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Identification of a polymorphism in the promoter region of *DRD4* associated with the human novelty seeking personality trait

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Polymorphism in the human dopamine D4 receptor gene (*DRD4*) exon III has been associated in some but not all studies of the human personality trait of Novelty Seeking. We searched for polymorphisms in the 5′ region of *DRD4* and identified six polymorphisms as follows: –1217G Ins/Del, –809G/A, –616C/G, –603T Ins/Del, –602(G)8–9, and –521C/T. Associations between these polymorphisms and personality traits measured by the Temperament and Character Inventory (TCI) were investigated in 86 healthy Japanese volunteers. The –521C/T polymorphism was significantly associated with Novelty Seeking (*P* = 0.0001). Subjects with the C/C genotype exhibited the highest Novelty Seeking scores and those with the T/T genotype exhibited the lowest. A transient expression method revealed that the T variant of the C-521T polymorphism reduces transcriptional efficiency. The present study suggests a contribution of dopamine D4 receptor availability to individual differences in Novelty Seeking behavior. *Molecular Psychiatry* (2000) **5,** 64–69.

Keywords: temperament; linkage disequilibrium; dopamine

Introduction

Novelty Seeking is a human personality trait characterized by impulsive, exploratory, or sensation-seeking behavior. Large twin studies have supported the heritability of Novelty Seeking.[1,2] Novelty Seeking is putatively linked to the dopaminergic systems since dopamine mediates exploratory behavior in experimental animals, the rewarding effects of amphetamines and cocaine are related to dopamine release, and the Novelty Seeking characteristic is low in dopamine-deficient patients with Parkinson's disease.[3,4] An association between Novelty Seeking and the presence of the 7-repeat allele or alleles of six or more repeats of a polymorphism in the exon III of the DRD4 gene has been reported.[5,6] The dopamine D4 receptor is distributed in brain regions associated with pleasure-seeking behavior, and a possible functional significance of the 7-repeat allele is suggested by differences in ligand affinity.[7] Some subsequent studies have replicated this association,[5,6,8,9] but others have not.[10–13]

To address whether there are functional polymorphisms in the 5′ region of *DRD4* associated with the Novelty Seeking personality trait, we screened 31 normal volunteers for nucleotide variants in the promoter region ranging from –1302 bp to –123 bp from the translation start site by single strand conformation polymorphism (SSCP) analysis. This region is known to contain a negative modulator between –770 and –679, and the promoter between –591 and –123 bp.[14]

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Methods

Subjects

Normal male volunteers (n = 86) aged 18–29 years (mean 22.8) were recruited from students and staff at University of Tsukuba and Tokyo Medical and Dental University. All subjects were Japanese and unrelated. Volunteers were asked to complete the Japanese version of the Temperament and Character Inventory (TCI).[15] The TCI is a 226-item, true–false questionnaire that measures seven dimensions of personality. It is an outgrowth of the Tridimensional Personality Questionnaire (TPQ).[3,16] The reliability and validity of the Japanese version of the TCI have been verified.[15]

Single strand conformation polymorphism (SSCP), sequencing, and genotyping

PCR was carried out using native *Pfu* polymerase (Stratagene, La Jolla, CA, USA) and primers listed in Table 1. Amplication consisted of incubation at 98°C for 1 min followed by 35 cycles at 98°C for 20 s, 68°C for 30 s, and 74°C for 2 min. To genotype the

