These data demonstrate an internal inconsistency of G-protein selectivity for the human 5-HT_{2C} (but not 5-HT_{1A}) receptor. The observation of distinct affinity states of G-protein–coupled receptors has been widely utilized to analyze G-protein coupling of receptors. Inverse agonists strongly prefer the uncoupled state, whereas agonist ligands strongly prefer the G-protein–coupled state, and thus the two types of ligand bind to distinct, but interconvertible populations of the receptor. In case of the 5-HT_{2C} receptor, the inverse agonist, [^{3}H]-mesulergine, binds to the G-protein–uncoupled form, while the agonist, [^{125}I]-DOI, preferentially binds to the G-protein–coupled form.[8] Thus, the proportion of [^{125}I]-DOI binding to [^{3}H]-mesulergine binding should reflect the ratio of G-protein–coupled form to uncoupled form. These results would suggest a G_q selectivity for this receptor. The data obtained from receptor-catalyzed GTPγS binding seem to indicate that $G\alpha_{i2}$ is strongly preferred by the human 5-HT_{2C} receptor as opposed to $G\alpha_q$. Houston *et al.*[16] suggests that there is a limitation to the utility of Sf9 cells for comparing the coupling of receptors to mammalian $G\alpha_s$ and $G\alpha_q$ since a large portion of the expressed $G\alpha_q$ was inactive. Fatty acylation regulates the cellular localization and function of G proteins: palmitoylation is required for both the signaling function and membrane attachment of $G\alpha_q$.[17,18] When expressed in Sf9 cells, G-protein α subunits accumulate in both cytosolic and membrane-bound pools. It is notable that palmitate is associated only with the latter.[19] The fact that most of the baculovirus-encoded $G\alpha_q$ localizes in cytosolic fractions from Sf9 cells (data not shown) might partially explain the poor coupling to $G\alpha_q$ coexpressed in Sf9 cells.

In Situ *Reconstitution of 5-HT_{2C} and 5-HT_{1A} Receptors with G Proteins*

FIGURE 3 presents the results for *in situ* reconstitution of G-protein activation by urea-extracted Sf9 membranes expressing 5-HT_{2C} or 5-HT_{1A} receptors. To avoid issues of incomplete or improper posttranslational modification of the Gα subunits, we utilized fractions of G proteins obtained from native membrane sources. As opposed to the coinfection data, these data for 5-HT_{2C} receptors reiterate our previous findings of G-protein selectivity. $G\alpha_q$ is robustly activated by the agonist-occupied receptors; there is a substantial "basal" activation of $G\alpha_q$ by the unoccupied receptor, consistent with findings for constitutive signaling in choroid plexus,[20] and the inverse agonist mianserin potently inhibits the basal activation of $G\alpha_q$ by 5-HT_{2C} receptors. Because we have employed an assay incorporating competing GDP as well as the tracer GTPγS in the present study, we were able to measure the weak activation of $G\alpha_i$ and $G\alpha_o$ by the 5-HT_{2C} receptors, which we had not detected in our earlier work.[10] In contrast, the 5-HT_{1A} receptor displays marginal activation of $G\alpha_q$, but robust activation of both $G\alpha_i$ and $G\alpha_o$, consistent with the established signaling pathway for this serotonin receptor subtype.

To conclude from these data, when G-protein heterotrimers and receptors are coexpressed in cellular systems such as the Sf9 cell, there may be a lack of fidelity of receptor/G-protein coupling. A convergent story for 5-HT receptor/G-protein interactions, and potentially authentic variation in these interactions, can be constructed from the behavior of these proteins in various cell systems, at different levels of expression, in *in vitro* reconstitution systems, and from *in vivo* transgene and receptor pharmacology.

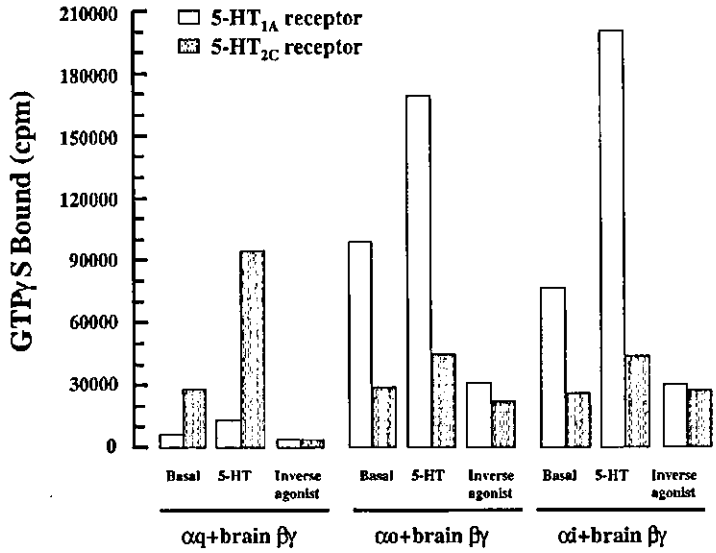

FIGURE 3. G-protein specificities of the 5-HT_{2C} and 5-HT_{1A} receptors. 5-HT_{2C} and 5-HT_{1A} receptor-catalyzed GTPγS binding to $G\alpha_q\beta\gamma$, $G\alpha_i\beta\gamma$, or $G\alpha_o\beta\gamma$ was assessed. Mianserin (10 μM) and spiperone (10 μM) were used as the inverse agonists for the 5-HT_{2C} and 5-HT_{1A} receptors, respectively. The GTPγS binding reactions were performed for 10 min at 30°C.

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こころの健康科学研究事業

気分障害の高精度候補領域解析および精神疾患ゲノムバンクの構築に関する研究

平成16年度 総括・分担研究報告書

主任研究者 吉川武男

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BRIEF REPORT

No Association Between the Val66Met Polymorphism of the Brain-Derived Neurotrophic Factor Gene and Bipolar Disorder in a Japanese Population: A Multicenter Study

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Background: *Two previous studies reported a significant association between a missense polymorphism (Val66Met) in the brain-derived neurotrophic factor (BDNF) gene and bipolar disorder; however, contradictory negative results have also been reported, necessitating further investigation.*

Methods: *We organized a multicenter study of a relatively large sample of 519 patients with bipolar disorder (according to DSM-IV criteria) and 588 control subjects matched for gender, age, and ethnicity (Japanese). Genotyping was done by polymerase chain reaction–based restriction fragment length polymorphism or direct sequencing.*

Results: *The genotype distributions and allele frequencies were similar among the patients and control subjects. Even if the possible relationships of the polymorphism with several clinical variables (i.e., bipolar I or II, presence of psychotic features, family history, and age of onset) were examined, no variable was related to the polymorphism.*

Conclusions: *The Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder, at least in a Japanese population.*

Key Words: Association study, bipolar disorder, brain-derived neurotrophic factor, genetics, single nucleotide polymorphism, susceptibility

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family and promotes the development, regeneration, survival, and maintenance of function of neurons (Maisonpierre et al 1991). It modulates synaptic plasticity and neurotransmitter release across multiple neurotransmitter systems, as well as the intracellular signal-transduction pathway (Thoenen 1995). Growing evidence has suggested important roles of BDNF in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents, such as mood stabilizers and antidepressants (reviewed by Duman 2002). In postmortem brains of patients with bipolar disorder, BDNF protein was reduced compared with control subjects (Knable et al 2004). Chronic electroconvulsive seizure or antidepressant drug treatments increase messenger ribonucleic acid of BDNF and its receptor, tyrosine kinase receptor B (Nibuya et al 1995). Lithium might also exert its neuroprotective effect through enhancing expression of BDNF and trkB (Hashimoto et al 2002).

