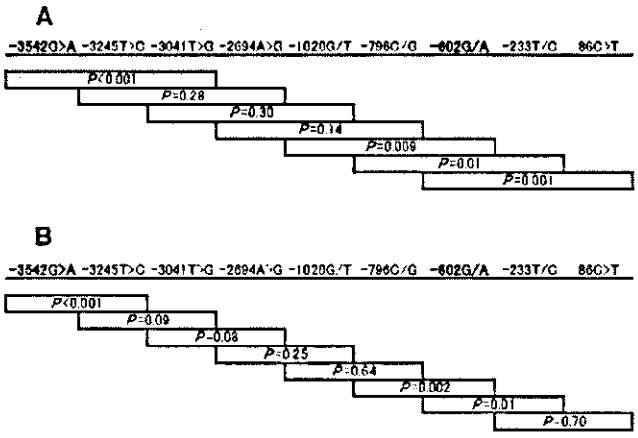

Figure 1. Haplotype associations in Japanese case control samples: **(a)** three-marker haplotype analysis; **(b)** two-marker haplotype analysis. *P* value indicates the global *P* analyzed using COCAPHASE program. Gray squares indicate statistically significant global *p* value.

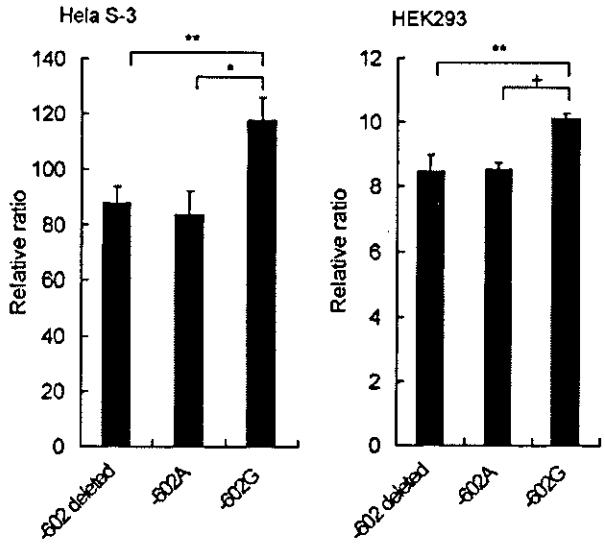

Figure 2. Promoter assay. Promoter activity of three kinds of reporter plasmids, having −602G or −602A and lacking the −602G> A site, were examined by the luciferase assay. Two kinds of cells, Hela-S3 and HEK293, were used. Four independent experiments were performed for each condition, and the mean and SEM values were presented. The transcription activity of the −602G construct was significantly higher than that of the −602A in both Hela-S3 and HEK293. The short construct lacking in the −602G> A site presented nearly equal activity of the −602A construct and had significantly smaller activity compared with −602G constructs. ** $p < .01$. * $p < .05$. + $p = .05$.

Sequencing of the PCR products was conducted using the BigDye terminator sequencing kit (Applied Biosystems) and an ABI 3700 DNA sequencer (Applied Biosystems).

Statistical Procedures

Deviations from Hardy–Weinberg equilibrium (HWE) were evaluated by use of the Arlequin program (http://anthropologie.Unige.Ch/arlequin/methods.html). Genotype distributions and allele frequencies between patients and control subjects were computed using Fisher's Exact Probability Test, which was applied using SPSS software (SPSS, Tokyo, Japan). For other analyses, UNPHASED programs (COCAPHASE and TDTPHASE; http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/) were used. The normalized LD coefficient D' and the squared correlation coefficient r^2 were calculated using COCAPHASE program. For TDT of NIMH trio samples, the McNemar Test was used. For the computation of haplotype frequencies, evaluation of haplotypic distributions, and TDT analysis of the multimarker haplotypes, the COCAPHASE and TDTPHASE programs were used. To evaluate the data appropriately, we reanalyzed the significant result using the permutation test implemented in COCAPHASE and TDTPHASE. Sequences were searched for potential transcription factor binding sites using the Match program (http://www.gene-regulation.de/).

Promoter Assay

A 1106-bp fragment (−1111 to −6) of the upstream from the initiation codon of the *NDUFV2* gene was amplified by PCR and cloned into the *MluI*/*BglII* site of pGL3-Basic vector (Promega, Madison, Wisconsin). Two kinds of reporter plasmids, having either −602G or −602A were prepared. A 586-bp fragment (−591 to −6) lacking the −602G> A site was also amplified and cloned into the same vector. Hela-S3 and HEK293 cells cultured in a 96-well plate were transfected using Superfect (Qiagen, Valencia, California) with .5 mg of the reporter plasmid, .05 mg of a reference plasmid (pRL-TK), and the pGL3-Basic vector carrying no insert. After 36 hours incubation, luciferase activities were measured with the aid of Dual-Glo luciferase assay system (Promega). Four independent experiments were performed for each condition, and the mean and SEM values were presented.

Results

We previously reported that −602G> A, among four polymorphisms (−796C> G, −795*T*> G, −602G> A, −233*T*> C) in the upstream region of *NDUFV2*, showed the significant association with BD (Washizuka et al 2003). In this study, four novel (−3542G> A, −3245*T*> C, −3041*T*> G, −2694A> G) and one known (−1020G> T) polymorphisms were detected by sequencing the upstream region extending up to 3963 bp of the transcription initiation site in the *NDUFV2*. We then genotyped these polymorphisms in Japanese patients with BD (n = 189) and control subjects (n = 222). The genotype frequencies of these five polymorphisms were in HWE in control subjects, although −3041*T*> C polymorphism was not in BDI patients, and −3245*T*> G was not in BDII patients. There was no significant difference of genotype frequencies of each SNP between male and female subjects. Detected polymorphisms in this study and in our previously reported polymorphisms (−796C> G, −795*T*> G, −602G> A, −233*T*> C, and 86C> T) were in strong linkage disequilibrium with each other (Table 1).

The genotype and allele distributions of the polymorphisms in Japanese population are shown in Table 2. The data for −602G> A polymorphism were cited from our previous paper (Washizuka et al 2003). Statistically significant differences in genotype distribution were observed between patients with BD and control subjects for −3041*T*> G (p = .001). The −3542G> A, −3245*T*> G, and −2694A> G polymorphisms showed significant genotypic association with BDII (p = .003, p = .002, and p = .006, respectively). These SNPs tended to be associated with BD or BDII even after Bonferroni correction. There was also a nominally significant difference in allelic distribution of −3041*T*> G polymorphism between patients with BDI and control subjects (p = .04).

