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Brief Research Communication

Fine Mapping of an Isodicentric Y Chromosomal Breakpoint From a Schizophrenic Patient

Kiyoshi Yoshitsugu,[1,2] Joanne M.A. Meerabux,[1] Kunihiko Asai,[3] and Takeo Yoshikawa[1]*

[1]*Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Wako, Saitama, Japan*
[2]*Department of Neuropsychiatry, Tokyo Medical and Dental University, Tokyo, Japan*
[3]*Asai Hospital, Chiba, Japan*

We report on a male schizophrenic patient who carried an isodicentric Y chromosome [idic(Y)] with a mosaic karyotype [mos 45,X/46,X,idic(Y)(q11)]. Although a potential association between sex chromosome abnormalities and a susceptibility to psychoses has been documented, there has only been one previous report of idic(Y) coincident with schizophrenia. The [45,X] karyotype is known to be associated with Turner syndrome (TS), but our patient lacked most of the phenotypic features of TS, except for short stature. To define the precise position of the breakpoint on the patient's abnormal Y chromosome, we carried out polymerase chain reaction (PCR) analysis, using primers for 15 marker loci along the chromosome. The breakpoint was localized to between the marker loci sY118 and sY119 on Yq in the 5M interval of the deletion map. This position represents the most centromeric breakpoint recorded for idic(Y). We cannot exclude the possibility that the development of schizophrenia is unrelated to the Y chromosome abnormality in this patient but we hope that this study will stimulate further cytogenetic and molecular genetic analyses of Y chromosome regions that may influence psychiatric traits. © 2003 Wiley-Liss, Inc.

KEY WORDS: cytogenetic abnormality; mosaic karyotype; STS-PCR; XYY; Turner syndrome; deletion map

INTRODUCTION

One of the most common structural changes of the Y chromosome is dicentricism [Hsu, 1994]. A dicentric chromosome is symmetrical at the position of the breakpoint, with the derivative Y chromosome containing two centromeres. Hsu [1994] reported 124 cases of isodicentric Y chromosomes [idic(Y)] from over 600 patients with rearrangements of the Y chromosome. These chromosomal rearrangements occurred mainly as mosaicisms.

Several groups have reported on the coincidence of sex chromosome anomalies with psychoses [Kaplan, 1970; Delisi et al., 1994; Crow, 1988; Kumra et al., 1998; Rajagopalan et al., 1998]. However, idic(Y) has not been documented previously in psychiatric patients with the exception of the study by Nanko et al. [1993]. They examined 7,974 inpatients from Japanese mental hospitals for sex chromosome abnormalities and found one idic(Y) patient with schizophrenia. We carried out cytogenetic analysis on 161 unrelated schizophrenics of Japanese origin and detected one patient with an idic(Y) karyotype [Toyota et al., 2001]. We report on the clinical characteristics of the patient and the molecular refinement of the breakpoint on chromosome Y.

The subject was a single Japanese male aged 68 years, 152.6 cm tall (mean stature at his age: 160.4 cm ± 5.9 cm; http://wwwdbtk.mhw.go.jp/toukei/index.html), and weighing 43.2 kg. He was the youngest of six children, born at full term with normal birth parameters. In his early years he achieved normal milestones. At 9 years, he left school and at 13 he began working as a builder to support his family. He quit this job 6 months later and remained under the care of his brothers. He was prone to impulsive violent

*Correspondence to: Takeo Yoshikawa, M.D., Ph.D., Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.
E-mail: takeo@brain.riken.go.jp

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outbursts and had difficulty in controlling his anger. He was twice sent to reform school and jail and was once detained at a reform home because of his delinquency, which consisted of repeated theft and arson. He was admitted to a psychiatric ward at the age of 32, where he remains to date. The medical record states that on admission he had auditory hallucinations, delusions of persecution and frequent tangentiality of thought, and was diagnosed as having paranoid schizophrenia. At that time, it was determined that he suffered from mild mental retardation, but there are no records of test scores. For this study, it was not possible to reliably determine his IQ because of reluctance and negativism on his part. There was no history of either drug or alcohol abuse or any history of mental illness in his family. He remains unmarried and has no children.

This study was approved by the Ethics Committees of RIKEN and Tokyo Medical and Dental University, with the participant giving informed consent.

GTG banding and fluorescence in situ hybridization (FISH) analyses using the sex determining region (SRY) and the DYZ3 probes were carried out on peripheral lymphocytes, as described elsewhere [Toyota et al., 2001]. Karyotyping was carried out on 30 metaphases from each sample.

The location of the breakpoint on the long arm of chromosome Y was determined by PCR amplification of sequence-tagged sites (STS-PCR). The reaction products were analyzed on 1.5 or 3.0% agarose gels containing ethidium bromide, and visualized under UV light. The following 15 STS were selected for investigation: sY14 (SRY), sY78 (DYZ3) [Nagafuchi et al., 1992], sY84, sY87, sY92, sY102, sY118, sY119, sY122, sY127, sY126, sY134, sY153, sY157 [Reijo et al., 1992], and sY255 [Vollrath et al., 1992]. We also referred to the reports of Foote et al. [1992], Jenderny et al. [1998], Reijo et al. [1996], and the following databases: STS-based map of the human genome (http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map), UniSTS (http://www.ncbi.nlm.nih.gov/genome/sts/). An STS was scored as absent after two amplification failures. Normal male and female samples were amplified as positive and negative controls, respectively.

Karyotype analysis showed two cell populations, [45,X] (11 metaphases) and [46,X,idic(Y)(q11)] (19 metaphases) (Fig. 1A). FISH experiments showed that the rearranged Y chromosome contained the markers SRY and DYZ3 (Fig. 1B,C), which are located on the short arm and centromeric region of Y respectively, thus confirming that the break occurred on the q-arm.

Vollrath et al. [1992] constructed the human Y chromosome deletion map, by testing 96 individuals with partial Y chromosomes for the presence or absence of marker loci. This map divides the Y chromosome into 43 intervals, denoting naturally occurring deletion sites. We used this physical map as a reference for our study of the Y chromosome because of its high resolution and functional link between chromosomal breaks and deletion events. Our STS-PCR analysis of the patient's DNA indicated that the breakpoint was located between sY118 and sY119 in the 5 M interval of the deletion map (<217 kb, based on the UniSTS) (Fig. 2).

A physical examination of the patient showed a left exotropia. Other neurological examinations detected no abnormalities. Laboratory tests for liver and renal functions, full blood counts, thyroid indices and urinalysis were normal. An electroencephalogram was within the normal range. A computed tomography scan and magnetic resonance imaging of the brain were normal. Abdominal echo tests showed no abnormal findings.

Our patient manifested psychopathological features including emotional blunting, social withdrawal, and illogically rigid thinking. His way of life was characterized by abulia and autism, occasionally suffering from violent outbursts. His mental state was assessed based on the criteria of DSM-IV [American Psychiatric Association, 1994], with the support of available medical records and information from his family and hospital staff. The current consensual diagnosis of two experienced psychiatrists (K.Y. and K.A.) was that of residual type schizophrenia, lacking positive symptoms such as delusions and hallucinations. Mild mental retardation was also suspected, but the patient's non-compliance made it difficult to properly assess his IQ. He is currently being treated with 300 mg of sulpiride daily.

Karyotypic and FISH analyses showed a mosaicism with an idic(Y)(q11), which is consistent with the report of Hsu [1994], stating that the majority of idic(Y)chromosomes occurred as mosaics. Nanko et al. [1993] reported a schizophrenic patient with a karyotype of [46,X,idic(Y)(q11)] but on this occasion without concomitant [45,X] cells. To our knowledge, our study is the second report of a patient with idic(Y) meeting the standardized diagnostic criteria for schizophrenia. We undertook STS-PCR analyses to refine the location of the breakpoint, and identified it as lying between sY118 and sY119 in the 5 M interval [Vollrath et al., 1992]. This is the most centromeric breakpoint recorded in idic(Y)(q11) cases [Stankiewicz et al., 2001].

Stuppia et al. [1996] noted from the examination of three carriers of mosaic idic(Y), that the greater the number of cells with an idic(Y), the more distal the breakpoint on Yq. In this study the percentage of idic(Y) per sample varied from 12–87% and all breakpoints were mapped within interval six of the deletion map (Fig. 2). Our patient's cell population contained 63.3% (19/30) of idic(Y) cells, but the breakpoint occurred at the more proximal 5 M interval, a location that is not consistent with Stuppia's prediction. Because the mechanism for the relationship between breakpoint positions and proportion of mosaic cells is not known, the significance of the discrepancy in our patient is unclear. Further studies of idic (Y) samples, analyzing other variables such as ethnicity are required.

