et al. 2004). Unregistered changes were considered for further confirmation. Genome position was based on the UCSC genome browser Human Mar. 2006 (hg18) assembly.

Fluorescence in situ hybridization

To confirm status of clones with a possibly abnormal copy number, FISH was performed, as previously described (Shimokawa et al. 2005). BAC DNA was labeled with SpectrumGreen™-11-deoxyuridine triphosphate (dUTP) or SpectrumOrange™-11-dUTP (Vysis, Downers Grove, IL, USA) by nick translation and denatured at 70°C for 10 min. Probe-hybridization mixtures (15 μ l) were applied on chromosomes, incubated at 37°C for 16–72 h, then washed and mounted in antifade solution (Vector, Burlingame, CA, USA) containing 4'-6'-diamidino-2-phenylindole (DAPI). Photographs were taken on an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). In ID394 and MZ102, we counted 100 interphase nuclei to validate the number of cells with X aneuploidy, as well as 30 metaphases.

Results and discussion

Six patients showed chromosomal abnormalities (10%, 6/59) (Table 1). As we could not obtain materials from most

Table 1 Summary of six patients with (sub)microscopic chromosomal rearrangements

| Patient | Gender | FH | Karyotype | Size of imbalance |
|---------|--------|-----|---|--|
| ID67 | M | No | 46,XY,der(13)t(12;13)(p12.1;p11).ish del(5)(p11p12) | 1.7 Mb deletion (chr.5) 23.1 Mb gain (chr.12) |
| MZ127 | F | Yes | 46,XX,ish dup(11)(p13p13) | 430 bp (?) gain (chr.11) |
| ID345 | M | No | 46,XY, ish del(17)(p12p12) | 1.3 Mb deletion (chr.17) |
| MZ102 | F | Yes | Mos45,X/46,XX | Whole X loss (mosaic) |
| ID394 | F | Yes | Mos45,X/46,XX | Whole X loss (mosaic) |
| ID391 | M | Yes | 46,X,idi(Y)(q11.2) | Yq12-qter deletion Yq11.23-Yq12 gain |

FH family history of schizophrenia and/or other psychiatric disorders

of their parents and sibs, heritability of the abnormalities could not fully be investigated. According to our experiences of microarray CGH analysis of more than 200 Japanese patients associated with mental-retardation-related disorders, all chromosomal abnormalities described here were never detected. Thus, it is less likely that the changes are polymorphisms.

In ID67, arr cgh 5p12p12(RP11-1037A10 → RP11-929P16) × 1, 12pterp12.1(GS-124K20 → RP11-12D15) × 3 was found. A 23.2-Mb copy number gain from 12pter to 12p12.1 (chr12: 0–23,176,547 bp) was detected (Fig. 1a). G-banded chromosomal analysis revealed that 12pter-12p12.1 was translocated to 13p11 (Fig. 1a). The 12p12.1 translocation breakpoint was localized between two BAC clones, RP11-35A22 and RP11-349E13, by FISH (chr12: 23,176,547–23,861,227 bp) (data not shown). Additionally, a 1.7-Mb submicroscopic deletion at 5p12 from RP11-1037A10 to centromeric sequence gap (chr5: 44,778 009–46,437 323 bp) was also found in this patient (Fig. 1a). The 12p trisomy is recognized as multiple congenital anomalies/mental retardation (MCA/MR) syndrome characterized by dysmorphic face, heavy birth weight, foot deformities, hypotonia, and mental retardation (Allen et al. 1996). A previous study suggested that partial duplication of 12pter-p13.2 is sufficient for recognizable phenotype of 12p trisomy (Rauch et al. 1996). The 23.1-Mb duplicated region contained at least 229 genes. Dysmorphic facial features of 12p trisomy (Rauch et al. 1996) were not recognized in this patient. It is interesting that ID67 also had a 1.7-Mb deletion at 5p12, containing two genes, *MRPS30* (the mitochondrial ribosomal protein S30 gene) and *HCN1* (the hyperpolarization-activated cyclic nucleotide-gated potassium channel 1 gene). It is worth noting linkage findings within the vicinity of this region in Costa Rican schizophrenia samples (Cooper-Casey et al. 2005). *HCN1* is an intriguing candidate gene. The general *Hcn1* loss in mice led to a defect in the learning of motor tasks, and specific deletion of the gene in forebrain neurons resulted in an unexpected enhancement of spatial learning and memory (Herrmann et al. 2007; Nolan

et al. 2003). ID67 (a 72-year-old male) developed psychotic symptoms (delusions, hallucinations, and psychomotor excitement) at age 20 years. He had received electroconvulsive therapy many times and continuous sleep therapy until antipsychotic medication (chlorpromazine) was introduced at age 23 years. Since the onset of the illness, he has spent most of his life in psychiatric hospitals because of exacerbations of psychotic episodes and marked deterioration of social functions. Intelligent quotient (IQ) at 72 years was 72. He had no family history of major psychosis within the first-degree relatives.

In MZ127, arr cgh 11p13p13(RP11-51J14) × 3 was recognized. Duplication of RP11-51J14 at 11p13 (chr11: 33,302,231–33,302,660 bp) was confirmed by FISH using LCL and peripheral blood lymphocytes (Fig. 1b). According to the genome browser, the size of RP11-51J14 is 430 bp, indicating that the reference sequence is somehow odd and may contain a deletion overlapping with RP11-51J14 as FISH signals of RP11-51J14 are strong enough to detect on a microscope, suggesting that its size is at least >10 kb. *HIPK3* (the homeodomain interactive protein kinase 3 gene) was corresponding to this clone. *HIPK3* is a Fas-associated death-domain (FADD)-interacting kinase involved in apoptosis (Curtin and Cotter 2003), remaining unknown in relation to schizophrenia. MZ127 (42-year-old woman) presented with epilepsy at age 12 years and has had recurrent depression and slight mania since age 29 years. She began to exhibit auditory hallucination, not synchronizing with mood swing, and was diagnosed as schizophrenia at 40 years. Her mother and sister suffered from major depression and schizophrenia, respectively. Her father committed suicide induced by depression.

