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Hiroshi Mitsuyasu · Naotsugu Hirata · Yasuyoshi Sakai
Hiroki Shibata · Yasuhiko Takeda · Hideaki Ninomiya
Hiroaki Kawasaki · Nobutada Tashiro
Yasuyuki Fukumaki

Association analysis of polymorphisms in the upstream region of the human dopamine D4 receptor gene (*DRD4*) with schizophrenia and personality traits

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Abstract The human dopamine D4 receptor (DRD4) is of major interest in molecular studies of schizophrenia and personality traits. We examined the association of schizophrenia and polymorphisms in the upstream region of the *DRD4* gene (−768G>A in the negative modulator region; −521C>T, −376C>T, and −291C>T in the cell type-specific promoter region; and −616C>G between the two regions) in 208 schizophrenic patients and 210 normal controls. No significant difference in genotype and allele frequencies was observed between the two groups, indicating that these polymorphisms do not make a major contribution to the pathogenesis of schizophrenia. We also studied the association of polymorphisms in the upstream region and a 48-bp repeat polymorphism in exon III of the *DRD4* gene with personality traits in 173 Japanese individuals who completed the temperament and character inventory (TCI). The −768G>A polymorphism was significantly associated with reward dependence ($P = 0.044$), while no significant association was observed between novelty seeking and polymorphisms in the upstream region or the exon III repeat polymorphism of the *DRD4* gene.

Key words Dopamine D4 receptor · Polymorphism · Association · Schizophrenia · Personality trait · TCI

Introduction

The dopaminergic neurotransmission system attracts attention because of its possible involvement in the etiology of several psychotic disorders and personality traits. Five distinct dopamine receptor genes have been identified, and have been classified into D1-like receptors (D1 and D5) and D2-like receptors (D2, D3, and D4) according to their structural and pharmacological characteristics. Of these, the human dopamine D4 receptor (DRD4) is considered to be a candidate for involvement in schizophrenia and personality traits. *DRD4* mRNA expression was observed in the human frontal cortex, amygdala, thalamus, cerebellum, pituitary, entorhinal cortex, and hippocampus (Hartman and Lanau 1997). An autoradiographic study with a D4-selective agent, [^{3}H]NGD-94-1, suggested high levels of radioligand binding in the normal post-mortem human hippocampus and entorhinal cortex, and low levels in the caudate, putamen, nucleus accumbens, and substantia nigra (Tarazi and Baldessarini 1999). Clozapine, an antipsychotic for the management of socially withdrawn and treatment-resistant schizophrenics, has a relatively high affinity to DRD4 (Van Tol et al. 1991). The density of DRD4 was sixfold elevated in the brains of schizophrenic patients (Seeman et al. 1993). Elevation of *DRD4* mRNA level was found in the frontal cortex of schizophrenic patients post-mortem compared with levels in controls (Stefanis et al. 1998). These observations suggest that allelic variations affecting transcription levels of the *DRD4* gene could be involved in the pathogenesis of schizophrenia.

Twin studies demonstrate that personality traits, measured by self-report questionnaires such as the tridimensional personality questionnaire (TPQ), are partially inherited, and 30%–60% of observed variance can be accounted for genetically (Cloninger 1987; Bouchard 1994). In the temperament and character inventory (TCI) that was developed from the TPQ, personality traits are divided into four temperaments: novelty seeking; harm avoidance; reward dependence; and persistence, based on a biopsychosocial view of personality. Association of the exon III repeat polymorphism in the *DRD4* gene with the personality trait of novelty seeking was reported in 1996 (Ebstein et al. 1996; Benjamin et al. 1996); however, conflicting results were also reported (Paterson et al. 1999). Association analysis of polymorphisms in the upstream region of the *DRD4* gene with personality

H. Mitsuyasu · N. Hirata · Y. Sakai · H. Shibata · Y. Takeda · Y. Fukumaki (✉)
Division of Disease Genes, Institute of Genetic Information, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
Tel. +81-92-642-6168; Fax +81-92-632-2375
e-mail: yfukumak@gen.kyushu-u.ac.jp

H. Mitsuyasu · Y. Takeda · H. Ninomiya · H. Kawasaki · N. Tashiro
Department of Neuropsychiatry, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

traits may be useful to evaluate this interesting observation.