Table 1 Primers used in this study

SSCP and direct sequencing *Amplified region*	*Sense primer*	*Antisense primer*
−1304 bp ~ −1023 bp	5′-TGCACAAGAGGGACTGAGCCTGGCT	5′-GCGCGCACATCCTGATGCTCTAGT
−1087 bp ~ −813 bp	5′-TCCTGGCACAGCCCCTCCACTCCAG	5′-TTCGGCTCGGCGAGGAGGCCAGGAGA
−878 bp ~ −563 bp	5′-TCTTCTGCACGTTTGGAACCTACCC	5′-CTCAACCGCCGACGCCTAGCTCATC
−627 bp ~ −343 bp	5′-CGGGGGCTGAGCACCAGAGGCTGCT	5′-GCATCGACGCCAGCGCCATCCTACC
−424 bp ~ −123 bp	5′-CCAGATACCAGGTGGACTAGGGTGA	5′-GGCCCCCGCGGCGCTCCACCGTGA
PCR-RFLP *Polymorphism/ variant*	*Sense primer*	*Antisense primer*
−1217G Ins/Del	5′-TGCACAAGAGGGACTGAGCCTGGCT	5′-GCGGCGCACATCCTGATGCTCTAGT
−1123C>T	5′-GCCCTGTCAGCGCCTCCGTCTAGGC	5′-GCGGCGCACATCCTGATGCTCTAGT
−809G/A	5′-CCTGGCCTCCTCGCGAGCCGAACGT	5′-CTCAACCGCCGACGCCTAGCTCATC
−616C/G	5′-TCTTCTGCACGTTTGGAACCTACCC	5′-CTCAACCGCCGACGCCTAGCTCACTC
−603T Ins/Del	5′-GCGGGGGCTGAGCACCAGAGGCAGC	5′-GCATCGACGCCAGCGCCATCCTACC
−602(G)8–9	5′-CTGAGCACCAGAGGCTGCGGGGG for −603T Ins	5′-GCATCGACGCCAGCGCCATCCTACC
−602(G)8–9	5′-CTGAGCACCAGAGGCTCGGGGG for −603T Del	5′-GCATCGACGCCAGCGCCATCCTACC
−521C/T	5′-CGGGGGCTGAGCACCAGAGGCTGCT	5′-GCATCGACGCCAGCGCCATCCTACC
pCAT plasmid construct *Plasmid*	*Sense primer*	*Antisense primer*
pCAT-D4C & T	5′-TTACGCGTGCGGGATGAGCTAGGCGTCGGC	5′-TTAGATCTGGCCCCCGCGGCGCTCCACCGTGA
pCAT-D4R	5′-TTACGCGTGGCCCCCGCGGCGCTCCACCGTGA	5′-TTAGATCTGCGGGATGAGCTAGGCGTCGGC

Sequences underlined are mismatch primers for PCR-RFLP or restriction enzyme linkers for pCAT plasmid construct.

−1123C>T, −809G/A, −603T Ins/Del, and −594G Ins/Del variants, PCR with mismatch primers was carried out using Exo- *Pfu* polymerase (Stratagene). The SSCP method was used to screen polymorphisms using PhastSystem (Pharmacia, Uppsala, Sweden). DNA sequencing was carried out using a Dye Terminator Cycle Sequencing Kit on an ABI PRISM 310 DNA Sequencer (Perkin-Elmer, Norwalk, CT, USA).

Plasmid constructs, cell culture, plasmid transfections and CAT assays

The region (−591 bp to −123 bp) containing the C-521 or −521T allele was amplified from the DNA of subjects homozygous for each allele by PCR with the *Mlu*I and *Bgl*II linker added primers (Table 1) and inserted into *Mlu*I/*Bgl*II-cut promoter-less vector pCAT-Basic (Promega, Madison, WI, USA) (pCAT-D4C and pCAT-D4T, respectively). The pCAT-D4R insert was the region containing the C allele but in the opposite orientation. The plasmid clones used were sequenced in full to rule out any sequence alterations. Human retinoblastoma Y-79 cells purchased from the American Type Culture Collection (ATCC) were grown in suspension in RPMI 1640 supplemented with 15% fetal bovine serum. Transfection was carried out using TransFast™ transfection Reagent (Promega). All plasmids used in transfections were prepared using EndoFree Plasmid kit (Qiagen, Valencia, CA, USA). After $2–3 \times 10^6$ Y-79 cells in 1 ml of serum-free medium were plated on a 60-mm dish, 1 ml of serum-free medium with 24 μl of TransFast Reagent, 6.5 μg of the test pCAT plasmid construct, and 1.3 μg pSV β-galactosidase control plasmid (Promega) was added. After 1 h, 10 ml of complete growth medium was added, and the transfected cells were incubated at 37°C for an additional 47 h.

