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ScienceDirect
Metabolism Clinical and Experimental 55 (2006) 1420–1425
Metabolism
Clinical and Experimental
www.elsevier.com/locate/metabol

Sequencing analysis of ghrelin gene 5′ flanking region: relations between the sequence variants, fasting plasma total ghrelin concentrations, and body mass index

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Received 27 September 2005; accepted 21 June 2006

Abstract

Ghrelin is a 28-amino-acid peptide with several functions linked to energy metabolism. Low ghrelin plasma concentrations are associated with obesity, hypertension, and type 2 diabetes mellitus, whereas high concentrations reflect states of negative energy balance. Several studies addressing the hormonal and neural regulation of ghrelin gene expression have been carried out, but the role of genetic factors in the regulation of ghrelin plasma levels remains unclear. To elucidate the role of genetic factors in the regulation of ghrelin expression, we screened 1657 nucleotides of the ghrelin gene 5′ flanking region (promoter and possible regulatory sites) for new sequential variations from patient samples with low (n = 50) and high (n = 50) fasting plasma total ghrelin concentrations (low- and high-ghrelin groups). Eleven single nucleotide polymorphisms (SNPs), 3 of which were rare variants (allelic frequency less than 1%) were found in our population. The genotype distribution patterns of the SNPs did not differ between the study groups, except for SNP −501A>C (P = .039). In addition, the SNP −501A>C was associated with body mass index (BMI) (P = .018). This variant was studied further in our large and well-defined Oulu Project Elucidating Risk for Atherosclerosis (OPERA) cohort (n = 1045) by the restriction fragment length polymorphism (RFLP) technique. No significant association of SNP −501A>C genotypes with fasting ghrelin plasma concentrations was found in the whole OPERA population. However, the association of this SNP with BMI and with waist circumference reached statistical significance in OPERA (P = .047 and .049, respectively), remaining of borderline significance for BMI after adjustments (P = .055). The results indicate that factors other than the 11 SNPs found in this study in the 5′ flanking region of ghrelin gene are the main determinants of ghrelin plasma levels. However, SNP −501 A>C genotype distribution seems to be different in subjects having the highest compared with those with the lowest ghrelin levels, and the SNP may be associated with BMI and waist circumference.

1. Introduction

Ghrelin is a 28-amino-acid, gut-derived peptide hormone with growth-hormone–releasing [1] and orexigenic activity [2]. It is known that ghrelin plasma concentrations vary in a pulsatile fashion, with concentrations increasing before and decreasing after meals, indicating that nutritional state might play a role in the determination of plasma ghrelin levels [3,4]. Ghrelin may play a role in the long-term regulation of body weight, as fasting plasma ghrelin concentrations are negatively correlated with body mass index (BMI) [4]. In addition, weight loss has been shown to increase the plasma levels of ghrelin in obese subjects [3]. Although the neuronal and hormonal regulation of ghrelin expression has been extensively studied, the regulation of ghrelin fasting plasma levels is not yet fully understood.

A twin study suggested that genetic factors could play a role in the determination of the ghrelin fasting plasma concentrations [5]. An Arg51Gln mutation in the coding region of ghrelin gene has been shown to associate with obesity [6] and low ghrelin plasma levels [7]. However, no genetic variations outside the coding region of the ghrelin gene with a regulatory function affecting ghrelin plasma levels have been published yet.

We hypothesized that variations in the upstream regions of the ghrelin gene might have an influence on the expression of the gene and thereby play a role in the

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0026-0495/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2006.06.014

determination of total ghrelin fasting plasma concentrations. Therefore, we screened putative upstream regulatory sites and the promoter area in the 5′ flanking region of the ghrelin gene for possible new functional variants from patient samples with low (n = 50) and high (n = 50) plasma ghrelin concentrations.