The BDNF gene is, therefore, an attractive candidate gene that might cause susceptibility to bipolar disorder or influence the clinical phenotype of the illness. Indeed, at least two previous studies reported a significant association between a missense polymorphism (Val66Met; National Center for Biotechnology Information Database of Single Nucleotide Polymorphisms reference number rs6265) of the BDNF gene and bipolar disorder (Neves-Pereira et al 2002; Sklar et al 2002); however, contradictory negative results have also been reported (Hong et al 2003; Nakata et al 2003). One possible reason for this inconsistency is the lack of statistical power due to small sample size. To draw any conclusion with respect to this possible association, we organized a multicenter study in which six laboratories combined their data to ensure adequate statistical power.

Methods and Materials

Subjects

Six laboratories (National Institute of Mental Health, two laboratories of the Brain Science Institute, Showa University, Tokyo Women's Medical College, and Fujita Health University) collected deoxyribonucleic acid (DNA) samples from patients with bipolar disorder and healthy control subjects. Each institute provided DNA samples of patients and control subjects matched for gender, age, and geographic area, which yielded a combined sample of 519 patients with bipolar disorder (244 male) and 588 control subjects (287 male). Mean age (± SD) for the patients was 49.3 ± 14.3 years and for the control subjects was 48.4 ± 12.7 years. All the patients and control subjects were Japanese and biologically unrelated. Consensus diagnosis of bipolar disorder was made for each patient by at least two experienced psychiatrists according to DSM-IV criteria (American Psychiatric Association 1994) on the basis of unstructured interviews and medical records. Among the patients, 347 were diagnosed as bipolar I

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Table 1. Genotype Distributions and Allele Frequencies for the Val66Met Polymorphism of the BDNF Gene Among the Patients with Bipolar Disorder and Control Subjects

	Genotype Distribution				Allele Frequency	
	n	Val/Val	Val/Met	Met/Met	*n*	Val	Met
Patients						
Total	519	188 (36.2)	239 (46.1)	92 (17.7)	1038	615 (59.2)	423 (40.8)
Bipolar I	347	123 (35.4)	166 (47.8)	58 (16.7)	694	412 (59.4)	282 (40.6)
Bipolar II	172	65 (37.8)	73 (42.4)	34 (19.8)	344	203 (59.0)	141 (41.0)
Control subjects	588	216 (36.7)	270 (45.9)	102 (17.3)	1176	702 (59.7)	474 (40.3)

Values in parentheses are percentages. Genotypewise comparisons: total patients vs. control subjects: χ^2 (2) = .0, p = .98; bipolar I vs. control subjects: χ^2 (2) = .3, p = .86; bipolar II vs. control subjects: χ^2 (2) = .8, p = .69. Allelewise comparisons: total patients vs. control subjects: χ^2 (1) = .0, p = .83; bipolar I vs. control subjects: χ^2 (1) = .0, p = .96; bipolar II vs. control subjects: χ^2 (1) = .0, p = .94.

and the remaining 172 as bipolar II. Control subjects were healthy volunteers who had no current or past contact with psychiatric services. The control subjects were recruited from the hospital staffs and their associates at each institution who showed good social functioning and reported themselves to be in good health. They were interviewed, and those individuals who had current or past contact with psychiatric services were excluded. Written informed consent for participation in the study was obtained from all subjects. The study protocol was approved by the institutional ethics committees.

Methods

Venous blood was drawn, and genomic DNA was extracted according to standard procedures. Genotyping was performed according to Neves-Pereira et al (2003). Briefly, the polymorphic site was amplified by polymerase chain reaction (PCR) and then digested with a restriction enzyme, Eco72I. The digested PCR products were visualized with gel electrophoresis and subsequent ethidium bromide staining. Genotyping for a portion of subjects was done by direct sequencing of PCR products encompassing the polymorphic site with an autosequencer (CEQ 8000; Beckman Coulter, Fullerton, California). Genotype data were read blind to the case–control status.

To examine the possible relationships of the Val66Met polymorphism with clinical variables, information on age of onset, family history, and presence of psychotic features (i.e., current or past episode with delusions or hallucinations) was obtained. We defined positive family history as having at least one first-degree relative with a history of contact with psychiatric services with a diagnosis of mood disorder or who was a suicide victim. Individuals with ambiguous clinical data were excluded from statistical analyses.

The presence of Hardy-Weinberg equilibrium for the genotype distributions in the patients and control subjects was examined with the χ^2 test for goodness of fit. The differences in the genotype and allele distributions between patients and control subjects were examined with the χ^2 test for independence. The possible relationships between the polymorphism and clinical variables were examined with the χ^2 test for independence or analysis of covariance (ANCOVA) within the patient group. All p values reported are two-tailed.

Results

Genotype and allele distributions of the Val66Met polymorphism in the patients and control subjects are shown in Table 1. The genotype distributions in the two groups were both in Hardy-Weinberg equilibrium [patients: $\chi^2(1) = 1.1$, $p = .29$; control subjects: $\chi^2(1) = 1.2$, $p = .27$]. The genotype and allele distributions for the patients were quite similar to those for the control group (see Table 1). The genotype and allele distributions of the patients with bipolar I and those with bipolar II were also similar.

When relationships between genotype and clinical variables were examined, genotype and allele distributions were not different according to presence of psychotic features (frequency of the Val66 allele for psychotic patients: .567; for nonpsychotic patients: .579) or family history (positive family history: .602; negative: .603). Age of onset was also similar, irrespective of the genotype (Val/Val: 35.3 ± 13.5 years; Val/Met: 37.7 ± 14.6 years; Met/Met: 36.3 ± 14.0 years). Even when ANCOVA controlling for age and gender was performed, there was no significant difference in age of onset across the three genotypic groups [$F(2) = .99$, $p = .37$].

Discussion

We tried to replicate the studies of Sklar et al (2002) and Neves-Pereira et al (2002), who found a significant association between the Val66Met polymorphism of the BDNF gene with bipolar disorder. They reported excess transmission of the Val66 allele to the patients in their family-based association studies. Contrary to these findings, the genotype and allele frequencies among the patients and control subjects were similar in our sample, which is in turn consistent with more recent studies (Hong et al 2003; Nakata et al 2003), suggesting that the Val66Met polymorphism of the BDNF gene is unrelated to the development of bipolar disorder in our sample. Because our study had adequate statistical power (more than 90% to detect an odds ratio of 1.33 or more in allelic association; power analysis was performed according to Armitage and Berry 1994), the potential type II error due to lack of statistical power is unlikely. One possible explanation for this inconsistency might be a differential effect of the polymorphism depending on ethnicity, given that the majority of the subjects of Sklar et al (2002) and Neves-Pereira et al (2002) were Caucasian, whereas those of Hong et al (2003), Nakata et al (2003), and in our study were Asian. Alternatively, the positive results of Sklar et al (2002) and Neves-Pereira et al (2002) might have arisen by chance.

