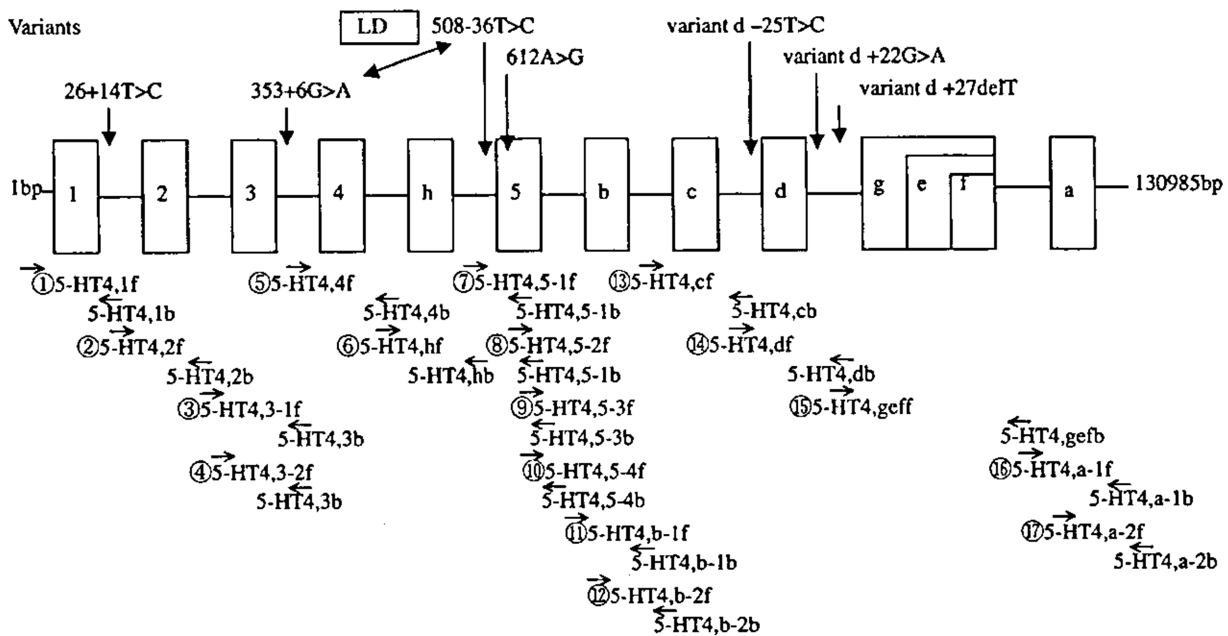

Fig. 1. Direct sequencing screen of the human 5-HT$_4$ receptor locus. The base pair (bp) designations are relative to the start codon. PCR and Sequencing primers are indicated by horizontal arrows and sequence variation by vertical arrows. Alphabetical coding regions are splice variants. We detected one synonymous DNA substitution and six SNPs in intron, which were designated '24 + 14 > C,' '353 + 6G > A,' '508 − 36T > C,' '612A > G,' 'variant d − 25tT > C,' 'variant d + 22G > A,' 'variant d + 27delT.'

thalamus and cerebellum [Medhurst et al., 2001; Vilaro et al., 2002]. These regions are important areas for cognitive function so that the discrepancies of the expression pattern of splice variants among individuals may reflect the diversity of the cognitive function.

Considering these findings, we selected *HTR4* as a candidate gene for vulnerability to schizophrenia and searched polymorphisms in the HTR4 coding regions, as well as intronic branch sites that may influence the alternative splicing. We identified a silent mutation and six intronic single nucleotide polymorphisms (SNPs), using the denaturing high-performance liquid chromatography (dHPLC) method [Spiegelman et al., 2000]. We also performed association and haplotype analysis to test that these SNPs have been associated with schizophrenia compared to ethnically matched controls.

SUBJECTS AND METHODS

Subjects

Subjects consisted of 189 patients with schizophrenia and 299 controls. The patients were recruited from psychiatric clinics of Fujita Health University Hospital. Consensus diagnosis according to DSM-IV by at least two experienced psychiatrists was made for each patient on the basis of unstructured interviews and information from medical records. None had severe medical complications or other Axis-I disorders according to DSM-IV. The controls, which were comprised of hospital staff and medical students, were not assessed for psychiatric symptoms by any structured interview method; however, they showed good social functioning and reported they were in good health. All subjects were unrelated to each other and ethically Japanese. After description of the study, written informed consent was obtained from each subject. This study was approved by the Fujita Health University Ethics Committee.