2. Subjects and methods

2.1. Subjects

The subjects were chosen from our Oulu Project Elucidating Risk for Atherosclerosis (OPERA) study, which is an epidemiologic case-control study addressing the risk factors and disease end points of atherosclerotic cardiovascular diseases. The series was collected during 1991-1993 at the Department of Internal Medicine of the University of Oulu. This study has been described in more detail elsewhere [8,9]. Fasting blood samples were collected and the genomic DNA was extracted. Fasting plasma total ghrelin concentrations were assessed [7] by radioimmunoassay (RIA) with a commercial Peptide Radioimmunoassay Kit (Phoenix Pharmaceuticals, Belmont, CA). The inter- and intra-assay coefficients of variation (CVs), as given by the manufacturer, were 7.5% and 4.0%, respectively. Interassay CV in our analyses was 12.2%. Fasting plasma leptin concentrations were measured by using a commercial double-antibody RIA (Human Leptin RIA Kit, Linco Research, St Charles, MO) with an intra-assay CV of 3.4% to 8.3% and an interassay CV of 3.0% to 6.2%. Plasma adiponectin concentrations were measured with enzyme-linked immunosorbent assay. The latter method was planned in our laboratory. Monoclonal anti–human adiponectin antibody (R&D Systems, Minneapolis, MN, Catalogue No. MAB10651) was used as a capture antibody and biotinylated monoclonal anti–human adiponectin antibody (R&D Systems, Minneapolis, MN Catalogue No. BAM1065) was used as a detection antibody. Both antibodies were used in a concentration of 2 μg/mL. For detection of biotin-labeled detection antibody, we used 1:18000 diluted alkaline phosphatase–labeled NeutrAvidin (Pierce, Rockford, IL, Catalogue No. 31002) and 30% Lumiphos530 (Lumigen, Southfield, MI, Catalogue No. P-501). The standard curve from 1.56 to 100 ng/mL was prepared from human recombinant adiponectin (Biovendor, Modrice, Czech Republic, Catalogue No. RD172023100). Plasma samples were diluted 1:500, and concentrations were measured as duplicates. The intra-assay variation of method was 13.9% and the interassay variation was 15.9% before and 6.5% after correction. All the subjects volunteered for the study, which was approved by the Ethical Committee of the University of Oulu. The study was conducted according to the principles of the Declaration of Helsinki.

We chose subjects with the highest (n = 50) and the lowest (n = 50) fasting plasma total ghrelin concentrations of our OPERA study to form 2 study groups, namely, a high-ghrelin group and a low-ghrelin group. Both groups were sex stratified, consisting of 25 men and 25 women. The groups did not differ from each other in body composition, which was determined by BMI, waist circumference, and plasma concentrations of leptin and adiponectin. The mean age in the high-ghrelin group was 51.8 years (SD, 4.37) and that in the low-ghrelin group was 53.9 years (SD, 5.52). The difference was statistically significant (P = .04) (Table 1) and it has been taken into account in the statistical analyses when appropriate.

2.2. Amplification of genomic DNA by polymerase chain reaction and sequencing of amplified DNA

Four overlapping fragments covering 1657 nucleotides upstream and 60 nucleotides downstream from the transcription start site of ghrelin gene were amplified from genomic DNA by polymerase chain reaction (PCR) using DyNazyme II DNA polymerase (Finnzymes, Espoo, Finland) or AmpliTaq Gold (PE Applied Biosystems, Foster City, CA) enzymes. The primers (Sigma-Genosys, The Woodlands, TX), fragment sizes, annealing temperatures, and number of cycles for each fragment are shown in Table 2. The PCR products were purified with Calf Intestinal Phosphatase (CIP, Finnzymes) and Exonuclease I (New England BioLabs, Beverly, MA) and sequenced in both directions by using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Buckinghamshire, UK). Detection of sequences was carried out with an ABI 377 sequencer (PE Applied Biosystems). The chromatograms and the sequences were analyzed by using Chromas 2.23 (Technelysium, Tenwantin

Table 1
Characteristics of the study subjects in the groups with low and high plasma ghrelin levels for the sequencing analysis study of the 5′ flanking region of the ghrelin gene