Concerning the possible effect of the polymorphism on clinical features, Rybakowski et al (2003) reported an earlier age of onset in Val/Val than Val/Met genotype (27 years vs. 38 years) among patients with bipolar disorder. They also found that the performance in all domains of the Wisconsin Card Sorting Test was significantly better for bipolar patients with Val/Val than for those with Val/Met genotype, suggesting a role of the Val66Met polymorphism in prefrontal cognitive function in bipolar disorder. This accords with the findings of Egan et al (2003), who

reported that the Met66 allele was associated with lower activity-dependent secretion of BDNF and poorer human memory and hippocampal function; however, we could not find any significant effect of the genotype on clinical variables of age of onset, subtype (bipolar I or II), psychotic features, or family history. Hong et al (2003) also failed to find significant difference in age of onset or suicidal history across genotypic groups in their Chinese subjects with bipolar disorder.

In conclusion, our results, together with previous two studies (Hong et al 2003; Nakata et al 2003), suggest that the Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder at least in Asian populations; however, the possibility remains that other variants of the BDNF gene might be associated with bipolar disorder in Asian populations, which requires further investigation.

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Association study of polymorphisms in the excitatory amino acid transporter 2 gene (*SLC1A2*) with schizophrenia

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Abstract

Background: The glutamatergic dysfunction hypothesis of schizophrenia suggests that genes involved in glutametergic transmission are candidates for schizophrenic susceptibility genes. We have been performing systematic association studies of schizophrenia with the glutamate receptor and transporter genes. In this study we report an association study of the excitatory amino acid transporter 2 gene, *SLC1A2* with schizophrenia.

Methods: We genotyped 100 Japanese schizophrenics and 100 controls recruited from the Kyushu area for 11 single nucleotide polymorphism (SNP) markers distributed in the *SLC1A2* region using the direct sequencing and pyrosequencing methods, and examined allele, genotype and haplotype association with schizophrenia.The positive finding observed in the Kyushu samples was re-examined using 100 Japanese schizophrenics and 100 controls recruited from the Aichi area.

Results: We found significant differences in genotype and allele frequencies of SNP2 between cases and controls (P = 0.013 and 0.008, respectively). After Bonferroni corrections, the two significant differences disappeared. We tested haplotype associations for all possible combinations of SNP pairs. SNP2 showed significant haplotype associations with the disease ($P = 9.4 \times 10^{-5}$, P = 0.0052 with Bonferroni correction, at the lowest) in 8 combinations. Moreover, the significant haplotype association of SNP2-SNP7 was replicated in the cumulative analysis of our two sample sets.

Conclusion: We concluded that at least one susceptibility locus for schizophrenia is probably located within or nearby *SLC1A2* in the Japanese population.

Background

Schizophrenia is a severe mental disorder characterized by hallucinations, delusions, disorganized thoughts, and various cognitive impairments. The life-time prevalence is about 1%, and genetic factors were known to play a critical role in its pathogenesis [1]. Based on the fact that

phencyclidine (PCP) induces schizophreniform psychosis, a glutamatergic dysfunction hypothesis has been proposed for the pathogenesis of schizophrenia [2-4]. This hypothesis has been supported by recent multiple reports of association of schizophrenia with glutamate receptor genes and with the genes related to glutamatergic transmission, such as *G72* and *NRG1* [5-10].

In addition, other synaptic elements related to glutamate, such as excitatory amino acid transporters (EAATs), also potentially affect glutamatergic neurotransmission. EAATs maintain extracellular glutamate concentrations within physiological levels by reuptaking the synaptically released glutamate. A deficient uptake has been implicated in the pathogenesis of ischemic brain damage [11] and may be involved in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) [12]. Recently significant increases of mRNA expression of EAAT1 and EAAT2 have been reported in the thalamus of schizophrenics, suggesting the possibility that an excessive glutamate uptake is involved in schizophrenia [13]. On the other hand, a significant decrease of EAAT2 mRNA expression was observed in the parahippocampal gyrus of schizophrenics [14]. Therefore the EAAT genes are reasonable candidates for schizophrenia, as well as glutamate receptor genes.

The EAATs family consists of five members (EAAT1-EAAT5). Their cellular localizations are different: EAAT1 and EAAT2 are astroglial, whereas EAAT3 EAAT4 and EAAT5 are neuronal [25]. Since EAAT2 accounts for approximately 90% of glutamate reuptake in the rodent forebrain [16,17], we focused on the EAAT2 gene (*SLC1A2*) in association studies of schizophrenia. *SLC1A2* has been mapped to 11p13-p12 [18] and consists of 11 exons spanning over 165 kb. In this study we tested associations of schizophrenia with 11 SNPs distributed in *SLC1A2* with an average interval of 15.9 kb. To enhance the detection power of the study, we also examined the haplotype associations of the SNPs with the disease.

Methods

Human subjects

Blood samples were obtained from unrelated Japanese individuals who had provided written informed consent. We used two Japanese sample sets in this study. In the first one, Kyushu samples, 100 schizophrenia patients (mean age 49.5; 44.0% female) were recruited from hospital in the Fukuoka and Oita areas and 100 healthy unrelated controls (mean age 51.2; 44.0% female) were recruited from the Fukuoka area. In the initial SNP selection process, we used another 16 Japanese samples which are recruited in the Fukuoka area and informed in the same way. In the second one, Aichi samples, 100 schizophrenia patients (mean age 34.4; 44% female) and 100 healthy unrelated controls (mean age 39.9; 45% female) were collected in the Aichi area about 600 km east of Fukuoka. All patients fulfilled the DSM-IV criteria for schizophrenia [19]. All of the case and control samples are ethnically Japanese. DNA samples were purified from whole peripheral blood by the method previously described [20]. This study was approved by the Ethics Committee of Kyushu University, Faculty of Medicine and the Fujita Health University Ethics Committee.

SNP selection in the SLC1A2 region

We retrieved the primary SNP information from the dbSNP database http://www.ncbi.nlm.nih.gov/SNP/. Assuming the same size of the half length of linkage disequilibrium (LD) (60 kb) as reported in Caucasians [21], we initially intended to select common SNPs every 50 kb in *SLC1A2*. We tested 22 candidate SNPs including all of the exonic SNPs, in the 16 healthy Japanese samples by the direct sequencing method. Out of the 22 SNPs we selected the following 7 common SNPs with minor allele frequencies over 10% for further analyses: SNP1, rs1923295; SNP3, rs4534557; SNP6, rs1885343; SNP8, rs752949; SNP9, rs1042113; SNP10, rs3838796; SNP11, rs1570216. We also identified a novel SNP, SNP7, in intron 1 (conting location: 34105026). After the LD analyses described below, we noticed LD gaps ($D' < 0.3$) of the initial SNP set and examined additional 20 candidate SNPs. Out of the 20 SNPs, we selected the following 3 SNPs to fill the LD gaps: SNP2, rs4755404; SNP4, rs4756224; SNP5, rs1923298. The locations of the total 11 SNPs are shown in Figure 1.

Genotyping

Eleven SNPs were amplified as 11 individual fragments by PCR using the primers shown in Table 1 - additional file 1. The reaction mixture for PCR was prepared in a total volume 10 μl with 5 ng of genomic DNA, 10 pmol of each primer (4 pmol of SNP3), 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP and 0.25 U of *Taq* DNA polymerase. An initial denaturing step of 1 min at 94°C was followed by 30, 35 or 40 cycles of 94°C for 30 sec, appropriate annealing temperature for 30 sec and 72°C for 30 sec. A final extension step was carried out at 72°C for 7 min. The nucleotide sequences of each primer, PCR conditions and genotyping methods for each SNP are shown in Table 1 - additional file 1. We genotyped SNP3 by pyrosequencing analysis on a PSQ™96MA Pyrosequencer according to the manufacturer's specifications with a biotinylated reverse primer (5'-CGCCTACTCCTGGTGACTTC-3'), and the sequencing primer (5'-CGCCCCCATGTGT-3'). The other 10 SNPs were genotyped by direct sequencing, as previously described [7]. The raw data of direct sequencing were compiled on PolyPhred [22].