Haplotype analysis consisting of all 10 polymorphisms revealed a statistically significant association in Japanese samples (global $p < .0001$). To explore which part of the *NDUFV2* gene

Table 3. Estimated Haplotype Frequencies of *NDUFV2* in Japanese Analyzed by Using the COCAPHASE Program

Haplotype[a]	Case	Frequency	Control	Frequency	χ^2	*P* Value	Common
A-A	244	.64	270	.61	.34	.55	+
G-A	3	.008	31	.07	20.35	<.0001	+
A-G	4	.01	37	.08	23.81	<.0001	+
G-G	127	.33	99	.22	10.79	.001	+
Global *P*						<.0001 (<.0001)[b]	

[a]Haplotypes of −3542G>A and −602G>A.
[b]The global *P* value in parentheses shows the global significance by permutation test.

contributes most to this overall association, we employed the sliding window approach in which each set of two or three consecutive polymorphisms were tested for association with BD (two- or three-marker haplotype analysis). This analysis showed evidence of association with BD in two limited regions around −3542G> A and −602G> A (most significant haplotype $p <$ .0001, global $p <$ .0001, and most significant haplotype $p = .008$, global $p = .001$, for the three-marker analysis, and global $p <$ .0001 and global $p = .002$, respectively, for the two-marker analysis; Figure 1).

Because these two SNPs were located at the putative promoter region, we supposed that these polymorphisms might alter the transcription activity. At the beginning, we prepared a 3983bp fragment containing those two polymorphisms and tried to ligate this fragment into the pGL3-basic vector; this was not successful, however. Then we examined whether the −602G> A polymorphism had functional significance. Based on our previous analysis indicating that the two major haplotypes, C-T-A-T and C-T-G-T (consisting of −796C> G, −795*T*> G, −602G> A, and −233*T*> C polymorphisms of *NDUFV2*) were associated with BD, constructs of these two haplotypes were analyzed. Promoter activity was examined in two cell lines, Hela-S3 and HEK293. The transcription activity of the −602G construct was significantly higher than that of the −602A both in Hela-S3 and HEK293 ($p = .03$ for Hela-S3, and $p = .05$ for HEK293). The short construct lacking in the −602G> A site presented nearly equal activity of the −602A construct but had significantly smaller activity compared with the −602G construct ($p = .0009$ and $p = .005$, respectively; Figure 2).

Because we could not experimentally examine the functional significance of the −3542G> A, we examined whether this site affects the putative binding sites of transcription factors using the Match program. The −3542G> A was predicted to be within the putative binding site for HSF (heat shock transcription factor). HSF1 is known to affect the expression of several other nuclear encoded mitochondrial complex I subunit genes (e.g., *NDUFB8, NDUFA10, NDUFAB1,* and *NDUFS1*). Recently, the binding sequence of HSF1 was well characterized (TTC[T/C][G/*A*]*GAANNT*-*TC*[T/*C*]; the bases similar to this site of *NDUFV2* promoter was italicized; Trinklein et al 2004). When the −3542 site is G, the core sequence of putative binding site for HSF1, GAA, is lost, and probability of binding was predicted to be decreased.

Thus, the frequency of haplotypes consisting of −3542G> A and −602G> A polymorphisms was also estimated. Distributions of haplotype frequencies differed significantly between patients with BD and control subjects (global $p <$ .0001) (Table 3). Among the haplotypes, the G-G haplotype was significantly more frequently seen in BD ($p = .001$), whereas G-A and A-G haplotypes were significantly less common in patients with BD compared with control subjects ($p <$ .0001). The results were basically similar when younger control subjects were excluded to match ages of the subjects.

We then performed a TDT in NIMH Genetics Initiative Bipolar Pedigrees. The distributions of genotypes of all 10 detected polymorphisms of the probands, fathers, and mothers were in HWE. We could not detect any allele that was significantly overtransmitted from patients to affected offspring in the NIMH trio samples of BD (Table 4).

We then examined the transmission of haplotypes consisting of −3542G> A and −602G> A polymorphisms from patients to affected offspring by using TDTPHASE program. We found significant association of the *NDUFV2* haplotypes with BD (global $p <$ .0001). Two haplotypes (G-A and A-G) tended to be undertransmitted in parents–proband trios of NIMH samples (nominal $p = .04$ and $p = .01$, respectively; Table 5).

Discussion

We identified four novel polymorphisms (−3542G> A, −3245*T*> G, −3041*T*> C, and −2694A> G) associated with BD in this study. Haplotype analysis revealed that two haplotype blocks surrounding the −3542G> A and −602G> A polymor-

Table 4. Transmission Disequilibrium Test in National Institute of Mental Health Initiative Bipolar Pedigrees

Polymorphism	Allele	Tr	Not Tr	Ratio	χ^2	*P*	Number of Trios
−3542G>A	G	20	27	.74	1.04	.30	85
−3245T>C	T	20	30	.67	2.00	.15	91
−3041T>G	T	39	44	.89	.30	.58	90
−2694A>G	A	19	26	.73	1.08	.29	81
−1020G>T	G	10	16	.63	1.38	.23	95
−796C>G	C	42	48	.88	.40	.52	94
−602G>A	G	31	24	1.29	.89	.34	98
−233T>C	T	43	50	.86	.52	.46	99
86C>T	C	34	25	1.36	1.37	.24	99

Tr, transmitted.

Table 5. Transmission of Haplotypes in National Institute of Mental Health Initiative Bipolar Pedigrees Analyzed by using TDTPHASE

Haplotype[a]	Tr	Frequency	Not Tr	Frequency	χ^2	*P* Value	Common
A-A	146	.84	137	.80	1.61	.20	+
G-A	<.01	<.01	3	.02	4.18	.04	+
A-G	<.01	<.01	4	.02	5.59	.01	+
G-G	26	.15	28	.16	.08	.76	+
Global *P*						.01 (.11)[b]	

[a]Haplotypes of −3542G>A and −602G>A.
[b]The global *P* value in parentheses shows the global significance by permutation test.

phisms were associated with BD. The haplotype of these two SNPs were significantly associated with BD in Japanese subjects. In NIMH trios, no individual SNP was associated, and the overtransmission of the risk haplotype in Japanese, G-G, was not observed. Although the observed trend of undertransmission of two haplotypes, G-A and A-G, might be due to the small number of trios, it is noteworthy that the trends of undertransmission of these two haplotypes seen in the NIMH bipolar trio samples were in the same direction to the significant decrease of these haplotypes in Japanese BD subjects.