SNP Identification

PCR amplification. Genomic DNA from the subjects was prepared using PUREGENER (Gentra systems, Minneapolis, MN). Amplification of genomic DNA was accomplished with the primer pairs listed in Table III. These primer pairs amplify 17 regions that cover the entire 5-HT$_4$ coding sequence and adjacent intronic sequences including branch sites. Positions of the exon-intron boundaries were predicted based on the published human sequences, and the position of the splice variants were based on each splice variant. DNA amplification was preformed using a GeneAmp^{TM} PCR System 9700 (Applied Biosystems Japan Ltd., Tokyo, Japan). The reaction mixture was in a 12 µl volume containing a 40 ng sample DNA, 6 nM of each primer, 200 µM each dNTP, 1× PCR Gold Buffer, 1.5 or 2.5 mM $MgCl_2$, and 0.3 U of AmpliTaq Gold^{TM} (Applied Biosystems Japan Ltd.). Initial denaturation at 95°C for 9 min was followed by 40 cycles of denaturation at 95°C for 15 sec, primer annealing at each suitable temperature for 20 sec, and primer extension at 72°C for 30 sec, and a final extension reaction was performed at 72°C for 7 min.

dHPLC analysis. The PCR products were denatured at 95°C for 4 min, then cooled to 25°C over 45 min. Only the heterozygous DNA, heteroduplices were

generated through hybridization. Each product was run on an automated WAVE™ dHPLC instrument equipped with a DNASep™ column (Transgenomic Japan Ltd., Tokyo, Japan). PCR products were separated at a flow rate 0.9 ml/min by means of a linear acetonitrile gradient. The column mobile phase consisted of a mixture of 0.1 M triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. DNA elution was monitored by UV detection. The product's recommended temperature and Gradient parameters were calculated with the WAVE™ Maker program (version 3.3.4) of the WAVE™ instrument (Table III).

Cycle sequencing. The PCR products were purified by two methods. One was as follows; the PCR products, which were predicted as heterozygous from the peak pattern of dHPLC analysis, were purified by WAVE™ instrument using an application of a Fragment Collector. We collected the peak on the Double Stranded Single Fragment method of WAVE™ condition, and then used Amplicon™ Microcon™-PCR Centrifugal Filter Devices (Millipore Japan Ltd., Tokyo, Japan) in order to concentrate the collection up to 20 ng/μl. The other method was performed with AdvanTage™ PCR Cloning Kit (Clontech Japan Ltd., Tokyo, Japan) and was carried out when the mutation was not found with a conventional method, although on the dHPLC analysis there was an apparent heteroduplex pattern. The PCR products were then sequenced with BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Japan Ltd.) according to the manufacturer's instructions with an AMI PRISM™ 377 DNA sequencing system (Applied Biosystems Japan Ltd.)

SNP Genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Table IV shows primer pairs, restriction enzymes, and condition used for PCR-RFLP assays. Assays for 353 + 6A and 508 − 36C were developed in which a base substitution was introduced adjacent to the codon of interest, creating an artificial restriction site to detect the presence of one allelic form [Haliassos et al., 1989]. One to three units of each restriction enzyme was added directly to the mixture after which the PCR amplicon was restriction enzyme digested in a total volume of 10 μl containing 1× recommended buffer for 3 hr at 37°C. DNA fragments were resolved by electrophoresis in a 6% acrylamide gel stained with ethidium bromide.

Primer extension using dHPLC. For 353 + 6A and 508 − 36C, PCR-RFLP was not suitable, because the primer sets need to be modified. To analyze these two SNPs, primer extension methods using dHPLC [Hoogendoorn et al., 1999] were developed. PCR for the fragments was performed the same conditions described above, although the total volume was 6 μl. Then PCR reactions were treated by 0.5 U of Shrimp Alkaline Phosphatase and 0.5 U of Exonucleases I (both from Amersham Japan Ltd., Tokyo, Japan) at 37°C for 60 min, after which the enzymes were deactivated by incubation at 80°C for 15 min in order to remove unincorporated primers and dNTPs. Primer extension reactions were carried out in 10 μl containing 30 ng input PCR product, 25 μM of the appropriate ddNTPs, 12.5 pmol primer and 0.25 U Thermo Sequenase (Amersham Japan Ltd.) in the buffer provided by the manufacturer. The reaction was carried out in a thermal cycler with an initial denaturation at 96°C then 50 cycle of 96°C for 10 sec, 43°C for 15 sec and 60°C for 1 min. At the end of thermal cycling, the reaction was heated to 96°C for 1 min and immediately placed on ice. PCR primer sets and nucleotide compositions are listed in Table IV. A total of 4 μl of primer extension reaction product was loaded at 70°C on an automated WAVE™ dHPLC instrument (Transgenomic Japan Ltd.).

Statistical Analysis

Deviation from the genotype counts predicted by Hardy-Weinberg equilibrium expectations was tested using an exact test, as described by Weir [Weir, 1996] and implemented in software written by Lewis and Zaykin (2001; Genetic Data Analysis (GDA), version 1.0 (d16c)). D (disequilibrium parameter), D′ (standardized disequilibrium coefficient measurement) and Δ^2 (measure of disequilibrium) for pairwise linkage disequilibrium (LD) were calculated.