Our patient carried a second population of cells with a [45,X] karyotype and was phenotypically male. This karyotype is diagnostic for TS [American Academy of Pediatrics, 1995]. Phenotypic women with TS are clinically characterized by three main features, short stature, gonadal dysgenesis and somatic stigmata such as widely spaced nipples, cubitus valgus and a webbed neck. Our subject was short in stature, but had no other TS-like physical features. His external genitals were

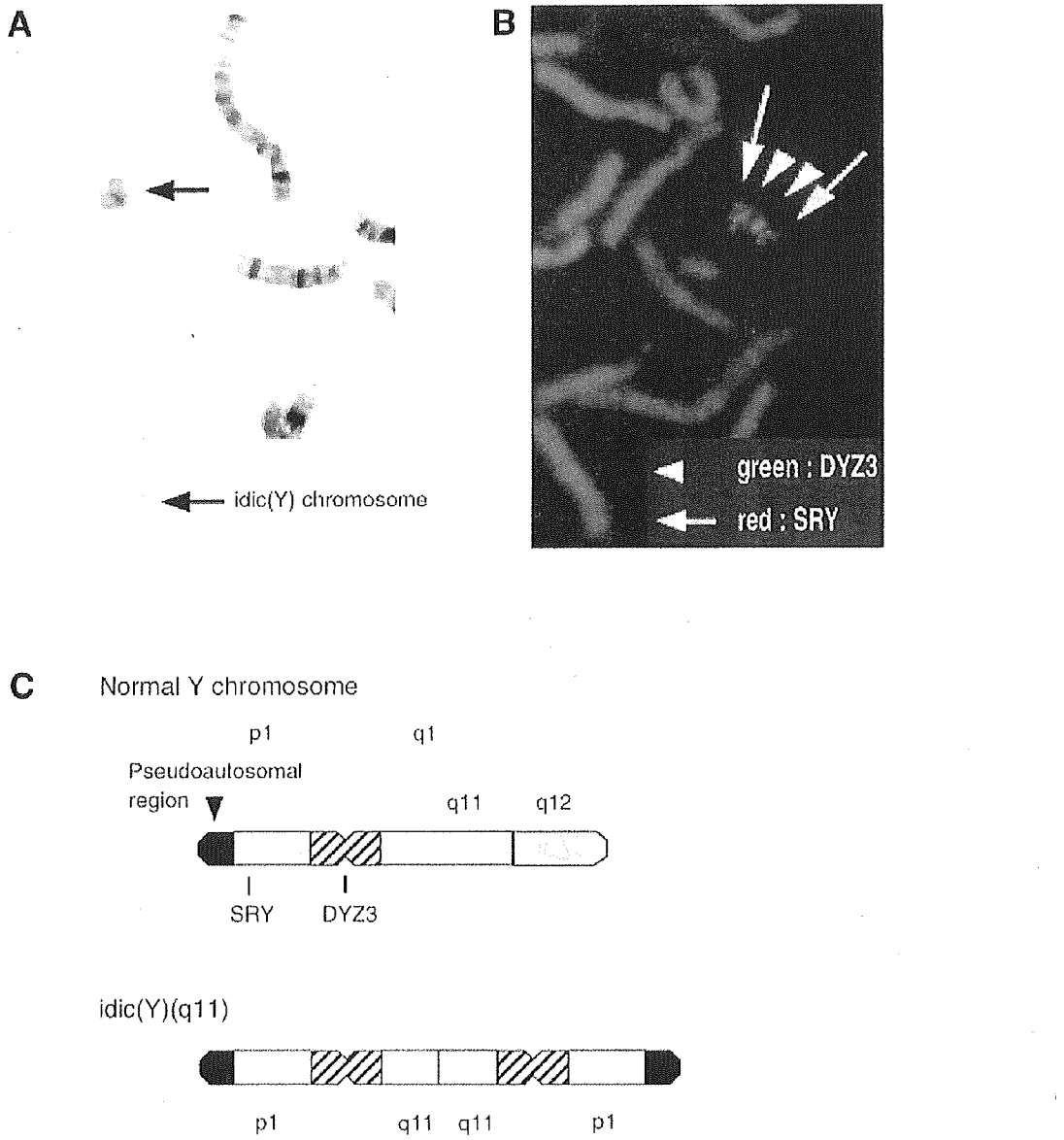

Fig. 1. Cytogenetic examination of an isodicentric Y chromosome. **A:** GTG-banding of an idic(Y) chromosome. **B:** Fluorescent in situ hybridization photograph of an idic(Y) chromosome, indicating the presence of the short arm marker SRY (red) and the centromeric marker, DYZ3 (green) [idic(Y)(q11)(DYZ3++,SRY++)]. **C:** Ideograms of a normal Y and an idic(Y)(q11) chromosome.

normal by visual inspection, although it could not be ascertained whether they were fully functional. Hsu [1994] postulated that in idic(Y) cases, the percentage of the concurrent [45,X] cells could explain phenotypic differences among patients. In our case, the proportion of idic(Y) cells was 63.3%. The unaffected Y chromosome (36.7%) and retained portion of the Y chromosome in the idic(Y) might have contributed to the male traits in this subject.

Crow [1988] suggested that the pseudoautosomal region of the sex chromosomes harbor genes that predispose to the major psychoses. Rajagopalan et al. [1998] reported a schizophrenic case with an XYY chromosomal anomaly. Ross et al. [2001] inferred a possible etiologic role for the Yp11.2/Xq21.3 regions after analyzing a male schizophrenic patient with the karyotype [46,X,t(X,Y)(p22.33;p11.2)]. Considering these reports, it is possible that the chromosome Y short arm or the retained proximal portion of the long arm of the idic(Y) may harbor a gene(s) that could confer or increase the risk for psychotic features in a dosage-dependent manner. We surveyed the genes at our breakpoint of Y chromosome using the Ensembl contig view (http://www.ensembl.org/). One gene,"51581" was identified, but its function and relevance to schizophrenia pathology remain elusive.

Patwardhan et al. [2002] investigated the neuroanatomical consequences of a supernumerary X chromo-

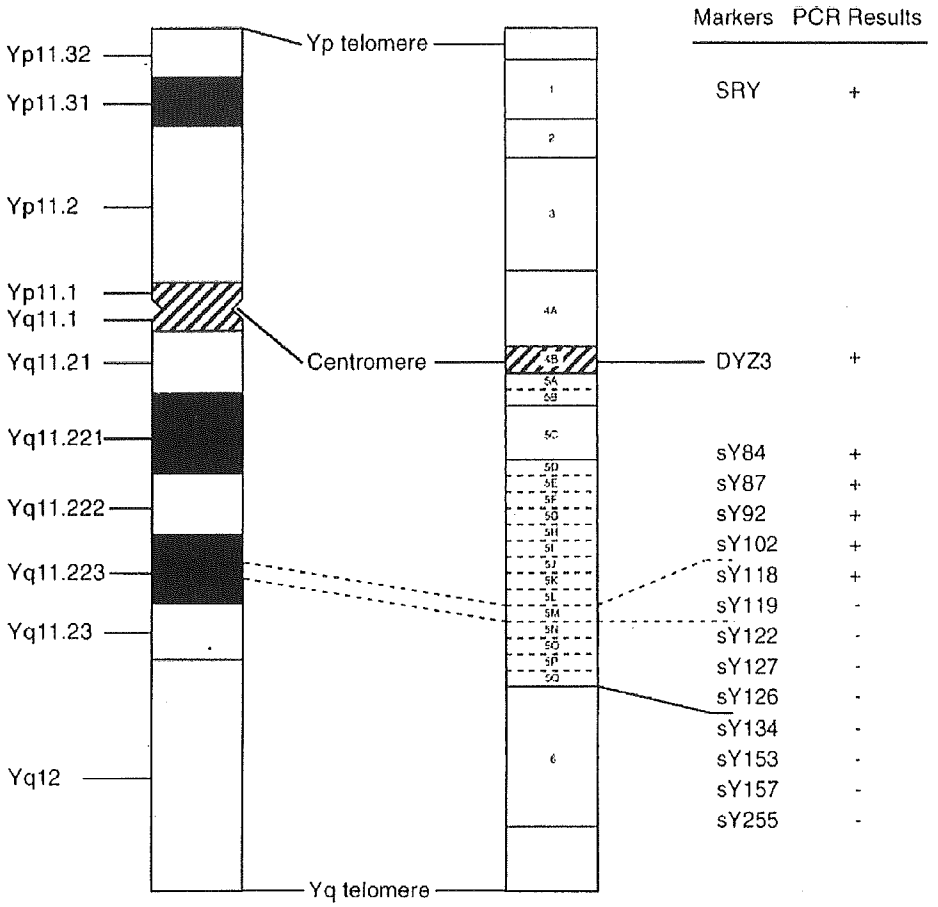

Fig. 2. STS mapping of the breakpoint on the idic(Y)(q11) chromosome. **Left:** Ideogram of a normal Y chromosome. Right: Schematic representation of the Y chromosome intervals defined by the deletion map [Vollrath et al., 1992]. The results of PCR-based testing for the presence (+) or absence (−) of 15 STS are shown at the far right.

some on the morphology of mesial temporal lobe structures by volumetric magnetic resonance imaging, and found that amygdala volumes were significantly reduced in men with 47,XXY, compared to control men. Warwick et al. [1999] reported no significant differences in lateral ventricles between the XYY group and controls. Although magnetic resonance imaging of the present case did not show distinct atrophy in any particular region of the brain, the continued collection of neuroimaging data on other idic(Y) cases would be of interest.

It is possible that our subject could have developed schizophrenia, independently of any factors related to the sex chromosome abnormality. Although increasing evidence suggests sex chromosomes as potential loci for susceptibility to psychoses, psychiatric studies of idic(Y) patients are rare. Accumulation of data on the relationship between the breakpoints and susceptibility to mental disorders is expected to narrow down the responsible region, on the Y chromosome. Therefore, we hope that this report will promote further studies on Y chromosome abnormalities and psychiatric illnesses.

