CLINICAL STUDY

Sequencing analysis of the ghrelin receptor (growth hormone secretagogue receptor type 1a) gene

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Abstract

Objectives and design: Ghrelin is a novel 28 amino acid peptide which is reported to have several endocrine and non-endocrine actions. It possesses strong growth hormone (GH)-releasing activity, which is mediated via the GH secretagogue receptor type 1a (GHS-R1a). We hypothesised that there might be functional sequential variations in the GHS-R1a gene affecting phenotypes linked to the GH/insulin-like growth factor-I (IGF-I)-axis.

Methods: To test our hypothesis we chose patients from our OPERA (Oulu Project Elucidating Risk of Atherosclerosis) study with low ($n = 96$) and high ($n = 96$) IGF-I levels, sequenced their GHS-R1a gene exons and performed association studies.

Results: We found five single-nucleotide polymorphisms (SNPs) which did not change the amino acid sequence. We were unable to detect associations between the SNPs and the IGF-I plasma concentrations, but instead we showed that SNP 171C > T was associated with the values of the area under the insulin curve (AUCIN) in an oral glucose tolerance test and with IGF-binding protein-1 (IGFBP-1) concentrations ($P < 0.05$). SNP 477G > A was associated with the low density lipoprotein and very low density lipoprotein cholesterol plasma levels and AUCIN values ($P < 0.05$).

Conclusions: This study was the first genomic screening of the GHS-R1a gene in a population. It suggests that genetic variations in the GHS-R1a gene are not the main regulators of IGF-I levels. However, the variants may be associated with IGFBP-1 concentrations and insulin metabolism.

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Introduction

Ghrelin is a novel 28 amino acid peptide hormone which was originally extracted from rat stomach (1) and which has subsequently been shown to be expressed at lower levels in the hypothalamus and pituitary and also in several peripheral tissues (2). Ghrelin at pharmacological doses strongly stimulates growth hormone (GH) release in rodents and humans (1, 3) acting synergistically with growth hormone-releasing hormone (GHRH) (4). Ghrelin is also adipogenic in rodents (5) and has orexigenic potency both in animals and in humans (6, 7). However, ghrelin is downregulated in human obesity (8). Ghrelin has been proposed to act as a link between the regulation of energy balance and GH secretion (9).

Ghrelin is an endogenous ligand for the type 1a growth hormone secretagogue receptor (GHS-R1a). GHS-R1a binds acylated ghrelin and other growth hormone secretagogues and mediates their stimulating activity on GH secretion (1, 10). This receptor is a member of the G-protein-coupled receptor superfamily and it was first detected from the hypothalamus,

pituitary and arcuate nucleus, where it is concentrated (10). It is also expressed at lower levels in the thyroid gland, pancreas, spleen, myocardium and adrenal glands (2), which supports the notion that, in addition to stimulating GH release, ghrelin also participates in other biological functions.

The biological activities of GH are mediated through insulin-like growth factor-I (IGF-I) and GH is characterised as the major driving force of IGF-I synthesis. IGF-I is produced mainly in the liver and released into the bloodstream, where it circulates as a free molecule or is bound to the IGF-binding protein (IGFBP) (11). Six different IGFBPs have been described (12), of which IGFBP-1 seems to affect IGF-I bioactivity effectively by regulating the amount of free IGF-I in the circulation. Decreased portal levels of insulin lead to elevated levels of IGFBP-1 which, in turn, reduces free IGF-I and diminishes the negative feedback on GH release in somatotrophs (13). The effects of ghrelin on the GH/IGF-I axis are not completely clear. Obesity is characterised by decreased ghrelin levels (8) and GH deficiency, while IGF-I levels are within the normal range or even elevated (14). Decreased IGF-I levels

have been associated with type 2 diabetes (15), diabetes-related complications and heart diseases (16, 17). Anorectic patients have elevated ghrelin (18) and IGFBP-1 levels but subnormal free IGF-I levels, while their GH levels are within the normal range (19). There is evidence that the role of ghrelin in physiology may be sexually dimorphic; decreased expression levels of the ghrelin receptor GHS-R1a gene in transgenic mice result in decreased GH and IGF-I levels more often in female than in male mice (20). This also suggests that a connection between GHS-R1a and IGF-I levels might exist. However, sequential variations in the GHS-R1a gene have not been described previously in a population-based study. We therefore screened the region of the GHS-R1a gene encoding the entire protein product to find sequence variations that might have an effect on protein structure and function and might be related to different phenotypes in the GH/IGF-I axis, e.g. IGF-I plasma levels.