Although the mechanism by which −602A> G changed the promoter activity is unknown, it would be of interest to note that the −602G polymorphism loses the putative binding site of a transcription factor, p300 (CCACTC). The finding that the −602G haplotype is more common in BD, although yielding a significantly higher promoter activity, is apparently inconsistent with our mitochondrial dysfunction hypothesis in BD; however, the direction of change of promoter activity in the luciferase assay cannot be directly compared with that in vivo. First, promoter activity can be affected by neighboring sequences because several transcription factors form a complex. When only a part of the promoter sequence is subcloned into the luciferase vector, as in this study, the promoter activity does not directly represent the activity in vivo. Second, regulation of gene expression is complex. Although we reported that mRNA expression of *NDUFV2* was decreased in the lymphoblastoid cells, Karry et al (2004) reported that the protein encoded by *NDUFV2* was up-regulated in autopsied BD brains. Thus, it cannot be concluded what kind of mitochondrial dysfunction is caused by polymorphisms of *NDUFV2* promoter. Even though the direction of the change may not represent the promoter activity in vivo, the results of promoter assay indicate that this region has some functional activity only when the −602 position is G.

We could not determine whether −3542G> A affects promoter activity, and this would be a worthwhile topic for future study. Because it has been reported that lithium enhances HSF1 activity (Carmichael et al 2002), it would be particularly interesting to examine the effects of HSF1.

It is most important to test whether the association of *NDUFV2* with BD is replicated using independent BD case–control or trio samples. In addition, 18p11 is a common linkage locus for BD and schizophrenia (Berrettini 2000; Lewis et al 2003), and mRNA and protein expression of *NDUFV2* is also altered in schizophrenia (Karry et al 2004). It would thus be interesting to examine the association between *NDUFV2* and schizophrenia.

In conclusion, the haplotypes consisting of −3542G> A and −602G> A polymorphisms in the upstream region of *NDUFV2* were associated with BD commonly in two ethnicities. Together with altered promoter activity, these findings indicate the role of *NDUFV2* as a genetic risk factor of bipolar disorder.

Data and biomaterials of the National Institute of Mental Health (NIMH) pedigrees were collected in four projects that participated in the NIMH Bipolar Disorder Genetics Initiative. From 1991 to 1998, the principal investigators and co-investigators were as follows: Indiana University, Indianapolis, Indiana, U01 MH46282, John Nurnberger, M.D., Ph.D., Marvin Miller, M.D., and Elizabeth Bowman, M.D.; Washington University, St. Louis, MO, U01 MH46280, Theodore Reich, M.D., Allison Goate, Ph.D., and John Rice, Ph.D.; Johns Hopkins University, Baltimore, Maryland U01 MH46274, J. Raymond DePaulo, Jr., M.D., Sylvia Simpson, M.D., MPH, and Colin Stine, Ph.D.; NIMH Intramural Research Program, Clinical Neurogenetics Branch, Bethesda, Maryland, Elliot Gershon, M.D., Diane Kazuba, B.A., and Elizabeth Maxwell, M.S.W.

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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.

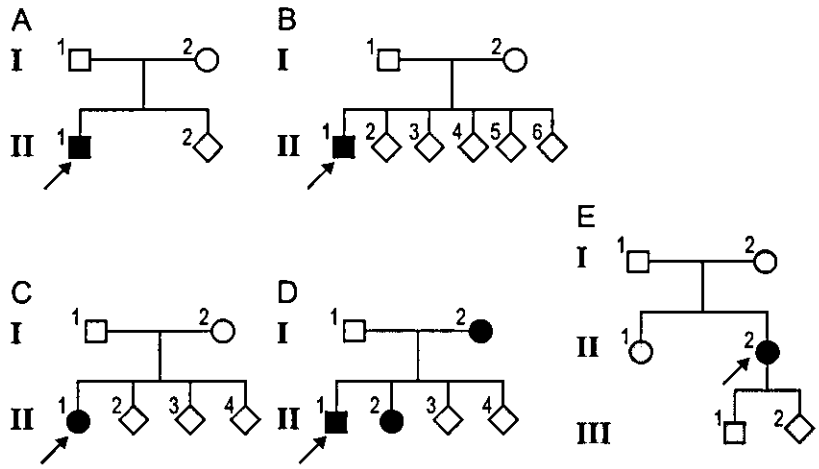

Fig. 1. Pedigrees of the probands with bipolar disorder and mitochondrial 3644C mutation. Arrows indicate the probands with bipolar disorder. Closed squares and circles indicate the patients with bipolar disorder or other mental disorders as follows: D, I-2 had a psychotic disorder not otherwise specified, 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.

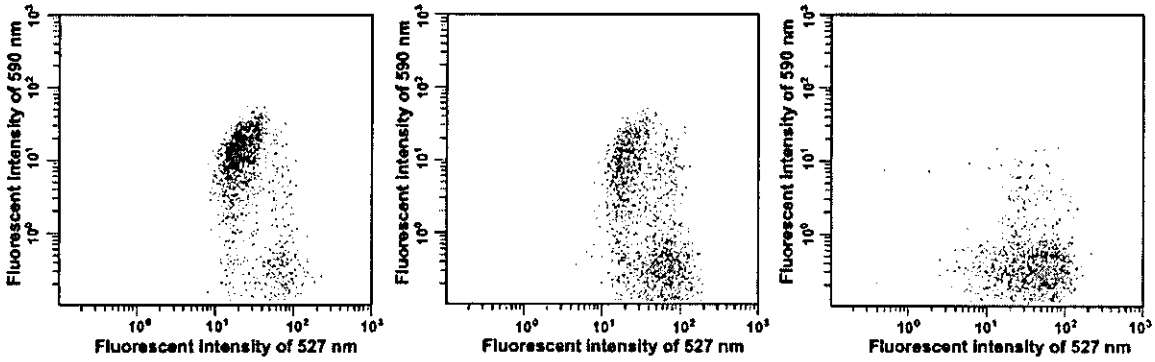

Fig. 2. Measurement of mitochondrial membrane potential using JC-1 and FACS. Vertical line, the fluorescence intensity of 590 nm, reflecting the aggregates and indicating high MMP. Horizontal line, the fluorescence intensity of 527 nm, reflecting the monomer and indicating low MMP. 10,000 cells were 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.

interview sessions by two senior psychiatrists and a consensus diagnosis was made. Their family history of mental disorder was assessed by interviewing the proband and available relatives. Control subjects were recruited from the staff or students of participating institutes and their friends, who reported themselves to be healthy. Written informed consent was obtained from all subjects. This study was approved by the ethics committees of RIKEN and all participating institutes.

The study subjects for the initial association study consisted of 199 patients with bipolar disorder (143 bipolar I and 56 bipolar II, 76 male and 123 female, 49.8 years of age on average) and 258 healthy volunteers (129 male and 129 female, 33.0 years of age on average). An additional independent sample set in COSMO consisted of 431 patients with bipolar disorder (214 male and 217 female, 49.5 years of age on average) and 476 healthy volunteers (226 male and 250 female, 50.4 years of age on average).