	Low-ghrelin group (n = 50)	High-ghrelin group (n = 50)	P
n (men/women)	25/25	25/25	1.0
Age (y)	53.9 (52.3-55.5)	51.8 (50.6-53.1)	.04
BMI (kg/m^2)	28.9 (27.5-30.4)	27.4 (26.1-28.7)	.117
Waist circumference (cm)	94.1 (90.2-98.1)	90.2 (85.7-94.7)	.189
Plasma leptin concentration (pg/mL)	11.4 (9.9-15.1)	7.2 (7.8-12.7)	.108
Plasma adiponectin concentration (mg/L)	15.3 (13.6-17.0)	15.4 (13.6-17.2)	.976
Plasma total ghrelin concentration (pg/mL)	253 (244-261)	1243 (1219-1267)	.000

The values of age, BMI, waist circumference, adiponectin and plasma total ghrelin concentration are means (95% CI), as these variables were normally distributed. Differences between the study groups were analyzed by ANOVA (P values shown). The values of waist to hip ratio and plasma leptin concentration are medians (95% CI) and the differences were analyzed by Mann-Whitney U test.

Table 2
Primers and annealing temperatures used in PCR amplification

Fragment	Left primer	Right primer	Annealing temperature (°C)	Number of cycles	Product size (bp)
1	5′ -cctctaaacacgggggatgt-3′	5′ -ccaagtccagccagagcat-3′	67	35	502
2	5′ -acacagcaacaaagctgcac-3′	5′ -cagggaggggggttagaggta-3′	61	35	509
3	5aggcatccgctaaaataggg-3′	5′ -ggaagaagcatgtgctccag-3′	60	35	501
4	5′ -tggagcctttgctaacatcc-3′	5′ -tcatttctgccaggtgtgac-3′	58	35	510

Fragments 1, 2, and 4 were amplified by using AmpliTaq Gold DNA polymerase (PE Applied Biosystems) and fragment 3 by using DyNAzyme (Finnzymes).

Qld, Australia) and Clustal W multiple sequence alignment program, version 1.8, [10] available on the Internet.

2.3. The restriction fragment length polymorphism study of single nucleotide polymorphism −501A>C

We studied the genotype distribution of single nucleotide polymorphism (SNP) −501A>C in the OPERA study (n = 1045) by restriction fragment length polymorphism (RFLP). The DNA fragment 2 (Table 2) containing the SNP was amplified by PCR as mentioned earlier. Thirty nanograms of the PCR product was digested with 0.025 U *Mwo*I restriction enzyme (New England Biolabs) in a reaction mixture containing 50 mmol/L TRIS-HCl, 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol (pH 7.9). The mixture was incubated at 60°C for 16 hours. The digestion of the DNA fragment consisting of 509 base pairs (bp) resulted in 136- and 372-bp fragments in the −501A/A genotypes, and in the −501C/C genotype, the 372-bp fragment was further digested into 241- and 141-bp fragments. The digestion fragments were separated and visualized on an ultraviolet transluminator after electrophoresis on a 2% agarose gel (QA agar) containing 2 ng/mL ethidium bromide.

2.4. Association studies and statistical analysis

Statistical analyses were performed by using the SPSS statistical package (Version 11.5, SPSS, Chicago, IL). The frequencies of the observed SNPs were compared between the study groups by χ^2 test. The associations of the observed SNPs with the fasting plasma ghrelin concentrations and the BMIs were studied by using one-way analysis of variance (ANOVA) and one-way analysis of covariance (ANCOVA). Transformations to meet the criteria for parametric tests were used when appropriate. Statistical significance was considered at $P < .05$.

3. Results

3.1. Sequence analysis of the ghrelin 5′ flanking region

Cycle sequencing of genomic DNA revealed 11 SNPs in the 5′ flanking region of the ghrelin gene. The mutations found were A to G at nucleotide −45 (−45A>G), (nucleotide number −1 corresponds to the first nucleotide upstream from the translation starting site), C to T at −113 (−113C>T), C to T at −213 (−213C>T), G to A at −473 (−473G>A), A to C at −501 (−501A>C), G to A at −604 (−604G>A), C to A at −887 (−887C>A), C to T at −994 (−994C>T), C to T at −1003 (−1003C>T), G to C at −1062 (−1062G>C), and G to C at −1500 (−1500G>C). None of these variants was located at the binding sites of known transcription factors identified by using TFSEARCH on the Internet [11].