Estimation of the haplotype frequencies was performed by the expectation-maximization (EM) algorithm. LDSUPPORT, which implements the EM algorithm, is available for academic use from N. Kamatani on request [Kitamura et al., 2002]. The genetic status of an individual concerning linked loci can be expressed by the combination of two haplotypes (diplotype configuration).

Association tests for single markers were performed using CLUMP [Sham and Curtis, 1995] that calculates empirical P values by Monte Carlo procedure.

Haplotypes were assessed using odds ratios (ORs) and were calculated using the statistical software JMP (version 5.0, SAS Institute, Tokyo, Japan). Hence, all ORs were calculated using the appropriate two-side test for the 2×2 contingency table. Multiple testing was corrected for eight tests, four examining association analysis and four examining haplotype analysis.

An effect size was estimated by power, which was calculated using a statistical program prepared by Ohashi J [Ohashi and Tokunaga, 2001]. We assumed a significance level at 0.05 and penetrance of haplotype A-T and G-C to 0.04 and 0.01, respectively.

RESULTS

We analyzed HTR4 locus from the 96 Japanese schizophrenic patients for sequence variation using dHPLC followed by direct sequencing. We detected a silent polymorphism in exon 5 and six intronic SNPs, which were designated to '612A > G,' '26 + 14T > C,' '353 + 6G > A,' '508 − 36T > C,' 'd − 25T > C,' 'd + 22G > A,' and 'd + 27 delT' (Fig. 1). An intronic SNP '508 − 36T > C' locates in the consensus sequences of the branch site at 5′ upstream form exon 5, that could effect on the alternative splicing.

TABLE I. Distribution of the HTR4 Genotypes in 198 Schizophrenic and 211 Control Subjects

	n	Genotype			Frequency of rare allele	Significance[a]
			26 + 14T > C			
		T/T	T/C	C/C	26 + 14C	
Schizophrenia	198	161 (81%)	36 (18%)	1 (1%)	0.096	NS
Control	211	179 (85%)	31 (15%)	1 (0%)	0.078	
			353 + 6G > A			
		G/G	G/A	A/A	353 + 6A	
Schizophrenia	198	110 (56%)	70 (36%)	18 (9%)	0.268	NS
Control	207	115 (56%)	75 (36%)	17 (8%)	0.263	
			508 − 36T > C			
		T/T	T/C	C/C	508 − 36C	
Schizophrenia	198	109 (55%)	69 (35%)	20 (10%)	0.275	NS
Control	225	130 (58%)	84 (37%)	11 (5%)	0.236	
			d − 25T > C			
		T/T	T/C	C/C	d − 25C	
Schizophrenia	198	58 (29%)	95 (50%)	45 (23%)	0.467	NS
Control	299	106 (35%)	135 (45%)	58 (19%)	0.42	

[a]Statistical analysis was performed using the computer program CLUMP.
NS, not significant.

For rapid genotyping, PCR-RFLP and primer extension assays were developed. Typing 96 subjects by both PCR-RFLP and primer extension yielded identical results. In the 189 Japanese, the frequencies of the rare alleles 612G, 26 + 14C, 353 + 6A, 508 − 36C, valiant d − 25C, alleles were 0.004, 0.1, 0.27, 0.28, 0.47, respectively. All genotype distributions were consistent with Hardy-Weinberg expressions in this ethnic population (Table I).

For ease of presentation and because we did not have sufficient resources to evaluate differences in frequency between rare genotypes, only those with a frequency >10% were compared. In the 299 normal controls, the frequencies of the rare alleles 26 + 14C, 353 + 6A, 508 − 36C, valiant d − 25C were 0.08, 0.26, 0.24, 0.42, respectively. No association was found between each variant and schizophrenia (Table I).

Following the concept of common disease-common variant common-origin hypothesis [Kruglyak, 1999], several mutations of genes may participate in the etiology of schizophrenia and a combination of the SNPs may have an important meaning for this disorder. We examined LD with the four SNPs, '26 + 14T > C,' '353 + 6G > A,' '508 − 36T > C,' 'd − 25T > C.' Among them, '353 + 6G > A' and '508 − 36T > C' were tightly linked in LD ($D' = 0.96$), therefore we analyzed haplotypes that consist of the two SNPs. Individual haplotypes were counted case and controls using LDSUPPORT software [Kitamura et al., 2002]. Estimated frequencies of four haplotypes were 71.7% (G-T), 26% (A-C), 1.5% (G-C), 0.8% (A-T), respectively in schizophrenic patients. We then compared case and control number to those with/without each haplotype. It was detected that haplotype A-T significantly decreased in schizophrenic patients than in the normal controls (Table II). Among 198 schizophrenics and 206 controls, there were 15 controls had A-T haplotype, but only two schizophrenics had the A-T haplotype (corrected $P = 0.014$, OR = 0.13, 95% CI [0.03 − 0.58], effect size denoted by power is 0.5).