Subjects and methods

Subjects

The subjects were chosen from our OPERA (Oulu Project Elucidating Risk of Atherosclerosis) study, which is an epidemiological 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 (21, 22). The subjects for this study were selected from the hypertensive cohort, which consisted of 600 age-stratified Finnish subjects (300 men and 300 women). Fasting blood samples were collected, and the oral glucose tolerance tests (OGTTs) were performed (with 75 g glucose) at the Department of Internal Medicine of the University of Oulu. Intima-media thicknesses (IMTs) were measured from the common carotid arteries by ultrasonography. The routine clinical laboratory tests were carried out in the Central Laboratory of Oulu University Hospital. Subsequently, several parameters have been measured using fasting plasma samples and the genomic DNA extracted from them. Measurements of the total IGF-I and IGFBP-1 concentrations were carried out using commercial kits (DSL-10-2800 ACTIVE Non Extraction IGF-I ELISA, Diagnostic Systems Laboratories, Inc., Webster, TX, USA and IGFBP-1 IOMA test, Oy Medix Biochemica Ab, Kauniainen, Finland). Ghrelin concentrations were assessed (23) by radioimmunoassay (RIA) using a commercial kit (Peptide Radioimmuno Assay Kit, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). Fasting insulin levels were measured by two-site immunoenzymometric assay (AIA-PACK IRI, Tosoh Corp., Tokyo, Japan), and fasting glucose levels were determined by the glucose dehydrogenase method (Diagnostica, Merck, Darmstadt, Germany).

Quantitative insulin sensitivity check indexes (QUICKI) were calculated as described elsewhere (24). The measurements of plasma triglyceride concentrations and very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels have been described in detail elsewhere (25). 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 1975, as revised in 2000.

Since we were also interested in studying the association of sequential variations with some diabetes-related parameters, we chose subjects from the hypertensive group of the OPERA study, which includes more diabetics than the control group. Another reason for choosing the study subjects from the hypertensive cohort was that our earlier results suggested that the effect of low ghrelin on blood pressure levels was stronger in a hypertensive state. When the hypertensive and non-hypertensive study cohorts were analysed separately, the association between ghrelin and blood pressure levels remained only in the hypertensive cohort (23). Therefore, ghrelin and its receptor as well as their genetic variation might play a specific role in hypertension.

Subjects with high ($n = 96$) and low ($n = 96$) total IGF-I plasma concentrations were chosen. The number of patients with type 2 diabetes was 11 in the group with high IGF-I levels and 16 in the group with low levels ($P = 0.299$). Both groups were sex-stratified, consisting of 48 men and 48 women. Physiological age-dependent reduction in IGF-I levels was taken into account by stratifying both of the study groups for age: the average age (mean) in the group with high IGF-I levels was 51.1 years and that in the group with low levels was 51.7 years. In the group with high IGF-I levels the mean concentration of IGF-I was 136.5 ng/ml (95% confidence interval (CI) = 127.4–145.5) and that of IGFBP-1 was 2.9 ng/ml (95% CI = 2.4–3.4). The mean IGF-I concentration in the group of low IGF-I levels was 30.2 ng/ml (95% CI = 27.0–33.4), and the mean IGFBP-1 concentration was 3.9 ng/ml (95% CI = 2.5–5.3). The difference in the IGFBP-1 levels between the groups with high and low IGF-I levels was not statistically significant ($P = 0.517$).

Amplification of genomic DNA by PCR and sequencing of amplified DNA

Two overlapping fragments covering the first exon and one fragment covering the second exon of the GHS-R1a gene were amplified from genomic DNA by PCR using DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) or AmpliTaq Gold (PE Applied Biosystems, Foster City, CA, USA) enzymes. The primers, (Sigma-Genosys Ltd., The Woodlands, TX,

Table 1 Primers and annealing temperatures used in PCR amplification. Sequencing was performed using the same primers.

Fragment	Forward primer	Reverse primer	Annealing temperature	Number of cycles	Product size (bp)
1	5' ctccctacgcgtctgcac 3'	5' ggaagcagatggcgaagtag 3'	56 °C	35	569
2	5' cagtgcagcgtgcacctacg 3'	5' agaaggcacaggagagaggat 3'	58 °C	30	491
3	5' ttgctgtgcatttcttgagc 3'	5' cttcctcccaagttctgctg 3'	56 °C	30	430

Fragment 1 was amplified using AmpliTaq Gold DNA polymerase (PE Applied Biosystems) and the other fragments were amplified using DyNAzyme (Finnzymes).