Table 3
The genotype frequencies and reference sequence numbers of the mutations found, their genotype distributions within the low- and high-ghrelin study groups, and associations with BMI

SNP	Reference sequence no.	Wild-type homozygotes (n)	Heterozygotes (n)	Variant homozygotes (n)	Genotype distribution between the study groups (*P*)	Association with BMI (*P*)
−45A>G	New	94	6	–	1.0	.960/.884
−113C>T	New	99	1	–	–	–
−213C>T	New	99	1	–	–	–
−473G>A	New	98	2	–	–	–
−501A>C	26802	62	29	9	.072[a]	.016/.018
−604G>A	27647	23	38	39	.917	.281/.409
−887C>A	4684678[b]	98	2	–	–	–
−994C>T	26312	76	19	5	.695	.067/.166
−1003C>T	New	99	1	–	–	–
−1062G>C	26311	76	19	5	.695	.067/.166
−1500G>C	3755777	47	40	13	.832	.605/.708

The nomenclature of the SNPs declares the nucleotide change and the position of the SNP (number −1 corresponds to the first nucleotide upstream from the translational starting point). The reference sequence numbers are shown for those SNPs that were entered into GenBank before. The differences in the genotype distributions between high- and low-ghrelin groups were studied by χ^2 test. The associations of the SNPs with BMI were studied by ANOVA/ANCOVA (adjusted for sex and age). In 5 of the SNPs found the low frequency of different genotypes did not allow meaningful statistical analysis to be performed.

[a] When A/A wild type was compared with C carriers (A/C and C/C genotypes combined), the difference in the genotype distribution between the study groups was statistically significant ($P = .039$).

[b] The nucleotide change in the GenBank reference sequence is G>A, and in our data, C>A.

The genotype distribution patterns were in agreement with Hardy-Weinberg equilibrium. The genotype frequencies and the GenBank reference sequence numbers of the SNPs, if available, are presented in Table 3. Five of the SNPs (−45A>G, −113C>T, −213C>T, −473G>A, and −1003C>T) had not been entered into GenBank before, and 3 of them (−113C>T, −213C>T, and −1003C>T) were rare variants (allelic frequency less than 1%). The χ^2 test was used to compare the genotype distributions between the study groups. *P* values of these statistical tests are shown in Table 3. Considering the genotype distribution of SNP −501A>C between the study groups, when A/C and C/C genotypes were combined, the difference in genotype distributions was significant ($P = .039$). A/A wild-type genotype in the low-ghrelin group and C carriers in the high-ghrelin group were more common than expected. However, there was no statistically significant difference between the mean fasting plasma total ghrelin concentrations of the SNP genotypes (data not shown). Considering the other SNPs of this study, no significant differences in the genotype distribution patterns between the study groups were observed.

Associations of the SNPs found with BMI were studied. A statistically significant association between the SNP −501A>C and BMI was observed ($P = .016$). The association remained significant after adjustment for age and sex ($P = .018$). Mean BMI of A/A wild-type subjects was 29.1 kg/m^2 (95% confidence interval [CI], 27.8-30.4); that of A/C heterozygotes, 27.3 kg/m^2 (95% CI, 25.7-28.8); and that of the C/C mutant genotype, 24.7 kg/m^2 (95% CI, 22.8-26.7). We also studied the association of this SNP with waist circumference and the result was in accordance with the association with BMI. There was a clear trend toward genotype A/A having the highest and genotype C/C having the lowest value of waist circumference. The mean waist circumference of the A/A genotype was 93.8 cm (95% CI, 89.9-97.6); that of the A/C heterozygotes, 92.3 cm (95% CI, 86.6-98.0); and that of the C/C genotype, 80.9 cm (95% CI, 73.8-88.0). However, these differences reached only a borderline statistical significance ($P = .053$) that was lost after adjustment for age and sex ($P = .082$) (data not shown elsewhere). The other SNPs found in this study were not associated with BMI or with waist circumference (Table 3).