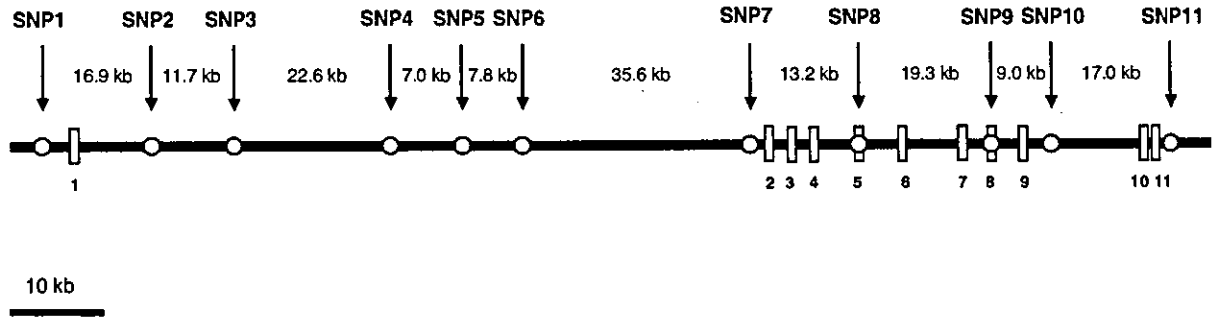

Figure 1
Genomic organization of *SLC1A2* and locations of the SNPs. Exons are shown as vertical bars with exon numbers. Eleven SNPs are indicated by circles. Distances between the SNPs are indicated above with kb.

Statistical analyses

To control genotyping errors, Hardy-Weinberg equilibrium (HWE) was checked in the control samples by the χ^2-test (d.f. = 1). We evaluated the statistical differences in genotype and allele frequencies between cases and controls by the χ^2-test (d.f. = 2) and the Fisher's exact probability test (d.f. = 1), respectively. The magnitude of LD was evaluated in D' and r^2 using the haplotype frequencies estimated by the EH program, version 1.14 [23]. Statistical analysis of the haplotype association was carried out as previously described [24]. The significance level for all statistical tests was 0.05.

Results

Genotyping and SNP association analysis

We selected 11 SNPs at average interval of 15.9 kb to cover the entire *SLC1A2* region with LD as described in Materials and Methods. Table 2 - additional file 2. shows the results of genotype and allele frequencies of SNPs in case and control samples. No significant deviation from HWE in control samples was observed (data not shown). SNP2 showed significant differences in genotype (P = 0.013) and allele (P = 0.008) frequencies between cases and controls. After Bonferroni corrections, these two P values became non-significance levels (P_{corr} = 0.143, P_{corr} = 0.088, respectively).

Pairwise linkage disequilibrium and haplotype association analyses

We compared the magnitude of LD for all possible pairs of the 11 SNPs in controls by calculating D' and r^2 (Table 3 - additional file 3., upper diagonal), because LD around common alleles can be measured with a modest sample size of 40–50 individuals to a precision equal to 10–20% of the asymptotic limit [19]. We observed relatively strong LD ($D' > 0.8$) in the seven combinations: SNP4-SNP5 (D' = 0.800), SNP7-SNP8 (D' = 0.877), SNP8-SNP9 (D' = 0.925), SNP4-SNP11 (D' = 0.838), SNP5-SNP11(D' = 0.999), SNP7-SNP11 (D' = 0.816), SNP9-SNP11 (D' = 0.819). Modest LD ($D' > 0.4$) was observed in the combinations of adjacent SNPs except for SNP5-SNP6 (D' = 0.286) in the control samples. However, modest LD was detected in cases in the SNP5-SNP6 combination (D' = 0.497).

We constructed pairwise haplotypes for all of the 55 possible SNP pairs (Table 3 - additional file 3., lower diagonal). We observed significant associations with schizophrenia in eight combinations: SNP2-SNP3 (P = 0.0021), SNP2-SNP4 (P = 0.0274), SNP2-SNP5 (P = 0.0054), SNP2-SNP6 (P = 0.0178), SNP2-SNP7 ($P = 9.4 \times 10^{-5}$), SNP2-SNP9 (P = 0.0354), SNP2-SNP10 (P = 0.0089) and SNP2-SNP11 (P = 0.0216). The combination of SNP2-SNP7 was the only one remained significant after Bonferroni correction (P_{corr} = 0.0052).

Cumulative analysis using the second sample set

In this study, we detected significant associations of one haplotype in the *SLC1A2* region with schizophrenia in the Kyushu samples. To confirm the positive finding, we investigated the second Japanese sample set recruited from the Aichi area. Although significant association of the disease was observed with neither genotype, allele frequencies of SNP2 (P = 0.195, P = 0.178, respectively), nor haplotypes of SNP2-SNP7 (P = 0.084) in the second sample set, the significant haplotype association of SNP2-

SNP7 was replicated in the cumulative analysis including the two sample sets ($P = 5.0 \times 10^{-4}$) (Table 4 - additional file 4.).

Discussion

SLC1A2 is located on the chromosomal region of 11p13-p12, to which no evidence has been reported for linkage of schizophrenia, [25,26]. However, there is still a possibility that *SLC1A2* is a candidate for schizophrenia susceptibility genes, because linkage studies could only detect genes with the large genotype relative risk [27]. We carried out the genotyping of 100 cases and 100 controls for 11 SNPs, which were selected to cover the entire *SLC1A2* region with LD. Since minor allele frequencies of each SNP we tested ranges from 0.220 to 0.485, the expected detection power of our case-control study is from 0.89 to 0.94 for the susceptibility gene assuming 2 for genotype relative risk [28].

Modest LD ($D' = 0.925 \sim 0.409$) was observed in the combinations of neighboring SNPs except for SNP5-SNP6 ($D' = 0.286$) in the control samples, suggesting that there may be a recombination hot spot present in the small region (7.8 kb) between the two SNPs (Table 3 - additional file 3.). We plotted the magnitude of LD with the physical distance for each pair of the SNPs, and estimated the average half-length of LD to be 31.8 kb by assuming a linear regression (Fig. 2). This is approximately half of the previously estimated size 60 kb in a United States population of north-European descent [21].

Significant associations of schizophrenia with genotype ($P = 0.013$) and allele ($P = 0.008$) frequencies of SNP2 (rs4755404) were detected (Table 2 - additional file 2.). However, none of these "single-marker" associations survived after Bonferroni corrections. An A-G transition in codon 206, causing a substitution of serine for asparagine, was identified in the exon 5 of *SLC1A2* in a heterozygous sporadic ALS patient [29]. Since located in a putative glycosylation site, the nonsynonymous SNP is potentially involved in the pathophysiology of schizophrenia through affecting the glycosylation status and the transport activity of *SLC1A2* [30]. No occurrence of the G allele of the SNP in 124 Italian schizophrenic and 50 control subjects has been reported [30]. We found also only A allele of the SNP in the 100 controls and 100 cases of the Kyushu samples (data not shown).