TABLE II. Association Between Schizophrenia and the Possession of Each HTR4 Haplotype (353 + 6G > A and 508 − 36T > C)

	Schizophrenia	Control	*P*	Corrected *P**	OR (95% CI)
A-C					
With	86	79	NS	NS	1.23 (0.83–1.84)
Without	112	127			
A-T					
With	2	15	0.001727	0.014	0.13 (0.03–0.58)
Without	196	191			
G-C					
With	4	2	NS	NS	2.1 (0.38–11.61)
Without	194	204			
G-T					
With	177	189	NS	NS	0.76 (0.39–1.48)
Without	21	17			

*The corrected *P* value was determined by multiplying the uncorrected *P* value by the number of tests displayed in this table added to those displayed in Table I (eight tests overall). Each corrected *P* value has been adjusted for eight tests.
NS, not significant.

TABLE III. Primers Used in dHPLC, PCR-RFLP, Primer Extension Analysis, and Temperature Used for dHPLC Analysis

Exon	Primer	5′–3′ sequence	Nucleotide position (5′–3′)	PCR product (bp)	PCR condition	dHPLC Temperature (°C)
Exon1 (nt1–26)	5-HT4-1f	ACT TCC CCC ATT TTA GGA CCC	$-63 \rightarrow -43$	151	Mg^{2+} 1.5 mM, pH 8.5, 63°C	56, 58
	5-HT4-1b	TAG AGT CTT CAT AGC AGA AAT GTT CTC A	$+62 \rightarrow +35$			
Exon2 (nt27–152)	5-HT4-2f	CTG ATG GTG AAG TTA CCT TTC TGA AG	$-66 \rightarrow -41$	255	Mg^{2+} 1.5 mM, pH 8.5, 60°C	59, 65
	5-HT4-2b	AAA AGG TTC CCT CCT GCT GCT	$+24 \rightarrow +4$			
Exon3 (nt153–353)	5-HT4-3.1f	CCC TTT TTC CCT TCA TCC CTC	$-56 \rightarrow -36$	312	Mg^{2+} 1.5 mM, pH 8.5, 60°C	58, 62
	5-HT4-3b	CAT CAA GTC ATG TCT CCA GCA TG	$+55 \rightarrow +33$			
	5-HT4-3.2f	TCT TAC ACT TTT TCA CTC ACA GGA AA	$-22 \rightarrow +4$	277	Mg^{2+} 3.5 mM, pH 8.5, 60°C	54, 61, 62
	5-HT4-3b	CAT CAA GTC ATG TCT CCA GCA TG	$+55 \rightarrow +33$			
Exon4 (nt354–507)						
	5-HT4-4f	TTG CCC ATG CCT ATG CTC TG	$-39 \rightarrow -20$	251	Mg^{2+} 1.5 mM, pH 8.5, 63°C	59, 62
	5-HT4-4b	GGA ACC CCA TGC AAA GTT GAT	$+58 \rightarrow +38$			
Exon h	5-HT4-hf	ACA GGG AGC TGC CCT TTC CT	$-68 \rightarrow -49$	155	Mg^{2+} 1.5 mM, pH 8.5, 60°C	61, 62
	5-HT4-hb	CCG GCA TTT CTT TCA GAA TCC	$+45 \rightarrow +25$			
Exon5 (nt508–1167)	5-HT4-5.1f	TCC CCA TTT TTC CCA CTT CTT	$-82 \rightarrow -62$	328	Mg^{2+} 1.5 mM, pH 8.5, 60°C	57, 62, 64
	5-HT4-5.1b	CAT GCG ATG AGT GCT ATG CT	$753 \rightarrow 734$			
	5-HT4-5.2f	ATA GAA AAG AGG AAG TTC AA	$508 \rightarrow 527$	246	Mg^{2+} 3.5 mM, pH 8.5, 57°C	59, 63, 66
	5-HT4-5b	CAT GCG ATG AGT GCT ATG CT	$753 \rightarrow 734$			
	5-HT4-5.3f	GCC CAT CAG ATC CAG ATG TT	$667 \rightarrow 686$	240	Mg^{2+} 1.5 mM, pH 8.5, 63°C	62, 64, 66
	5-HT4-5.3b	TAT AGC CGA GCC AGA GGA AA	$907 \rightarrow 888$			
	5-HT4-5.4f	CAT AGA CTA CAC TGT CCC TG	$852 \rightarrow 871$	255	Mg^{2+} 3.5 mM, pH 8.5, 60°C	59, 62
	5-HT4-5.4b	CAA ATC AAT GAA CTC CCT TA	$+30 \rightarrow +11$			
Exon b	5-HT4-b.1f	GGT GGG CTC TTT CAG GAG ATG	$-75 \rightarrow -54$	190	Mg^{2+} 1.5 mM, pH 8.5, 62°C	60, 65
	5-HT4-b.1b	TCT TCT GGG TCA TTG TCC CAG	$+25 \rightarrow +5$			
	5-HT4-b.2f	GGA TGC AGT GGA GTG TGG TG	$1 \rightarrow 20$	159	Mg^{2+} 1.5 mM, pH 8.5, 63°C	65, 66
	5-HT4-b.2b	AAG CAG CAG CTT AGG ACC TGG CCC	$+68 \rightarrow +45$			
Exon c	5-HT4-cf	CTG TGG TTT AAT AGC ATC TCA GGA TTA	$-98 \rightarrow -72$	290	Mg^{2+} 1.5 mM, pH 8.5, 58°C	54, 57, 58
	5-HT4-cb	ACG AAT TCT GAA TAG CAT TTC TCT TTC	$+32 \rightarrow +6$			
Exon d	5-HT4-df	GTT CTT CTC CTG TGA CAT TTT GAT A	$-76 \rightarrow -50$	151	Mg^{2+} 1.5 mM, pH 8.5, 60°C	58, 59, 61
	5-HT4-db	CAA AAA CCT GTG TTG GGC ACT	$+59 \rightarrow +39$			
Exon gef	5-HT4-geff	TTC CCA AAT TCT GCT TGG CT	$-58 \rightarrow -39$	180	Mg^{2+} 1.5 mM, pH 8.5, 60°C	62, 63
	5-HT4-gefb	AAT AGG CAG ACA CAG ACA GAC TCA CA	$+61 \rightarrow +36$			
Exon a	5-HT4-a.1f	TGA CTT CGG TGC AGT TGG AG	$-62 \rightarrow -43$	174	Mg^{2+} 1.5 mM, pH 8.5, 60°C	62, 63
	5-HT4-a.1b	CTA AGT TGT GAG CCA TGT CCT CA	$+24 \rightarrow +2$			
	5-HT4-a.2f	TAC ACC GTT CTG CAC AGG GG	$2 \rightarrow 21$	156	Mg^{2+} 1.5 mM, pH 8.5, 60°C	61, 62
	5-HT4-a.2b	ATG CCA GGG TGA CCT GTT CA	$+69 \rightarrow +50$			
PCR-RFLP	5-HT4-353 + 6(g-a)	CCG CTA TGC ACA TTG TTC GGT	$-27 \rightarrow -7$	250	Mg^{2+} 1.5 mM, pH 8.5, 60°C, with 5-HT4-3.2f	
	5-HT4-508 − 36(t-c)	TTT TCA CTT TTT CTT TCC TTT TTA GC	$-62 \rightarrow -37$	308	Mg^{2+} 1.5 mM, pH 8.5, 60°C, with 5-HT4-5.1b	
Primer extension	HT4-PE-353 + 6G > A	CAT TTC TCT GGA TAG GTA AG				
	5-HT4-PE-508 − 36T > C	CTT TTT CTT TCC TTT TTA CC				

