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Short communication

Identification of polymorphic loci in the promoter region of the serotonin 5-HT_{2C} receptor gene and their association with obesity and Type II diabetes

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Abstract

Aims/hypothesis. Polymorphisms in the upstream region of the 5-HT_{2C} receptor gene could play a part in the development of obesity.
Methods. We screened the upstream region from 27 men by the single strand conformational polymorphism analysis and PCR-direct sequencing and then genotyped 466 non-obese (body mass index < 28 kg/m^2) and 123 obese (≥ 28 kg/m^2) men including 138 patients with Type II (non-insulin-dependent) diabetes mellitus.
Results. Three loci of single nucleotide substitution (G→A at –995, C→T at –759, G→C at –697) and a (GT)n dinucleotide repeat polymorphism at –1,027 were identified. The frequency of –995/–759 and –697 variants was higher in non-obese subjects and that of –995/–759 variants in non-diabetic subjects. In the dinucleotide repeat locus, five alleles were detected including *Z* containing 17 repeats. The *Z–6* allele was more common in non-obese subjects and the *Z + 2* allele in obese subjects. Haplotype 3 (*Z–6*, –995A, –759T, –697C) was associated with leanness ($p = 0.02$) and the absence of diabetes ($p = 0.033$) and haplotype 9 (*Z + 2*, –995G, –759C, –697G) with obesity ($p = 0.007$). Haplotype 2 (*Z–6*, –995G, –759C, –697C) tended to be more common in non-obese subjects. A luciferase reporter assay showed that haplotype 2 and haplotype 3 had 1.44- or 2.58-fold higher promoter activities than the most common haplotype 6 (*Z*, –995G, –759C, –697G).
Conclusion/interpretation. The haplotypes containing the nucleotide substitutions could be associated with higher transcription levels of the gene and thereby with resistance to obesity and Type II diabetes. Promoter polymorphisms of the 5-HT_{2C} receptor gene may play an important part in genetic predisposition to the disorders. [Diabetologia (2000) 43: 373–376]

Keywords Serotonin, 5-HT_{2C} receptor, promoter, polymorphism, obesity.

Serotonin is a neurotransmitter involved in a large number of psychophysiological processes including the regulation of appetite. At least seven types of serotonin receptors have been characterised [1] and two or more variants are recognised for type 1, 2, and 5. Several lines of evidence have indicated that the type 2C serotonin receptor (5-HT_{2C}R), plays a pivotal role in the regulation of food intake [2]. Further *5-HT_{2C}R* transcripts have been detected in the hypothalamus including the paraventricular nucleus, lesions of which result in obesity [3]. A targeted mutation of the *5-HT_{2C}R* gene in mice caused a chronic hyperphagia leading to obesity and hyperinsulinaemia [4, 5].

The *5-HT_{2C}R* gene has been mapped to human chromosome X band q24 [6]. A Cys→Ser missense mutation has been identified at codon 23 of the *5-HT_{2C}R* gene. The *Cys23Ser* mutation was not, howev-

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Abbreviations: SSCP, Single strand conformational polymorphism; OR, odds ratio.

Table 1. Association of single nucleotide substitution polymorphisms of the *5-$HT_{2C}R$* gene with obesity and Type II diabetes

Polymorphic loci	Number	–995/–759		–697	
		Wild type	Variant	Wild type	Variant
BMI					
< 28 kg/m^2	466	408	58 (0.12)[a]	388	78 (0.17)[b]
≥ 28	123	117	6 (0.05)	114	9 (0.07)
Glucose tolerance					
Non-diabetic	451	395	56 (0.12)[c]	379	72 (0.16)
BMI < 28	395	341	54 (0.14)	327	68 (0.17)
BMI ≥ 28	56	54	2 (0.04)	52	4 (0.07)
Diabetic	138	130	8 (0.06)	123	15 (0.11)
BMI < 28	71	67	4 (0.06)	61	10 (0.14)
BMI ≥ 28	67	63	4 (0.06)	62	5 (0.07)

[a] $p = 0.02$ (OR 2.8, 95 % CI 1.2–6.4), [b] $p = 0.009$ (OR 2.5, 95 % CI 1.3–5.1) vs obese subject with body mass index ≥ 28. [c] $p = 0.03$ (OR 2.3, 95 % CI 1.1–4.9) vs diabetic subjects

er, associated with human obesity [7]. The 5′-flanking region of the human *5-$HT_{2C}R$* gene contains regulatory regions as well as a putative transcription factor binding region [8]. Transcription of the human *5-$HT_{2C}R$* gene is initiated at multiple sites, with the major transcription initiation site located at –703 [8]. In this study we tested a hypothesis that polymorphisms in the upstream region of the *5-$HT_{2C}R$* gene alters the expression level of the receptor and thereby play a part in the development of obesity.