Six patients with bipolar disorder with somatic symptoms suggestive of mitochondrial disorders were chosen from the first sample set for examination of the entire mtDNA. They had been recruited in our bipolar disorder study based on our inclusion criteria, having DSM-IV bipolar disorder by consensus diagnoses after two nonstructured interview sessions with senior psychiatrists, and exclusion criteria, having no clinically remarkable neurological diseases, head trauma, or comorbid Axis II diagnoses. Characteristics of these six subjects are listed below and summarized in the Table 1.

The transmitochondrial cybrids for the following functional analyses were generated from 24 subjects in the initial samples, including two patients with 3644C.

Case reports

Case 1, 38-year-old female, is a patient with bipolar I disorder without psychotic features. She had the first episode of mania with psychomotor agitation and confusion at age 17. At age 27, she was admitted to a hospital due to bilateral optic neuritis. She had no other symptoms suggestive of multiple sclerosis. Her optic neuritis was improved by steroid therapy, and final diagnosis was idiopathic optic neuropathy. Because she had no relatives with optic neuropathy and her symptoms were reversible, Leber disease was not considered by the attendant ophthalmologist.

Case 2 is a 61-year-old male diagnosed as having bipolar I disorder. At age 30, he had the onset of mania with mood-incongruent psychotic features. At age 60, after being discharged from a psychiatric hospital, he was admitted to a hospital due to stroke. Brain imaging revealed infarctions in the cerebellum and the brain stem. During this hospitalization, chest X-ray showed enlarged heart and he was diagnosed as idiopathic dilated cardiomyopathy. He also had renal failure. His attendant physician did not suspect mitochondrial disease.

Case 3 is a 56-year-old male diagnosed with bipolar I disorder. At age 49, he had the onset of depression characterized by depressive mood, fatigability, retardation, insomnia, and suicidal thought. At age 52, he suddenly became manic. During this manic episode, he caused a motor vehicle accident. He was admitted to a psychiatric ward for the treatment of mania. After the first admission, he had generalized tonic clonic seizures. Although electroencephalography (EEG) recording showed no signs of epilepsy, he was clinically diagnosed as having epilepsy. At age 54, he complained of swollen eyelid, and medical examination did not show any signs of renal failure. He also complained of muscle weakness and had an episode of falling down due to muscle weakness. A neurologist saw this patient and assessed that ptosis may be present but fluctuating and was not pathological. He also showed some tendency of disturbed movement of the eyes, but it was also fluctuating and he did not have diplopia. Muscle weakness was not objectively present. Based on these clinical examinations, the neurologist ruled out mitochondrial disease from differential diagnosis and judged that further investigation was not necessary. Electrocardiogram indicated supraventricular extrasystole, but it was not clinically remarkable.

Case 4 is a 46-year-old female diagnosed with bipolar I disorder. At age 24, she had the onset of mania. At age 40, she began rapid cycling. During her psychiatric hospitalization, EEG recording showed epileptic abnormality. However, she did not have any signs or symptoms of epilepsy and was not diagnosed as epileptic.

Case 5 is 57-year-old male diagnosed as having bipolar I disorder. He had the onset of a manic episode at age 50. Since his clinical representation resembled confusion caused by organic mental disorder, he received lumbar puncture by a neurologist during psychiatric hospitalization, which showed elevated cerebrospinal fluid protein levels. The neurologist also noted muscle weakness and slight ptosis on the left eyelid. However, these symptoms were improved without any treatment and his subsequent manic episodes were typical manic syndrome without any additional neurological features or psychotic features. He was finally diagnosed as having bipolar I disorder. After the onset of bipolar disorder, he was diagnosed as having non-insulin-dependent diabetes mellitus. His cranial magnetic resonance image showed multiple subcortical silent infarction.

Case 6 is a 38-year-old male having bipolar I disorder. His clinical record was published elsewhere [37]. He complained of ptosis during antipsychotic treatment. Both a neurologist and an ophthalmologist examined and diagnosed him as not having any mitochondrial disease, since his sign was transient and not clinically remarkable.

MtDNA sequencing

Total DNA was extracted from peripheral blood leukocytes by standard protocols. Entire mtDNA sequenc-

ing was performed as previously described [38] with some modifications. In short, each DNA sample was diluted to 10 μg/ml, and nested PCR was performed. PCR was initially performed to obtain two long PCR products, 6 and 11 kb of mtDNA; the second PCR was designed as a set of three overlapping fragments from the first 6-kb PCR product and six fragments from the first 11-kb PCR product. After the second PCR, the products were treated with a SeqDirect PCR Cleaning kit (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocol. Both strands of these fragments were then sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and ABI Prism 3700 DNA sequencer (Applied Biosystems). Each mtDNA site was read at least three times, including at least once for each strand.

Genotyping

The two base substitutions of mtDNA, 3644T→C and 13928G→T, were genotyped using the PCR-RFLP method and sequencing. The enzymes and experimental conditions for the PCR-RFLP were as follows: 3644 was genotyped by primers 5′-GTAGAATGATGGCTAGGGTGACT-3′ and 5′-TCTAGCCACCTCTAGCCTAGACG-3′ and the restriction enzyme *Tai*I (Fermentas), 13928 by 5′-CATACTCGGATTC-TACGCTA-3′, 5′-TTTAGGTAATAGCTTTTCTA-3′, and *Nhe*I (Takara Bio, Inc., Shiga, Japan). MtDNAs 5178C→A and 10398A→G were genotyped to determine the haplogroups. Genotypes of 5178C→A and 10398A→G were examined as previously described [10].

Generation of cybrids

The 143B.TK$^-$ $\rho^0$206 cell line, lacking mtDNA and established by King and Attardi [39], was used for generating cybrids. Platelets of patients and controls were separated from peripheral blood and fused with $\rho^0$206 cells using 40% polyethylene glycol 1500 (Sigma), as previously described [40]. We used DMEM (Gibco BRL) containing 10% FBS (fetal bovine serum; Gibco BRL), penicillin/streptomycin, pyruvate (Gibco BRL), and uridine (Sigma) as the growth medium for ρ^0 cells. For the selection of transmitochondrial cybrid cell lines, we used DMEM containing 10% dialyzed FBS, penicillin/streptomycin, and pyruvate. After the harvest of individual cybrid cell lines, the integration of mtDNA was confirmed by Southern blot analysis using 18S ribosomal RNA repeating units as a reference [41]. The identity of the mtDNA of the cybrids with that of the donor was verified by sequencing the D loop and genotyping several polymorphisms. For Southern blot analysis, we used the ECL Labeling and Detection System according to the manufacturer's protocol (Amersham Biosciences Corp., NJ, USA). Cybrids were stored in liquid nitrogen for further experiments.