Our previous study of *DRD4* gene expression, using cultured cells, revealed that the region between −591 and −123 relative to the first nucleotide of the initiation codon contains the promoter of the *DRD4* gene, which is responsible for the cell type-specific expression of the gene, and the negative modulator region is located between −770 and −679 (Kamakura et al. 1997). We have identified ten polymorphisms in the upstream region of the *DRD4* gene, spanning the negative modulator and the cell type-specific promoter regions (Mitsuyasu et al. 1999). In the present study, we investigated the association between schizophrenia and polymorphisms in the upstream region of the *DRD4* gene. We also examined the association between personality traits and polymorphisms in the upstream region and an exon III repeat polymorphism of the *DRD4* gene.

Subjects and methods

Subjects

The Ethics Committee of the Faculty of Medicine of Kyushu University approved the study protocol. Two hundred and eight schizophrenic patients (116 men and 92 women) were recruited from seven hospitals in the Fukuoka area, one hospital in the Nagasaki area, and one hospital in the Oita area, Japan. The diagnosis of schizophrenia was made according to DSM-IV criteria (American Psychiatric Association, 1994). The mean age of the schizophrenic patients was 51.5 years (SD, 13.9). There were 210 controls (120 men were recruited from the personnel of the Japanese Self-Defense Forces and 90 women volunteers were recruited from the staff of two hospitals). The mean age of the controls was 50.5 years (SD, 5.0). In the association study for personality traits, 173 subjects (all men; mean age, 52.2 years; SD, 0.8) were recruited from the Japanese Self-Defense Forces personnel. All patients and the control subjects gave their informed consent. All patients and controls were ethnically Japanese.

Genotyping

Genomic DNA was extracted from leukocytes by salting out with saturated sodium chloride solution, as described by Lahiri and Nurnberger (1991). The fragment spanning between −947 bp and −156 bp of the *DRD4* gene was amplified by polymerase chain reaction (PCR), using the sense primer, D4neg3 (5′-CAGGTCACAGGTCACCCCT CTT-3′, −947/−926) and the antisense primer, D4neg4 (5′-TTGCTCATCTTGGAATTTTGCG-3′, −156/−177). The PCR was performed in a volume of 100 μl containing 100 ng genomic DNA, 20 pmol of each primer, 10 μl of KOD Dash 10 × PCR Buffer, 200 μM each of dNTPs, 10% dimethylsulfoxide (DMSO), and 2.5 units of KOD Dash (Toyobo, Osaka, Japan). The cycle conditions were 94°C for 1 min, then 30 cycles of 94°C for 30 s, 61°C for 2 s, and 74°C for 30 s, with a final extension step of 5 min at 74°C, in a GeneAmp 2400 Thermocycler (PE Biosystems, Foster City, CA, USA). The PCR products were electrophoresed on a 1.5% agarose gel containing 1 × Tris-borate/ethylenediamine tetraacetic acid (EDTA) electrophoresis buffer (TBE), and visualized by ethidium bromide staining.

We have done genotyping of nine polymorphisms out of ten in the upstream region of the *DRD4* gene previously reported: they are −809G>A, −768G>A, −616C>G, −603T>del, −602G>del, −600G>C, −521C>T, −376C>T, and −291C>T (Mitsuyasu et al. 1999). The genotypes of the 173 men from the Self-Defense Forces personnel were determined by direct DNA sequencing. Cycle sequencing was done in a volume of 20 μl containing 50–100 ng of concentrated PCR product DNA, with a Microcon YM-100 unit (Millipore, Bedford, MA, USA), with 3.2 pmol of either primer D4neg3 (sense) or D4neg4 (antisense) in the thermocycler with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). The cycle sequencing protocol was as recommended by the manufacturer, except that 28 cycles were performed. Sequences were analyzed in an ABI PRISM model 377 DNA Sequencer (PE Biosystems).