The cells were harvested, suspended in 100 μl of 0.25 M Tris-HCl (pH 7.5), and disrupted by three freeze-thawing cycles. The suspension of disrupted cells was incubated at 65°C for 10 min to inactivate deacetylase, and then centrifuged at 14 000 g for 5 min at 4°C. The cell lysate was incubated with 0.5 mM acetyl coenzyme A, 3.7 kBq ^{14}C-chloramphenicol (Amersham) and 0.25 M Tris-HCl (pH 7.8) in a final volume of 80 μl at 37°C for 3 h. The reaction mixture was extracted with ethyl acetate and applied to a thin-layer chromatography plate. The radioactive ratio between acetylated and non-acetylated chloramphenicol was quantitated by FUJIX BAS 2000 (Fuji Photo Film Co Ltd). The experiments were repeated.

Results

SSCP analysis and direct sequencing using primers listed in Table 1 revealed seven nucleotide variants: −1217G Ins/Del, −1123C>T, −809G>A, −616C>G, −603T Ins/Del, −602(G)8–9, −521C>T (Figure 1). They

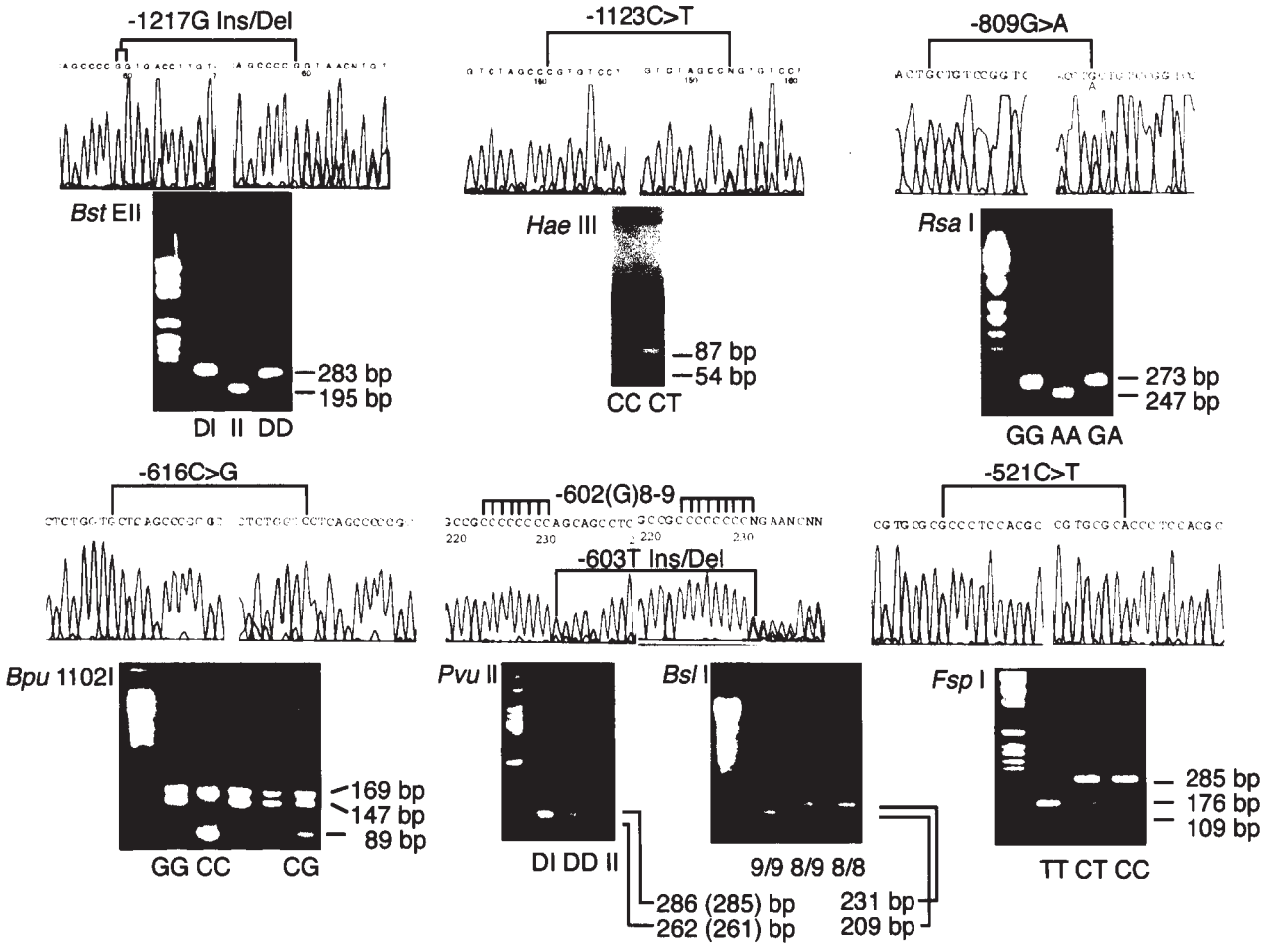

Figure 1 Direct sequencing of the identified variants in the 5′ region of the dopamine D4 receptor gene and restriction fragment length polymorphism patterns.

were genotyped by PCR restriction fragment length polymorphism analysis using primers listed in Table 1 and restriction enzymes shown in Figure 1. Genotyping them in 100 unrelated Japanese subjects, the –1123T variant was observed in only one subject and, except for –1123C>T, six were polymorphic among these Japanese subjects (Figure 2). Only weak linkage disequilibria between the –809G/A and the exon III VNTR polymorphisms, between the –809G/A and –616C/G polymorphisms, between the –616C/G and –603T Ins/Del polymorphisms, and between the –603T Ins/Del and –602(G)8–9 polymorphisms were observed in our Japanese subjects (Figure 2). Except for these nucleotide variants, our sequencing data of the nucleotide sequence in the 5′ flanking region of *DRD4* were identical to the sequence reported by Kamakura.[14]