Measurement of MMP using JC-1

MMP was estimated using JC-1 (Molecular Probes, Eugene, OR) and flow cytometry. Cybrids stored in liquid nitrogen were thawed and incubated in an atmosphere of 5% CO_2 at 37°C in DMEM containing 10% FBS, penicillin/streptomycin, and pyruvate. Cells (1×10^6) were trypsinized and harvested in 10 ml of DMEM containing 10% FBS, washed with PBS (phosphate-buffered saline) once, and stained with DMEM containing 5 μg/ml JC-1 for 15 min at 37°C. Cells were then washed with PBS and subjected to analysis using a FACS (Epics Elite cell sorter; Beckman Coulter, Fullerton, CA, USA) as previously described [42]. The excitation wavelength was 488 nm by argon ion laser. Emissions at 590 and 527 nm were isolated by each photomultiplier detector and 10,000 cells were measured for each experiment. The experiment was performed in triplicate for each cell line. The cells with polarized mitochondria were defined by an intensity ratio of 590 nm/527 nm above 0.2.

Activities of enzymes in the electron-transport chain

For the sample preparation of the mitochondrial fraction, each line of cybrids was amplified until the cell count was 5×10^7. Cybrids were trypsinized and harvested in DMEM. After being washed once with PBS and once with isolation buffer [210 mM D-mannitol, 71 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin (fatty acid free), 5 mM Hepes, pH 7.2], the cells were suspended in 5 ml of isolation buffer. Using a chilled Dounce glass homogenizer with a loose fitting pestle, 20 passes were applied to the cell suspension on ice, which was centrifuged at 700*g* for 7 min at 4°C. The supernatant was centrifuged at 10,000*g* for 7 min at 4°C, and the mitochondrial pellet was obtained. The pellet was suspended in 250 mM sucrose, divided into aliquots, and kept at −80°C until use. Activities of complexes I, III, and IV were measured as previously described [43]. Rotenone-sensitive complex I activity was measured by the change in absorption of decylubiquinone. All samples were measured within 1 month from preparation. The activity of each complex was corrected by citrate synthase activity. All the chemical products for these assays were obtained from Sigma. We used a UVmini1240 spectrophotometer (Shimadzu, Kyoto, Japan) for this experiment.

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Brief Reports

Possible relationship between mitochondrial DNA polymorphisms and lithium response in bipolar disorder

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Abstract

Although many investigators have been searching for genetic markers to predict lithium response in bipolar disorders, no genetic predictor has been established yet. We previously reported the association of mitochondrial DNA (mtDNA) 5178 and 10398 polymorphisms with bipolar disorder. The objective of this study is to clarify whether these mtDNA polymorphisms can predict response to maintenance lithium treatment in bipolar patients. We examined these polymorphisms and some clinical variables in 54 bipolar patients. A logistic regression analysis was performed and revealed that patients carrying the 10398A polymorphism showed a significantly better response to lithium (p=0.03). Some clinical variables such as sex, age at onset, and rapid cycling also showed a significant association with lithium response in univariate analysis ([chi]^2 test, p<0.05). Our findings suggest that the mtDNA 10398 polymorphism might be related to maintenance lithium treatment response.

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Key Words: Bipolar disorder; lithium response; mitochondrial DNA; pharmacogenetics; treatment.

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Association of Mitochondrial Complex I Subunit Gene *NDUFV2* at 18p11 With Bipolar Disorder

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Linkage of bipolar disorder with 18p11 has been replicated by several investigators. A nuclear-encoded mitochondrial complex I subunit gene, *NDUFV2*, is one of the candidate genes in this locus, since the possible pathophysiological significance of mitochondrial dysfunction in bipolar disorder has been suggested. The objective of our study was to clarify the association between the *NDUFV2* gene and bipolar disorder. We performed the real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) for *NDUFV2* mRNA expression in lymphoblastoid cell lines derived from patients with bipolar disorder and healthy controls. We also screened novel polymorphisms using denaturing high performance liquid chromatography (D-HPLC) and PCR-direct sequencing method. Detected five single nucleotide polymorphisms (SNPs) were genotyped. A decrease of the expression level of *NDUFV2* gene was found in patients with bipolar I disorder compared with controls ($P = 0.006$). We also found that the haplotype frequencies of the four polymorphisms in the upstream region of *NDUFV2* were significantly different between bipolar disorders and controls ($P = 0.0001$). Our findings suggest that polymorphisms of the *NDUFV2* gene may be one of the genetic risk factors for bipolar disorder.

KEY WORDS: **bipolar disorder; gene expression; polymorphism; haplotype; mitochondria**

INTRODUCTION

Genetic factors are known to contribute to the etiology of bipolar disorder from twin, adoption, and family studies [Goodwin and Jamison, 1990; Gershon and Cloninger, 1994], and linkage studies have pointed out several candidate loci, such as 1q21-42, 4p16, 10q21-26, 12q23-24, 13q11-32, 18p11, and 22q11-12 [Craddock and Owen, 1994; DeLisi et al., 2000]. However, no pathogenetic gene has been identified in these loci as yet.

We have reported altered brain energy metabolism in patients with bipolar disorder using phosphorus-31 magnetic resonance spectroscopy (^{31}P-MRS) [Kato et al., 1993, 1994; Murashita et al., 2000]. The response pattern of phosphocreatine in the occipital lobe of patients with bipolar disorder to photic stimulation [Murashita et al., 2000] was similar to that in patients with chronic progressive external ophthalmoplegia (CPEO), a mitochondrial disease, without CNS involvement [Rango et al., 2001]. Affective symptoms were sometimes found in patients with mitochondrial diseases or mitochondrial DNA (mtDNA) mutations [Kato, 2001]. Especially, Suomalainen et al. [1992] reported a family with CPEO, caused by multiple deletions of mtDNA, in which depression is one of the major clinical features. We also reported that the 4,977 bp deletion in mtDNA was slightly increased in the autopsied brains of patients with bipolar disorder [Kato et al., 1997]. These findings suggested that bipolar disorder might be associated with mitochondrial dysfunction.

mtDNA polymorphisms in bipolar disorder were also examined by three groups from the UK [Kirk et al., 1999], Japan [Kato et al., 2000, 2001], and USA [McMahon et al., 2000]. The m.5178C genotype was associated with bipolar disorder in Japanese [Kato et al., 2000], although the m.5178C > A polymorphism is rare in Caucasians. The m.10398A genotype was also significantly associated with bipolar disorder in Japanese [Kato et al., 2001], and a similar tendency was also observed in Caucasians

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[McMahon et al., 2000]. Both the m.5178C and m.10398A polymorphisms cause amino acid substitution in subunits (ND2 or ND3) of the mitochondrial complex I (NADH: ubiquinone oxidoreductase). Complex I catalyzes the transfer of electrons from NADH to ubiquinone. It also transports protons from the mitochondrial matrix to outside the mitochondria, which causes a mitochondrial membrane potential [Brini et al., 1999; Smeitink et al., 2001]. This complex is the largest and most complicated enzyme in the mitochondrial electron transport chain, consisting of at least 43 subunits. Only 7 subunits of complex I are coded in the mtDNA, and the others are coded in the nuclear genome [Smeitink et al., 2001].