In pairwise haplotype association analyses, SNP2 consistently showed significant haplotype associations. The *P* value of the combination SNP2-SNP7 was still significant even after Bonferroni correction ($P = 9.4 \times 10^{-5}$, $P_{corr} = 0.0052$). In our second sample set, the Aichi sample, no significant association of SNP2 was observed in any of the analyses of genotypes, alleles and haplotypes. Cumulative

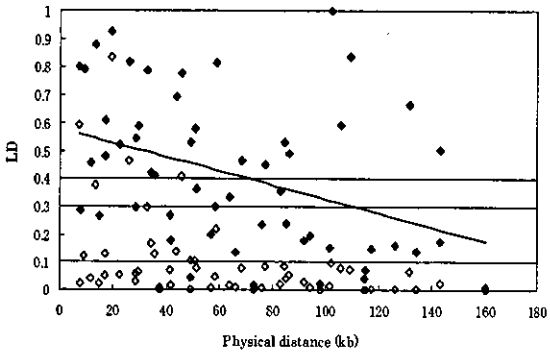

Figure 2
A plot of pairwise linkage disequilibrium (LD) vs. physical distance between the SNPs in the *SLC1A2* region. *D'* were plotted with filled diamonds, and r^2 with open diamonds. From the regression line, the half-length of LD was estimated to be 31.8 kb in the *SLC1A2* region.

analyses of the two sample sets, however, provide the replication of the significant haplotype association of SNP2-SNP7 with schizophrenia ($P = 5.0 \times 10^{-4}$). The frequency of the G-C haplotype in schizophrenics (26.6%) was notably higher than in controls (5.6%), suggesting that the G-C haplotype may be a risk haplotype for schizophrenia. We observed that the G-C haplotype frequency of schizophrenics (20.0%) was only slightly higher than controls (14.2%) in the Aichi sample, suggesting a less contribution of this locus on schizophrenia pathogenesis in the Aichi sample, although no apparent difference in clinical subtypes between both sample sets studied in this paper. The positive association reported here needs to be validated in larger sample sets, and it would also be worthwhile to search for functional SNPs in the region spanning SNP2-SNP7.

Conclusion

We concluded that at least one susceptibility locus for schizophrenia is probably located within or nearby *SLC1A2* in the Japanese population.

Competing interests

None declared.

List of abbreviations used

SNP; single nucleotide polymorphism

DSM-IV; dianostic and statistical manual of mental disorders, 4th edn

PCR; polymerase chain reaction

HWE; Hardy-Weinberg equilibrium

LD; linkage disequilibrium

EAAT; excitatory amino acid transporter

Authors' contributions

XD carried out genotyping, statistical analyses and drafted the manuscript: HS participated in design of this study and statistical analyses: HN, NT, NI and NO participated in collecting specimens and clinical data: YF conceived of the study and participated in its design and coordination.

Additional material

Additional file 1

Table 1

PCR primers for genotyping of SNPs in SLC1A2.

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Additional file 2

Table 2

Genotype and allele frequencies of SNPs in SLC1A2 in Kyushu samples.

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Additional file 3

Table 3

Pairwise linkage disequilibrium and haplotype association in SLC1A2.

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Additional file 4

Table 4

Association analysis of the SNP2-SNP7 haplotype using two sample sets.

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Mitochondrial DNA 3644T→C mutation associated with bipolar disorder

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Abstract

Mitochondrial dysfunction associated with mutant mitochondrial DNA (mtDNA) has been suggested in bipolar disorder, and comorbidity with neurodegenerative diseases was often noted. We examined the entire sequence of mtDNA in six subjects with bipolar disorder having comorbid somatic symptoms suggestive of mitochondrial disorders and found several uncharacterized homoplasmic nonsynonymous nucleotide substitutions of mtDNA. Of these, 3644C was found in 5 of 199 patients with bipolar disorder but in none of 258 controls ($p = 0.015$). The association was significant in the extended samples [bipolar disorder, 9/630 (1.43%); controls, 1/734 (0.14%); $p = 0.007$]. On the other hand, only 5 of 25 family members with this mutation developed bipolar disorder, of which 4 patients with 3644C had comorbid physical symptoms. The 3644T→C mutation converts amino acid 113, valine, to alanine in the NADH-ubiquinone dehydrogenase subunit I, a subunit of complex I, and 113 valine is well conserved from *Drosophila* to 61 mammalian species. Using transmitochondrial cybrids, 3644T→C was shown to decrease mitochondrial membrane potential and complex I activity compared with haplogroup-matched controls. According to human mitochondrial genome polymorphism databases, 3644C was not found in centenarians but was found in 3% of patients with Alzheimer disease and 2% with Parkinson disease. The result of modest functional impairment caused by 3644T→C suggests that this mutation could increase the risk for bipolar disorder.

Keywords: Bipolar disorder; MtDNA 3644T→C; Association study; Mitochondrial membrane potential; Complex I activity

Bipolar disorder is a major mental disorder characterized by recurrent manic and depressive episodes affecting about 1% of the population. The contribution of multiple genetic factors in the etiology of bipolar disorder is known from studies of twins, adoptions, and families. Although recent

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studies suggested several candidate polymorphisms, such as Val 311 of the brain-derived neurotrophic factor [1,2] and the −116G polymorphism of X-box binding protein 1 [3], the pathophysiological mechanisms of bipolar disorder have not yet been totally elucidated. Mitochondrial dysfunction in bipolar disorder was initially suggested by altered brain energy metabolism detected by ^{31}P magnetic resonance spectroscopy [4] and was recently supported by the altered gene expressions of mitochondria-related genes revealed by DNA microarray analysis in the postmortem brain [5]. The comorbidity of bipolar disorder or depression and a mitochondrial disorder, chronic-progressive external ophthalmoplegia (CPEO) [6–8], also suggests that mitochondrial dysfunction can cause bipolar disorder. It was pointed out that some families of bipolar disorder were seen in the maternal lineage [9], suggesting that mitochondrial DNA may have a pathophysiological role in bipolar disorder. The authors previously reported an association between bipolar disorder and two mitochondrial DNA (mtDNA) polymorphisms, 5178C and 10398A, in Japanese subjects [10]. A similar trend of association with 10398A was also reported in Caucasians [11]. These two polymorphisms convert amino acids in the subunits of complex I (NADH:ubiquinone oxidoreductase). NDUFV2, a nuclear-encoded complex I subunit gene, was also associated with bipolar disorder [12]. These results suggest that other genetic variations of complex I subunits in mtDNA are also risk factors for bipolar disorder.

Human mtDNA is inherited only maternally and encodes 13 protein subunits of the respiratory chain, including 7 complex I subunit genes, 22 tRNAs, and rRNAs [13]. It has been reported [14] that heteroplasmic tRNA mutations of mtDNA are related to neuromuscular diseases such as mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), and myoclonus epilepsy with ragged-red fibers. Large-scale deletions are related to CPEO. On the other hand, there are missense mutations of mtDNA related to diseases, such as neurogenic muscle weakness, ataxia, and retinitis pigmentosa; Leigh encephalopathy; and Leber hereditary optic neuropathy (LHON). Most are heteroplasmic, a mixture of mutant and wild-type mtDNA, but sometimes these mutations can be homoplasmic in patients. The homoplasmic mutation of 1555A→G in the rRNA coding region related to inherited hearing loss caused by aminoglycoside toxicity is well described [15,16]. Alterations in mtDNA have also been studied in patients with Parkinson disease and Alzheimer disease [17,18]. The phenotypes of mitochondrial diseases are diverse and overlapping. The same mtDNA mutation can produce quite different phenotypes, while different mutations can produce similar phenotypes. The mutations or polymorphisms associated with bipolar disorder, if any, may also cause overlapping phenotypes and become a risk factor for other disorders.