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Distribution of Haplotypes Derived From Three Common Variants of the *NR4A2* Gene in Japanese Patients With Schizophrenia

Yoshimi Iwayama-Shigeno,[1] Kazuo Yamada,[1] Tomoko Toyota,[1,2] Hiromitsu Shimizu,[3] Eiji Hattori,[1] Kiyoshi Yoshitsugu,[1] Tetsuya Fujisawa,[1] Yukako Yoshida,[1] Toshio Kobayashi,[2] Michio Toru,[2] Akeo Kurumaji,[2] Sevilla Detera-Wadleigh,[4] and Takeo Yoshikawa[1]*

[1]*Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, Japan*
[2]*Department of Neuropsychiatry, Tokyo Medical and Dental University, Tokyo, Japan*
[3]*Department of Neuropsychiatry, Hokushin General Hospital, Nagano, Japan*
[4]*National Institute of Mental Health, Intramural Research Program, National Institutes of Health, Bethesda, Maryland*

Dysregulation in dopaminergic neurotransmission might play a role in the pathogenesis of schizophrenia, and therefore genetic components of the dopamine (DA) pathway may confer risk. The *NR4A2* (Nurr1) gene is essential for the development and maintenance of mesencephalic DA-synthesizing neurons. Moreover, Nurr1 forms a heterodimer with the retinoid X receptor and disturbances in the retinoid-signaling cascade may be involved in susceptibility to schizophrenia. To investigate the potential genetic contribution of *NR4A2*, we performed a case-control association study using three common variants in the gene [−2922(C)2-3, IVS6+17~+18insG, EX8+657 (CA)9-10] that were in strong linkage disequilibrium with each other. We did not detect a significant allelic or genotypic association. Haplotypes derived from all three polymorphisms generated similar results. These data do not support the notion that the *NR4A2* gene plays a major role in risk for schizophrenia among Japanese individuals. © 2003 Wiley-Liss, Inc.

KEY WORDS: **dopamine; NGFI-B family; retinoid; polymorphism; heterogeneity**

INTRODUCTION

Several investigators have postulated that the dysregulation of retinoid-activated transcription mechanisms underlies vulnerability to schizophrenia. Symptoms caused by retinoid toxicity or its absence are similar to those of schizophrenia, such as a thought disorder, enlarged ventricles, and several major and minor congenital malformations [Goodman, 1998]. Retinoids regulate several candidate genes for schizophrenia, such as those for glutamate receptors [Ray and Gottlieb, 1993; Hardy et al., 1994], nicotine receptors [Berrard et al., 1993], tyrosine hydroxylase and choline acetyltransferase [Kobayashi et al., 1994], and the dopamine (DA) D2 receptor [Samad et al., 1997].

Among genes related to the retinoid cascade, the human ortholog of murine Nurr1, *NR4A2*, is an intriguing candidate susceptibility gene for schizophrenia because it is functionally linked to the DA pathway. It encodes an orphan receptor of the NGFI-B/Nurr77 steroid/thyroid hormone receptor superfamily. Although a putative ligand has not been identified, the NR4A2 protein is thought to act as a ligand-activated transcription factor [Torii et al., 1999]. The expression of Nurr1 starts at embryonic day 10.5, predominantly in the central nervous system including the limbic area and in the ventral midbrain that contains DA; expression in the latter region is retained during adulthood [Zetterström et al., 1996]. Nurr1 knockout mice born alive at birth could not retain milk in the stomach and died within 12–48 h [Zetterström et al., 1997]. Null mice were depleted of DA and tyrosine hydroxylase immunoreactivity in the central dopaminergic region [Zetterström et al., 1996]. Heterozygous mice had reduced DA levels and increased vulnerability to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [Le et al., 1999]. In addition, Nurr1 elicited increased expression of the human DA transporter gene in the mature brain, whereas other members of the NGFI-B subfamily of nuclear receptors had lesser effects or none [Sacchetti et al., 2001].

*Correspondence to: Takeo Yoshikawa, M.D., Ph.D., Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute (BSI), 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan.
E-mail: takeo@brain.riken.go.jp

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These diverse biological actions render the *NR4A2* gene a compelling target for investigation. To date, several studies including ours [Kobayashi et al., 2000] have identified seven polymorphisms in the gene: −469delG, M97V, H103R, ΔY122, −2922(C)2-3, IVS6 + 17∼+18insG, and EX8 + 657(CA)9-10 [Buervenich et al., 2000; Chen et al., 2001; Ishiguro et al., 2002]. Among these, the latter three variants are common (frequency of minor alleles >15%) in both Caucasian and Asian populations. The possible involvement of *NR4A2* in schizophrenia investigated using combinations of these polymorphisms has failed to reveal a significant association. However, none of these studies used all three of the common variants. The present study analyzes the three common variants and the haplotypes that they define to identify an association with schizophrenia.

MATERIALS AND METHODS

Samples

One hundred and eighty unrelated schizophrenic patients (91 males, mean age 48.3 ± 11.9 years; 89 females, mean age 46.4 ± 13.6 years) were recruited from central Japan. Each patient was diagnosed by the consensus of at least two experienced psychiatrists according to the DSM-IV criteria. The control group consisted of 180 unrelated volunteers (90 males, mean age 44.2 ± 8.5 years; 90 females, mean age 41.8 ± 13.4 years) with no history of psychiatric diseases according to brief interviews. All participants were ethnically Japanese. Written informed consent was obtained from all participants and the study protocol was approved by the Committee on Ethics at RIKEN.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures. The −2922(C)2-3 and IVS6 + 17∼+18insG polymorphisms were genotyped based on PCR-restriction fragment length polymorphisms (PCR-RFLP) [Buervenich et al., 2000; Chen et al., 2001; Ishiguro et al., 2002]. Both strands of ambiguous genotypes were directly sequenced using the BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA) and an ABI 3700 DNA analyzer (PE Applied Biosystems). The EX8 + 657 (CA)9-10 polymorphism was genotyped using a fluorescent dye-labeled sense primer (5′-GTTGCAGTTCA-GACAAATG; 5′ end at nt EX8 + 621) and antisense primer (5′-TACAGCAGAAAACTTTGAAG; 5′ end at nt EX8 + 739) on an ABI 3700 DNA analyzer (PE Applied Biosystems). Thermal cycling for the (CA)n repeat polymorphism included an initial denaturation at 95°C for 5 min and 35 cycles of 15 s at 94°C, 15 s at 50°C, and 30 s at 72°C, followed by a final extension at 72°C for 10 min. Genotypes were analyzed using GENESCAN 3.5.2 and GENOTYPER 3.6 software (PE Applied Biosystems). Several PCR products were subcloned and sequenced to confirm the accuracy of the genotyping. Samples with genotypes that could not be unambiguously resolved were excluded from the current study.

Statistics

Deviations from Hardy–Weinberg equilibrium and estimates of haplotype frequency were computed using the expectation-maximization algorithm implemented in Arlequin 2000 (http://lgb.unige.ch/arlequin/). Evidence of linkage disequilibrium (LD) (D value), the normalized LD statistics ($D' = D/D_{max}$), and the squared correlation coefficient (r^2), were also calculated using the Arlequin program. Allele distribution between patients and controls were compared using Fisher's exact test. Differences in genotype and haplotype distributions were assessed by the CLUMP program that determines an empirical P value using the Monte-Carlo method with 10,000 simulations [Sham and Curtis, 1995]. Significance level was set at 5%. Power calculations were performed using the program Sample-Power 2.0 (SPSS Japan, Inc., Tokyo, Japan).

RESULTS

Analysis of the Three *NR4A2* Variants in Schizophrenics and Controls

We examined the three common variants individually in both the schizophrenic and control groups. A significant deviation from the Hardy–Weinberg equilibrium was not found in either patients or controls for the three genotypes ($P = 0.764$ and 0.876 for −2922(C)2-3; $P = 0.884$ and 0.650 for IVS6 + 17∼+18insG; and $P = 0.838$ and 0.647 for EX8 + 657(CA)9-10 in schizophrenics and controls, respectively.

We then compared the allelic and genotypic distribution of the three variants (Table I). This analysis also revealed no significant differences in the allele [$P = 0.144$ for −2922(C)2-3; $P = 0.203$ for IVS6 + 17∼+18insG; $P = 0.318$ for EX8 + 657(CA)9-10] and genotype ($P = 0.320$ for −2922(C)2-3; $P = 0.365$ for IVS6 + 17∼+18insG; $P = 0.433$ for EX8 + 657(CA)9-10) frequencies in the schizophrenic and control groups in this Japanese cohort.

Linkage Disequilibrium Between Polymorphisms and Haplotype Distribution

We uncovered evidence of strong LD between the three common polymorphic sites (Table II). We constructed four sets of haplotypes. Three were derived from various combinations of two variants and one was derived from a combination of all three polymorphisms. An estimation of haplotype frequencies did not reveal any significant differences between the schizophrenic and control groups (Table I). The frequencies of the haplotypes constructed from combinations of the −2922(C)2-3 and EX8 + 657(CA)9-10 polymorphisms in the present study were quite different from those in another study of Japanese schizophrenic and control groups (Ishiguro et al., 2002; $P < e^{-16}$ for our vs. their controls, by extended Fischer's exact test; $P < e^{-27}$ for our vs. their schizophrenics).