Of those, *NDUFV2* [de Coo et al., 1995] (GenBank accession no.: NT_010859) is located at 18p11, the locus reported to be linked with bipolar disorder. Berrettini et al. [1994] initially reported significant evidence for a susceptibility locus for bipolar disorder on the pericentromeric portion of chromosome 18. This finding was confirmed by several other investigators [Stine et al., 1995; Gershon et al., 1996; Berrettini et al., 1997; Nothen et al., 1999; Turecki et al., 1999]. Recent meta-analysis study also supported the linkage of chromosome 18 with bipolar disorder [Segurado et al., 2002]. The analysis of densely mapped markers on chromosome 18 localized the maximal peak of excess allele sharing on 18p11.2 [Detera-Wadleigh et al., 1999].

Based on these evidences, we hypothesized that polymorphisms of *NDUFV2*, a nuclear-encoded mitochondrial complex I 24-kDa subunit gene, may be genetic risk factors of bipolar disorder. In this study, we genotyped a previously reported mutation, screened new mutations, and presented evidence for the association of bipolar disorder with the polymorphisms in the upstream region of *NDUFV2* gene, as well as decreased expression of this gene in cultured lymphoblastoid cells from patients with bipolar disorder.

MATERIALS AND METHODS

Subjects

The subjects examined by denaturing high performance liquid chromatography (D-HPLC) were 96 unrelated patients (47.2 ± 13.7 [mean ± SD]-years old, 65 females and 31 males, 68 with bipolar I disorder (BPI) and 28 with bipolar II disorder (BPII)).

The subjects examined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) or direct sequencing were 189 unrelated patients including the 96 patients noted above (117 females and 72 males, 136 with BPI and 53 with BPII, 49.8 ± 13.8-years old) who were followed up at the hospitals or clinics participating in this study. Two hundred twenty-two unrelated control subjects (117 females and 105 males, 30.2 ± 8.3-years old) were recruited from hospital staff and students.

Measurement of the *NDUFV2* mRNA expression level by reverse transcription PCR (RT-PCR) was performed in 32 subjects whose lymphoblastoid cells were available. They were 13 unrelated patients with BPI (6 females and 7 males, 52.5 ± 12.4-years old), 8 unrelated patients with BPII (6 females and 2 males, 57.0 ± 10.7-years old), and 11 unrelated control subjects (3 females and 8 males, 51.1 ± 10.0-years old).

Consensus diagnosis by at least two senior psychiatrists according to the DSM-IV criteria [American Psychiatric Association, 1994] was made for each patient using a non-structured interview and scrutinizing medical records. Control subjects were not assessed for psychiatric symptoms by any structured interview method, but they showed good social functioning and reported themselves to be in good health. All the subjects were Japanese. Written informed consent was obtained from all the subjects. The ethical committees of the Brain Science Institute and all other participating institutes approved this study.

PCR-RFLP

Total leukocyte DNA was extracted from peripheral blood by the salting-out extraction method or phenol-chloroform method. The 86C > T polymorphism in exon 2 was genotyped by PCR-RFLP using Mae III (Roche Diagnostics GmbH, Mannheim, Germany) according to the previous report [Hattori et al., 1998]. For PCR reaction, iCycler (Bio-Rad, Inc., Hercules, CA) was used.

PCR

Eleven fragments including three fragments in the upstream region and eight on the all exons (1 to 8) of the *NDUFV2* gene (Fig. 1) were amplified using 11 sets of primers (Table I). These fragments except for exon 1 and the third upstream region were amplified in a 25 μl reaction volume containing 25 ng of template DNA, 0.1 μM of each primer, 100 μM of each dNTP, 2.5 μl of 10× Ex-Taq buffer (Takara Co., Shiga, Japan), and 1.2 U of Ex-Taq DNA polymerase (Takara). Exon 1 was amplified in a 50 μl reaction volume using 20 ng of template DNA, 0.3 μM of each primer, 300 μM of each dNTP, 1 mM $MgSO_4$, 5 μl of 10× Pfx Amplification buffer (Invitrogen Japan K.K., Tokyo, Japan), and 1.25 U of PLATINUM Pfx DNA polymerase (Invitrogen). The third upstream region was amplified in a 15 μl reaction volume using 15 ng of template DNA, 0.2 μM of each primer, 100 μM of each dNTP, 7.5 μl of 2× GC buffer I (Takara), and 0.75 U of LA-Taq DNA polymerase (Takara). The PCR parameters were shown in Table I.

D-HPLC

D-HPLC was performed according to the manufacture's protocol (Transgenomic, Inc., Omaha, NE). The experiments were performed at three temperatures, including the optimum condition and one degree higher or lower than that. The values for the gradient ranges and mobile-phase temperatures used are reported in Table I.

Sequencing

PCR fragments were sequenced using the BigDye terminator cycle sequencing kit (Applied BioSystems, Foster City, CA) on an ABI 3700 DNA sequencer (Applied BioSystems).

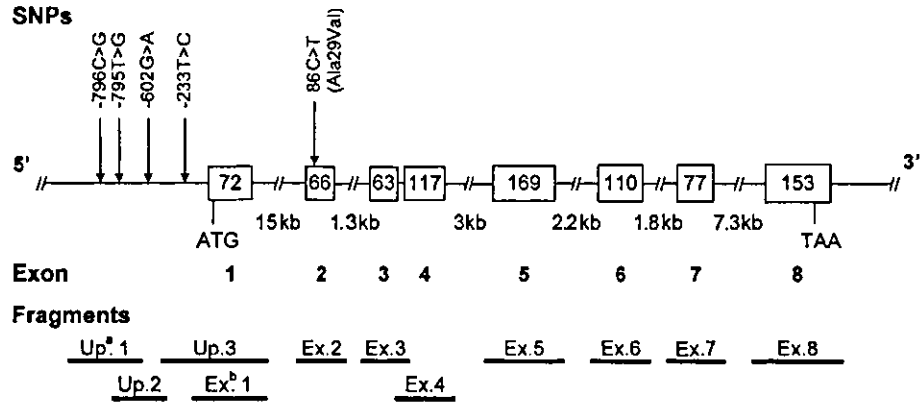

Fig. 1. Genomic structure and locations of the polymorphic sites of the *NDUFV2* gene. [a]The PCR fragments of upstream region. [b]The PCR fragments of exon.