In ID345, arr cgh 17p12p12(RP11-78J16 → RP11-103P10) × 1 was found, as previously described (Ozeki et al. 2008). The deletion from RP11-246F16 to RP11-103P10 (chr17: 14,061,460–15,374,745 bp) is 1.4 Mb, compatible with the common deletion found in approximately 85% of hereditary neuropathy with liability to pressure palsies (HNPP; OMIM #162500) (Stogbauer et al.

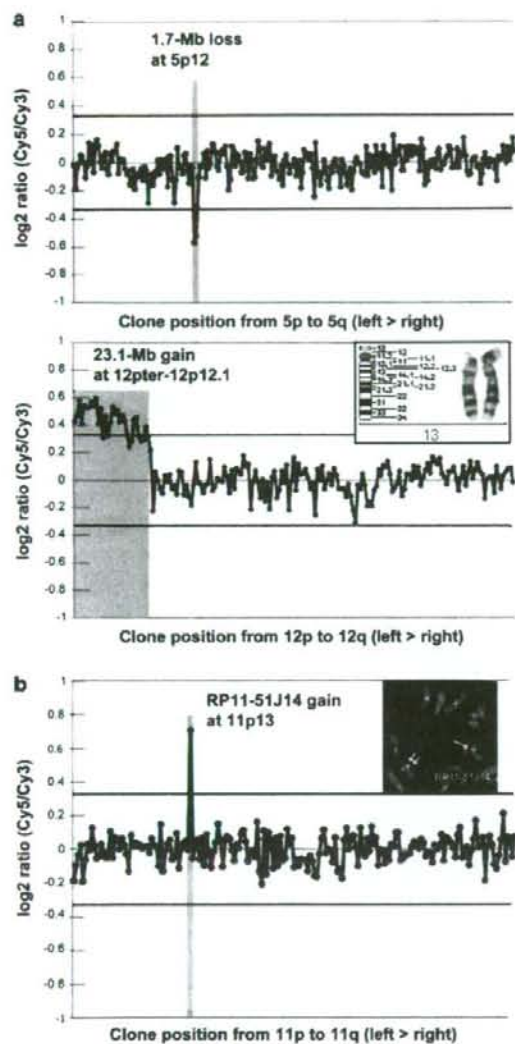


Fig. 1 Results of microarray comparative genomic hybridization (CGH) in ID67 (a) and MZ127 (b). Chromosomes 5 (upper) and 12 (lower) are displayed (a). The karyotype is arr cgh 5p12p12(RP11-1037A10 → RP11-929P16) × 1, 12pterp12.1(GS-124K20 → RP11-12D15) × 3. Partial karyotype clearly shows a 12pter-p12.1 segment is translocated to 13p11. Chromosome 11 is presented (b). The karyotype is arr cgh 11p13p13(RP11-51J14) × 3. RP11-51J14 at 11p13 is duplicated

2000). The deletion was also identified in his father's chromosomes from peripheral blood lymphocytes. He suffered from auditory hallucination and delusion of persecution and received antipsychotic treatment at age 19. Neurological examination did not reveal any manifestations

of HNPP (Ozeki et al. 2008). Pareyson et al. (1996) reported that about 25% of individuals with HNPP deletion are asymptomatic. The peripheral myelin protein 22 gene (*PMP22*) may be a candidate that is not only expressed in the peripheral nervous system but also in the central nervous system (Ohsawa et al. 2006), this being supported by linkage studies of psychotic bipolar disorder (Park et al. 2004) and schizophrenia (Owen et al. 2004). No family history regarding psychiatric disorders was observed in ID345.

Entire X chromosome copy number aberration was suspected in two patients, ID394 and MZ102 (data not shown). FISH analysis using RP11-65B15 at Xq23 revealed mosaic monosomy of chromosome X: mos45,X[41]/46,XX[59] in ID394 and mos45,X[84]/46,XX[16] in MZ102. X aneuploidy is well known to be seen in elderly normal females (Stone and Sandberg 1995). ID394 and MZ102 were 67 and 38 years old, respectively. The fraction of cells with X monosomy was very high (84% and 41%) in lymphoblastoid cell lines of these patients. Reevaluation of peripheral blood lymphocytes showed mos 45,X[7]/46,XX[98] in ID394 and mos 45,X[4]/46,XX[96] in MZ102. These findings may support involvement of X-chromosomal abnormalities in schizophrenia (Kumra et al. 1998; Kunugi et al. 1999), but mosaic X monosomy is also found in age-matched normal controls (Toyota et al. 2001). ID394 (a 67-year-old woman) developed psychotic symptoms (paranoid delusion and hallucinations) at age 31 years when she delivered her second child. Since then, she had been admitted to a psychiatric hospital three times (each for a few months). She quit her job as a pharmacist after the onset of the illness and has lived as a housewife. She has been managed by antipsychotic medications without major exacerbation for the past decade. The second child developed schizophrenia-like symptoms, including social withdrawal and lack of volition. MZ102 (a 38-year-old woman) exhibited psychomotor excitement and was diagnosed as having schizophrenia at age 23 years. Her father showed psychotic disorder, and her uncle had schizophrenia. In ID391, arr cgh Ypterq11.23(GS-98C4 → RP11-214M24) × 3, Yq11.23qter(RP11-263C17 → RP11-80F8) × 1 was identified. FISH analysis using BACs, RP11-74L17 at PAR1, RP11-375P13 at Yp11.2, RP11-655E20 at Yq11.2, and RP11-80F8 at Yq12 revealed the isodicentric Y chromosome [46,X,idel(Y)(q11.2)] (data not shown). Previously, two cases of idic(Yp) were reported in schizophrenia, although idic(Yp) is one of the most common rearrangements in the Y chromosome (Nanko et al. 1993; Yoshitsugu et al. 2003). ID391 (a 29-year-old man) developed hallucinations and abnormal sense of self at age 21 years, when he was admitted to a psychiatric hospital for 3 months. Since then, his illness has been well controlled by antipsychotic medication. He quit university after the onset

of illness and has not obtained a job, suggesting deterioration of functioning. His younger sister (apparently without the Y chromosome) has schizophrenia. Thus, contribution of sex chromosomal abnormalities found in this study is less likely.