TABLE I. Genotype, Allele, and Haplotype Frequencies of the *NR4A2* Gene Polymorphisms

Polymorphism	n	Genotype count (frequency)			*P*	Allele count (frequency)		*P*
−2922(C)2-3		C2/C2	C2/C3	C3/C3		C2	C3	
Schizophrenia	175	44 (0.25)	90 (0.51)	41 (0.23)	0.32	178 (0.51)	172 (0.49)	0.144
Controls	165	52 (0.32)	83 (0.50)	30 (0.18)		187 (0.57)	143 (0.43)	
IVS6 + 17∼ + 18insG		DelG/DelG	DelG/InsG	InsG/InsG		DelG	InsG	
Schizophrenia	179	42 (0.23)	91 (0.51)	46 (0.26)	0.365	175 (0.49)	183 (0.51)	0.203
Controls	179	36 (0.20)	85 (0.47)	58 (0.32)		157 (0.44)	201 (0.56)	
EX8 + 657(CA)9-10		CA9/CA9	CA9/CA10	CA10/CA10		CA9	CA10	
Schizophrenia	172	11 (0.06)	62 (0.36)	99 (0.58)	0.433	84 (0.24)	260 (0.76)	0.318
Controls	173	6 (0.03)	61 (0.35)	106 (0.61)		73 (0.21)	273 (0.79)	
					Estimated haplotype frequency			
			−2922(C)-IVS6					
	n	C2-DelG	C2-InsG	C3-DelG	C3-InsG	*P*		
Schizophrenia	330	0.012	0.497	0.480	0.012	0.103		
Controls	348	0.031	0.536	0.411	0.022			
			IVS6-EX8					
	n	DelG-CA9	DelG-CA10	InsG-CA9	InsG-CA10	*P*		
Schizophrenia	346	0.243	0.246	0.000	0.512	0.175		
Controls	342	0.203	0.233	0.008	0.556			
			−2922(C)-EX8					
	n	C2-CA9	C2-CA10	C3-CA9	C3-CA10	*P*		
Schizophrenia	318	0.012	0.497	0.231	0.261	0.292		
Controls	334	0.022	0.544	0.180	0.254			
					−2922(C)-IVS6-EX8			
	n	C2-DelG-CA9	C2-DelG-CA10	C2-InsG-CA10	C3-DelG-CA9	C3-DelG-CA10	C3-InsG-CA9	C3-InsG-CA10	*P*
Schizophrenia	318	0.009	0.003	0.497	0.232	0.247	0.000	0.012	0.413
Controls	332	0.022	0.004	0.540	0.176	0.238	0.003	0.016	

TABLE II. Pairwise Linkage Disequilibrium Between Polymorphisms of the *NR4A2* Gene in Controls (Schizophrenics)

	−2922(C)2-3	IVS6 + 17∼+18insG	EX8 + 657(CA)9-10
−2922(C)2-3		$D = 0.220$ (0.238) $D' = 0.912$ (0.954) $r^2 = 0.802$ (0.910) $P < 0.0001$ (0.0001)	$D = 0.092$ (0.112) $D' = 0.807$ (0.903) $r^2 = 0.214$ (0.271) $P < 0.0001$ (0.0001)
IVS6 + 17∼+18InsG	Total number of gametes 330 (348)		$D = 0.110$ (0.124) $D' = 0.927$ (1.000) $r^2 = 0.297$ (0.336) $P < 0.0001$ (0.0001)
EX8 + 657(CA)9-10	Total number of gametes 318 (334)	Total number of gametes 346 (342)	

D values indicate deviation from linkage disequilibrium and the D' ($=D/D_{max}$) is the normalized linkage disequilibrium statistic. r^2 shows the squared correlation coefficient. These values were calculated by using control (schizophrenia) samples.

Power Analysis

We performed power calculations based on Cohen's method [Cohen, 1988]. When an effect size index of 0.1 (that corresponds to "weak" gene effect) is assumed, the present sample size had ≥75, ≥36, and between 43% (haplotype defined by two polymorphisms) and 59% (haplotype defined by three polymorphisms) power to detect a significant ($\alpha < 0.05$) allelic, genotype, and haplotype association, respectively. Given an effect size index of 0.2 (that corresponds to a "weak to moderate" gene effect), our sample had over 90% power to detect a significant ($\alpha < 0.05$) allelic, genotype, and haplotype association.

DISCUSSION

All the prior association studies between the *NR4A2* gene and schizophrenia were conducted using one or two of the common variants [−2922(C)2-3, IVS6 + 17∼+ 18insG, and EX8 + 657(CA)9-10], and failed to uncover evidence of a correlation [Buervenich et al., 2000; Chen et al., 2001; Ishiguro et al., 2002]. Because the three common polymorphisms are all biallelic, we suspected that the failure to detect a positive association might have been partly due to the polymorphisms being insufficiently informative. Therefore, we investigated the haplotypes defined by the three polymorphisms in our Japanese schizophrenia and control groups. Generally, haplotype comparisons are statistically more powerful than individual genetic markers, because the level of heterozygosity is increased and haplotypes could be used to tease out complex patterns of LD. However, the present haplotype analysis did not detect evidence of an association with schizophrenia. We showed that our sample size had reasonable power to detect association even when these variants had small to medium effects ($w = 0.2$) on susceptibility. However, we might have missed the real association, especially when a gene might have only a minor effect ($w = 0.1$) on overall risk.

Haplotype analysis is far more vulnerable to mistyping (especially misclassification of low frequency alleles) than a single locus analysis, often yielding confounding results on genetic association tests. The discrepancy in haplotypic distribution of the *NR4A2* between the present study and that by Ishiguro et al. [2002], which both investigated the same ethnic population, might be derived from incomplete typing accuracy. To avoid laboratory errors, we confirmed the genotypes of all the samples that gave equivocal PCR-RFLP profiles by sequencing. On the other hand, haplotypes are more strongly influenced by population stratification than a single locus that could generate a type I error. Thus, the above inconsistency might imply the existence of confounding biases including an admixture within the Japanese population.

Disturbed DA neurotransmission has long been implicated in the pathogenesis of schizophrenia, and a large number of association studies on DA-related candidate genes have been reported. However, with few exceptions, the results so far have not been successfully confirmed [Waterworth et al., 2002]. Possible dysregulation of the retinoid cascade, a closely related signaling pathway to that of the DA system, has been recently examined at the molecular level. However, the present data along with the previous ones do not support major roles of the genes for NR4A2, RXRB, and PPARA in the development of schizophrenia. Studies of other ethnic populations and other components in retinoid pathway are required.

The present study focused only upon common polymorphisms according to the "common disease-common variant hypothesis." This notion is derived from the general assumption that susceptibility mutations for complex diseases should have an ancient origin and that they should therefore be distributed at a high frequency [Pritchard, 2001]. It also implies that multiple risk-conferring alleles should present in an individual to render susceptibility to a common disease; thus the alleles would be much more common than the disease. In addition, a few common single nucleotide polymorphisms (SNPs), referred to as "haplotype tag SNPs," might be sufficient to determine the common haplotypes [Johnson et al., 2001]. Therefore, the present study excludes a major contribution of the *NR4A2* gene to schizophrenia susceptibility in our Japanese cohort, although the involvement of other rare variants and rare haplotypes of the gene cannot be excluded.

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Transcriptional Activities of Cholecystokinin Promoter Haplotypes and Their Relevance to Panic Disorder Susceptibility

Mitsuru Ebihara, Hisako Ohba, Eiji Hattori, Kazuo Yamada, and Takeo Yoshikawa*
Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, Japan

We previously identified a polymorphic compound short tandem repeat (STR) in the 5′-regulatory region of the cholecystokinin (CCK) gene, and showed that when the STRs were classified into three groups based on length and linkage disequilibrium behavior with neighboring variants, the medium class allele was significantly associated with panic disorder. The present study examined the transcriptional activity of the CCK promoter construct containing the STR and downstream −188A/G variation. The STRs acted as transcriptional repressors with a similar potency among the three classes, but the long (L) class STR exhibited a synergistic effect on decreasing promoter activity when combined with −188G. The haplotype composed of the L class of STR and −188G was significantly less frequent in panic disorder ($P = 0.0032$; odds ratio, 95% confidence interval = 0.06, 0.01–0.69). These results suggest that the L-(−188G) haplotype may act as a protective factor against panic by reducing the expression of anxiogenic CCK.

KEY WORDS: **luciferase assay; microsatellite; repressor; synergism; protective factor**

INTRODUCTION

The neuropeptide cholecystokinin (CCK) has been suggested as one of the molecular candidates underlying the pathophysiology of panic disorder. The carboxy terminal tetrapeptide of CCK (CCK-4) provokes panic attacks in individuals with panic disorder and in normal controls [de Montigny, 1989; Bradwejn et al., 1990], with panic disorder patients being more sensitive to CCK-4 than controls [Bradwejn et al., 1991]. The CCK gene is located on chromosome 3p22.1 (http://genome.ucsc.edu/cgi-bin/hgGateway), where linkage to panic disorder has been reported [Crowe et al., 2001].

Polymorphism screenings have identified several potential functional variants in the 5′-upstream stretch of the CCK gene that might be able to alter gene expression: −36C > T [Bowen et al., 1998], −188A > G [Fujii et al., 1999; Hattori et al., 2001], −345G > C [Hattori et al., 2001], and the complex short tandem repeat (STR) cluster located over 1.7 kb upstream from the transcription start site [Hattori et al., 2001] (Fig. 1A). Among these, the functional consequences of the C to T transition at nt −36 induce no in vivo transcriptional change [Hansen et al., 2000]. We originally reported the −345G > C transversion, but its heterozygosity was fairly low (frequency of the minor allele was 1%). Our test for an association of −36C > T and −188A > G with panic disorder did not establish significant roles for either transversion [Hattori et al., 2001].

The compound STR is comprised of a variable number of discrete tetranucleotide repeat units (Fig. 1A). By comparing the size distribution of the STRs between panic and control samples, and by taking into account linkage disequilibrium (LD) behavior between the STR and neighboring SNPs, we validated from a genetic viewpoint that the STR could be grouped into three classes according to length (L, long; M, medium; S, short). We then demonstrated that the M class allele of the STR were over-represented in panic patients [Hattori et al., 2001]. However, the precise mechanism of this putative association has remained elusive. Therefore, in the present study we aimed to examine the effects of STR length and of the −188 A to G transition on transcriptional activity, and to re-evaluate genetic data based on the functional results.