TABLE IV. SNPs Typing by PCR-RFLP and Primer Extension

SNPs	PCR-RFLP primers	PCR product (bp)	Nucleotide position	Substitution	Enzyme for RFLP	Alleles	Fragment size (bp)	PCR primer for primer extension	Extension primer	Composition of primer extension reaction	Variant alleles
26 + 14T > C	5-HT4, 1f	151	26 + 14	(tct-tcg)	BsrGI	26 + 14t	104 + 48				
	5-HT4, 1b					26 + 14c	152				
353 + 6G > A	5-HT4-3.2f	250	353 + 6	(agg-aga)	AvaII	353 + 6g	229 + 21	5-HT4-3.2f	5-HT4 = PE = 353 + 6G > A	ddG,ddA	G/A
	5-HT4-353 + 6(g-a)					353 + 6a	250	5-HT4-3.2b			
508 − 36T > C	5-HT4-508 − 36(t-c)	309	508 − 36	(cct-ccc)	AluI	508 − 36t	185 + 98 + 25	5-HT4-5.1f	5-HT4 = PE = 508 − 36T > C	ddC,ddT	T/C
	5-HT4-5.1b					508 − 36c	210 + 98	5-HT4-5.1b			
d − 25C < T	5-HT4-df	151	− 25	(tac-tat)	HpyCH4IV	d − 25c	100 + 51				
	5-HT4-db					d − 25t	151				

DISCUSSION

The results of the present study may lead to two interpretations that (1) haplotype A-T inhibits the occurrence of schizophrenia, or (2) haplotype A-T is just a marker, and there are true common disease variations relating to schizophrenia within the areas constructed in the LD. For example, within 1,000 kb around HTR4, many genes are addressed including protein phosphatase 2 (formerly 2A), dihydropyrimidinase-like 3, interleukin 17B, calcium/calmodulin-dependent protein kinase IIα (CaMKII), and casein kinase α1. Among them, CaMKII is required for hippocampal LTP and spatial learning [Rotenberg et al., 1996].