USA), fragment sizes, annealing temperatures and the number of cycles for each fragment are shown in Table 1. The PCR products were purified with calf intestinal phosphatase, (CIP; Finnzymes) and Exonuclease I (New England BioLabs Inc., Beverly, MA, USA) and sequenced in both directions using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Bucks, UK). Detection of sequences was carried out with an ABI 377 sequencer (PE Applied Biosystems).

Association studies and statistical analysis

Statistical analysis was performed using the SPSS statistical package (versions 9.0 and 11.5; SPSS Inc., Chicago, IL, USA). The frequencies of the observed single-nucleotide polymorphisms (SNPs) were compared in the groups with low and high IGF-I plasma levels by a chi-square test. The associations of the observed SNPs with the body mass indexes (BMIs), IMTs and metabolic parameters were studied by using one-way analysis of variance (ANOVA), Mann–Whitney's U-test, *t*-test and analysis of covariance (ANCOVA) when appropriate. Appropriate transformations were used to meet the criteria of the parametric tests. Significance was considered at $P < 0.05$.

Results

Sequence analysis of the GHS-R1a gene exons

Cycle sequencing of genomic DNA revealed five SNPs in the first exon and none in the second exon of the GHS-R1a gene. The found mutations were C to T at nucleotide 60 (60C > T), (nucleotide number 1 corresponds to the first nucleotide of the first codon), C to T at nucleotide 171 (171C > T), C to G at nucleotide 447 (447C > G), G to A at nucleotide 477 (477G > A) and C to A at nucleotide 531 (531C > A). The location of the SNPs is illustrated in Fig. 1 (adapted from the original paper (26)), and the genotype and allele frequencies are presented in Table 2. All of the SNPs have been entered into the GenBank previously and the access numbers are indicated in Table 2. None of these mutations changes the coded amino acid. The distribution patterns were in agreement with Hardy–Weinberg equilibrium.

Association studies

The frequencies of the discovered SNPs were compared between the groups with high and low IGF-I levels by a chi-square test. The allele frequencies were equally distributed between the groups (data not shown). However, SNP 171C > T was associated with the IGFBP-1 and area under the insulin curve (AUCIN) values, showing statistical significance in ANOVA ($P = 0.009$ and $P = 0.020$ respectively; Table 3). When adjustments for age, BMI and sex were carried out, significance was maintained (ANCOVA, $P = 0.038$ and $P = 0.038$ respectively; Table 3). The wild-type C/C homozygotes had the highest AUCIN values and the lowest IGFBP-1 plasma levels compared with the heterozygotes or variant homozygotes. The differences were statistically significant between the wild-type homozygotes and heterozygotes in pair-wise comparisons (AUCIN: $P = 0.011$ and $P = 0.032$ after Bonferroni correction; IGFBP-1: $P = 0.012$ and $P = 0.037$ after Bonferroni correction; Table 3).

SNP 477G > A was associated with the AUCIN values. There was a significant difference in the AUCIN values between the 477G > A genotypes (ANOVA, $P = 0.007$, Table 3). Significance was maintained

Figure 1 Genomic screening for sequence variations in the type 1a growth hormone secretagogue receptor (GHS-R1a) gene revealed five single-nucleotide polymorphisms (SNPs). The SNPs were found in the codons of amino acids, which are indicated by arrows in this presentation of the predicted structure of the receptor. The structure of the receptor was illustrated by adapting the information from the original paper (26).

Table 2 Genotype and allele frequencies for the sequence variations observed in the study.

SNP	dbSNP rs#	Genotype frequency (n)			Allele frequency (%)	
		C/C	C/T	T/T	C	T
60C > T	rs2232165	189	3	—	99.2	0.8
171C > T	rs495225	C/C	C/T	T/T	C	T
		20	81	91	31.5	68.5
447C > G	rs2232169	C/C	C/G	G/G	C	G
		189	3	—	99.2	0.8
477G > A	rs572169	G/G	G/A	A/A	G	A
		81	85	26	64.3	35.7
531C > A	rs4988509	C/C	C/A	A/A	C	A
		188	4	—	99.0	1.0

The nomenclature of the SNPs shows the bases taking part in nucleotide substitutions and the numbers stand for the nucleotides at which the substitutions have taken place; number one corresponds to the first nucleotide of the first codon. The GenBank access number for the SNPs are shown in the column entitled dbSNP rs#.

after adjustments for age, sex and BMI (ANCOVA, $P = 0.049$). In the pairwise comparisons, the difference was significant between the wild-type G/G homozygotes and G/A heterozygotes ($P = 0.014$, $P = 0.042$ after Bonferroni correction; Table 3). The wild-type homozygotes had the highest AUCIN values.