3.2. RFLP study for the distribution of SNP −501A>C genotypes in the OPERA study

The distribution of SNP −501A>C genotypes was further examined by RFLP technique in our large OPERA cohort. The genotype distribution was in agreement with Hardy-Weinberg equilibrium. There was no difference in the number of men and women or in the mean ages between the genotypes. No significant differences in the total ghrelin plasma concentrations between the SNP −501A>C genotypes were observed (Table 4).

We found a statistically significant difference in the mean BMIs between the SNP−501A>C genotypes when A/A and A/C carriers were combined (A carriers) and compared with C/C mutant homozygotes in the OPERA study ($P = .047$, Table 4). The mean BMI of A carriers was 27.8 kg/m^2 (95% CI, 27.5-28.1) and that of C/C homozygotes was 26.8 kg/m^2 (95% CI, 26.0-27.6). The mean waist circumference of A carriers was 90.8 cm (95% CI, 90.0-91.7) and that of C/C homozygotes was 88.0 cm (95% CI, 85.7-90.4). After adjustment for age and sex, the difference in the BMIs between A carriers and C/C homozygotes reached borderline statistical significance ($P = .055$, Table 4).

To study the association of the SNP −501A>C with other obesity-related parameters, we also carried out association studies for SNP −501A>C with waist circumference and with plasma leptin and adiponectin concentrations. No differences in the plasma levels of leptin and adiponectin between the genotypes were observed. However, there was a significant difference in the waist circumference between the

Table 4
Main phenotype characteristics of the ghrelin 5′ flanking region SNP −501A>C genotypes in OPERA study

	−501A>C genotype			*P*	Combined −501A>C genotype		*P*
	A/A	A/C	C/C		A/A and A/C	C/C	
n (men/women)	557 (273/284)	382 (193/189)	96 (46/50)	.857	939 (466/473)	96 (46 /50)	.749
Age (y)	51.2 (50.6-51.6)	51.6 (51.1-52.2)	50.9 (49.6-52.1)	.375	51.3 (51.0-51.7)	50.9 (49.6-52.1)	.445
BMI (kg/m^2)	27.8 (27.4-28.2)	27.8 (27.4-28.3)	26.8 (26.0-27.6)	.136/.159[a]	27.8 (27.5-28.1)	26.8 (26.0-27.6)	.047/.055[a]
Waist circumference (cm)	90.6 (89.5-91.7)	91.1 (89.8-92.5)	88.0 (85.7-90.4)	.119	90.8 (90.0-91.7)	88.0 (85.7-90.4)	.049/.266[a]
Plasma leptin concentration (pg/mL)	8.1 (9.8-11.3)	8.5 (9.8-11.4)	8.1 (9.2-12.8)	.911	8.2 (10.1-11.1)	8.1 (9.2-12.8)	.989
Plasma adiponectin concentration (mg/L)	15.7 (15.2-16.2)	15.3 (14.7-16.0)	16.3 (15.0-17.6)	.374	15.5 (15.1-15.9)	16.3 (15.0-17.6)	.260
Plasma total ghrelin concentration (pg/mL)	667.4 (646.1-688.7)	667.0 (642.2-691.8)	683.9 (629.8-738.1)	.828/.913[b]	667.2 (651.1-683.3)	683.9 (629.8-738.1)	.540/.669[b]

The values for age, BMI, waist circumference, plasma adiponectin, and ghrelin concentration are means (95% CI) and medians (95% CI) for plasma leptin concentration. *P* values are probabilities for the difference between the genotypes in ANOVA or in ANCOVA adjusted for [a]age and sex, and [b]for age, sex, and BMI.

genotypes when genotypes were combined as before (P = .049). The mean waist circumference of A carriers was 90.8 cm (95% CI, 90.0-91.7) and that of C/C homozygotes, 88.0 cm (95% CI, 85.7-90.4). After adjustment for age and sex the association of the combined genotypes with waist circumference was lost (P = .266, Table 4). In addition, we analyzed men and women separately without combining the genotypes. The difference in waist circumference between the −501A>C genotypes was significant in men but not in women (P = .042 and .588, respectively). In men, the mean waist circumference of the A/A genotype was 97.9 cm (95% CI, 96.6-99.3); that of the A/C genotype, 97.7 cm (95% CI, 96.1-99.1); and that of the C/C genotype, 93.6 cm (95% CI, 90.8-96.4) (data not shown elsewhere).