Four microarray CGH studies of schizophrenia were reported: 1,440 BAC microarray for 30 patients, 2,460 BAC microarray for 35 patients, a tiling-path microarray consisting of ~36,000 BACs for 93 patients, and high-resolution microarrays (85,000–2,100,000 oligos) for 150 patients (Kirov et al. 2008; Moon et al. 2006; Walsh et al. 2008; Wilson et al. 2006). We could not replicate any similar abnormalities, though microarray platforms were all different in terms of clones and genome coverage. In this study, (sub)microscopic rearrangements were detected in 10% of patients. Similarly, 15% of patients analyzed by high-resolution microarrays were found to possess submicroscopic chromosomal changes (Walsh et al. 2008). Various kinds of recurrent and unique submicroscopic changes were found in 10–17% of idiopathic mental retardation and 7% of autism by microarray CGH analysis (Miyake et al. 2006; Sebat et al. 2007; Zahir and Friedman 2007). Importantly, a 22q13 deletion (in autism) involving SH3 and multiple ankyrin repeat domains 3 (*SHANK3*), whose point mutation was related to autism (Durand et al. 2007), strongly supports this approach as one of the most powerful and straightforward strategies in neuropsychiatric disorders.

In conclusion, microarray technologies could provide good opportunity to identify chromosomal copy number changes in relation to mental and psychiatric disorders, and genome-wide copy number survey should be considered in genetic studies of these disorders.

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Dose-dependent effect of the Val66Met polymorphism of the brain-derived neurotrophic factor gene on memory-related hippocampal activity

Ryota Hashimoto^{a,b,c,*}, Yoshiya Moriguchi^d, Fumio Yamashita^d, Takeyuki Mori^{d,e},
Kiyotaka Nemoto^d, Takeya Okada^c, Hiroaki Hori^c, Hiroko Noguchi^c,
Hiroshi Kunugi^c, Takashi Ohnishi^{c,d,e}

^aThe Osaka-Hamamatsu Joint Research Center For Child Mental Development, Osaka University Graduate School of Medicine, Japan

^bDepartment of Psychiatry, Osaka University Graduate School of Medicine, Japan

^cDepartment of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan

^dDepartment of Radiology, National Center Hospital for Mental, Nervous, and Muscular Disorders, National Center of Neurology and Psychiatry, Japan

^eDepartment of Investigative Radiology, Research Institute, National Cardiovascular Center, Japan

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Abstract

Brain-derived neurotrophic factor (BDNF) plays a critical role in activity-dependent neuroplasticity underlying learning and memory in the hippocampus. Recent human studies have indicated that a common single nucleotide polymorphism of the BDNF gene, the Val66Met polymorphism, has impact on episodic memory, hippocampal morphology and memory-related hippocampal activity measured by functional magnetic resonance imaging (fMRI). However, two issues remain to be clarified: (1) whether the genotype effect of this polymorphism on memory-related brain activity is allele dose dependent and (2) whether the effect of this polymorphism in Asian population is the same as effects observed in Caucasian sample. To clarify these issues, we studied the relationship of the Val66Met polymorphism genotype and hippocampal activity during episodic memory task using fMRI in healthy 58 biologically unrelated Japanese. Although there was no genotype effect on episodic memory function obtained by behavioral assessments, fMRI measurements revealed a significantly negative correlation between the dose of Met-BDNF allele and encoding related brain activity in the bilateral hippocampi and right parahippocampal gyrus. There was no genotype effect on retrieval related brain activity. These data indicated a genetic mechanism for normal variation in human memory and suggest effects of BDNF signaling on hippocampal function in humans.

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1. Introduction

Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, has important roles in hippocampal plasticity and hippocampal-related learning and memory through long-term potentiation (LTP) (Poo, 2001). It is also important for long-term developmental phenomena, such as

neuronal survival, differentiation, and activity-dependent refinement of synaptic architecture (Huang and Reichardt, 2001; Malcangio and Lessmann, 2003). A common single nucleotide polymorphism (SNP) of the BDNF gene producing an amino acid substitution of valine to methionine (Val66Met) affects intracellular packaging and activity-dependent secretion of BDNF (Egan et al., 2003; Chen et al., 2004). A previous study revealed that normal individuals with the Met-BDNF allele had poorer episodic memory accompanying with lesser engagement of the hippocampi during memory task observed in a functional magnetic resonance imaging (fMRI) when compared to individuals homozygous for the Val-BDNF allele (Hariri et al., 2003). They also demonstrated that Met-BDNF

* Corresponding author at: The Osaka-Hamamatsu Joint Research Center For Child Mental Development, Osaka University Graduate School of Medicine, D3, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 3074; fax: +81 6 6879 3059.

E-mail address: hashimor@psy.med.osaka-u.ac.jp (R. Hashimoto).

carriers had smaller hippocampus when compared to individuals homozygous for the Val-BDNF allele (Pezawas et al., 2004; Bueller et al., 2006).