Subjects and methods

Subjects. The subjects consisted of 589 men aged 51 ± 10 years having body mass index (BMI) of 25.2 ± 4.0 kg/m^2 recruited from people who attended the Kumamoto Red Cross Health Care Centre and patients treated at the Kurume University Hospital. Of those 138 were diagnosed as having Type II (non-insulin-dependent) diabetes mellitus based on clinical findings and glucose tolerance evaluated by oral treatment with 75 g glucose according to the World Health Organization criteria of 1980. The subjects included 123 obese (BMI ≥ 28) and 466 non-obese (BMI < 28) men.

Genetic analysis. We amplified two overlapping fragments of the *5-$HT_{2C}R$* gene regulatory region from genomic DNA by PCR using two pairs of primers: a sense primer 5′-CTTGAAGGGAGTTTCAAAGC-3′ (–1133 to –1114) and an antisense primer 5′-CCGGTCTCTTAGTGCATCTG-3′ (–846 to –827); a sense primer 5′-ATCTCCACCATGGGTCTCGC-3′ (–885 to –866) and an antisense primer 5′-CAATCTAGCCGCTCCAAAGG-3′ (–653 to –634). The region was screened for mutations using single strand conformational polymorphism (SSCP) analysis. To identify the mutations noted, the PCR-amplified products were sequenced directly or cloned in a PCR compatible TA vector (pCR2.1, Invitrogen, Carlsbad, Calif., USA).

Luciferase reporter assay. The promoter region was amplified using a forward primer 5′-GGTACCTTGAAGGGAGTTTCAA-AGC-3′ (–1133 to –1114) and a reverse primer 5′-CTCGAGTATGCAATCGGCAGGTAAGG-3′ (–600 to –581). The PCR products were TA-cloned and ligated into a KpnI/XhoI site of the pGL3-Enhancer vector (Promega, Madison, Wis., USA). The pGL3 constructs were transfected into a mouse embryonal carcinoma cell line (P19, Dainippon, Tokyo, Japan) by the cationic lipid method (Tfx-50, Promega). Cells were harvested and lysed 48 h after transfection. The relative promoter activity was calculated by a formula (light unit of sample construct – light unit of empty vector) / (light unit of pGL3-Control vector – light unit of empty vector).

Statistical analysis. Values are given as means and SD. The chi-squared test was used to compare frequencies. A p value less than 0.05 was considered statistically significant. Relative risk was estimated by the odds ratios (ORs) and their 95 % confidence intervals (CIs). Differences between group means were estimated by the Student's unpaired t test.

Results

Mutations consisting of changes in a single base pair were found in the promoter region between positions 995 and 697 upstream from the ATG translation initiation site; a G→A substitution at –995, a C→T substitution at –759, and a G→C substitution at –697. Furthermore, a (GT)n dinucleotide repeat polymorphism was identified at –1,027. The G→A substitution at position –995 abolishes an RsaI restriction site. Both the C→T substitution at –759 and the G→C substitution at –697 abolish each AciI restriction site. In the following studies to genotype 589 men, including the 27 subjects whose genes were screened by the SSCP analysis, these single nucleotide substitutions were detected by the PCR-restriction fragment length polymorphism (RFLP) technique. The length of the dinucleotide repeat was determined by PCR-direct sequencing using the first set of primers.

All subjects carrying the G→A substitution at –995 also had the C→T substitution at –759. The allele frequencies of the –995/–759 variant and –697 variant were 0.11 and 0.15, respectively (Table 1). We found a significant excess of the –995/–759 variant and the –697 variant in non-obese subjects (OR 2.8, 95 % CI 1.2–6.4, $p = 0.02$ and OR 2.5, 95 % CI

Table 2. Association of *5-HT_{2C}R* haplotypes with obesity and Type II diabetes

Haplotype	Polymorphic loci				Body mass index (kg/m^2)[a]		Glucose tolerance		Total
	(GT) n	–995	–759	–697	< 28	≥ 28	Non-diabetic	Diabetic	
1	Z–6	wild	wild	wild	1 (0.002)	0	0	1 (0.007)	1 (0.002)
2	Z–6	wild	wild	variant	19 (0.041)	2 (0.016)	15 (0.033)	6 (0.043)	21 (0.036)
3	Z–6	variant	variant	variant	57 (0.122)[b]	6 (0.049)	55 (0.122)[c]	8 (0.058)	63 (0.107)
	Z–6 total				77 (0.165)[d]	8 (0.065)	70 (0.155)	15 (0.109)	85 (0.144)
4	Z–2	wild	wild	wild	1 (0.002)	0	1 (0.002)	0	1 (0.002)
5	Z–2	wild	wild	variant	1 (0.002)	0	1 (0.002)	0	1 (0.002)
	Z–2 total				2 (0.004)	0	2 (0.004)	0	2 (0.003)
6	Z	wild	wild	wild	350 (0.751)	97 (0.789)	341 (0.756)	106 (0.768)	447 (0.759)
7	Z	wild	wild	variant	0	1 (0.008)	0	1 (0.007)	1 (0.002)
8	Z	variant	variant	variant	1 (0.002)	0	1 (0.002)	0	1 (0.002)
	Z total				351 (0.753)	98 (0.797)	342 (0.758)	107 (0.775)	449 (0.762)
9	Z+2	wild	wild	wild	30 (0.064)	17 (0.138)[e]	32 (0.071)	15 (0.109)	47 (0.080)
10	Z+4	wild	wild	wild	6 (0.013)	0	5 (0.011)	1 (0.007)	6 (0.010)
Total					466	123	451	138	589