The genotypes of the rest of the controls and the schizophrenic patients were determined by the PCR-restriction fragment length polymorphism method, as previously described (Mitsuyasu et al. 1999). The PCR products were digested with *Nar*I, *Ava*II, *BssH*II, *Rsa*I, and *Ava*I to determine the genotypes of the following polymorphisms: −768G>A, −616C>G, −521C>T, −376C>T, and −291C>T, respectively. Electrophoreses of the digests were carried out on a 2% agarose gel and visualization was with ethidium bromide staining, except for *Ava*I digests, which were electrophoresed on a 3% agarose gel.

We examined the exon III repeat polymorphism of the *DRD4* gene in the Self-Defense Forces personnel using a previously described method (Muramatsu et al. 1996), with slight modification. PCR amplification was performed in a volume of 25 μl containing 50 mM KCl; 10 mM Tris-HCl (pH 9.0); 100 ng genomic DNA; 10 pmol of each primer (sense primer, 5′-AGGTGGCACGTCGCGCCAAGCT GCA-3′; antisense primer, 5′-TCTGCGGTGGAGTCTG GGGTGGGAG-3′); 100 μM each of dATP, dTTP, dCTP, and 7-deaza-2′-deoxyguanosine 5′-triphosphate; 1.5 mM $MgCl_2$; 10% DMSO; and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA). The cycle conditions were 94°C for 1 min, then 30 cycles of 94°C for 15 s, 65°C for 30 s, and 72°C for 15 s, with a final extension step of 7 min at 72°C, in a GeneAmp 2400 thermocycler. The PCR products were electrophoresed on a 3% agarose gel containing 1 × TBE, and visualized by ethidium bromide staining.

Personality questionnaire

We used the Japanese version of the temperament and character inventory (TCI) (Cloninger et al. 1993), which is a 240-item questionnaire measuring seven dimensions of personality (Kijima et al. 1996). The TCI was developed from the TPQ (Cloninger 1987). The reliability and validity of

the Japanese version of the TCI have already been verified (Kijima et al. 1996). The 173 individuals from the Self-Defense Forces personnel filled out this personality questionnaire.

Statistical analysis

Significant differences in genotype and allele frequencies between the schizophrenic patients and control group were evaluated using the χ^2 test, with the significance level of 0.05. Associations between the TCI test scores and *DRD4* polymorphisms were assessed by the Kruskal-Wallis test or the Mann-Whitney *U*-test. We used the estimate haplotype frequencies (EH) program (Otto 1999) to evaluate linkage disequilibrium.

Results

Association of polymorphisms with schizophrenia

We determined genotype and allele frequencies of one polymorphism, −768G>A, in the negative modulator region, three polymorphisms, −521C>T, −376C>T, and −291C>T, in the cell type-specific promoter region, and one polymorphism, −616C>G, between the two regions for the association analysis in the 208 schizophrenic patients and the 210 normal controls. The frequencies of these polymorphisms in the *DRD4* gene in the two groups are presented in Table 1. The distribution of these five polymorphisms was in Hardy-Weinberg equilibrium (data not shown). We observed no significant difference in genotype and allele frequencies between the two groups.

There were also no significant differences in these frequencies among schizophrenic subtypes (paranoid, disorganized, catatonic, undifferentiated and residual) (data not shown). Average age of onset did not show any association with the polymorphisms.

Association of polymorphisms with personality traits

To examine the association between personality traits and nine polymorphisms in the upstream region of the *DRD4* gene and the exon III repeat polymorphism, we genotyped the subjects from the Self-Defense Forces personnel. As shown in Table 2, the Mann-Whitney *U*-test revealed a significant association between the −768G>A polymorphism and reward dependence scores ($P = 0.044$). Reward dependence scores in subjects with the −768G/A genotype were significantly lower than the scores in those with the −768G/G genotype. The Kruskal-Wallis test revealed significant associations between the −602G>del polymorphism and novelty seeking scores ($P = 0.037$). The novelty seeking scores of subjects with the −602G/G genotype were significantly higher than the scores of those with the −602del/del genotype. However, these differences were not significant after Bonferroni correction ($P = 0.1095$).