MATERIALS AND METHODS

Samples and Genetic Analysis

The details of the patients with panic disorder (diagnosed based on DSM III-R) (n = 73) and control

*Correspondence to: Dr. Takeo Yoshikawa, M.D., Ph.D., Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute (BSI), 2-1 Hirosawa, Wako-city, Saitama 351-0198 Japan. E-mail: takeo@brain.riken.go.jp

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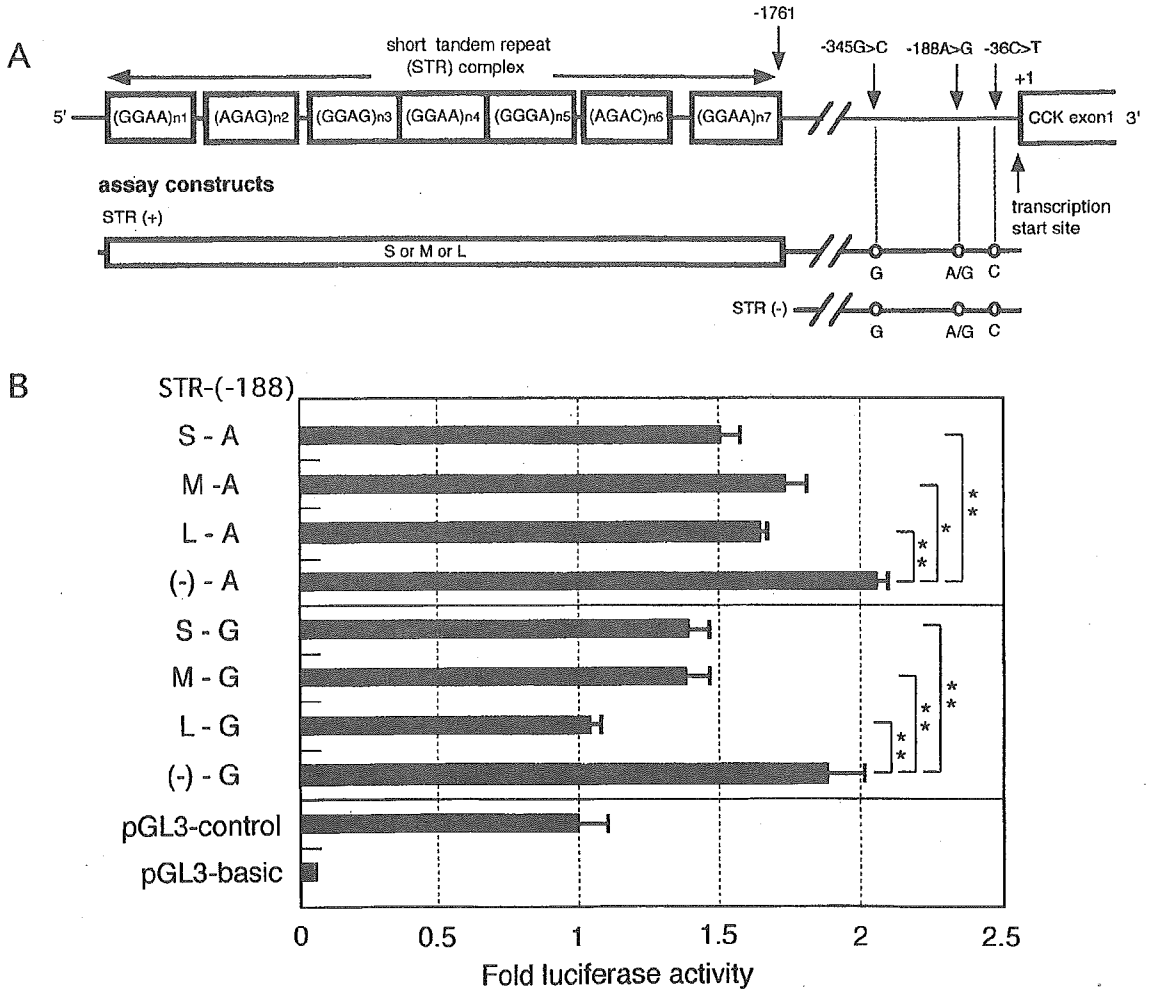

Fig. 1. **A**: Structures of 5′-upstream regions of the human cholecystokinin (CCK) gene and promoter construct used for transient assays. A short tandem repeat (STR) complex, its polymorphic constituents and three single nucleotide polymorphisms are shown. **B**: Transient reporter gene assay of the human CCK promoter. The y-axis shows the constructs and control vectors. S, M, and L denote class of STR, and (–) means a deleted STR. A and G correspond to −188A and −188G, respectively. Luciferase activities of constructs are expressed relative to the activity of pGL3-control. Values represent means ± SE of at least three independent transfections, each with triplicate determinations. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Dunnett's multiple comparison test.

subjects (n = 253), as well as the methods of genotyping are described elsewhere [Hattori et al., 2001]. Differences in genotype and haplotype distribution were assessed by the Monte-Carlo method using the CLUMP program [Sham and Curtis, 1995] with 10,000 simulations. Allelic frequencies were evaluated by chi-square statistics. Odds ratios with a 95% confidence interval (CI) were estimated for the effects of protective allele and haplotype. Tests for Hardy–Weinberg equilibrium, the calculation of LD between two loci, and the estimation of haplotype frequencies were performed using Arlequin software (http://lgb.unige.ch/arlequin/).

Preparation of Plasmid Constructs Used for the Promoter Assay

The transcription start site of the CCK gene was determined by a primer extension method [Nielsen et al., 1996]. A fragment containing the L, M, or S class of STR with −188G and −36C in the CCK promoter (Fig. 1A) was amplified from genomic DNA by nested PCR, using Pyrobest DNA polymerase (Takara, Tokyo, Japan) and MasterAmp KN buffer (Epicentre Technologies, Madison, WI). The primer sets were: 5′-ATGCCACTGTACTCCAGCCTGGGCG (5′ end at nt −2204) and 5′-GAGCCAAGTTCAGGGAGGACCA (5′end at nt + 14) for the first PCR, and 5′-CCTACGCGTGGGCGACAGAGTGAGACTCTGTCTC (3′ end at nt −2160; underline shows an added *Mlu*I recognition site), and 5′-CCTAGATCTGAGGACCAGCGGGCGGCTGTCT (3′ end at nt −22; underline shows an inserted *Bgl*II recognition sequence) for the second PCR. We used the upstream primer, 5′-CCTACGCGTTCAGACCTACTGAATTAGAAGCTCT (3′ end at nt −1715; underline shows an *Mlu*I site), and a downstream primer that was the same as the above nested primer to generate STR-less clones. All these amplicons were digested with *Mlu*I and *Bgl*II, and the fragments were subcloned into the

pGL3-basic vector digested with *MluI/BglII*. The internal control reporter vector was pRL-TK (Promega, Madison, WI). The pGL3-control vector used as the positive control consists of the SV40 promoter, enhancer sequences, and the luciferase gene. To substitute the G at position −188 with A nucleotide, we performed site-directed mutagenesis according to the rapid PCR-based method [Costa et al., 1996].

Luciferase Assay

NB1 neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium and RPMI1640 (1:1), supplemented with 10% FBS. Transfection was performed using LipofectAMINE2000 (Invitrogen, Carlsbad, CA) according to the supplied instructions. The transcriptional assay was performed using the PicaGene Dual SeaPansy kit (Toyo Ink, Tokyo, Japan). The promoter activity was assessed from at least three independent transfections, each performed in triplicate.

RESULTS AND DISCUSSION

We defined the STR as starting at the 5′ end of (GGAA)n1 and finishing at the 3′ end of (GGAA)n7 (Fig. 1A). The size of each STR class varied with: 346, 350, 354, and 358 bp representing S, 370 and 374 bp representing M, and 382, 386, 390, and 394 bp representing L. We prepared six plasmid constructs to represent all possible haplotypes defined by the three STR classes and the −188A > G. The tetranucleotide components of analyzed STRs were chosen from the most frequent allele in each class of the STR.: n1 = 12, n2 = 2, n3 = 2, n4 = 11, n5 = 0, n6 = 1, and n7 = 10 (total 358 bp) for S; n1 = 13, n2 = 1, n3 = 3, n4 = 9, n5 = 1, n6 = 0, and n7 = 14 (total 370 bp) for M; n1 = 13, n2 = 2, n3 = 3, n4 = 11, n5 = 1, n6 = 0, and n7 = 14 (total 382 bp) for L (Fig. 1A). In addition, we generated two other derivatives that had no STR and differed only at the −188 position in order to discriminate the *cis*-acting effects of the STR and −188A > G (Fig. 1A). Figure 1B shows the results of firefly luciferase assays using these constructs. The 5′ flanking sequences of CCK gene directed transcriptional activities equal to or more than that of the positive control (pGL3-control). Among the constructs with −188A, the activity of the STR-less plasmid was significantly higher than that with either of the STRs ($P = 0.0006$ by ANOVA). Promoter activity was also enhanced by deleting the STR from constructs with −188G ($P < 0.0001$ by ANOVA). These results suggest that the STR plays an important role as a negative regulatory element. Comparisons between the S-A (construct with the S class STR and −188A) and the S-G, and between the M-A and the M-G, showed that transcription was slightly reduced by substituting −188A with −188G (Fig. 1B). A search of databases including TRANSFAC (http://transfac.gbf.de/TRANSFAC/) and TFSEARCH (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html) hit no compelling mammalian *cis*-acting elements in the vicinity encompassing −188A. However, when the A nucleotide was replaced with G, two putative consensus motifs were generated: one for CAC-binding protein (GGGAGGG, underline shows the nt −188) [Mignotte et al., 1989] and the other for Sp1 (GGGAGG) [Kadonaga et al., 1987]. Since the former is an erythroid-specific factor and the latter is a ubiquitous transcription activating factor, the functional significance of −188A > G variation in the present results is obscure. The L-G construct suppressed transcription by over 25% compared with the S-G and M-G plasmids ($P = 0.032$ by ANOVA) (Fig. 1B). This finding implies that the L class STR and −188G synergistically interact to repress the transcriptional ability of the CCK promoter.