Although these results strongly suggest that the Val66Met polymorphism of the BDNF gene should contribute inter-individual differences of human episodic memory function and memory-related brain structures and brain activity, at least two issues remain to be clarified. First, because of relatively small sample size, particularly homozygous for Met-BDNF allele, the previous study might demonstrate different brain activity related to memory task between homozygous for Val-BDNF allele and Met-BDNF carriers (Hariri et al., 2003). It has been unclear whether the genotype effect of the Val66Met polymorphism on memory-related brain activity is dose dependent. Indeed, their behavioral study with larger sample demonstrated dose-dependent effect of the Val66Met polymorphism on episodic memory performance in normal individuals (Egan et al., 2003). Second, some studies suggested racial differences of effects of the Val66Met polymorphism, such as discrepancy in associations between BDNF polymorphism and the prevalence of neuropsychiatric diseases (Sklar et al., 2002; Kunugi et al., 2004). In terms of brain morphology, our previous structural MR study demonstrated that effects of the Val66Met polymorphism on brain morphology in Japanese were different from those in Caucasians; i.e. the effect of the Val66Met polymorphism was noted in not hippocampal volume but volumes of the parahippocampal gyrus and caudate nucleus (Nemoto et al., 2006). Taken together, it would be possible that genotype effects of the Val66Met polymorphism on brain activity related to episodic memory task in Asian population may differ from those in Caucasians.

To clarify these two issues, we performed fMRI measurements with memory task in larger samples consisted of normal Japanese individuals.

2. Methods

2.1. Subjects

Fifty-eight healthy subjects participated in the study. Written informed consent was obtained from all the subjects in accordance with ethical guidelines

in place at local ethical committee. All the subjects were recruited from local advertisements and underwent a Wechsler Adult Intelligence Scale-Revised (WAIS-R), Wechsler Memory Scale-Revised (WMS-R) and MRI scanning. They were also screened by a questionnaire on medical history and excluded if they had neurological, psychiatric or medical conditions that could potentially affect the central nervous system, such as substance abuse or dependence, atypical headache, head trauma with loss of consciousness, asymptomatic or symptomatic cerebral infarction detected by T2 weighted MRI, hypertension, chronic lung disease, kidney disease, chronic hepatic disease, cancer, or diabetes mellitus. All the subjects were biologically unrelated Japanese. Table 1 shows characteristics of subjects. The age range of the sample was 22–63 years (mean 36.4, standard deviation [S.D.] 10.6). According to genotypes, subjects were categorized into three groups; the homozygous Val-BDNF group ($n = 17$, male:female = 5:12, one was left-handed), the Val/Met-BDNF group ($n = 29$, male:female = 5:24, two were left-handed), and the remaining, homozygous Met-BDNF group ($n = 12$, male:female = 2:10, all were right-handed). The genotype distribution of this SNP was not deviated with Hardy-Weinberg equilibrium (chi-square = 0.003, $p = 0.95$). The one way analysis of variance revealed that there was no significant difference of age, education years, full scale IQ, or scores of WMS-R between groups (all $p > 0.05$). The chi-square test also revealed that there was no significant difference of distribution of gender ratio (d.f. = 2, chi-square = 1.1, $p = 0.57$), handedness (d.f. = 2, chi-square = 0.85, $p = 0.65$) or the ApoE genotype (d.f. = 2, chi-square = 2.9, $p = 0.24$).

2.2. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. Genotyping the Val66Met SNP (dbSNP accession: rs6265) was performed with the TaqMan 5'-exonuclease allelic discrimination assay, described previously (Nemoto et al., 2006). Primers and probes for detection of the SNP (TaqMan SNP Genotyping assays on demand) were purchased from Applied Biosystems (ABI, Foster City, CA, USA). PCR cycling conditions were: at 95 °C for 10 min, 45 cycles of 92 °C for 15 s and 60 °C for 1 min. ApoE genotypes were determined by the polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) method. We used the primers designed by Hixson and Vernier (Hixson and Vernier, 1990). The procedures have been previously described in detail (Asada et al., 1996). Hha I-digested PCR fragments were subjected to gel electrophoresis and visualized by staining with ethidium bromide.

2.3. fMRI study

Cerebral activation was measured with fMRI using blood oxygen level-dependent contrast. After automatic shimming, a time course series of 145 volumes was obtained using single-shot gradient-refocused echo-planar imaging (TR = 4000 ms, TE = 60 ms, flip angle = 90°, in-plane resolution

Table 1
Characteristics of subjects

| | Val/Val-BDNF | Val/Met-BDNF | Met/Met-BDNF | Genotype F (p) | Chi square (p) |
|--|--------------|--------------|---------------|--------------------|--------------------|
| Number of subjects | 17 | 29 | 12 | | |
| Age | 37.6 (2.9) | 35.1 (10.1) | 38.0 (10.6) | 0.47 (0.63) | |
| Gender (M/F) | 5/12 | 5/24 | 2/10 | | 1.1 (0.57) |
| Handedness (R/L) | 16/1 | 27/2 | 12/0 | | 0.85 (0.65) |
| Education | 15.4 (2.7) | 16.4 (2.4) | 15.9 (2.7) | 0.97 (0.36) | |
| ApoE epsilon4 allele (non-carrier/carrier) | 15/2 | 21/8 | 11/1 | | 2.9 (0.24) |
| Full scale IQ (WAIS-R) | 108.9 (13.7) | 108.3 (12.2) | 110.8 (13.2) | 0.12 (0.89) | |
| WMS-R | | | | | |
| Verbal memory | 117 (12.4) | 108.3 (12.8) | 113.08 (11.8) | 2.51 (0.09) | |
| Visual memory | 109.5 (11.2) | 108.9 (9.1) | 106.3 (9.1) | 0.41 (0.67) | |
| General memory | 116.8 (11.7) | 109.4 (12.0) | 112.7 (10.7) | 2.05 (0.14) | |
| Attention/concentration | 106.6 (16.3) | 104.1 (14.2) | 105.9 (15.9) | 0.14 (0.97) | |
| Delayed recall | 116.9 (13.5) | 110.4 (11.9) | 108.3 (16.7) | 1.81 (0.17) | |

WMS-R (Wechsler memory scale-revised); mean value (standard deviation).