The exon III repeat polymorphism genotypes were classified into two groups: the short (S) group, containing alleles with two to four repeats and the long (L) group, containing one or two alleles with five to seven repeats. In our subjects, the four-repeat allele was observed most frequently (80.9%), followed by the two-repeat allele (11.8%). In contrast, the three-repeat (0.3%), six-repeat (1.2%), and seven-repeat (1.2%) alleles were rare. There was no significant association between any TCI scores and the exon III repeat polymorphism of the *DRD4* gene.

Linkage disequilibrium between polymorphisms

We evaluated linkage disequilibria between the nine polymorphisms, based on the data obtained from the TCI analysis. D' values (i.e., D/Dmax if $D > 0$, D/Dmin if $D < 0$) (Hartl and Clark 1997) between polymorphisms were calculated where *P* values were less than 0.05 by the χ^2 test, using the EH program (Otto 1999). D' values were 0.868 between −521C>T and −291C>T, 0.614 between −768G>A and −291C>T, 0.568 between −616C>G and −603T>del,

Table 1. Genotypes and allele frequencies of *DRD4* polymorphisms in schizophrenic patients and controls

Position	*n*	Genotype (frequency)			Number of allele (frequency)		Genotype			Allele		
							df	χ^2	*P* value	df	χ^2	*P* value
−291		C/C	C/T	T/T	C	T						
Control	210	157 (0.748)	49 (0.233)	4 (0.019)	363 (0.864)	57 (0.136)	2	0.94	0.6264	1	0.555	0.4563
Schizophrenic	208	151 (0.726)	50 (0.240)	7 (0.034)	352 (0.846)	64 (0.154)						
−376		C/C	C/T	T/T	C	T						
Control	210	168 (0.800)	41 (0.195)	1 (0.005)	377 (0.898)	43 (0.102)	2	2.79	0.248	1	2.402	0.1212
Schizophrenic	208	179 (0.861)	28 (0.135)	1 (0.005)	386 (0.928)	30 (0.072)						
−521		C/C	C/T	T/T	C	T						
Control	210	25 (0.119)	110 (0.524)	75 (0.357)	160 (0.381)	260 (0.619)	2	2.05	0.3584	1	0.923	0.3368
Schizophrenic	208	25 (0.120)	122 (0.587)	61 (0.293)	172 (0.413)	244 (0.587)						
−616		C/C	C/G	G/G	C	G						
Control	210	25 (0.119)	85 (0.405)	100 (0.476)	135 (0.321)	285 (0.679)	2	1.18	0.5551	1	1.258	0.262
Schizophrenic	208	30 (0.144)	89 (0.428)	89 (0.428)	149 (0.358)	267 (0.642)						
−768		G/G	G/A	A/A	G	A						
Control	210	203 (0.967)	7 (0.033)	0 (0.000)	413 (0.983)	7 (0.017)	1	0.32	0.5693	1	0.319	0.5722
Schizophrenic	208	203 (0.976)	5 (0.024)	0 (0.000)	411 (0.988)	5 (0.012)						

df, Degrees of freedom

Table 2. Temperament and character inventory (TCI) scores classified by *DRD4* polymorphisms