Genetic linkage results must be received with caution until replicated, or until convergent findings become available. One limitation of the study is the small effect size (power = 0.5), although this result is consistent with the expected multigenic and multifactorial origins of schizophrenia. Another limitation is that population stratification can lead to spurious associations between a phenotype and an unlinked genetic locus [Altshuler et al., 1998]. Despite the precaution of ethnic matching in this study, there may have been ethnic stratification bias between patients and controls. To avoid false positive results due to stratification, methods using control loci [Pritchard and Rosenberg, 1999] are available for follow-up studies.

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ORIGINAL RESEARCH ARTICLE

CNR1, central cannabinoid receptor gene, associated with susceptibility to hebephrenic schizophrenia

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To examine the cannabinoid hypothesis for pathogenesis of schizophrenia, we examined two kinds of polymorphisms of the CNR1 gene, which encodes human CB1 receptor, a subclass of central cannabinoid receptors, in schizophrenics and age-matched controls in the Japanese population. Allelic and genotypic distributions of polymorphism 1359G/A at codon 453 in the coding region and AAT triplet repeats in the 3′ flanking region in the Japanese population were quite different from those in Caucasians. Although the polymorphism 1359G/A was not associated with schizophrenia, the triplet repeat polymorphism of the CNR1 gene was significantly associated with schizophrenia, especially the hebephrenic subtype (P = 0.0028). Hebephrenic schizophrenia showed significantly increased rate of the 9 repeat allele (P = 0.032, OR = 2.30, 95% CI (1.91–2.69)), and decreased rate of the 17 repeat allele (P = 0.011, OR = 0.208, 95% CI (0.098–0.439)). The present findings indicated that certain alleles or genotypes of the CNR1 gene may confer a susceptibility of schizophrenia, especially of the hebephrenic type.
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The cannabinoid hypothesis for the pathogenesis of schizophrenia is supposed to be based on the clinical facts that abuse of cannabis could precipitate the psychotic state, with hallucinations and delusions resembling schizophrenia[1–4] and worsen positive symptoms of schizophrenia,[5,6] even under regular medication of antipsychotics.[7] Cannabinoid consumption could result in poor outcome and liability to relapse for schizophrenics.[8–10] In addition, heavy cannabis users may develop an amotivational syndrome, reminiscent of some of the negative symptoms of schizophrenia.[11] A Swedish cohort study showed that cannabis use before 18 years of age raises the incidence rate of schizophrenia six-fold.[12] Another study showed that administration of delta-9-hydrocannabinol to normal volunteers induced cognitive impairment of three dimensions resembling closely that of schizophrenic patients.[13] The hallucinogenic action of cannabis and marijuana mediated the central cannabinoid receptor, G protein-coupled receptor CB1, which was discovered in 1988.[14] CB1 receptors were expressed abundantly throughout the brain, especially in substantia nigra, globus pallidus, hippocampus and cerebellum.[15,16] CB1 receptors are encoded by the CNR1 gene (MIM114610), which was cloned by Matsuda *et al* in 1992.[17] CB1 is located at 6q14–q15, which was included in a schizophrenia susceptibility locus, 6q13–q26, revealed by Cao *et al*[18] using two independent series of pedigrees, which was designated by Schizophrenia 5 (SCZ5, OMIM 603175). Recently, two polymorphisms, AAT repeats microsatellite in the 3′ flanking region and 1359 G/A polymorphism at codon 453 in the coding exon of the CNR1 gene, were reported.[19,20] To examine the cannabinoid hypothesis for schizophrenia, we examined these two polymorphisms in the CNR1 gene of schizophrenia in the Japanese population.

Genotypic distribution and allelic frequency of 1359 A/G and AAT repeat polymorphisms are summarized in Tables 1 and 2, respectively. Distributions of the alleles of the two kinds of polymorphisms of the CNR1 gene in both groups were within the values expected from Hardy–Weinberg equilibrium. GG, GA and AA of the 1359 G/A polymorphism genotype in controls were 94.2%, 4.4% and 1.5%, respectively. The genotypic distributions were not significantly different between controls and schizophrenia (G = 0.69, df = 1, P = 0.41), or among controls, hebephrenic and paranoid type schizophrenia (G = 2.39, df = 2, P = 0.30). The allelic frequency of the G allele and the A allele in controls was 96.4% and 3.7%, respectively. The allelic frequencies were not significantly different between controls and schizophrenia (G = 0.01, df = 1, P = 0.90), or among controls, hebephrenic and paranoid type schizophrenia (G = 1.70, df = 2, P = 0.43).