To investigate associations between these polymorphisms and personality traits, we genotyped 86 Japanese male volunteers who had been asked to complete the Japanese version of the Temperament and Character Inventory (TCI).[15] Analysis of variance (ANOVA) revealed a significant association between the –521C/T polymorphism and Novelty Seeking scores ($P = 0.0001$) (Table 2). Subjects with the C/C genotype exhibited the highest Novelty Seeking scores and those with the T/T genotype exhibited the lowest. The polymorphism accounts for 20% of total variance. There was a slight negative correlation between Novelty Seeking and Persistence scores; the association with Persistence scores was not significant after correction for Novelty Seeking scores ($P = 0.09$). The other polymorphisms were not significantly associated with TCI scores (data not shown). The mean Novelty Seeking score in our subjects was similar to that reported by Ono *et al*.[8] There was no significant correlation between subjects' ages and Novelty Seeking scores, probably because our subjects comprised only young adults.

The influence of this polymorphism on *DRD4* expression was assessed by chloramphenicol acetyltransferase (CAT) assay using human retinoblastoma (Y-79) cells in which *DRD4* is expressed naturally.[14] The region between –591 bp and –123 bp containing the –521C or –521T allele was inserted into promoterless vector pCAT-Basic (Promega) (pCAT-D4C and pCAT-D4T, respectively). The region between –591 and –123 is known to give the highest transcriptional activity in Y-79 cells. Figure 3 shows the relative CAT