3.44 mm × 3.44 mm, FOV = 22 cm, contiguous 4-mm slices to cover the entire brain) with a 1.5 T MAGNETOM Vision plus MR scanner (Siemens, Erlangen, Germany) using the standard head coil. The first five volumes of each fMRI scan were discarded because of non-steady magnetization, with the remaining 140 volumes used for the analysis. Head motion was minimized by placing tight but comfortable foam padding around the subject's head. Before the collection of fMRI data for each subject, we acquired a reference EPI scan and visually inspected it for artifacts (i.e. ghosting) as well as for good signal across the entire volume of acquisition, including the medial temporal lobes.

The fMRI paradigm consisted of the encoding and subsequent retrieval of novel, complex scenes. Stimuli were presented in a blocked paradigm; seven encoding blocks were followed by seven retrieval blocks in an interleaved design with a passive viewing of scrambled picture condition (control condition). During encoding blocks, subjects viewed five images, presented serially for 4 s each, and were asked to remember them. All scenes were of neutral emotional valence and were derived from the International Affective Picture System (Lang et al., 1997). During subsequent retrieval blocks, subjects again viewed five images, presented serially for 3 s each, and determined whether each scene was "new" (presented during the encoding blocks) or "old" (not presented during the encoding blocks). In each retrieval block, half of the scenes were "old" and remaining half were "new". During the interleaved rest blocks, subjects were instructed to fixate on a centrally

presented cross-hair. During scanning, all subjects responded by button presses with their dominant hand, allowing for the determination of accuracy and reaction time.

Analysis of the fMRI data were completed using statistical parametric mapping 2 (SPM2). Images for each subject were realigned to the first volume in the time series to correct for head motion, spatially normalized into a standard stereotaxic space (Montreal Neurological Institute template) using a 12 parameter affine model and smoothed with a Gaussian filter, set at 8 mm full-width at half-maximum. Voxel-wise signal intensities were ratio normalized to the whole-brain global mean. Task-related activations were calculated using a *t*-statistic, producing a statistical image for the contrasts of encoding versus control and retrieval versus control for each subject. These individual contrast images were then used in second-level random effects models, which account for both scan-to-scan and subject-to-subject variability, to determine task-specific regional responses at the group level for the entire sample (main effects of tasks). We used $p < 0.001$, corrected for multiple comparisons with false discovery rate (FDR) < 0.05 as a statistical threshold for these whole-brain search. To test dose-dependent genotype effects of the BDNF gene, we performed correlational analysis. In this analysis, we treated Met-BDNF gene dose (i.e. the number of Met-BDNF allele in a person's BDNF genotype) as a covariate of interest and treated the genotype of the ApoE gene, age, and gender as nuisance variables. Because

Table 2
Brain activity related to encoding and retrieval

| Brain regions | Brodmann area | Cluster size | Talairach coordinates | | | Corrected <i>p</i> value | <i>T</i> value |
|---|---------------|--------------|-----------------------|----------|----------|--------------------------|----------------|
| | | | <i>x</i> | <i>y</i> | <i>z</i> | | |
| Activity related to encoding (Fig. 1) | | | | | | | |
| Rt. Middle frontal gyrus | 46 | 36 | 44 | 17 | 21 | $<5.0 \times 10^{-4}$ | 4.5 |
| Lt. Middle frontal gyrus | 46 | 38 | -44 | 17 | 25 | 5.0×10^{-3} | 3.8 |
| Rt. Parahippocampal gyrus, hippocampus | 30/36/19 | 2617 | 36 | -32 | -22 | $<5.0 \times 10^{-4}$ | 11.8 |
| Lt. Parahippocampal gyrus, hippocampus | 30/36/19 | 2617 | -24 | -39 | -11 | $<5.0 \times 10^{-4}$ | 8.9 |
| Rt. Precuneus | 19 | 2617 | 32 | -72 | 33 | $<5.0 \times 10^{-4}$ | 8.3 |
| Lt. Precuneus | 19 | 2617 | -28 | -76 | 37 | $<5.0 \times 10^{-4}$ | 7.5 |
| Lt. Superior parietal lobule | 7 | 2617 | -24 | -72 | 44 | $<5.0 \times 10^{-4}$ | 6.9 |
| Rt. Fusiform gyrus | 19/37 | 2617 | 28 | -39 | -11 | $<5.0 \times 10^{-4}$ | 10.5 |
| Rt. Middle temporal gyrus | 19 | 2617 | 40 | -81 | 19 | $<5.0 \times 10^{-4}$ | 13.1 |
| Rt. Angular gyrus | 39 | 2617 | 36 | -76 | 30 | $<5.0 \times 10^{-4}$ | 9.7 |
| Lt. Fusiform gyrus | 37 | 2617 | -48 | -59 | -17 | $<5.0 \times 10^{-4}$ | 13.7 |
| Lt. Middle temporal gyrus | 19 | 2617 | -40 | -81 | 19 | $<5.0 \times 10^{-4}$ | 9.7 |
| Rt. Lingual gyrus | 19 | 2617 | 32 | -58 | -4 | $<5.0 \times 10^{-4}$ | 7.7 |
| Rt. Middle occipital gyrus | 37 | 2617 | 44 | -70 | 7 | $<5.0 \times 10^{-4}$ | 11.6 |
| Lt. Superior occipital gyrus | 39 | 2617 | -32 | -76 | 30 | $<5.0 \times 10^{-4}$ | 8.2 |
| Lt. Middle occipital gyrus | 18/19 | 2617 | -36 | -86 | -2 | $<5.0 \times 10^{-4}$ | 17.5 |
| Activity related to retrieval (Fig. 2) | | | | | | | |
| Lt. Middle frontal gyrus | 9 | 74 | -40 | 13 | 25 | $<5.0 \times 10^{-4}$ | 6.4 |
| Rt. Middle frontal gyrus | 9/46 | 56 | 44 | 13 | 25 | $<5.0 \times 10^{-4}$ | 5.4 |
| Rt. Parahippocampal gyrus, hippocampus | 19/30/36/37 | 2393 | 16 | -50 | 3 | $<5.0 \times 10^{-4}$ | 9.4 |
| Rt. Posterior cingulate | 29 | 2393 | 4 | -42 | 6 | $<5.0 \times 10^{-4}$ | 9.2 |
| Lt. Parahippocampal gyrus, hippocampus | 30/35/36/37 | 2393 | -24 | -47 | -8 | $<5.0 \times 10^{-4}$ | 6.2 |
| Lt. Posterior cingulate | 29 | 2393 | -4 | -42 | 6 | $<5.0 \times 10^{-4}$ | 10.5 |
| Rt. Precuneus | 7 | 2393 | 4 | -71 | 51 | $<5.0 \times 10^{-4}$ | 7.3 |
| Lt. Precuneus | 7 | 2393 | -4 | -75 | 52 | $<5.0 \times 10^{-4}$ | 6.4 |
| Rt. Fusiform gyrus | 20/37/39 | 2393 | 36 | -55 | -14 | $<5.0 \times 10^{-4}$ | 14.8 |
| Lt. Fusiform gyrus | 37 | 2393 | -44 | -59 | -17 | $<5.0 \times 10^{-4}$ | 13.0 |
| Rt. Middle occipital gyrus | 18/19 | 2393 | 40 | -85 | 4 | $<5.0 \times 10^{-4}$ | 18.1 |
| Lt. Lingual gyrus | 18 | 2393 | -8 | -82 | -13 | $<5.0 \times 10^{-4}$ | 9.9 |
| Lt. Middle occipital gyrus | 19 | 2393 | -28 | -89 | 15 | $<5.0 \times 10^{-4}$ | 15.6 |
| Lt. Inferior occipital gyrus | 18 | 2393 | -40 | -82 | -6 | $<5.0 \times 10^{-4}$ | 15.0 |
| Genotype effects on brain activity related to encoding | | | | | | | |
| R Parahippocampal gyrus, hippocampus | | 44 | 27 | -35 | -5 | $<5.0 \times 10^{-4}$ | 4.8 |
| L Hippocampus | | 52 | -36 | -20 | -9 | 2.0×10^{-3} | 4.0 |
| Genotype effects on brain activity related to retrieval | | | | | | | |
| Not significant | | | | | | | |