Position	Genotype	*n*	NS	HA	RD	P
−291	C/C	131	18.6 ± 3.9	16.6 ± 5.6	16.2 ± 3.0	5.0 ± 1.8
	C/T	38	17.6 ± 4.3	18.7 ± 5.6	15.1 ± 3.4	5.0 ± 1.7
	T/T	4	21.0 ± 9.0	16.0 ± 6.1	17.8 ± 2.2	5.5 ± 3.1
	P[a]		0.604	0.060	0.090	0.622
−376	C/C	133	18.7 ± 4.4	17.0 ± 5.7	16.0 ± 3.3	5.0 ± 1.8
	C/T	39	17.7 ± 3.3	17.1 ± 5.9	15.8 ± 2.7	5.1 ± 1.8
	T/T	1	15.0	22.0	17.0	4.0
	P[a]		0.333	0.590	0.888	0.751
−521	C/C	22	19.2 ± 3.7	18.0 ± 5.8	15.5 ± 3.1	5.0 ± 1.6
	C/T	93	18.7 ± 4.2	16.4 ± 5.8	16.1 ± 2.8	5.0 ± 1.8
	T/T	58	17.8 ± 4.3	17.7 ± 5.4	16.0 ± 3.7	4.9 ± 1.8
	P[a]		0.362	0.262	0.824	0.991
−600	G/G	164	18.4 ± 4.2	17.1 ± 5.7	15.9 ± 3.2	5.0 ± 1.8
	G/C	6	19.8 ± 5.0	15.2 ± 3.8	17.0 ± 1.5	5.2 ± 1.7
	C/C	3	17.7 ± 4.2	17.7 ± 6.7	15.7 ± 4.5	4.3 ± 3.1
	P[a]		0.626	0.613	0.696	0.932
−602	G/G	69	19.1 ± 4.8	16.9 ± 5.9	15.9 ± 3.0	4.9 ± 1.9
	G/del	72	18.6 ± 3.6	17.5 ± 4.8	16.1 ± 3.2	5.2 ± 1.6
	del/del	31	16.7 ± 3.7	16.5 ± 7.0	15.7 ± 3.3	4.8 ± 1.9
	P[a]		0.037	0.854	0.604	0.690
−603	T/T	26	18.2 ± 3.7	18.0 ± 6.0	15.8 ± 4.2	5.3 ± 1.5
	T/del	72	18.6 ± 3.9	16.9 ± 5.6	15.5 ± 2.9	4.9 ± 1.8
	del/del	75	18.5 ± 4.6	16.8 ± 5.7	16.5 ± 2.9	4.9 ± 1.8
	P[a]		0.874	0.589	0.253	0.685
−616	C/C	20	18.9 ± 3.9	17.7 ± 4.8	15.8 ± 2.9	5.3 ± 1.8
	C/G	88	18.4 ± 3.9	17.1 ± 5.7	15.7 ± 3.1	4.9 ± 1.8
	G/G	65	18.4 ± 4.7	16.8 ± 6.0	16.4 ± 3.2	5.0 ± 1.7
	P[a]		0.772	0.834	0.640	0.593
−768	G/G	166	18.5 ± 4.2	16.9 ± 5.7	16.1 ± 3.0	5.0 ± 1.8
	G/A	7	17.3 ± 3.1	20.3 ± 3.8	12.6 ± 4.8	5.4 ± 2.2
	P[b]		0.565	0.092	0.044	0.617
−809	G/G	106	18.5 ± 3.9	17.4 ± 5.4	15.8 ± 3.4	4.9 ± 1.9
	G/A	53	18.1 ± 4.3	16.2 ± 6.2	16.3 ± 2.9	5.2 ± 1.4
	A/A	14	19.3 ± 6.0	17.3 ± 5.7	16.1 ± 2.2	5.1 ± 2.3
	P[a]		0.645	0.445	0.578	0.643
Exon III	S	151	18.4 ± 4.1	17.1 ± 5.7	15.9 ± 3.2	5.0 ± 1.8
	L	22	18.9 ± 4.6	16.4 ± 5.9	16.2 ± 2.7	5.0 ± 1.6
	P[b]		0.653	0.627	0.891	0.835

Scores are shown as means ± SD. NS, novelty seeking; HA, harm avoidance; RD, reward dependence; P, persistence; S, genotypes with only the short *DRD4* alleles with two to four exon III repeats; L, genotypes with one or two copies of the long *DRD4* alleles with five to seven exon III repeats

[a] Kruskal-Wallis test

[b] Mann-Whitney *U*-test

0.441 between −603T>del and −602G>del, 0.335 between −809G>A and −291C>T, 0.255 between −809G>A and −376C>T, 0.200 between −616C>G and −602G>del, 0.173 between −809G>A and −616C>G, 0.129 between −616C>G and −521C>T, 0.086 between −521C>T and −376C>T, 0.084 between −602G>del and −376C>T, 0.044 between −809G>A and −600G>C, 0.043 between −603T>del and −600G>C, and 0.021 between −376C>T and −291C>T.