Allelic frequencies of AAT repeats of the CNR1 gene were shown in Table 2. Nine kinds of allele, $(AAT)_9$, $(AAT)_{10}$, $(AAT)_{12}$–$(AAT)_{18}$ repeat alleles were found. The most frequent allele of Japanese controls was $(AAT)_{15}$ allele (34.8%), followed by $(AAT)_{16}$ allele (28.7%), $(AAT)_{14}$ allele (16.9%) and $(AAT)_{17}$ allele (7.1 %). Alleles of $(AAT)_9$ and $(AAT)_{12}$ repeat were relatively rare and those of $(AAT)_{10}$ and $(AAT)_{18}$ repeat were few. The allelic distributions were significantly different between controls and schizophrenia (z = 1.995, P = 0.046). As to subcategories of schizophrenia, hebephrenic type, but not paranoid type schizophrenia

Table 1 Allelic and genotypic frequencies of the 1359G/A polymorphism at codon 453 in the CRN1 gene coding region

Group	*n*	*Genotype*			*Allele*	
		GG	*GA*	*AA*	*G*	*A*
Control	137	94.2%	4.4%	1.5%	96.4%	3.7%
All schizophrenia	116	93.1%	6.9%	0.0%	96.6%	3.5%
Hebephrenic	61	90.2%	9.8%	0.0%	95.1%	4.9%
Paranoid	55	96.4%	3.6%	0.0%	98.2%	1.8%

Table 2 Allele frequencies of AAT triplet repeats in the 3′ flanking region of the CNR1 gene

Group	*Numbers of AAT repeats*									
	n	*9*	*10*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
Control	296	5.1%	0.0%	5.7%	1.4%	16.9%	34.8%	28.7%	7.1%	0.3%
All schizophrenia	242	9.9%*	0.4%	5.8%	0.4%	18.6%	33.9%	27.7%	3.3%*	0.0%
Hebephrenic	128	10.9%*	0.0%	7.0%	0.8%	21.1%	35.9%	22.7%	1.6%*	0.0%
Paranoid	110	9.1%	0.0%	3.6%	0.0%	15.5%	31.8%	34.5%	5.5%	0.0%

*$P < 0.05$.

were significantly different from controls (among the three groups, H = 10.17, P = 0.006; hebephrenic *vs* controls, z = 2.99, P = 0.0028; paranoid *vs* controls, z = 0.24, P = 0.81). Schizophrenia in all and hebephrenic type schizophrenia also showed a significantly increased rate of $(AAT)_{10}$ allele (schizophrenia in all, G = 4.58, df = 1, P = 0.032; hebephrenia, G = 4.39, df = 1, P = 0.036), and significantly decreased rate of $(AAT)_{18}$ allele (schizophrenia in all, G = 3.85, df = 1, P = 0.049, hebephrenia, G = 6.41, df = 1, P = 0.011). The odds ratios of hebephrenia for the $(AAT)_{10}$ allele and the $(AAT)_{18}$ allele were 2.30 (95% CI (1.91–2.69)) and 0.208 (95% CI (0.098–0.439)), respectively.

In the present study, we found that genotypic and allelic distributions of 1359A/G and AAT repeat polymorphism of the CNR1 gene in Japanese controls were quite different from those in the Caucasian population. Thus, Gadzicki *et al*[19] reported that G and A allele frequencies of 1359G/A polymorphism were 76% and 24%, respectively in the German Caucasian population. Heterozygosities of this polymorphism in the Japanese and Caucasian population were 0.069 and 0.365. As to the AAT repeat polymorphism, Comings *et al*[21] reported that the most frequent allele of the polymorphism in non-Hispanic Caucasians living in the USA was $(AAT)_{13}$ repeat, followed by $(AAT)_{17}$, $(AAT)_{16}$ and $(AAT)_{15}$. The allele of $(AAT)_{13}$ repeats was as rare as 1.4% in the Japanese population. The allelic distribution of AAT repeats in the Japanese population was similar but not consistent with that in the Han Chinese population reported by Li *et al*.[22] These results indicated that genotypic and allelic distributions of the two polymorphisms of the CNR1 gene could differ greatly among different races.

We have found that AAT repeats but not the 1359G/A polymorphism of the CNR1 gene were significantly associated with schizophrenia for the first time. As to subcategories of schizophrenia, hebephrenic type showed a significant association with AAT repeats polymorphism whereas the paranoid type did not. Tsai *et al* (2000)[23] reported no association between AAT repeats of the CNR1 gene and Chinese schizophrenics. Certain reasons for this inconsistency are unknown, however, it is possible that racial difference, in the Japanese and Chinese population, may affect it. Alternatively, the composition of schizophrenic patients used in their study may considerably differ from that of the present study, although they did not show the ratio of each subtype of schizophrenia. If the paranoid type of schizophrenics was predominant in their subjects, a significant association may be ignored, because the present study showed that the paranoid type of schizophrenia did not associate with the CNR1 gene.