of our *a priori* hypothesis regarding the differential response of the hippocampus and parahippocampal gyrus, a statistical threshold of $p < 0.05$, a small volume correction for multiple comparisons within the *a priori* volume of interest, was used to identify dose-dependent genotype effects on brain activity. For this hypothesis-driven analysis, we used the Wake Forest University PickAtlas (Maldjian et al., 2003). Whole-brain correlational analyses were also calculated using second-level random effect models. Because we had no *a priori* hypotheses regarding the activity of brain regions outside of the memory-related areas, we used a statistical threshold of $p < 0.05$, corrected for multiple comparisons across all supra-threshold voxels, for these whole-brain correlational analyses.

3. Results

3.1. Task performance during fMRI measurements

The percentage of correct answers (S.D.) for the retrieval task in each genotype was as follows; 98.9% (2.13) for Val/Val-BDNF group, 97.2% (6.06) for Val/Met-BDNF group, and 96.7% (3.89) for Met/Met-BDNF group, respectively. There was no significant genotype effect on the percentage of correct answers of the retrieval task ($F = 0.8$, $p = 0.45$). The mean reaction time (S.D.) of the retrieval task in each genotype was as follows; 1239 (232) ms for Val/Val-BDNF group, 1227 (175) ms for Val/Met-BDNF group, and 1312 (219) ms for Met/Met-BDNF group, respectively. There was no significant genotype effect on reaction time for the retrieval task ($F = 0.76$, $p = 0.48$).

3.2. Results of brain activity

When all subjects were treated one group, we found a significant activation in the bilateral hippocampus, parahippocampal gyri, visual association areas, parietal association areas including precune and the dorsolateral prefrontal cortices during encoding period (Table 2 and Fig. 1). During retrieval period, a significant activation was also found in the bilateral hippocampus, parahippocampal gyri, posterior cingulate cortex (PCC), visual association areas, parietal association areas and the dorsolateral prefrontal cortices (Table 2 and Fig. 2).

Regarding brain activity related to encoding, a significantly negative correlation between the dose of Met-BDNF gene and brain activity was noted in the bilateral hippocampi and right parahippocampal gyrus (Table 2 and Figs. 3 and 4). On the other hand, a weak negative correlation between dose of Met-BDNF gene and brain activity related retrieval in the bilateral parahippocampal gyri at a lenient statistical threshold (uncorrected $p < 0.05$), however, no voxels could survive after the correction for multiple comparisons within the ROI (corrected $p > 0.05$). Since the previous study demonstrated genotype effect on retrieval related hippocampal activity in the group comparison (Val/Val BDNF subjects versus Met-BDNF carriers), we additionally performed the same analysis to estimate genotype effect on retrieval related activity. With a lenient statistical threshold (uncorrected $p < 0.05$), Val/Val-BDNF group showed stronger activation during retrieval in the