In this study, we hypothesized that there are some homoplasmic mutations or polymorphisms increasing the risk for bipolar disorder and other signs and symptoms related to mitochondrial impairment. To identify such nucleotide substitutions of mtDNA, we sequenced the entire 16.6-kb mtDNA of patients with comorbidity of bipolar disorder and somatic symptoms frequently associated with mitochondrial disorders. Among newly identified nonsynonymous nucleotide substitutions in these patients, the 3644T→C at NADH-ubiquinone dehydrogenase subunit I (ND1), decreasing mitochondrial membrane potential and complex I activity, was associated with bipolar disorder. The comorbidity with bipolar disorder was present in most of these cases but their phenotypes were various. It was suggested that this mutation could increase risks for bipolar disorder with syndromic comorbidity.

Results and discussion

Unreported homoplasmic mtDNA base substitutions in patients

We examined the entire mtDNA sequence of six patients with bipolar disorder and somatic symptoms suggestive of mitochondrial disorders, such as ptosis, optic neuropathy, cardiomyopathy, and myoclonus (Table 1). None of them could be diagnosed as known mitochondrial diseases, such as MELAS, CPEO, and LHON, because of the reasons as described under Case reports. Five of them had a family history of mood disorder compatible with maternal inheritance. Every patient had several base substitutions compared with the revised Cambridge Reference Sequence [13,19]. The average number of base substitutions in each individual was 32.5 ± 6.9 (mean ± SD), and that of nonsynonymous base substitutions was 5.5 ± 2.1. We consulted the MITOMAP database (http://www.mitomap.org/) [20,21], and two mutations were provisionally reported in relation to mitochondrial diseases, 11084A→G (MELAS) and 12311T→C (CPEO). We also found four nonsynonymous nucleotide substitutions, 3644T→C, 4705T→C, 13651A→G, and 13928G→T, which were not registered in the MITOMAP, all of which were in the complex I subunits. We confirmed that these base substitutions were homoplasmic by the PCR restriction-length polymorphism method (PCR-RFLP).

To identify the mtDNA base substitutions having pathophysiological significance, we examined whether these base substitutions were found in 96 Japanese centenarians using the mtSNP database (Human Mitochondrial Genome Polymorphism Database in Japan, http://www.giib.or.jp/mtsnp/index_e.html) [22]. We regarded the base substitutions found in centenarians as having minimum pathophysiological significance. Base substitutions 4705T→C, 11084A→G, 12311T→C, and 13651A→G were found in centenarians, while two base substitutions, 3364T→C and 13928G→T, were not found in centenarians.

Table 1
Patients and unreported nucleotide substitutions of mitochondria DNA

Case	Diagnosis	Gender	Age at onset	Clinical manifestations		MtDNA substitutions	
				Physical symptoms	Family history	Unreported	Provisionally disease related
1	Bipolar I disorder	F	17	Optic neuritis	Mo, depression	13651A→G	
2	Bipolar I disorder	M	30	Cerebral infarction Dilated cardiomyopathy	Bro, bipolar disorder MoSib, psychotic NOS		
3	Bipolar I disorder	M	50	Ptosis Epilepsy Cardiac arrhythmia	MoSib, depression		12311T→C (CPEO)
4	Bipolar I disorder	F	24	Epileptic EEG	Bro, bipolar disorder Sis, NOS		11084A→G (MELAS)
5	Bipolar I disorder	M	57	Ptosis Muscle weakness NIDDM Multiple cerebral infarction	Sporadic	3644T→C	
6	Bipolar I disorder	M	35	Ptosis	Sib, depression	4705T→C 13928G→T	

Abbreviations: Mo, mother; Bro, brother; Sis, sister; Sib, sibling; MoSib, mother's sibling; psychotic NOS, psychotic disorder not otherwise specified.

Association study of mtDNA base substitutions

To know whether these two base substitutions, 3644T→C and 13928G→T, are associated with bipolar disorder, we used two sets of the study subjects. The initial association study consisted of 199 patients with bipolar disorder and 258 healthy volunteers. An additional independent sample set in COSMO (Collaborative Study of Mood Disorder) consisted of 431 patients with bipolar disorder and 476 healthy volunteers, was also used. To examine whether there is a hidden population structure, we performed stratification analysis on the initial samples using eight polymorphisms [3] using the method of Pritchard et al. [23], and no subpopulation was found for either patients or controls. We performed a similar stratification analysis using 20 SNPs in 169 Japanese samples, including COSMO samples, and found no subpopulation. We further analyzed the stratification in 169 Japanese samples using 374 microsatellite markers and found no hidden subpopulation (Yamada et al., manuscript in preparation). Thus, we concluded that there is no hidden subpopulation in our Japanese samples. Six patients examined for the entire mtDNA sequence were included in the first sample set, because they developed comorbid somatic symptoms after the diagnosis of bipolar disorder.

We genotyped at 3644 and 13928 by PCR-RFLP in the initial sample set (Table 2). Base 3644C was found in 5 of 199 Japanese patients with bipolar disorder in the first sample set, including the proband (case 5 in Table 1, II-1 of family A in Fig. 1), but in none of the controls ($p = 0.015$) (Table 2). Among other 4 patients, 1 had non-insulin-dependent diabetes mellitus (NIDDM), 1 had headache, and 1 had tremor suggestive of neurological impairment. In their family members, only 5 of 25 members in the same maternal lineages, who were assumed to have the same genotype, 3644C, developed

Table 2
Association study using independent sample sets and haplogroups

Base at 3644:	All samples					Haplogroup D (5178A/10398G)				
	T		C		*p* value	T		C		*p* value
Initial sample set										
Patients	97.5%	(194)	2.5%	(5)		94.4%	(68)	5.6%	(4)	
Controls	100.0%	(258)	0.0%	(0)	0.015*	100.0%	(97)	0.0%	(0)	0.003*
Independent sample set										
Patients	99.1%	(427)	0.9%	(4)		98.3%	(171)	1.7%	(3)	
Controls	99.8%	(475)	0.2%	(1)	0.197	100.0%	(192)	0.0%	(0)	0.106
Total sample set										
Patients	98.6%	(621)	1.4%	(9)		97.2%	(239)	2.8%	(7)	
Controls	99.9%	(733)	0.1%	(1)	0.007*	100.0%	(289)	0.0%	(0)	0.004*

Each number in parentheses shows the real number of subjects. The *p* value was given by Fisher's exact test.
* Statistically significant.

