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BRIEF REPORT — POLYMORPHISM REPORT

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Novel polymorphisms in the upstream region of the human dopamine D4 receptor (*DRD4*) gene

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Abstract We found nine novel polymorphisms in the upstream region of the human dopamine D4 receptor (*DRD4*) gene of Japanese by direct sequencing. These polymorphisms are −809G > A, −768G > A, −616C > G, −603T > del, −602G > del, −600G > C, −376C > T, −291C > T, and −128G > T. One known polymorphism, −521C > T, was also recognized. Six of these sites were identified as restriction fragment length polymorphisms (RFLPs).

Key words Dopamine D4 receptor gene · Promoter · Polymorphisms · RFLP

Introduction

The human dopamine D4 receptor (DRD4) is of great interest in molecular studies of human personality and psychiatric disorders such as schizophrenia, mood disorders, attention deficit hyperactivity disorder, Tourette's syndrome, alcoholism, and substance abuse.

The density of DRD4 was found to be six-fold elevated in the brains of schizophrenic patients (Seeman et al. 1993). DRD4 mRNA was elevated in the frontal cortex of schizophrenics in postmortem studies compared with controls (Stefanis et al. 1998), suggesting that there are allelic variations in transcription levels of the *DRD4* gene.

Our previous study of *DRD4* gene transcription revealed that the region between −591 and −123 relative to the first nucleotide of the initiation codon contained the promoter of the *DRD4* gene, which is responsible for the cell type-specific expression of the gene, while the negative modulator was located between −770 and −679 (Kamakura et al. 1997). Based on these results we tried to detect polymorphisms in the upstream region of the *DRD4* gene spanning the negative modulator and the cell type-specific promoter region.

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Source and description

DNA samples

Ten-ml venous blood samples were drawn from unrelated Japanese individuals into heparin-containing tubes. Genomic DNA was extracted by a rapid non-enzymatic method. Leukocyte DNA was salted out with saturated NaCl solution (Lahiri and Nurnberger 1991).

PCR primers

Two sets of primers were used to amplify the upstream region (regions A and B) of the *DRD4* gene (Fig. 1). Region A was amplified by polymerase chain reaction (PCR), using the sense primer, D4pos3 (5′-CTCAGGTCTTTCTGCGTCTGGC-3′, −472/−451) and the antisense primer, D4pos4 (5′-GGCTCACCTCGGAGTAGACGAA-3′, +292/+271). Region B was amplified by PCR using the sense primer, D4neg3 (5′-CAGGTCACAGGTCACCCCTCTT-3′, −947/−926) and the antisense primer, D4neg4 (5′-TTGCTCATCTTGGAATTTTGCG-3′, −156/−177). The sense primer, D4pos1 (5′-GGAGGTTTTGCCAGATACCA-3′, −434/−415) was used to determine the nucleotide sequence of region A.

PCR conditions

The first PCR to amplify region A was performed in a volume of 20 μl containing 60 ng genomic DNA, 4 pmol of each primer (D4pos3, D4pos4), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM each of dNTPs, 7.5% dimethylsulfoxide (DMSO), and 0.5 U AmpliTaq Gold (PE Biosystems, Foster City, CA, USA). The cycle conditions were 95°C for 10 min, then 40 cycles of 94.5°C for 20 s,