Transformation of B-Lymphocytes by Epstein–Barr virus

Lymphocytes from peripheral blood were transformed by Epstein–Barr virus using standard techniques as described before [Kato et al., 2002]. After transformation, the culture medium was changed three times a week with RPMI1640 and 10% FBS. For mRNA quantification, the cells were frozen once and kept in liquid nitrogen until use. The cells were recultured for more than 1 week after they were thawed, and the culture medium was changed everyday for 3 days before the usage in order to randomize the cell cycle.

Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR for *NDUFV2* mRNA expression relative to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was performed according to the manufacturer's protocol (User Bulletin no. 2, SYBR Green PCR Master Mix and RT-PCR protocol, Applied Biosystems). Total RNA was isolated by standard methods (Trizol reagent, Invitrogen; RNeasy spin column, Qiagen, Valencia, CA). It was treated with DNase I (Takara) and reverse transcribed using the Superscript First Strand Synthesis System (Invitrogen) with oligo-$(dT)_{12-18}$ primers. The cDNA product was stored at −20°C after being treated with RNase H. The primers for RT-PCR are as below:

GAPDH: 5′-CATTGATGGCAACAATATCCACT, and 5′-CAACGGATTTGGTCGTATTGG.
NDUFV2: 5′-CAAAACCAGGGCCAAGGA, and 5′-CCCTTGGGTGGTTCAGTCAA.

The RT-PCR products were checked by 4% agarose gel electrophoresis and confirmed as showing a single band. An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA. Measurement of ΔCt [Ct (*NDUFV2*) − Ct (*GAPDH*)] by quantitative RT-PCR was performed for each sample in quadruplicate. The differences between bipolar disorder patients and controls in all the experiments on the ratio of each mRNA level for *GAPDH* were examined by the Student's *t*-test.

Statistical Analyses

For statistical analysis, the Chi-square test and the Student's *t*-test were applied using SPSS software (SPSS, Tokyo, Japan). The CLUMP program [Sham and Curtis, 1995] was used for the Monte Carlo method. Sequences were searched for potential transcription factor binding sites at the following website (http://transfac.gbf.de/TRANSFAC). Linkage disequilibrium between two loci and estimates of haplotype frequencies were assessed using Arlequin software (http://anthropologie.unige.Ch/arlequin/methods.html).

RESULTS

86C > T (Ala29Val) Polymorphism in Exon 2

The 86C > T (Ala29Val) polymorphism in exon 2 of the *NDUFV2* gene was reported to be associated with Parkinson's disease [Hattori et al., 1998]. We genotyped this polymorphism by a PCR-RFLP method among patients with BPI (n = 136), BPII (n = 53), and controls (n = 222). However, there was no significant difference of genotype distribution ($\chi^2 = 2.14$, df = 4, $P = 0.710$) or allele frequency ($\chi^2 = 0.83$, df = 2, $P = 0.661$) among these three diagnostic groups (control, BPI, BPII) (Table II).

D-HPLC Analysis of Coding Regions

In order to search for other mutations causing amino acid substitution or abnormal splicing, all exons and intron-exon boundaries of the *NDUFV2* gene were screened in 96 patients with bipolar disorder by D-HPLC. The D-HPLC pattern suggesting heteroduplex was found only in exon 2, and this was found to be caused by the 86C > T polymorphism.

RT-PCR

Since no novel polymorphism causing amino acid substitution or abnormal splicing was found by D-HPLC, we supposed that the altered gene expression of *NDUFV2* might be associated with bipolar disorder. Thus, we quantified the expression level of the *NDUFV2* gene

TABLE I. PCR and D-HPLC Analysis Conditions of the *NDUFV2* Gene

Fragment	Size (bp)	Forward primer	Reverse primer	PCR[a]	Temp.[b] (°C)	%B buffer[c] (start/end)
Up.[d] 1	384	5′-ATTCATAGGGGTTGCAATGG	5′-GAAGCTGCATGGTCGTTCTT	A	55	56/65
Up. 2	444	5′-ATTTCCAGAGATAACCAAACAAACA	5′-CTGGATTTATAGGGGTGAGCTTC	A	55	57/66
Up. 3	521	5′-AGCAATATAAGAGGAGGTGGCTAAT	5′-CAAAACTAAGGGAGACACGGAG	B	—[e]	—
Exon 1	285	5′-GCGCGGCTGGGGAAGGTGAACAGTGT	5′-CAGTATGGCAACCCTCCCTCGG	C	66	54/63
Exon 2	291	5′-CTCCTGAAATAGAGATGGATAGGGT	5′-ATTTTTACAATGGATTTACCCCAAT	A	53	54/63
Exon 3	281	5′-TTGATGGAAGGATAGGGTGG	5′-AAGAAAGAACATCATTTTATACATC	A	52	54/63
Exon 4	333	5′-GATGTATAAAATGATGTTCTTTCTTT	5′-CACCTCTATGCATTAAGGGAATG	A	56	55/64
Exon 5	415	5′-TGTTTTCTGCTCTTGAAGAACTTTT	5′-AAGTACAGATCCAATAAGATGGCAG	A	53	57/66
Exon 6	293	5′-ATAACCTGGTCCTTAGAGTGTT	5′-GAAACCAACATCTAAGGACACA	A	52	56/65
Exon 7	343	5′-TTTACAGATTTCAGCCGTAGAAAGT	5′-CTGAAAGATTAATTTAGGTGAGCCA	A	54	55/64
Exon 8	372	5′-TAGGAAGAGAAAATCTTGCATCAGT	5′-GCTACGGTTAGCATGAGGATGTA	A	54	56/65

The parameters of the PCR were as follows: A: 94°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec, for 35 cycles. First denaturation at 95°C for 2 min and final extension at 72°C for 3 min were performed before and after these cycles; B: 94°C for 15 sec, 55°C for 30 sec, and 68°C for 30 sec, for 35 cycles. First denaturation at 94°C for 2 min was performed before these cycles; C: 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, for 30 cycles. First denaturation at 95°C for 2 min and final extension at 72°C for 1 min were performed before and after these cycles.

[a]PCR condition.
[b]Oven temperature.
[c]0.1 M triethylamine acetate with 25% acetonitrile (pH 7.0).
[d]The upstream region of *NDUFV2* gene.
[e]All samples were sequenced.

using a quantitative RT-PCR method in lymphoblastoid cell lines from patients with bipolar disorders and healthy volunteers. This experiment revealed that the expression level of *NDUFV2* in the lymphoblastoid cell lines from patients with bipolar disorder was decreased compared with controls ($P = 0.03$ by the Student's t-test). Especially, there was a significant difference between BPI and controls ($P = 0.006$) (Fig. 2).