[a] $p = 0.005$, lean vs obese subjects by the chi squared test (rare haplotypes 1, 4, 5 and 7 were excluded). [b] $p = 0.02$ (OR 2.7, 95% CI 1.2–6.3). [c] $p = 0.033$ (OR 2.3, 95% CI 1.1–4.8). [d] $p = 0.005$, *Z–6* total vs others (OR 2.8, 95% CI 1.4–5.9). [e] $p = 0.007$ (OR 2.3, 95% CI 1.3–4.3)

1.3–5.1, $p = 0.009$, respectively). The frequency of –995/–759 variants was also higher in non-diabetic subjects when compared with diabetic patients (0.14 vs 0.06, $p = 0.03$). Although the association could be attributable to the greater proportion of diabetic subjects in the obese group, the frequency of –995/–759 variants tended to be higher in non-diabetic lean subjects than in lean patients with diabetes (0.14 vs 0.06, $p = 0.06$). In the dinucleotide repeat polymorphic locus, five different alleles were detected including *Z*, the most common allele containing 17 repeats, and *Z–6*, *Z–2*, *Z + 2*, and *Z + 4*, which differed from *Z* by –6, –2, + 2, and + 4 nucleotides, respectively. The *Z–6* allele frequency was higher in lean subjects than in obese subjects (OR 2.8, 95% CI 1.4–5.9, $p = 0.005$), whereas the *Z + 2* allele was more common in obese subjects (OR 2.3, 95% CI 1.3–4.3, $p = 0.007$). The variants at –995, –759, and –697 were in linkage disequilibrium with the *Z–6* allele. We detected ten *5-HT_{2C}R* haplotypes including five rare haplotypes observed in only one subject (Table 2). Haplotype-based analysis showed a clear association of the polymorphisms with obesity ($p = 0.005$). Haplotype 3 was associated with leanness (OR 2.7, 95% CI 1.2–6.3, $p = 0.02$) and haplotype 9 with obesity (OR = 2.3, 95% CI 1.3–4.3, $p = 0.007$). A significant association was also observed between haplotype 3 and glucose tolerance; the haplotype was more common in non-diabetic subjects (0.122 vs 0.058, $p = 0.033$). In addition, the frequency of haplotype 2 was higher in lean subjects (4.1% vs 1.6%), although the difference did not reach statistical significance.

We generated a panel of luciferase reporter gene constructs containing haplotype 2, 3, 6 or 9. The relative luciferase activity of lysates of P19 cells transfected with haplotype 2 or haplotype 3 constructs was 1.44- or 2.58-fold higher than that of cells transfected with the haplotype 6 construct ($p = 0.02$ and $p = 0.0006$, respectively). On the contrary, no difference was obtained in the promoter activity between the haplotype 9 construct and the haplotype 6 construct.

Discussion

In this study we identified three loci of single nucleotide substitution and a dinucleotide repeat polymorphic site in the promoter region of the *5-HT_{2C}R* gene. The single nucleotide substitutions were more common in non-obese or non-diabetic subjects. The variant alleles were in linkage disequilibrium with the *Z–6* allele. Haplotype analysis showed that the frequency of haplotype 3 (*Z–6*, –995A, –759T, –697C) containing all of the substitutions was significantly higher in non-obese or non-diabetic subjects, indicating that the haplotype could be associated with resistance to obesity and Type II diabetes.

The luciferase reporter assay showed a pronounced increase in the promoter activity of haplotype 3 compared with the wild type (*Z*, –995G, –759C, –697G). In addition, haplotype 2 (*Z–6*, –995G, –759C, –697C) showed a slightly but significantly higher promoter activity, in accordance with the tendency that the haplotype was more common in non-obese subjects. Hence both the –995/–759 substitution and the –697 substitution could be involved in the promoter activity, although the latter could be more important. These data are consistent with the hypothesis that naturally occurring *5-HT_{2C}R* promoter mutations alter transcription levels and could play a part in the neural regulation of appetite. On the other hand, haplotype 9, which was associated with obesity, contained the *Z + 2* allele but not the substi-

tution mutations. No significant difference was obtained in the promoter activity between haplotype 9 and haplotype 6. This observation raises a possibility that there could be other functional loci in linkage disequilibrium with the (GT)n dinucleotide repeat locus.

We analysed DNA samples only from male subjects, because the *5-HT_{2C}R* gene is located on chromosome X. In mice, a locus associated with adiposity and overweight has been identified on chromosome X [9]. We identified four polymorphic loci in the promoter region of the *5-HT_{2C}R* gene. The nucleotide substitutions could be associated with the transcription level of the *5-HT_{2C}R* gene and contribute to the genetic resistance to obesity and Type II diabetes. The dinucleotide repeat polymorphism could be a useful genetic marker for susceptibility to obesity.

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