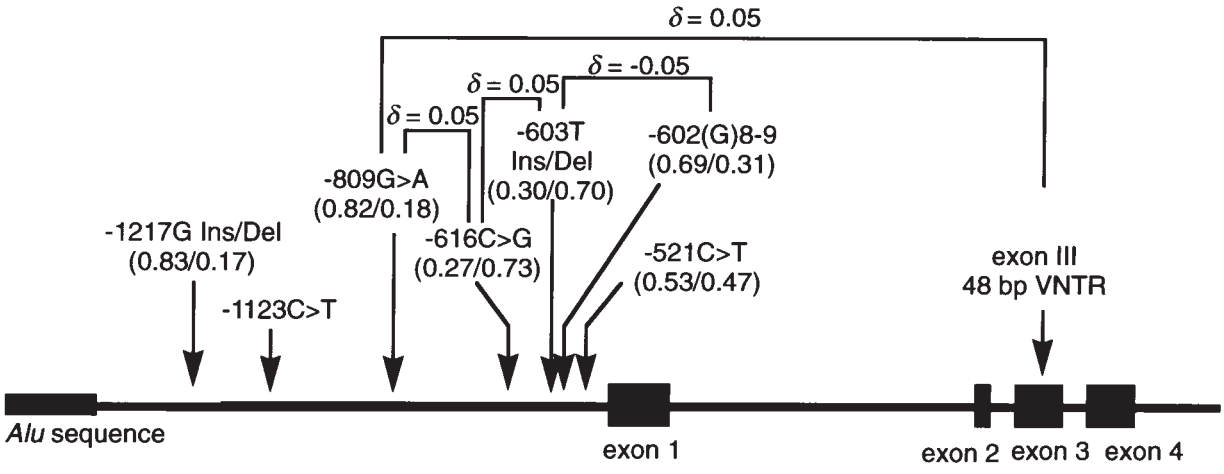

Figure 2 Schematic diagram of the human dopamine D4 receptor gene and location of identified variants in the 5′ region. The positions of the variants in the 5′ region are numbered by reference to the translation start site. Numbers in parentheses under the polymorphisms show allele frequencies of each polymorphism that were determined in 100 unrelated Japanese subjects. δ values shown on the bars between pairs of polymorphisms indicate deviation from linkage equilibrium (δ = h – p1p2; h, haplotype frequency; p1, p2, frequencies of two alleles at two gene loci[31]). The alleles of the exon III VNTR polymorphism were classified as the long (≥5 repeats) or short (<5 repeats) alleles. Only δ values between two polymorphisms where *P* values were less than 0.05 by χ^2 test for linkage disequilibrium are shown.

Table 2 TCI tetra-dimensional temperament scores in subject groups classified by DRD4 genotypes

Genotype	*Novelty seeking*	*Harm avoidance*	*Reward dependence*	*Persistence*
C/C (*n* = 28)	25.1 (4.5)	18.0 (7.2)	14.5 (3.7)	3.5 (1.7)
C/T (*n* = 36)	21.7 (4.2)	16.7 (5.8)	14.9 (3.5)	4.9 (1.7)
T/T (*n* = 22)	19.1 (5.6)	20.9 (7.4)	14.8 (3.6)	4.8 (2.1)
F[a]	10.39	2.71	0.089	5.27
P	0.0001	0.07	0.91	0.007

TCI results are given as mean raw scores (SD).
[a]*F* value by one-way ANOVA.

activity of each construct in Y-79 cells. Compared with pCAT-Basic, transcriptional activity was observed with pCAT-D4C and pCAT-D4T but not with pCAT-D4R, which carries the region containing the C allele but in the opposite orientation. Compared with pCAT-D4C, pCAT-D4T was approximately 40% less active.

Discussion

The present study revealed the 5′ region of DRD4 to be highly polymorphic. The –521C/T polymorphism is particularly interesting because it locates in the region regulating cell-type specific gene expression[14] and because pCAT containing the –521T allele gave significantly less transcriptional activity than that containing the –521C allele. The human dopamine D4 receptor has received considerable attention because of its high affinity for the atypical antipsychotic clozapine and reported elevation of this receptor[17] and mRNA[18] in the post-mortem schizophrenic brain. Associations of the *DRD4* exon III VNTR polymorphism with substance abuse,[19,20] attention deficit hyperactivity disorder,[21,22] and Tourette syndrome[23] have been reported. The polymorphisms identified in this study could be candidates for these mental disorders.