Since the above functional analyses highlighted the L class STR-(−188G) haplotype and favored the dichotomy of STR into (S + M) (referred to as SM hereafter) and L categories, we reanalyzed the CCK genotype data [Hattori et al., 2001] by incorporating this new STR classification into panic and control samples. The biallelic STR showed a trend of LD with −188A > G ($P = 0.075$), but not with −36C > T ($P = 0.33$) in panic cohorts. The genotypic distribution of two STR clusters significantly differed between panic and control groups ($P = 0.030$) (Table I). Allelic distribution also significantly differed between the two groups ($P = 0.015$) (Table I). The genotypic distribution of the classified STR alleles did not deviate from the Hardy–Weinberg equilibrium in either panic or control samples ($P = 0.67$

TABLE I. Genotypic, Allelic, and Haplotype Distributions of the Biallelic Short Tandem Repeat

	Genotype counts (frequency)[a]				Allele counts[a]		
	SM/SM	SM/L	L/L	*P*-value	SM	L	*P*-value
Panic disorder (n = 73)	66 (0.90)	7 (0.10)	0 (0.00)	0.030[b]	139 (0.95)	7 (0.05)	0.015[b]
Control (n = 253)	198 (0.78)	51 (0.20)	4 (0.02)		447 (0.88)	59 (0.12)	

	Estimated haplotype (STR/−188) frequency				
	SM-A	SM-G	L-A	L-G	*P*-value
Panic disorder (n = 73)	0.554	0.404	0.037	0.005[c]	0.0249
Control (n = 253)	0.483	0.400	0.045	0.072	

[a]The alleles of the short tandem repeat were divided into SM (346–374 bp) and L (382–394 bp) classes according to their length.
[b]Odds ratio (and 95% confidence interval) for L allele vs. SM is 0.382 (0.19–0.87), $\chi^2 = 5.87$, d.f. = 1.
[c]The haplotype L-G was significantly less frequent in panic compared to control subjects; $P = 0.0032$ by Fischer's exact test, odds ratio (and 95% CI) = 0.06 (0.01–0.69).

and 0.73, respectively). These and the previous data agree, supporting the notion that the STR is genetically relevant to anxiety disorder.

A comparison of the haplotypes defined by the SM and L alleles of STR and the −188A > G showed that their distribution significantly differed between panic and control groups ($P = 0.025$) (Table I). The L-(−188G) haplotype was significantly less frequent in panic than in control groups ($P = 0.003$ by Fisher's exact test; odds ratio, 95% CI = 0.06, 0.01–0.69). These results suggest that a haplotype composed of the L allele of STR and −188G would confer a protective effect against panic development by suppressing CCK gene expression in the brain. The accumulation of M class STR in panic subjects [Hattori et al., 2001] might be construed as a reflection of negligible amounts of the L class allele in these patients. We did not examine the transcriptional effect of the C to T transition at nt −36 in the context of the haplotype, because of the scarcity of LD between the STR and −36C > T in panic samples.

Increasing evidence demonstrates that microsatellite repeats play a regulatory role in transcriptional control [Gogos and Karayiorgou, 1996; Meloni et al., 1998; Shimajiri et al., 1999; Croager et al., 2000]. We have shown here, that the tetranucleotide repeat complex of the CCK gene functions as a negatively acting *cis*-element, like the CCAT microsatellite in the CD30 gene [Croager et al., 2000], and provide evidence for a potential synergistic interaction between the repeat sequence and a distant sequence motif(s). Further studies are required, to identify DNA binding protein(s) and to elucidate the detailed underlying mechanisms.

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Table 1 Correlation between the number of repeats of the genotype's longest allele with the WURS and TCI scales

		Total wurs (n=107)	*Factor 1 (emotional difficulties)*	*Factor 2 (behavior disorders)*	*Factor 3 (hyperactivity)*	*Factor 4 (attention deficit)*
Longest allele in genotype	Pearson correlation	0.246	0.179	0.217	0.157	0.277
	Sig. (2-tailed)	**0.011**	0.065	**0.025**	0.105	**0.004**
	TCI (n=99)	*Novelty seeking*	*Harm avoidance*	*Recompense dependence*	*Cooperation*	*Self-directiveness*
Longest allele in genotype	Pearson correlation.	0.308	0.072	0.132	0.133	0.188
	Sig. (2-tailed)	**0.002**	**0.480**	0.194	0.189	**0.062**

Differences were regarded as significant only when $P<0.05$

Further studies are needed to determine the functional basis of the observed association.

G Ponce*[,1], J Hoenicka*[,2], G Rubio[1], I Ampuero[2], MA Jiménez-Arriero[1], R Rodríguez-Jiménez[1], T. Palomo[1] and JA Ramos[2]

[1]*Unidad de conductas adictivas, Servicio de Psiquiatría, Hospital 12 de octubre. Madrid, Spain;* [2]*Banco de Tejidos para investigaciones Neurologicas, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain*

Correspondence should be addressed to J Hoenicka.
E-mail: jhoenicka@cbm.uam.es
*These authors contributed equally to this work.

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Two-step association analyses of the chromosome 18p11.2 region in schizophrenia detect a locus encompassing *C18orf1*

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SIR – The pericentromeric region of chromosome 18, specifically 18p11.2, is described as schizophrenia susceptibility locus[1,2] (the maximum LOD score to date is 3.1 by Schwab *et al*[1]), in addition to being a strong candidate region for bipolar disorder.[3] We have previously cloned two novel brain-derived transcripts from this region: the gene for a second human *myo*-inositol monophosphatase (*IMPA2*)[4] and a gene of unknown function, *C18orf1*.[5] Our prior genetic analysis of *IMPA2* generated evidence for its association with Japanese schizophrenia in a case–control context.[6] Owing to an inherent risk for producing false-positive results in case–control designs, we performed a family-based linkage disequilibrium (LD) study as the first step towards identifying relevant genetic loci around 18p11.2, and then followed this up using independent case–control samples. The ethics committee of RIKEN approved the present study, and written informed consent was obtained from all participants.

First, we genotyped 80 independent trios, each composed of schizophrenic offspring (based on DSM-IV) and their parents, using 15 markers that included seven microsatellites and eight single-nucleotide polymorphisms (SNPs) (Table 1, except for 6409T>C). These markers, chosen at even intervals as long as possible, were selected from the 14 Mb region spanning 18p11.22 and the proximal q-arm,

Table 1 Summary of the markers examined and P values evaluated by ETDT

Marker	*Physical location (kb)*[a]	*Genetic location (cM)*[b]	*Cytogenetic location*[c]	*Type of polymorphism*[a]	*Location within the gene*	*Heterozygosity*[d]	*ETDTP value*	
							Allele-wise	*Genotype-wise*
D18S1158	11 183	38.1	18p11.22	(CA)n		0.635	0.900	0.904
D18S378	11 560		18p11.22	(GAAA)n		0.660	0.174	0.346
GNAL.PCR5[e]	11 830		18p11.22	(CA)n	*GNAL* –intron4	0.465	0.538	0.541
GNAL-intron5[f]	11 830	40.4	18p11.22	(CA)n	*GNAL* –intron5	0.643	0.730	0.546
IVS1 15G>A[g]	12 040		18p11.22	SNP	*IMPA2* –intron1	0.447	0.293	0.293
800C>T[g]	12 090		18p11.22	SNP	*IMPA2* –exon6	0.209	0.168	0.168
D18S1116	12 527	40.4	18p11.21	(CA)n		0.586	0.157	0.299
D18S852[h]	13 371		18p11.21	(GCT)n	*C18orf1* –3′UTR	0.236	**0.010**	**0.010**
6409T>C[i]	13 371		18p11.21	SNP	*C18orf1* –3′UTR	0.260	**0.046**	**0.046**
D18S40	13 392		18p11.21	(CA)n		0.746	**0.023**	0.052
D18S37	14 000		18p11.21	(CA)n		0.391	0.243	0.240
IMS-JST031657[j]	14 147		18p11.21	SNP		0.319	1.000	1.000
IMS-JST031830[j]	14 438		18p11.21	SNP		0.355	0.302	0.302
D18S1114	14 637	42.9	18p11.21	(CA)n		0.725	0.180	0.121
IMS-JST037630[j]	15 238		18p11.21	SNP		0.372	1.000	1.000
IMS-JST031660[j]	25 259		18q11.2	SNP		0.431	0.710	0.710

[a]The locations are based on the NCBI database (http://www.ncbi.nlm.nih.gov/) except for *D18S378*, which is from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).
[b]Based on the Genethon database (http://www/genethon.fr/).
[c]Derived from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hggateway).
[d]Calculated using the genotype data from the parents of 80 trios.
[e]Derived from the Genome Database (ID 569 516) (http://www.gdb.org/gdb/).
[f]See Schwab *et al.*[1]
[g]See Yoshikawa *et al.*[6]
[h]See Yoshikawa *et al.*[5]
[i]See Yoshikawa *et al*, the A of initiation ATG codon is counted as +1.
[j]These SNPs were derived from the JSNP database (http://snp.ims.u-tokyo.ac.jp/).