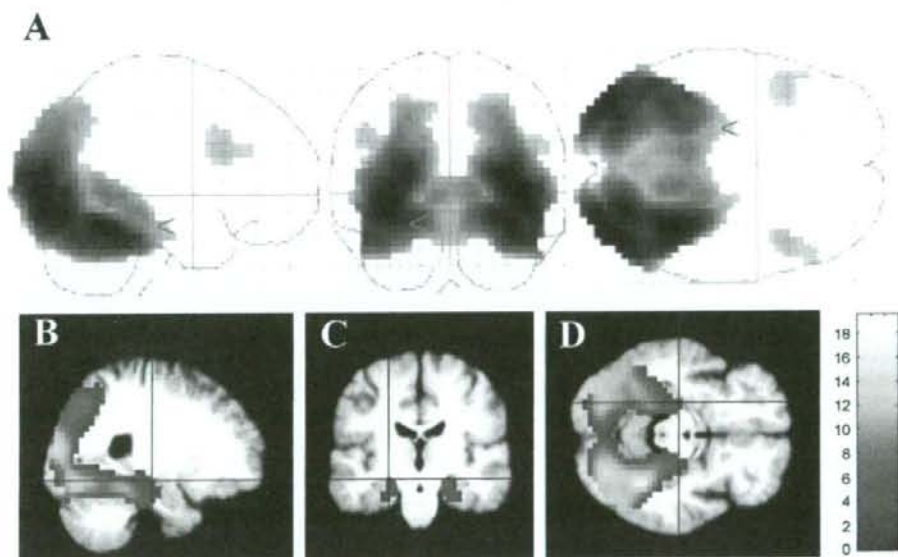


Fig. 1. Brain activity associates with encoding in the whole group. (A) The SPM (t) is displayed in a standard format as a maximum-intensity projection (MIP) viewed from the right, the back and the top of the brain as SPM2 "glass brain" representation. The anatomical space corresponds to the atlas of Talairach and Tournoux. Representation in stereotaxic space of brain areas with significant activation was demonstrated. Task-related activations were calculated using a t -statistic of SPM2. A significant activation associated with encoding is noted in the bilateral hippocampus, parahippocampal gyri, visual association areas, parietal association areas including precune and the dorsolateral prefrontal cortices ($p < 0.05$, corrected for multiple comparisons) and anatomical localization, as projected on (B) sagittal, (C) coronal, and (D) axial sections of a normal MRI, spatially normalized into the Montreal Neurological Institute template. The scale of T value is indicated on the bottom right.

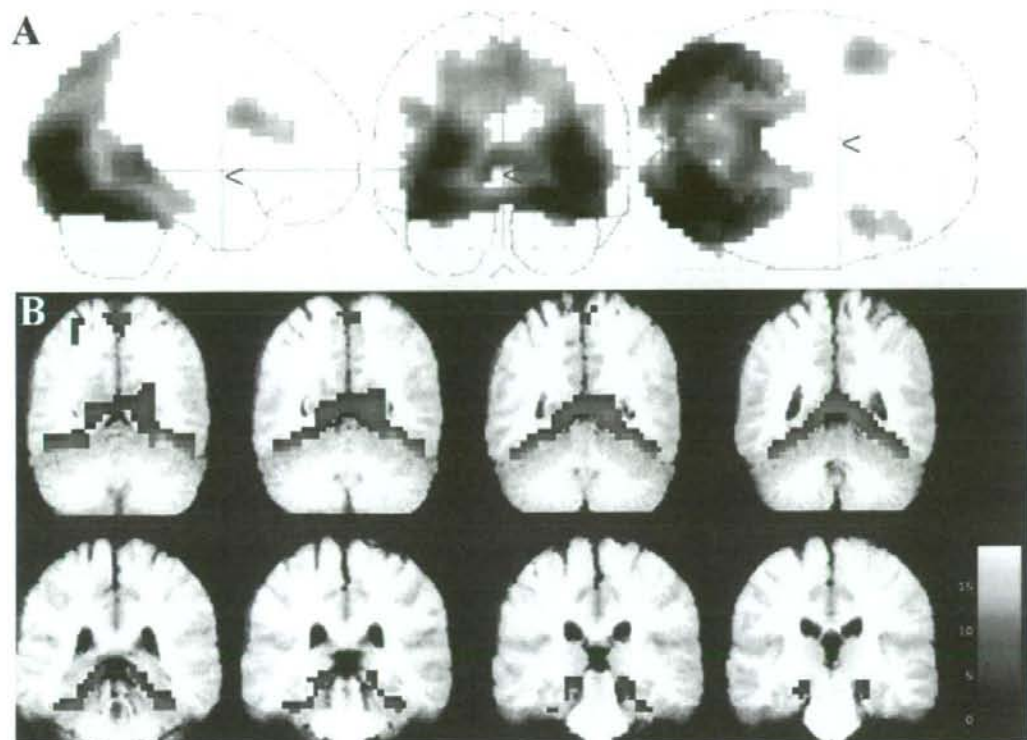


Fig. 2. Brain activity associates with retrieval in the whole group. (A) The SPM t is displayed in a standard format as a MIP viewed from the right, the back and the top of the brain as SPM2 "glass brain" representation. The anatomical space corresponds to the atlas of Talairach and Tournoux. Representation in stereotaxic space of brain areas with significant activation was demonstrated. Task-related activations were calculated using a t -statistic of SPM2. A significant activation was also found in the bilateral hippocampus, parahippocampal gyri, posterior cingulate cortex, visual association areas, parietal association areas and the dorsolateral prefrontal cortices ($p < 0.05$, corrected for multiple comparisons) and anatomical localization, as projected on (B) coronal sections of a normal MRI, spatially normalized into the Montreal Neurological Institute template. The scale of T value is indicated on the bottom right.

bilateral parahippocampal gyri and hippocampi. However, no voxels could survive after the correction for multiple comparisons within the ROI (corrected $p > 0.05$). Whole brain correlational analyses demonstrated that the Val66Met polymorphism had no impact on activity within the distributed cortical network, such as visual association areas, parietal association areas and the dorsolateral prefrontal cortex (corrected $p > 0.05$).

As age range of our subjects is relatively broad, age might be a possible confounding factor. Thus, we analyzed the data including age as a covariate. We further re-analyzed the data using subjects leaving out the subjects older than 55 years (Val/Val = 3, Val/Met = 1 and Met/Met = 0) and obtained essentially same results in all analysis (data not shown).

4. Discussion

In the whole subjects' analysis, we found significant bilateral activation in the hippocampus and parahippocampal gyrus during both encoding and retrieval. The data was well concordant with previous fMRI studies of episodic memory.

We also replicated significant bilateral activations in the inferotemporal, parietal, and frontal cortices, a distributed network critical for visuospatial information processing (Ungerleider and Haxby, 1994) during both encoding and retrieval. In addition, we found significant activation in the bilateral PCC during retrieval. Several studies have indicated that the PCC have prominent response properties associated with episodic memory, particularly, successful retrieval (Wagner et al., 2005). A significant activation in the PCC associated with retrieval in the present study is well consistent with previous studies.