The hebephrenic type schizophrenia, which has been shown to be associated with the CNR1 gene in the present study, is characterized by predominant negative symptoms such as blunted affect, disorganized thought and deterioration of personality. Such symptomatic features of hebephrenic schizophrenia bear resemblance to chronic cannabinoid psychoses. Although acute use of cannabis can induce diverse types of psychotic state, such as panic reaction, confusional state, paranoid state and mania,[24–26] chronic cannabis users often develop an 'amotivational syndrome'[27–30] typified by a diminution of ambition, productivity and motivation, which are often observed in hebephrenic schizophrenics. In addition, cannabinoid use affects cognitive function such as information processing and planning tasks.[31] Such cognitive dysfunc-

tion is prominent in chronic schizophrenics like hebephrenics. Therefore, the endogenous cannabinoid system may be activated in the brain of patients with schizophrenia, especially with the hebephrenic type. A knockout study showed that central actions of cannabinoids are mediated via CB1 receptors encoded by the CNR1 gene.[32] The CB1 receptor also mediates actions of endogenous cannabimimetic ligands, endocannabinoids like annadamide.[33] It is possible that neurotransduction of CB1 receptors may be enhanced in the schizophrenic brain. A recent postmortem study supported this possibility which showed that receptor density of CB1 receptors in dorsolateral prefrontal cortex was increased in schizophrenics compared with controls.[34] The present study has revealed an association of the CNR1 gene polymorphism with hebephrenic schizophrenia. The finding would support genetically the hypothesis that changes in the endogenous cannabinoid system of the brain may be involved in the pathogenesis of schizophrenia. The AAT repeat polymorphism locates in downstream about 16 kb of the CNR1 coding region. If the polymorphism affects transcription efficiency of the CNR1 gene as an enhancer, however, it is not still known, the present finding is very significant for the cannabinoid hypothesis of schizophrenia. Alternatively, the present finding may indicate an association with schizophrenia of other genes located close to the AAT repeat polymorphism of the CNR1 gene, although no genes or EST were registered in GenBank in the 3 prime region of the CNR1 gene to date. To confirm the significance of the study for the cannabinoid hypothesis of schizophrenia, the relationship of an allele containing a certain number of AAT repeats of the CNR1 gene and the transcription rate of the CNR1 gene should be specified. In addition, the present significant findings should be replicated by genomic control methods[35] or family based control methods to avoid false positive results due to population stratification.

Methods

Subjects

The subjects were 121 patients with schizophrenia (F20, 74 males and 48 females, age 44.7 ± 13.2 years) meeting ICD-10-DCR criteria who were outpatients or inpatients of psychiatric hospitals, and 148 age-matched normal controls (70 males and 78 females, age 44.5 ± 16.0 years) who had no known history of psychiatric disease in their families. Diagnosis was made by trained psychiatrists by interview. As to the subcategory of schizophrenia, hebephrenic (F20.1), paranoid (F20.0), catatonic (F20.2), and undifferentiated type (F20.3) accounted for 63, 55, two, and one patients, respectively. All subjects were Japanese, born and living in the middle western area of Japan. No subject abused any illicit drugs, such as cannabis and methamphetamine. This study was performed after approval by the ethics committee of Okayama University Medical School, Zikei Hospital and Takaoka Hospital, and all subjects provided written informed consent for the use of their DNA samples for this research.

CNR1 genotyping

Genomic DNA was extracted from peripheral leukocytes by the standard phenol/$CHCl_3$ method. The region contained the 1359 G/A polymorphism amplified by PCR, using a mismatch primer set according to Gadzicki *et al*[19] to create Msp I recognition sites (5'-GAAAGCTGCATCAAGAGCCC-3', 5'-TTTTCCTGTG CTGCCAGGG-3'). PCR was performed in a final volume of 15 μl with 10% dimethyl sulfoxide and 1 unit of Supertaq (Sawady Co, Japan) in the reaction mixture. PCR conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min, and 72°C for 5 min. The PCR products were analyzed on 3% agarose gel after digestion with Msp I. The region containing the AAT triplet repeat polymorphism was amplified according to Dawson *et al*[20] (5'-GCTGCTTCTGTTAACCCTGC-3', 5'-TACATCTCCG TGTGATGTTCC-3'). For analysis of polymorphic AAT repeats, the forward primer was 5'-end-labelled with Texas Red, and PCR products and the Texas red-labelled size standard were electrophoretically run on 6% polyacrylamide gel using an SQ5500 DNA sequencer (Hitachi Co, Japan) and each length was calculated using Fragyls 2 (Hitachi Co, Japan) computer software. Repeat numbers of AAT of the CNR1 gene were confirmed by direct sequencing using PCR samples from subjects homozygous for the repeat polymorphism of $(AAT)_9$, $(AAT)_{14}$, $(AAT)_{16}$ and $(AAT)_{17}$. All genotyping was carried out in a blinded fashion with control and patient samples mixed randomly. Statistical analysis between control and schizophrenia for the 1359 G/A polymorphism and each repeat allele of the AAT repeat polymorphism was done using the log-likelihood ratio test, and that for the overall allele of repeat polymorphism was done using the Mann–Whitney test. When comparison was made among three groups, control, hebephrenic type and paranoid type, the F test using the log-likelihood ratio test or the Kruskal–Wallis test was examined at first. If it was significant, each pair was examined as above mentioned.

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