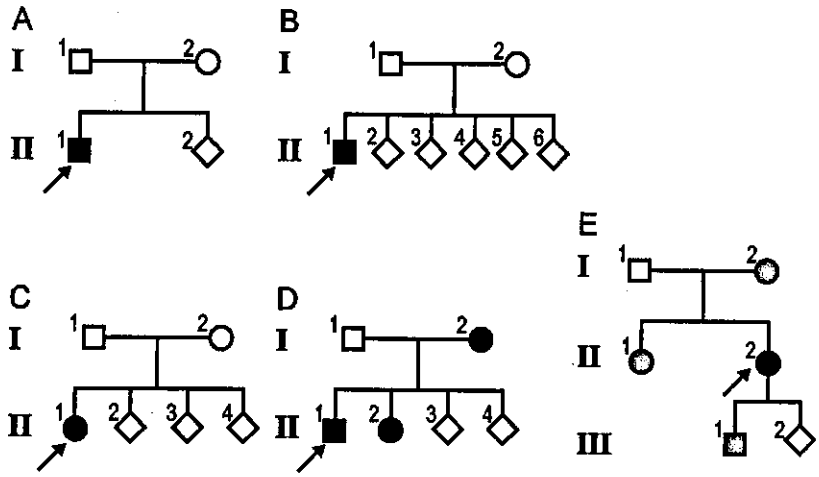

Fig. 1. Pedigrees of the probands with bipolar disorder and mitochondrial 3644C mutation. Arrows indicate the probands with bipolar disorder. Closed squares and circles indicate the patients with bipolar disorder or other mental disorders as follows: D, I-2 had a psychotic disorder not otherwise specified, II-2 had schizotypal personality disorder. Their comorbidities were as follows: A, II-1 had muscle weakness, ptosis, and NIDDM (case 5 in Table 1); C, II-1, had essential tremor; D, II-1, had NIDDM; E, I-2, II-1, II-2, and III-1 had headache. To maintain the anonymity of the pedigrees, the sexes of the unaffected siblings are not shown.

bipolar disorder, of which 4 patients with 3644C had comorbid physical symptoms and one had only bipolar disorder. Mutation 3644T→C converts amino acid 113 valine in the putative third transmembrane region of ND1, the protein subunit of complex I, to alanine. This 113 valine is well conserved from *Drosophila* to 61 mammalian species. There was no difference in the frequency of 13928G→T [13 of 199 patients with bipolar disorder (6.5%) and 19 of 258 controls (7.4%), $p = 0.804$ by Fisher's exact test]. Mutation 13928G→T changes the 531 serine into isoleucine in the ND5 subunit and it was not conserved even among mammalian species.

We further analyzed 3644T→C as a candidate risk factor for bipolar disorder using the independent sample set obtained from COSMO. While 4 additional individuals having 3644C were found among the patients, only 1 of the 476 controls had 3644C. Although this difference in frequency was not statistically significant ($p = 0.197$), this is likely due to the low statistical power to detect the difference (0.29). In the analysis of total samples having higher statistical power (0.79), 3644C was significantly more common in bipolar disorder than in the controls ($p = 0.007$) (Table 2).

Since mtDNA is highly polymorphic, other polymorphisms possibly confounded the association analysis. To minimize the effects of other polymorphisms, we categorized these samples into mitochondrial DNA haplogroups and the association analysis was repeated in each haplogroup. Seven patients with 3644C were assigned to the Asian haplogroup D characterized by 5178A/10398G [22], which we reported as an anti-risk haplotype for bipolar disorder [10]. The 3644C was significantly associated with bipolar disorder in haplogroup D (Table 2). On the other hand, only 1 control subject and 2 patients with 3644C were classified into haplogroup M characterized by 5178C/10398G, and no association was found in haplogroup M [2 of 187 patients (1.1%) and 1 of 233 controls (0.4%), $p = 0.588$].

We concluded that 3644C was associated with bipolar disorder for the following reasons: 3644T→C was associated with bipolar disorder in the initial case-control study; this substitution converts well-conserved amino acid 113 valine to alanine in ND1. A similar trend was observed in the independent samples, although there was no significant difference, possibly due to the small number of subjects replicating the association. In the analysis of the total sample set having enough statistical power to detect a difference, 3644C was significantly associated with bipolar disorder. The significant association between 3644C and bipolar disorder remained in haplogroup-matched case-control analysis.

We called 3644C a "mutation," because its frequency was very low (0.14% in 734 controls and 0.7% in 1364 total samples examined), it converted a well-conserved amino acid, and it appeared in at least two independent haplogroups. However, this mutation is not sufficient to cause bipolar disorder because 3644C was found in 1 healthy volunteer, and only 5 of 25 members in the same maternal lineages, all of whom were assumed to have 3644C, developed bipolar disorder. Among these patients, comorbidity in 4 patients with bipolar disorder was heterogeneous: 2 had NIDDM, 1 headache, and 1 tremor suggestive of neurological impairment. The other patient had only bipolar disorder. It means that 3644C cannot be a risk factor for comorbid symptoms seen in these patients but could be a risk factor for bipolar disorder, if not a causative mutation. Bipolar disorder is a multigenic disease and one type of mutation in mtDNA can cause various phenotypes. We

postulate that synergistic effects of other risk factors and 3644C could cause bipolar disorder.

Functional analyses in cybrids with 3644C

To evaluate the functional consequences of 3644T→C, we generated cell lines of the transmitochondrial hybrids, "cybrids," using the platelets derived from the subjects. Different from heteroplasmic mutations in the regions of tRNAs and protein subunits, functional impairment associated with homoplasmic mutation has not been well established. In the case of heteroplasmic mutation, two cybrid cell lines with different nucleotides at one particular position of mtDNA could be generated and analyzed. On the other hand, in the case of homoplasmic mutation, it was impossible to identify such a pair of cell lines. To minimize the effects of other polymorphisms, we compared cybrids with 3644C with haplogroup-matched controls for functional studies. A total of 24 cybrid cell lines were obtained from the initial sample set, and 9 cybrid cell lines belonged to haplogroup D, 5178A/10398G (Table 3). Among the 9 cell lines, only 2 were from patients with 3644C (II-1 in family D and II-2 in family E, in Fig. 1) and 7 were from subjects with 3644T (3 patients with bipolar disorder and 4 controls). We could not obtain other samples with 3644C because of ethical reasons.

Mitochondrial membrane potential (MMP) was measured using JC-1, a fluorescent cationic dye, which accumulates in mitochondria and changes its emission from wavelength 527 nm (monomer) to 590 nm (aggregates) depending on the mitochondrial membrane potential, and a fluorescence-activated cell sorter (FACS), and it distinguished well the difference between control cybrids and $\rho^0$206 cells lacking mtDNA: while 82.9 ± 9.9% (mean ± SD, N = 12) of the cybrids from control subjects were polarized, only 13.2 ± 7.7 (mean ± SE of three measurements) of the $\rho^0$206 cells were polarized (Fig. 2, left and right, respectively). This indicated that our measurement method is sensitive enough to detect the difference in MMP. The percentage of polarized cells was significantly decreased in cybrids with 3644C [51.7 ± 6.6 and 67.0 ± 4.3% (means ± SE), respectively] compared with haplogroup-matched cybrids (df = 8, p = 0.04 by Mann-Whitney U test) (Table 3). There was no significant difference between cybrids of bipolar disorder and controls nor between cybrids of other haplogroups.

Subsequently, the activities of complexes I (rotenone-insensitive), III, and IV in the electron-transport chain were measured using the citrate synthase activity as the reference (Table 4). The activity of $\rho^0$206 cells was measured to assess nonspecific activity. The 3644C group consisted of two cybrid cell lines. While there was no significant difference between complex III and complex IV activities ($p > 0.1$), complex I activity of the two cybrids with 3644C tended to be lower than four haplogroup-matched control cybrids (df = 5, p = 0.06 by Mann-Whitney U test). Decreased MMP could be explained by reduced complex I activity since MMP is maintained by the efflux of protons from the mitochondrial matrix, in which complex I plays an important role. MMP generated by the proton gradient is the driving force of not only ATP synthesis but also Ca^{2+} uptake across the mitochondrial inner membrane. We hypothesized that impaired mitochondrial Ca^{2+} uptake caused altered calcium signaling in bipolar disorder. Our result of decreased MMP in cybrids with 3644C supports our hypothesis.