with the exception of the sparsely represented pericentromeric region and two markers each from the *GNAL* and *IMPA2* genes (the average marker density on 18p is 1/280 kb). This marker spacing is valid for an initial genetic scan, given the recently reported LD distance of 0.5–2 cM retained in the Japanese population.[7] We analyzed genotype data using the extended transmission disequilibrium test (ETDT),[8] which calculates the allele wise TDT statistics that determine the preferential transmission of specific alleles, and the genotype-wise TDT statistic that evaluates the deviation of allele transmission from each parental genotype. We simulated the empirical significance levels of the ETDT results using the MCETDT program, (http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html). P values indicating significantly distorted transmission were obtained from markers *D18S852* and *D18S40* (Table 1). Since *D18S852* consists of a GCT triplet repeat present within the 3′-untranslated region of the *C18orf1* gene, we tested another polymorphism, 6409T>C, which we had identified from the same region.[5] This SNP also showed a modest but significant association in both allele- and genotype-wise TDT statistics (Table 1). However, all three markers lost significance if a Bonferroni correction for multiple testing was applied. To compute the statistical power obtained from the present family-based association study, we used the TDT Power Calculator program.[9] Our sample size had a power of 0.821 to detect significant association, based on a model assuming a dominant trait with an allele frequency of 0.2, a penetrance of 0.4 and a phenocopy risk of 0.4%.[10]

In the second stage, to ascertain whether the association of the three markers could be replicated, we analyzed 214 schizophrenics and 313 mentally healthy controls. The samples partially overlapped with those used in the previous study,[6] and are thus regarded as extended samples. None of the markers were significant ($P>0.05$). Since these markers were in LD with each other ($P<0.05$ by Arlequin at http://lgb.unige.ch/arlequin/), we compared haplotypic distributions (estimated by Arlequin) that were defined by the combinations of either any two or all three of *D18S852*, 6409T>C and *D18S40*, with the aim of increasing statistical power. The analysis revealed that the haplotype constructed from *D18S852* and 6409T>C was strongly associated with schizophrenia (nominal $P=0.00001$ by CLUMP at http://www.mds.gmw.ac.uk/statgen/dcurtis/software.html; $P=0.00008$, corrected for multiple comparisons). The *D18S852*–

6409T > *C D18S40* haplotype was also associated with schizophrenia ($P=0.012$), while the 6409T > *C-D18S40*–($P=0.274$) and *D18S852–D18S40* ($P=0.138$) were not. The *C18orf1* protein is predicted to have a putative type Ib transmembrane domain, a low-density lipoprotein receptor class A domain, potential binding sites for src homology 3 and tryptophan tryptophan–domains. It may therefore function by interacting with signaling molecules.[5]

From the data presented here, *C18orf1* as well as *GNAL* and *IMPA2* on the short arm of chromosome 18 (within a 1.5 Mb region, Table 1) show association with schizophrenia or functional psychoses.[1,6] Recent examples showing fine mapping of complex diseases have demonstrated the significance of clustered positive markers around causal variants frequently interspersed with non significant markers.[11] In this context, the present findings support the idea that the proximal area of 18p is a schizophrenia susceptibility locus, and indicate that the genomic region surrounding the *C18orf1* gene warrants further scrutiny.

M Kikuchi[1,2], K Yamada[1], T Toyota[1,2], M Itokawa[1], E Hattori[1], K Yoshitsugu[1], H Shimizu[3] and T Yoshikawa[1]

[1]*Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan;* [2]*Department of Neuropsychiatry, Tokyo Medical and Dental University, Tokyo, Japan;* [3]*Hokushin General Hospital, Nagano, Japan*

Correspondence should be addressed to T Yoshikawa.
E-mail: takeo@brain.riken.go.jp

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Hypothalamic–pituitary–adrenal (HPA) system activity in depression and infection with Borna disease virus and *Chlamydia pneumoniae*

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SIR – The pathophysiology of severe depression may be associated with mutual immune and hypothalamic–pituitary–adrenal (HPA) system activation,[1,2] by which infection-induced immune response is triggering the HPA system, thereby changing behavior,[3] and stress hormones, in turn, the immune system.[4] In this context, we investigated infections with two independent agents (Borna disease virus (BDV) and *Chlamydia pneumoniae* (CP)), one of which already under debate to specifically contribute to depression.[5–8] Our study provides the first clinical evidence that in depressed patients, infection with Bornavirus is associated with activation of the HPA system.

The study enrolled 48 patients with a major depressive episode (DSM-IV), giving informed consent and presenting with at least 18 points in the Hamilton Depression Scale (HAMD) after a 6-day wash-out, who were kept off psychotropic medication for 1 week (except lorazepam and zolpidem) and subsequently treated with 150 mg amitriptyline or 40 mg paroxetine for weeks 1–4. Excluded were patients with schizophrenia, bipolar or current substance-related disorders. Saliva cortisol was measured daily at 8.00, 16.00 and 22.00 h during weeks −1 to 4, and mean daily concentrations were calculated each week. Citrated blood was drawn weekly for 6 weeks. IgG, IgM and IgA antibodies to CP were analyzed twice (weeks −1 and 4) using a commercially available assay. BDV structural proteins p40 and p24 and specific circulating immune complexes (CICs), together indicating antigenemia as a result of viral replication, were determined by enzyme immune assays (EIAs) using specific monoclonal antibodies.[5,8] BDV antigen-positive scoring requested moderate or high reactivity in the BDV-CIC and/or plasma antigen EIA. Nonreactivity in either assay was considered BDV negative. Laboratories (endocrinology, microbiology) were mutually kept blind as to their results. Analysis of variance with repeated measures (ANOVA-rm) and *t*-tests were used to compare HPA system activity between subgroup of patients.

A total of 16 patients were BDV antigen positive at one or more time points, while 17 did not show positive BDV antigen in any of 6-weekly blood samples. In all, 15 subjects were excluded because BDV antigen results did not allow clear stratification. BDV antigen-positive and -negative subgroups did not differ with respect to age (54 ± 12 *vs* 52 ± 13 years), pre-treatment, severity (HAMD: 24 ± 4 *vs* 24 ± 5) or subtype of depression, antidepressive treatment (seven paroxetine/nine amitriptyline *vs* nine paroxetine/eight amitriptyline) or use of lorazepam during the study or HAMD score at day 28 (15 ± 9 *vs* 11 ± 6). A higher proportion of women (13/22) than men (3/11) was BDV antigen positive.

Using ANOVA-rm, we found increased baseline cortisol in the BDV antigen positive compared to the negative group (effect of group: $F_{1,60}=5.79$, $P<0.03$; effect of time: $F_{2,60}=128.24$, $P<0.0001$; 8.00 h:

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Analysis of a cluster of polymorphisms in *AKT1* gene in bipolar pedigrees: a family-based association study

Tomoko Toyota[a,b], Kazuo Yamada[a], Sevilla D. Detera-Wadleigh[b], Takeo Yoshikawa[a,*]

[a]*Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Wako, Saitama 351-0198, Japan*
[b]*Mood and Anxiety Disorders Program, National Institute of Mental Health, Bethesda, MD, USA*

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Abstract

We have previously performed a genome scan in 22 multiplex pedigrees with bipolar disorder and detected a moderate linkage signal on distal portion of chromosome 14q22–32. One of the large pedigrees displayed a parametric lod score >3 at a marker on 14q23–32. Upon inspection of genes located in this region revealed *AKT1*, a kinase that activates a lithium-responsive cell-survival pathway. Because lithium is an effective mood stabilizer for bipolar disorder patients, *AKT1* is an interesting candidate for further investigation. We screened the gene for possible mutations and detected 14 polymorphisms. Seven polymorphic sites were clustered in a small segment spanning exon 14 and downstream intron. Transmission of haplotypes constructed from this cluster showed a weak evidence of association between the *AKT1* and bipolar disorder.

Keywords: Affective disorder; Linkage; Candidate gene; Lithium; Phosphoinositide signaling pathway; Haplotype block; Pedigree disequilibrium test

Bipolar affective disorder is characterized by episodic, relapsing and debilitating oscillations of elation and depression, afflicting approximately 1% of the world population with usual onset in young adulthood [10]. Although the exact etiology remains elusive, complex inheritance mechanisms involving multiple genes and gene-environment interactions have been suggested [5]. To identify chromosomal loci contributing to vulnerability, we have previously conducted a linkage analysis in 22 multiply affected mood disorder pedigrees consisting of 393 individuals of European descent [7]. The results highlighted several loci. On 14q32.1–q32.2, *D14S1434* gave a lod score of 1.79 for the entire series and *D14S617* yielded a lod >3 in one of the largest pedigrees by a pairwise parametric analysis [7]. Nonparametric multipoint analysis was supportive of this finding, therefore making 14q a region of potential relevance for susceptibility to bipolar disorder.

Many genes are encoded by the region including *AKT1*.The product of the gene is a serine/threonine kinase [3,17], also known as protein kinase B [18]. Recently, Detera-Wadleigh noted that the map positions of multiple molecular components of lithium-perturbed pathways overlap with reported linkage regions for bipolar disorder, and that scrutiny of such genes for relevance to etiology is warranted [8]. Lithium is a well-established mood stabilizer and a potent prophylactic medication in bipolar disorder [1]. Several lines of evidence suggest that AKT1 is a principal target of lithium: (1) expression of *AKT1* is increased in neuronal cells by lithium treatment [4]; (2) AKT1 is an important mediator of the phosphoinositide signal transduction system because it is located downstream of phosphoinositide 3-kinase (PI3-K) cascade and its activation generates phosphorylation of many cellular proteins, including glycogen synthase kinase-3β (GSK-3β) which is another major target of lithium, that are involved in processes of metabolism, apoptosis and proliferation in not only somatic cells but also in neuronal system [3,17]; (3) activities of PI3-K and GSK-3β are also known to be stimulated [2] and inhibited [11] by lithium, respectively. AKT1 is also known to transduce nerve growth factor- and NT3-mediated signals and elicit axon increase and branching [13], and a deficit in these mechanisms might be involved in affective disorder pathology [16].