Discussion

The present study failed to detect a positive association between schizophrenia and five polymorphisms (−768G >A, −616C >G, −521C >T, −376C >T, and −291C >T) in the upstream region of the *DRD4* gene. We could not replicate the previous finding of association between −521C>T and Japanese schizophrenic patients (Okuyama et al. 1999). The allele C frequencies of controls and schizophrenic patients in the study by Okuyama et al. were 0.41 and 0.48, respectively, while these frequencies were 0.381 and 0.413 in this study. This discrepancy in the frequencies may be due to regional differences in the subjects. The *DRD4* gene shows a high degree of genetic variation in human populations. These include a −11C>T, a +31G>C (11Gly>Arg) (Cichon et al. 1995), a 12-base pair (bp) repeat in exon I (Catalano et al. 1993), a 13-bp deletion in exon I (Nothen et al. 1994), a (G)n mononucleotide repeat polymorphism in intron I (Petronis et al. 1994), a +581T>G (194Val>Gly) (Seeman et al. 1994), a 48-bp repeat polymorphism in exon III (Van Tol et al. 1992), and polymorphisms in the upstream region of the gene mentioned in this study (Mitsuyasu et al. 1999). To date, none of these polymorphisms have shown evidence of a strong genetic association with schizophrenia. These results do not support the notion that polymorphisms in the *DRD4* gene have a major role in susceptibility to schizophrenia.

Cloninger et al. (1993) proposed a seven-dimensional personality model, consisting of four temperament scales and three character scales, which can be evaluated by the TCI, an outgrowth of the TPQ (Cloninger 1987). Association of the exon III repeat polymorphism in the *DRD4* gene with the personality trait of novelty seeking was reported by two groups in 1996 (Ebstein et al. 1996; Benjamin et al. 1996). After that, four studies replicated this finding (Ono et al. 1997; Ebstein et al. 1997; Noble et al. 1998; Tomitaka et al. 1999), but seven studies failed to replicate the finding. Our findings showed no association between the exon III repeat polymorphism and novelty seeking. The distribution of this polymorphism did not differ from that previously reported in the Japanese population (Muramatsu et al. 1996; Ono et al. 1997; Tomitaka et al. 1999). Because novelty seeking scores are negatively correlated with age (Ebstein and Belmaker 1997), the lack of association in this study may reflect the high mean age, or the sex, of the subjects. The mean ages of the subjects in two previous reports on Japanese population samples (Ono et al. 1997; Tomitaka et al. 1999) were 18.7 and 25.0 years, respectively, and the subjects were women.

The −768G>A polymorphism showed significant association with reward dependence ($P = 0.044$). Subjects with the G/G genotype had a higher reward dependence score than those with the G/A genotype. However, this observation should be interpreted with caution, because the frequency of this polymorphism was rather rare, and there was no significant association of novelty seeking with the −291C>T polymorphism ($P = 0.090$), which showed linkage disequilibrium ($D' = 0.614$) to the −768G>A polymorphism. Because the −768G>A polymorphism is located within the negative modulator region, the transcription of the *DRD4* gene may be affected by this substitution. Noradrenaline was originally hypothesized to be the major neuromodulator of reward dependence (Cloninger 1987). There is close interaction between dopaminergic and adrenergic pathways in the brain (Eshel et al. 1990). The function of recombinant DRD4 expressed in cultured cells was activated by adrenaline, and by noradrenaline in addition to dopamine, indicating that DRD4 may be involved in coordinating signaling among the catecholamine neurotransmitter systems (Hartman and Lanau 1997). These observations suggest that DRD4 could modulate noradrenergic transmission pathways that are related to the personality trait of reward dependence.

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