Interestingly, the mtSNP database [22] showed that while 3644C was not found in 96 centenarians, it was found in 3.1% (3/96) of patients with Alzheimer disease and 2.0% (2/96) of patients with Parkinson disease. These findings suggested a possibility that 3644C is a risk factor common to bipolar disorder and neurodegenerative disorders, rather than a causative mutation only for bipolar disorder. If 3644C is also a risk factor for neurodegener-

Table 3
Mitochondrial membrane potential (MMP) of 24 cybrid cell lines

	N	Age	(C/B)	Gender	MMP
Diagnosis					
Control	12	48.0 ± 9.2		6/6	82.9 ± 9.9
Bipolar disorder	12	41.8 ±11.4		6/6	77.2 ±11.0
Bipolar disorder with 3644T	10	40.7 ±12.0		5/5	80.8 ± 7.0
Bipolar disorder with 3644C	2	42, 53		1/1	59.4 ±10.9*
Haplogroup					
10398A–5178C–3644T	7	43.6 ± 10.6	4/3	3/4	81.9 ± 8.6
10398G–5178C–3644T	8	41.6 ± 10.7	4/4	4/4	80.8 ± 9.5
10398G–5178A–3644T	7	48.9 ± 10.5	4/3	3/4	83.3 ± 8.5
10398G–5178A–3644C	2	42, 53	0/2	1/1	59.4 ±10.9**
ρ^0 cells	1				13.21

C/B, numbers of control/bipolar disorder; gender, number of men/women. The p value was given by the Mann–Whitney U test.
* p = 0.03 vs 3644T, 0.08 vs controls.
** p = 0.04 vs 3644T, 0.03 vs all other haplogroups.

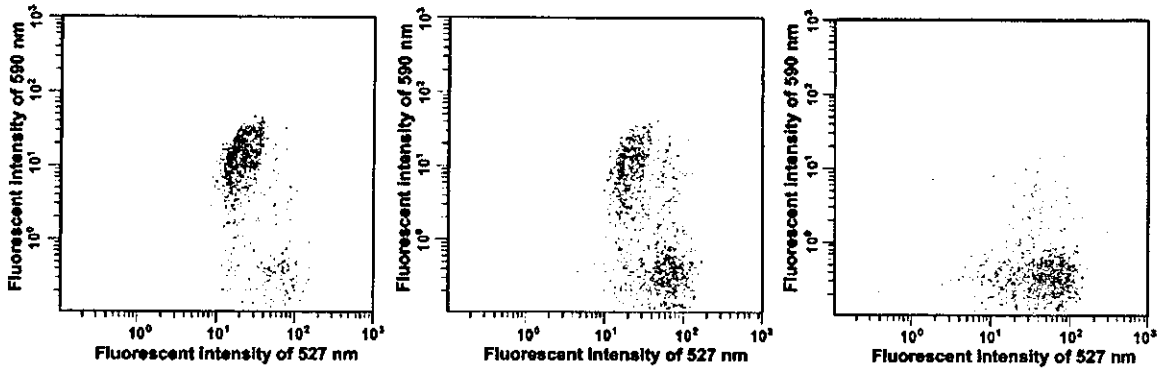

Fig. 2. Measurement of mitochondrial membrane potential using JC-1 and FACS. Vertical line, the fluorescence intensity of 590 nm, reflecting the aggregates and indicating high MMP. Horizontal line, the fluorescence intensity of 527 nm, reflecting the monomer and indicating low MMP. 10,000 cells were examined for one cell line. Representative results of one experiment each from three cell lines are shown. Left, control cybrids whose haplogroups were matched with the cybrids with 3644C; middle, cybrids with 3644C; right, $\rho^0$206 cells. While most control cybrids were polarized, having high 590/527 nm, most $\rho^0$206 cells were depolarized. The cybrids with 3644C were intermediate, having both polarized and depolarized cells.

ative disorders, the mechanism might be explained by a disruption of MMP that causes apoptosis. It is also compatible with the reduction of complex I activity in platelets or altered calcium signaling in cybrids derived from patients with Parkinson disease or Alzheimer disease [24–26]. Neuropathological studies of bipolar disorder also showed a decreased number of neurons in postmortem brains [27,28]. It was pointed out that having bipolar disorder increases the risk of Alzheimer disease [29,30] and Parkinson disease [31]. Two mood stabilizers, lithium and valproate, are known to have antiapoptotic effects by increasing Bcl-2 [32]. These findings are also compatible with the possibility that 3644C is a risk factor common to bipolar disorder and neurodegenerative disorders.

One might have a concern that 3644C is not a risk factor for bipolar disorder but associated with physical symptoms. Although the initial patient had several physical symptoms suggestive of mitochondrial disorder such as ptosis, muscle weakness, NIDDM, and cerebral infarction, other patients carrying 3644C had no or one nonspecific comorbid symptom. Thus, the apparent association between bipolar disorder and 3644C cannot be explained by the secondary phenomenon due to physical symptoms. However, it cannot be ruled out that these patients carrying 3644C have some subtle mitochondria-related symptoms that were not clinically apparent. In fact, there are reports of patients with pathogenic mtDNA mutations such as 3243A→G who showed psychotic symptoms at first and developed mitochondrial diseases later [33,34]. It might be possible that detailed physical examinations, for example, glucose tolerance test or close neurological examinations, would reveal subtle comorbid somatic symptoms. Needless to say, we need to address whether the 3664C substitution is associated with somatic symptoms alone. In the future, it is needed to look carefully at the phenotype and the clinical course of these subjects and investigate whether 3644C is associated with bipolar disorder or a bipolar disorder-somatic symptom subtype.

Functional impairment was reported also in the homoplasmic mutation, 1555A→G, in maternally inherited hearing loss [15,16]. The 11778A mutation of LHON, which is usually heteroplasmic but sometimes homoplasmic, was also shown to cause a modest reduction in complex I activity [35]. It was pointed out that the nuclear background potentially affects the expression of mtDNA polymorphisms [36]. Further study using cybrids with another nuclear background would be interesting. The mechanism of how the V113A amino acid substitution caused by 3644T→C in ND1 decreases complex I activity cannot be explained since the structure and function of each protein subunit are not yet well known. In particular, it remains unclear how complex I translocates protons across the mitochondrial inner membrane coupled to electron transfer. In summary, 3644T→C is a rare base substitution of mtDNA but induces modest impairment of complex I activity and becomes a risk factor for bipolar disorder.

Materials and methods

Subjects

Patients with bipolar disorder were diagnosed according to the DSM-III-R or DSM-IV criteria by at least two

Table 4
Enzyme activities of electron-transport chain of cybrids with 3644C and controls

	Control (mean ± SD), $N = 4$	3644C (mean ± SD), $N = 2$	ρ^0cells	p value*
Complex I/CS	14.47 ± 5.43	7.64 ± 0.08	4.82	0.06
Complex III/CS	32.15 ± 12.78	21.12 ± 2.72	7.36	0.36
Complex IV/CS	39.74 ± 11.24	29.21 ± 6.97	0.76	0.36

* The p value was calculated using the Mann–Whitney U test.