D-HPLC and Sequencing Analysis of Upstream Regions

We further screened the upstream region of the *NDUFV2* gene to identify the polymorphisms causing the decreased expression of *NDUFV2* in bipolar disorder. Of the three fragments in the upstream region, two were examined in 96 patients by D-HPLC, and one was sequenced in all 411 subjects for both genotyping and mutation screening. Different D-HPLC patterns were found in two PCR fragments. These were confirmed by direct sequencing and all of them were previously reported polymorphisms (−602G > A, −795T > G, −796C > G). Sequencing analysis also showed a previously reported polymorphism (−233T > C). We did not detect the −413C > A polymorphism in the SNP database, probably because this SNP is extremely rare in Japanese. In total, there were four polymorphisms in the upstream region extending up to 1,010 bp of the transcription initiation site in the *NDUFV2* gene (Fig. 1).

Polymorphisms in the Upstream Region

The association of these four polymorphisms in the upstream region with bipolar disorders was examined. Table II shows the genotype distributions and allele frequencies for the detected polymorphisms among the patients with BPI, BPII, and controls. The genotype frequencies of these polymorphisms were in Hardy-Weinberg equilibrium in both groups of patients with bipolar disorder and controls. All polymorphisms were shown to be in strong linkage disequilibrium (LD) with each other using Arlequin software (The range of D' value: 0.78–1.00., D' is the normalized LD statistic, which lies in the range {0,1} with the greater value indicating stronger LD). There was a significant difference in genotype distribution ($\chi^2 = 11.36$, df = 4, $P = 0.023$) of the −602G > A polymorphism among the three diagnostic groups. A higher occurrence of the G/G genotype in BPII compared with controls was found ($\chi^2 = 11.46$, df = 2, $P = 0.003$). There was no significant difference in allele frequency among the three diagnostic groups ($\chi^2 = 1.98$, df = 2, $P = 0.371$). The −602G > A polymorphism was predicted to create a binding site of p300 transcription factor co-activator using the TRANSFAC software.

Only one of 32 subjects examined by RT-PCR had the G/G genotype of −602G > A polymorphism. The *NDUFV2* expression in this patient with G/G was within the range of the value in other subjects but somewhat lower (0.14) than subjects with A/A (0.19 ± 0.04).

No significant differences in genotype distributions or allele frequencies of the other three polymorphisms were found among the three diagnostic groups.

TABLE II. Genotype Distributions and Allele Frequencies of the 86C > T (Ala29Val), −796C > G −602G > A, and −233T > C Polymorphisms of the *NDUFV2* Gene

Polymorphisms	Subject counts (percentage)				*P* value[b]
	Controls	Bipolar total	BPI	BPII	
Missense 86C > T					
Genotype					
C/C	81 (0.37)	65 (0.34)	46 (0.34)	19 (0.36)	
C/T	112 (0.50)	98 (0.52)	74 (0.54)	24 (0.45)	0.68
T/T	29 (0.13)	26 (0.14)	16 (0.12)	10 (0.19)	
Allele					
C	274 (0.62)	228 (0.60)	166 (0.61)	62 (0.58)	
T	170 (0.38)	150 (0.40)	106 (0.39)	44 (0.42)	0.83
Upstream −796C > G[a]					
Genotype					
C/C	72 (0.32)	62 (0.33)	40 (0.29)	22 (0.42)	
C/G	117 (0.53)	98 (0.52)	73 (0.54)	25 (0.47)	0.60
G/G	33 (0.15)	29 (0.15)	23 (0.17)	6 (0.11)	
Allele					
C	261 (0.59)	222 (0.59)	153 (0.56)	69 (0.65)	
G	183 (0.41)	156 (0.41)	119 (0.44)	37 (0.35)	0.29
−602G > A					
Genotype					
G/G	17 (0.08)	27 (0.14)	15 (0.11)	12 (0.23)[c]	
G/A	106 (0.48)	77 (0.41)	60 (0.44)	17 (0.32)	0.02
A/A	99 (0.44)	85 (0.45)	61 (0.45)	24 (0.45)	
Allele					
G	140 (0.32)	131 (0.35)	90 (0.33)	41 (0.39)	
A	304 (0.68)	247 (0.65)	182 (0.67)	65 (0.61)	0.37
−233T > C					
Genotype					
T/T	112 (0.51)	94 (0.50)	62 (0.46)	32 (0.60)	
T/C	94 (0.42)	79 (0.42)	63 (0.46)	16 (0.30)	0.33
C/C	16 (0.07)	16 (0.08)	11 (0.08)	5 (0.10)	
Allele					
T	318 (0.72)	267 (0.71)	187 (0.69)	80 (0.75)	
C	126 (0.28)	111 (0.29)	85 (0.31)	26 (0.25)	0.41

[a]The data for the −795T > G polymorphism is not shown because the distribution of the −795T > G polymorphism was completely linked with −796C > G.
[b]Differences in genotype distributions or allele frequencies among patients with BPI, BPII, and controls.
[c]Odds ratio of G/G as a risk for BPII was 2.01, 95% confidence interval: 1.05–3.81.

Haplotype Analysis

The frequency of the haplotypes consisting of −796C > G, −795T > G, −602G > A, and −233T > C were predicted using Arlequin software (Table III). The predicted haplotype distributions were significantly different between patients with bipolar disorders and controls, and between patients with BPI and controls (Monte Carlo method, 10,000 simulations, $P = 0.0001$ and 0.001, respectively). In major two haplotypes (CTAT and CTGT) which account for 56.3% of all haplotypes, the CTAT haplotype was significantly rare in patients with bipolar disorder compared with controls ($\chi^2 = 10.12$, $df = 1$, $P = 0.001$. Odds ratio of CTAT as a risk for bipolar disorder was 0.73, 95% confidence interval: 0.60–0.90).

The relationship between the haplotype of the upstream region of *NDUFV2* and its expression level could not be assessed.

DISCUSSION

We revealed that the gene expression level of *NDUFV2* in lymphoblastoid cell lines from patients with bipolar disorder was decreased compared with controls. A mutation search of the upstream region revealed that the CTAT haplotype was found to be significantly less common in patients with bipolar disorder than controls. This may at least partly explain the decreased expression, although we could not examine the association between the haplotypes and the expression levels. Most of 32 subjects whose gene expression levels were examined, were not homozygous for these polymorphisms, and the exact haplotype could not be determined in each subject. The difference in the gene expression level of *NDUFV2* cannot be explained solely by the −602G > A polymorphism, which was associated only with BPII. There may be other functional polymorphisms linked with these haplotypes in the further upstream region or intron of *NDUFV2*. Such polymorphisms associated mainly with BPI may affect the gene expression.

Why is the gene expression level of *NDUFV2* decreased in bipolar disorder? Recently, it has been clarified that mitochondria play an important role in intracellular calcium signaling systems [Babcock and Hille, 1998], and the inhibition of complex I results in altered intracellular calcium signaling [Sherer et al., 2001]. Reduced expression of the *NDUFV2* gene may cause