In terms of effects of the Val66Met polymorphism of the BDNF gene on memory-related brain activity, we obtained two main findings: (1) the Val66Met polymorphism of the BDNF gene affects encoding related neuronal activity in the hippocampal regions with a dose-dependent manner; i.e. a negative correlation between the number of Met allele and the degree of activation in the bilateral hippocampi and parahippocampal gyri during encoding and (2) the Val66Met polymorphism of the BDNF gene did not affect retrieval related neuronal activity in the hippocampal regions. Further, this

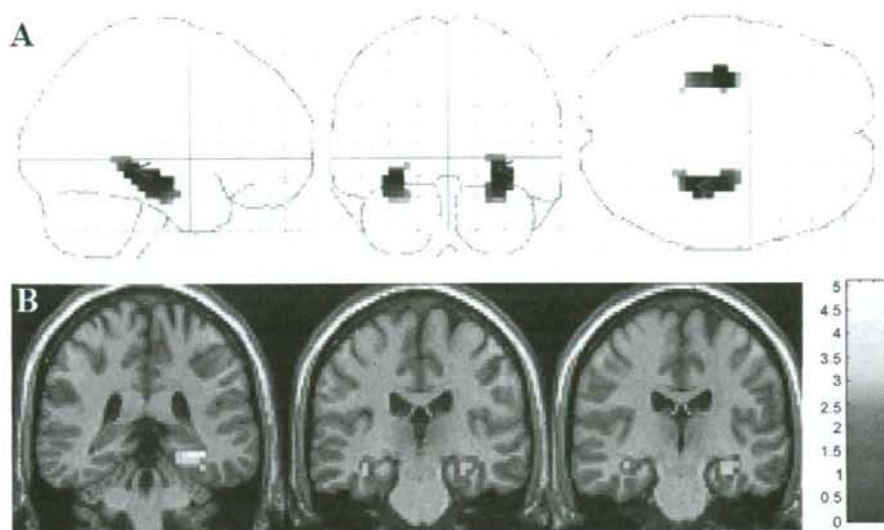


Fig. 3. Genotype effect of the Val66Met polymorphism of the BDNF gene on encoding related hippocampal activity. (A) The SPM (t) is displayed in a standard format as a MIP viewed from the right, the back and the top of the brain as SPM2 "glass brain" representation. The anatomical space corresponds to the atlas of Talairach and Tournoux. Representation in stereotaxic space of brain areas with significant activation was demonstrated. A significant negative correlation between numbers of Met allele and degree of activation is noted in the bilateral hippocampal regions calculated by correlational analysis of SPM2 ($p < 0.05$, corrected for multiple comparisons) and anatomical localization, as projected on (B) coronal sections of a normal MRI, spatially normalized into the Montreal Neurological Institute template.

polymorphism had no effects on activity within the other distributed cortical network, such as visual association areas, parietal association areas and the dorsolateral prefrontal cortex involved with general visuo-spatial information processing. Consistent with the previous fMRI study of the Val66Met polymorphism (Hariri et al., 2003), our data also suggest that this polymorphism could have impact on memory-related neuronal activity of the hippocampal region in an Asian sample. In addition, our data indicate Met-BDNF dose-dependent

effects of the Val66Met polymorphism on hippocampal activity during encoding. The specific effect of BDNF on hippocampal activity is well concordant with the expression pattern of BDNF in the brain, which is highest in the hippocampus (Murer et al., 2001). This effect of the Val66Met polymorphism on BOLD response in the hippocampal region may be mediated through alterations in activity-dependent hippocampal processes requiring BDNF-regulated secretion, particularly the abnormal intracellular trafficking and regulated secretion of BDNF in Met carriers, which may result in impaired hippocampal LTP that could underlie encoding (Egan et al., 2003; Chen et al., 2004). Most neuroimaging studies also have demonstrated genotype effects on hippocampal region, i.e. Met allele is associated with reduced hippocampal volume and impaired hippocampal function (Hariri et al., 2003; Pezawas et al., 2004; Bueller et al., 2006). However, owing to the relatively small sample size and the low frequency of the Met allele, these studies, including the previous memory fMRI study, used combined Met/Met and Val/Met subjects (Hariri et al., 2003; Pezawas et al., 2004; Bueller et al., 2006). On the other hand, our study with a relatively larger sample, particularly individuals with Met-allele, revealed dose-dependent genotype effects of the Val66Met polymorphism on encoding-related hippocampal activity. Our data support and strengthen the results of the previous fMRI study.

The previous study demonstrated that the Val66Met polymorphism affected both on encoding and retrieval related hippocampal activity (Hariri et al., 2003), however, we could not find genotype effects on retrieval related hippocampal activity. Considering the possible underlying mechanism of

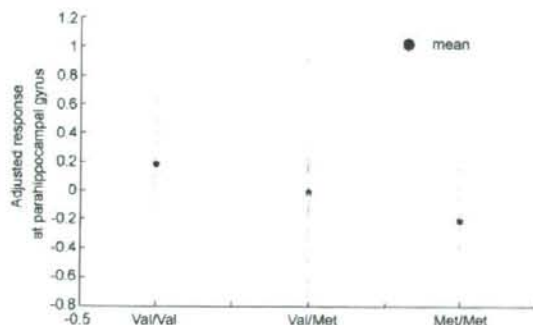


Fig. 4. Dose-dependent effect of Met allele on hippocampal activation. Activation in parahippocampal gyrus during encoding task was calculated in each genotype individual (Val/Val = 17, Val/Met = 29 and Met/Met = 12) by the SPM2. X-axis: adjusted response at parahippocampal gyrus; Y-axis: genotype group. A gray dot indicates each individual response and a black dot indicates a mean response in the genotype groups. Negative correlation between activation associated with encoding and genotype was calculated by correlational analysis of SPM2 ($p < 0.05$, corrected for multiple comparisons).