The association between *DRD4* exon III polymorphism and Novelty Seeking has been controversial.[5,6,8–13,24] Moreover, the group that reported a receptor molecule with different binding characteristics produced by the 7-repeat allele have pursued their findings in a subsequent study and concluded that the polymorphic repeat sequence has little influence on binding profiles.[25] The allele frequencies of this polymorphism differ considerably between populations, and the 7-repeat allele is quite rare among the Chinese and Japanese.[26] Instead, the 5-repeat allele is the third most common among the Japanese, and, when alleles with five or more repeats were grouped with the L allele, the L allele was associated with increased Novelty Seeking scores in Japanese subjects.[8] We did not find a significant association between high Novelty Seeking scores and alleles of five or more repeats in our subjects (data not shown), nor did we observe significant linkage disequilibrium between the –521C/T polymorphism and exon III alleles of five or more repeats (Figure 2).

High Novelty Seeking scores were reported to be

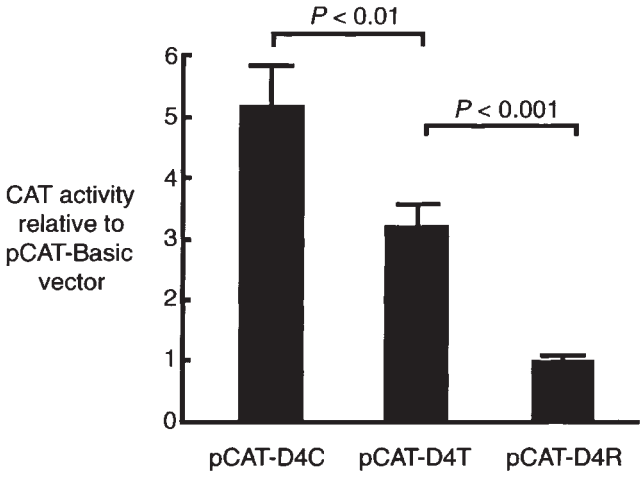

Figure 3 Transient transfection analysis in Y-79 cells. The DNA fragment –591 bp to –123 bp from the translation start site of *DRD4* that contains –521C, –521T, or –521C in the opposite orientation was cloned into the pCAT-Basic vector (pCAT-D4C, pCAT-D4T, and pCAT-D4R, respectively). Transfection efficiencies were normalized according to β-galactosidase activity. Bars show promoter activity expressed as the ratio of each pCAT construct to pCAT-Basic in Y-79 cells. Data shown are the mean ± SEM of two independent experiments consisting of five plates each.

associated with the A2 allele of the dopamine D2 receptor gene (*DRD2*).[9] PET studies indicate that the A2 allele is associated with increased dopamine D2 receptor binding in the striatum in Caucasians.[27,28] High Novelty Seeking scores were reported to be associated with the Gly9 homozygous genotype in comparison to the other genotypes of the Ser9Gly polymorphism of the dopamine D3 receptor gene in recovered bipolar patients.[29] A significantly higher dopamine binding affinity for the Gly9 homozygote than that for the other genotypes was detected in CHO cells doubly infected with the Semliki Forest virus system containing the dopamine D3 receptor gene.[30] In this study, higher Novelty Seeking scores were associated with the C allele of the –521C/T polymorphism. The CAT assay suggested that the C allele is transcriptionally more active than the T allele. The reported findings together with our data lead us to speculate that high D2-group dopamine receptor availability might be associated with high Novelty Seeking.

The –521C/T polymorphism is in one of the CpG dinucleotides in a GC-rich region but does not appear to comply with any transcription factor consensus sequence. Changes in transcriptional activity in reporter gene systems do not necessarily mean true changes in the receptor characteristics *in vivo*. Direct evidence would be needed for a conclusion on the role of –521C/T in Novelty Seeking personality, though the measurement of D4 receptor binding is still not straightforward. Further studies are needed to evaluate the contribution of DRD4 to individual differences in Novelty Seeking behavior.

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