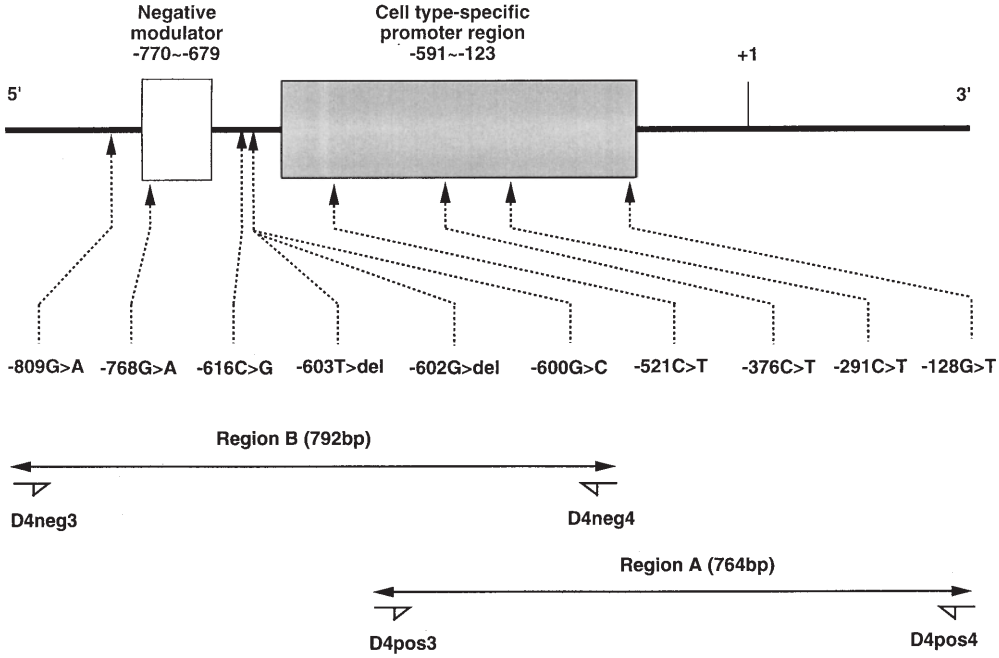

Fig. 1. Locations of ten polymorphisms in the upstream region of the human *DRD4* gene. Ten polymorphic sites (−809G > A, −768G > A, −616C > G, −603T > del, −602G > del, −600G > C, −521C > T, −376C > T, −291C > T, −128G > T) are covered by regions A and B. Each position is shown relative to the first nucleotide of the initiation codon, indicated as +1. The cell type-specific promoter region between −591 and −123 (*gray box*) contains the promoter of the *DRD4* gene. This region is responsible for the cell type-specific expression of the gene and contains transcription initiation sites located between −501 and −436. The *open box* indicates the negative modulator. The *open arrowheads* indicate the orientation of two sets of oligonucleotide primers, D4pos3/D4pos4 and D4neg3/D4neg4 for amplification of regions A and B, respectively

61.0°C for 5 s, and 72°C for 1 min 45 s, with a final extension step of 7 min at 72°C, on a Perkin Elmer 2400 Thermocycler. The second PCR was performed in a volume of 20 μl containing 1.5 μl of the first PCR product, 4 pmol of each primer (D4pos3, D4pos4), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM each of dNTPs, 7.5% DMSO, and 0.5 U AmpliTaq Gold. The cycle conditions were as the same as those for the first PCR except that 22 cycles rather than 40 cycles were performed.

PCR to amplify region B was performed in a volume of 50 μl containing 100 ng genomic DNA, 25 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM $MgCl_2$, 200 μM each of dNTPs, 5.0% DMSO, and 1.25 U AmpliTaq Gold. The cycle conditions were 95°C for 10 min, then 40 cycles of 95°C for 20 s, 57°C for 5 s, and 72°C for 1 min 45 s, with a final extension step of 7 min at 72°C. The PCR products were electrophoresed on a 1.5% agarose gel containing 1 × Tris-borate/EDTA electrophoresis buffer (TBE), and visualized by ethidium bromide staining.

DNA sequencing

The PCR products were concentrated and purified with a Microcon YM-100 unit (Millipore Bedford, MA, USA). Cycle sequencing of region A was done in a volume of 10 μl containing 50–100 ng of concentrated PCR product DNA, primer D4pos1 (sense), on a Thermocycler with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, USA). The cycle sequencing protocol was as recommended by the manufacturer, except for an annealing temperature of 52.5°C and performance of 32 cycles.

Cycle sequencing of region B was done in a volume of 10 μl containing 50–100 ng of concentrated PCR product DNA, primer D4neg3 (sense) or D4neg4 (antisense) on the Thermocycler with the Kit. The cycle sequencing protocol was as recommended by the manufacturer except for an annealing temperature 57°C and performance of 32 cycles. Sequences were analyzed and collected on a Model ABI Prism 377 DNA Sequencer (PE Biosystems, Foster City, CA, USA).

Polymorphisms and allele frequencies

We determined the nucleotide sequence of regions A and B of the *DRD4* gene from 80 and 67 unrelated Japanese individuals, respectively. Nine novel polymorphic sites and one known site (−521C > T) (Okuyama et al. 1999) were identified in the upstream region of the *DRD4* gene. The genotype and allele frequencies of −809G > A, −768G > A, −616C > G, −603T > del, −602G > del, −600G > C, −521C > T, −376C > T, −291C > T in 67 and −128G > T in 80 Japanese are summarized in Table 1. The allele frequency of −521C > T was the same as that reported by Okuyama et al. (1999).