In the present study, we first determined the genomic organization of the *AKT1* gene, by aligning the cDNA

* Corresponding author. Tel.: +81-48-467-5968; fax: +81-48-467-5916.
E-mail address: takeo@brain.riken.go.jp (T. Yoshikawa).

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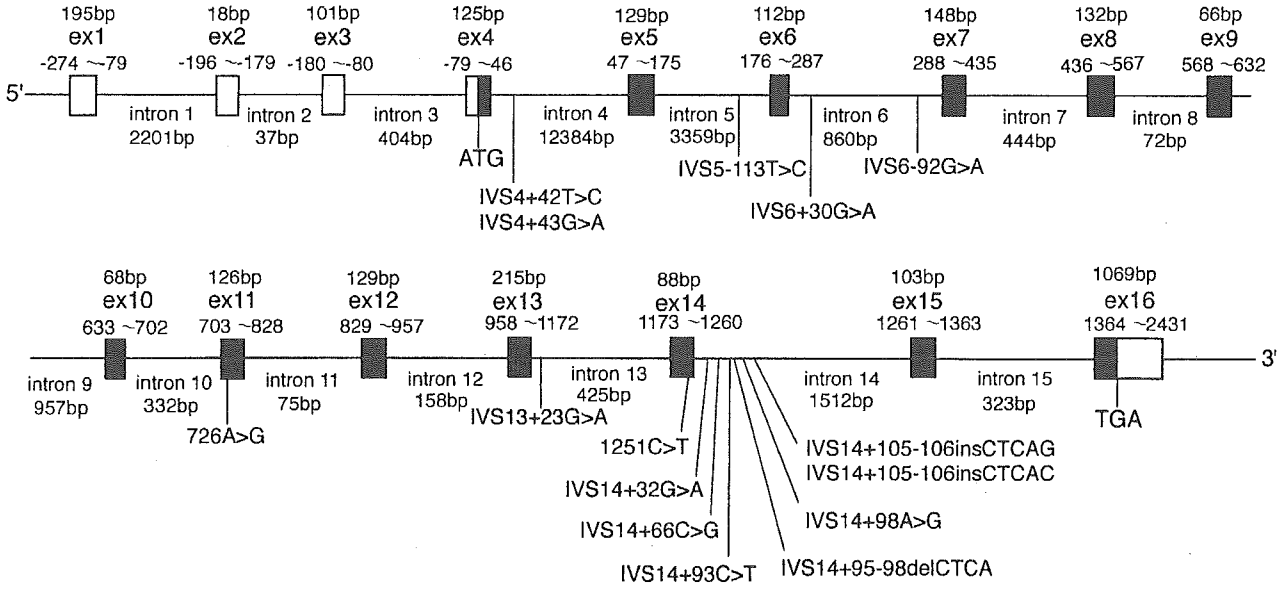

Fig. 1. Genomic structure and polymorphic sites in the *AKT1* gene. The sizes of exons and introns are shown but they are not drawn to size. Protein-coding exons are shown as black blocks and white boxes indicate untranslated regions.

sequence (GenBank Acc. No. XM_032231) with the sequence of a bacterial artificial chromosome clone (GenBank Acc. No. AL590327). The gene was found to be approximately 26 kb in size consisting of 16 exons, with the protein coded for by exons 4–16 (Fig. 1). This structure is different from that reported by Matsubara et al. [15], which lacked exons 1 and 2. We screened all exons and flanking introns in 22 unrelated bipolar patients and detected a total of 14 polymorphisms composed of 12 single nucleotide polymorphisms (SNPs) and two insertion/deletion variants. The most downstream polymorphic sites in intron 14 had three alleles, that is, the insertion of either -CTCAC- or -CTCAG- or absence of insertion (Fig. 1). No missense mutations were detected. Interestingly, seven variants were clustered in a 132 bp genomic stretch [hitherto referred to as 'HV (highly variable)' region] encompassing exon 14 and the 5′ portion of intron 14.

Recent large-scale genome analyses of SNP patterns have revealed that the human genome can be parsed objectively into haplotype blocks, sizable regions over which there is little evidence for historical recombination and within which only a few common haplotypes are observed [6,9]. Therefore haplotype-based methods could be a powerful approach to disease gene mapping, based on the association between causal mutations and the ancestral haplotype on which they arose [9]. Gabriel et al. [9] reported that half of the human genome exists in blocks of 44 kb or larger in European samples and that within each block, a very small number of common haplotypes (3–5) typically capture ~90% of all chromosomes. Based on these

Table 1
Haplotypes defined by the HV region of *AKT1* gene and their frequencies

Haplotype No.	Polymorphism[a]							Number of haplotypes (% frequency)[b]
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	
1	C	G	C	C	–	G	insCTCAG	540 (70)
2	C	G	C	C	–	A	–	159 (21)
3	C	G	G	C	–	A	–	39 (5)
4	C	G	C	C	del	NA[c]	–	23 (3)
5	T	G	C	T	–	G	insCTCAG	3 (0.4)
6	C	G	C	C	–	A	insCTCAC	2 (0.3)
7	C	A	C	C	–	G	insCTCAG	2 (0.3)

[a] No. 1, 1251C > T; No. 2, IVS14 + 32G > A; No. 3, IVS14 + 66C > G; No. 4, IVS14 + 93C > T; No. 5, IVS + 95-98delCTCA; No. 6, IVS14 + 98A > G; No. 7, IVS14 + 105 − 106ins (CTCAC) or (CTCAG)

[b] The haplotpes for nine individuals out of 393 individuals could not be unequivocally determined, and these data are not included.

[c] NA, not applicable.

Table 2
PDT analysis of haplotype transmission in the bipolar pedigrees under the affection status model I (II)

Haplotype[a]	Parent-to-offspring		Transmission in disordant sibs		Z score	*P* value
	Transmitted	Non-transmitted	Affected	Non-affected		
1	52 (73)	60 (83)	130 (139)	198 (179)	−1.5 (−1.3)	0.136 (0.200)
2	21 (27)	13 (20)	49 (52)	58 (51)	2.1 (2.1)	0.039 (0.038)
3	6 (12)	5 (8)	8 (14)	16 (13)	−0.9 (0.6)	0.345 (0.555)
4	1 (2)	2 (3)	7 (7)	12 (9)	−0.4 (−0.9)	0.654 (0.361)

[a] Only four common haplotypes were analyzed. See Table 1 for the structure of each haplotype.

potential advantages and the feasibility of haplotype determination of the *AKT1* HV region by one PCR amplification, we focused on the haplotypes in this region. We used the following primers to amplify the HV region: forward, 5′-GGGCCCTACATCACAGGAGGAA (5′ end at IVS13 − 88); reverse, 5′-GCAGCAAGGCCCTCCTTGTA (5′ end at IVS13 + 144). The genotypes were determined by the combination of direct sequencing of the PCR products and sequencing after subcloning into TA vectors (Invitrogen). Sequencing was done using the BigDye terminator cycle sequencing kit (Applied Biosystems) and an ABI 3700 DNA analyzer (Applied Biosystems). The haplotype pattern of each individual was determined by both visual inspection and the use of GENEHUNTER v. 2 [12].

The frequencies of haplotypes detected in the 384 members of the 22 pedigrees are shown in Table 1. There were seven different haplotypes, four of which were 'common' (frequency ≥ 3%). These common haplotypes are defined by four different variants, and their sum represented 99% of the total chromosomes. These values approximate the estimates by Gabriel et al. [9]. We then examined transmission pattern of haplotypes in the 22 pedigrees by employing the pedigree disequilibrium test (PDT) (Table 2) [14]. PDT can utilize data from all informative individuals among the families, and it is a valid test of association even when several nuclear families from an extended pedigree are related. The PDT program provides two statistics, PDT-sum and PDT-ave. The former gives more weight to larger families, while the latter places equal weight to all families. The 22 pedigree panel consists of extended families therefore we calculated the PDT-sum. Given the uncertainty regarding the spectrum of inherited mood disorder illnesses, we created two disease categories (affection status models: ASM) consisting of a restricted definition of affected (model one: ASM I) and a broad model (model two: ASM II) [7]. ASM I included bipolar disorder and schizoaffective disorder with manic phases, and ASM II was composed of ASM I plus recurrent major depression. There were 117 affected and 276 unaffected individuals in the ASM I category, and 156 affected and 237 unaffected in the ASM II [7]. This analysis showed that haplotype 2 was preferentially transmitted to affected offspring under both ASM I (nominal $P = 0.039$; the global P value that takes multi-allele correction into account was 0.129) and ASM II (nominal $P = 0.038$, global $P = 0.150$).

In this study, we conducted a haplotype transmission analysis of *AKT1* gene in the large bipolar pedigrees. The major attraction of haplotype methods is the idea that common haplotypes capture most of the genetic variation across sizable regions and that these haplotypes (and the undiscovered variants they contain) can be tested with the use of a small number of haplotype tag SNPs [9]. Although the considerable subset of haplotype blocks in European descendants extend to over 50 kb [9], it remains elusive whether there is any linkage disequilibrium gaps in the intervals between the 5′ end of *AKT1* gene and the HV region (~23 kb), and between the 3′ end of the gene and the HV region (~3 kb). However, the current results of a possible association between the *AKT1* gene haplotype and mood disorders warrant a comprehensive search for the disease-causing allele(s) on chromosome 14q in larger samples.

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