4. Discussion

In this study we found 11 SNPs in the ghrelin gene ′5 flanking area. Five of them were new variants. None of them associated with plasma ghrelin concentrations. However, there was a significant difference in the genotype distribution of one of the SNPs, SNP −501A>C, between the high and low ghrelin study groups when genotypes were combined. The same SNP associated also with BMI. The C carriers were more common in high ghrelin group than expected and the C/C genotype had the lowest mean BMI.

To investigate the role of the SNP −501A>C more, some further analysis were performed in a larger population (n = 1045). Fasting plasma total ghrelin concentrations were not associated with SNP −501A>C genotypes, suggesting that this SNP is not playing a major role in the overall determination of fasting ghrelin plasma levels. Despite this finding, it is still theoretically possible that the SNP might have a function in the regulation of ghrelin gene expression. On one hand, it must be remembered that the plasma concentration of any peptide hormone is not a perfect reflection of the rate of its gene expression because events such as storage and release of the hormone take part in the determination of the plasma hormone concentration. Therefore, eg, different activity of the cellular processes that occur after gene expression might be able to mask at least mild interindividual differences in the rate of gene expression. In vitro studies might elucidate the role of this SNP and ghrelin gene expression more. On the other hand, it must be noted that fasting ghrelin plasma concentration reflects only the level of ghrelin in circulation at one moment and does not necessarily tell if there is something unusual in the diurnal secretory profile of ghrelin. We admit that fasting ghrelin concentrations have been shown to correlate strongly with the 24-hour integrated area under the curve of ghrelin at least in a population of small sample size [3], but still it can be speculated that some individuals might have some genetic or other factors that affect this correlation, and the association of such a factor might be missed in studies such as ours when only fasting plasma ghrelin levels were assessed.

There was a significant difference in the mean BMIs of the SNP −501A>C genotypes in our larger study population when genotypes were combined, and it reached borderline significance after adjustments. The SNP was also associated with waist circumference. Our results suggested that the ghrelin 5′ flanking region SNP −501A>C genotypes A/A and A/C might contribute to the tendency to higher BMI and greater waist circumference than the C/C genotype. It could be worth studying this suggested association in more detail, eg, whether this SNP associated with more specific parameters of obesity such as with the amount of fat and lean muscle mass. Anyhow, we admit, that it is difficult to explain the possible mechanism linking the ghrelin 5′ flanking region SNP −501A>C with these obesity-related parameters. It is possible that this SNP could be in linkage disequilibrium with a certain unidentified genetic factor, which could be associated with BMI and waist circumference.

We conclude that the 11 SNPs in the 5′ flanking region of ghrelin gene found in this study are not the main factors in the regulation of fasting ghrelin plasma levels in our population. To our knowledge, this was the first study to describe ghrelin 5′ flanking region variants of ghrelin gene in relation to ghrelin plasma levels and some obesity-related parameters in a human population. Our results indicate that SNP −501A>C might be associated with BMI and waist circumference. In addition, because the genotype frequencies differ in a statistically significant way between the groups having the highest and lowest ghrelin plasma concentrations of our study population, it can be suggested that in some cases the SNP might associate with plasma ghrelin levels. Further studies are needed to understand the regulation of ghrelin gene expression, the determination of ghrelin plasma levels, and the suggested association of the ghrelin 5′ flanking area SNP −501A>C with obesity-related parameters in detail.

Acknowledgment

This study was supported by the Research Council for Health of the Academy of Finland and the Finnish Foundation for Cardiovascular Research.

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