Six of these polymorphic sites were also recognized as the following restriction fragment length polymorphisms

Table 1. Genotype and allele frequencies in the upstream region of the *DRD4* gene

Position	Genotype frequency			Allele frequency	
−128	G/G	G/T	T/T	G	T
	0.975	0.000	0.025	0.975	0.025
−291	C/C	C/T	T/T	C	T
	0.761	0.164	0.075	0.843	0.157
−376	C/C	C/T	T/T	C	T
	0.836	0.134	0.030	0.903	0.097
−521	C/C	C/T	T/T	C	T
	0.179	0.463	0.358	0.410	0.590
−600	G/G	G/C	C/C	G	C
	0.910	0.060	0.030	0.940	0.060
−602	G/G	G/del	del/del	G	del
	0.463	0.224	0.313	0.575	0.425
−603	T/T	T/del	del/del	T	del
	0.358	0.194	0.448	0.455	0.545
−616	C/C	C/G	G/G	C	G
	0.149	0.269	0.582	0.284	0.716
−768	G/G	G/A	A/A	G	A
	0.970	0.015	0.015	0.978	0.022
−809	G/G	G/A	A/A	G	A
	0.582	0.254	0.164	0.709	0.291

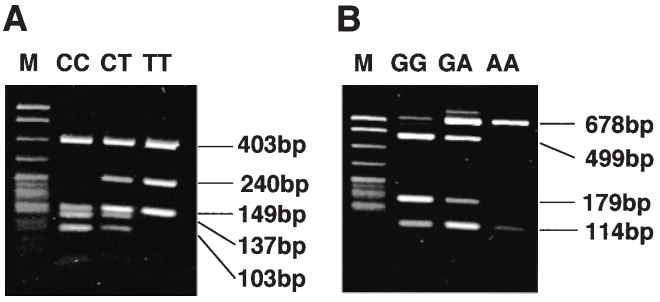

Fig. 2A, B. Genotyping of the −291C > T and −768G > A polymorphisms. Digestion products were electrophoresed on a 3.0% agarose gel and visualized by ethidium bromide staining. **A** Genotyping of the −291C > T polymorphism by digestion of region B PCR products with *Ava*I. *Lane CC*, Homozygote for the C allele; *lane CT*, heterozygote; lane *TT*, homozygote for the T allele; *lane M*, DNA molecular weight marker pBR322 DNA — *Msp*I digests (New England BioLabs, Beverly, MA, USA). **B** Genotyping of the −768G > A polymorphism by digestion of region B PCR products with *Nar*I. *Lane GG*, Homozygote for the G allele; *lane GA*, heterozygote; *lane AA*, homozygote for the A allele; *lane M*, as in **A**

(RFLPs):

- *Sac*II RFLP: PCR products of region A were digested with *Sac*II. Allele G-128 was digested in five fragments, of 344, 232, 102, 47, and 39 bp, respectively, whereas allele T-128 was digested in four fragments, of 446, 232, 47, and 39 bp.
- *Ava*I RFLP: PCR products of region B were digested with *Ava*I. Allele C-291 was digested in four fragments, of 403, 149, 137, and 103 bp, whereas allele T-291 was digested in three fragments, of 403, 240, and 149 bp.
- *Rsa*I RFLP: PCR products of region B were digested with *Rsa*I. Allele T-376 was digested in two fragments, of 571 and 221 bp, whereas allele C-376 was uncut.
- *Bss*H II RFLP: PCR products of region B were digested with *Bss*H II. Allele C-521 was digested in three fragments, of 426, 184, and 182 bp, whereas allele T-521 was digested in two fragments, of 608 and 184 bp.
- *Ava*II RFLP: PCR products of region B were digested with *Ava*II. Allele G-616 was digested in five fragments, of 334, 178, 146, 127, and 7 bp, whereas allele C-616 was digested in four fragments, of 512, 146, 127, and 7 bp.
- *Nar*I RFLP: PCR products of region B were digested with *Nar*I. Allele G-768 was digested in three fragments, of 499, 179, and 114 bp, whereas allele A-768 was digested in two fragments, of 678 and 114 bp.

As representatives, the genotyping of −291C > T and −768G > A polymorphisms based on RFLPs is shown in Fig. 2A, B.

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