SNP 477G > A was also associated with the plasma concentrations of VLDL and HDL cholesterol. There were significant differences in the VLDL and HDL cholesterol levels between the genotypes (ANOVA, $P = 0.021$ and $P = 0.032$ respectively; Table 3). After adjustment for age, BMI, sex and alcohol consumption,

the statistical difference in the HDL concentrations was of borderline significance (ANCOVA, $P = 0.05$) and the difference in the pairwise comparisons was significant between the variant homozygotes and heterozygotes ($P = 0.021$, Table 3). HDL cholesterol plasma concentrations were the lowest among the variant homozygotes. However, statistical significance disappeared when Bonferroni correction was performed ($P = 0.064$; Table 3). The statistical significance of the VLDL association disappeared after adjustments for age, sex and BMI (ANCOVA, $P = 0.071$; Table 3).

Association studies were also performed for SNPs and BMI, plasma ghrelin and fasting insulin concentrations, QUICKI, triglyceride, LDL cholesterol plasma concentrations and carotid intima-media thicknesses. None of the SNPs showed statistically significant associations with any of these parameters. SNPs 60C > T, 447C > G and 531C > A were not associated with any parameters studied.

Discussion

The GH/IGF-I axis has an important role in normal growth and metabolism. A GH pulse in the circulation has been thought to be the major driving force for IGF-I synthesis and secretion. Some association studies between ghrelin, an endogenous ligand for the GH secretagogue receptor, and IGF-I levels exist. In a recent study, serum IGF-I levels were not altered by ghrelin administration (27). Fasting, which is characterised by increased ghrelin secretion, has been shown to lead to a decrease in IGF-I levels (28).

Table 3 BMI and metabolic parameters in relation to GHS-R1a 171C > T and 477G > A. SNPs in the study group consisting of 96 male and 96 female hypertensive patients. The values are shown as means (95% CI).

	171C > T				477G > A			
	C/C (n = 20)	C/T (n = 81)	T/T (n = 91)	P	G/G (n = 81)	G/A (n = 85)	A/A (n = 26)	P
BMI (kg/m ²)	29.6 (27.8–31.4)	28.4 (27.4–29.5)	29.0 (28.1–30.0)	ns	29.5 (28.6–30.4)	28.1 (27.1–29.1)	29.2 (26.9–31.4)	ns
Fasting insulin (mU/l)	20.1 (13.8–26.7)	17.4 (14.1–20.8)	15.7 (13.7–17.7)	ns	17.9 (15.1–20.8)	15.9 (13.2–18.7)	16.6 (12.0–21.3)	ns
IGF-I (mmol/l)	100.7 (66.1–135.3)	89.4 (75.4–103.4)	74.3 (62.0–86.6)	ns	84.0 (69.3–98.7)	86.4 (72.7–100.1)	71.8 (52.4–91.1)	ns
Ghrelin (mmol/l)	201.0 (161.8–240.3)	198.9 (181.9–215.8)	213.7 (197.7–229.7)	ns	204.6 (187.0–222.3)	207.2 (190.9–223.5)	207.2 (175.5–238.9)	ns
AUCIN (mmol/h)	247.1 (133.4–360.7)	148.0 (123.4–172.7)	164.5 (141.2–187.8)	0.020 ^a	192.6 (157.7–227.6)	136.5 (116.2–156.9)	177.7 (123.8–231.5)	0.007 ^a
IGFBP-1 (ng/ml)	1.9 (1.3–2.5)	4.3 (2.7–5.8)	3.0 (2.4–3.5)	0.009 ^a	3.7 (2.1–5.3)	3.0 (2.5–3.5)	3.6 (2.0–5.1)	ns
VLDL-chol (mmol/l)	0.62 (0.48–0.77)	0.53 (0.43–0.63)	0.56 (0.47–0.65)	ns	0.62 (0.51–0.72)	0.47 (0.39–0.55)	0.63 (0.45–0.80)	0.021 ^a
HDL-chol (mmol/l)	1.32 (1.14–1.15)	1.28 (1.20–1.37)	1.29 (1.22–1.37)	ns	1.25 (1.17–1.33)	1.36 (1.28–1.45)	1.18 (1.24–1.34)	0.032 ^a

chol, cholesterol.

^aOne-way ANOVA, no adjustments. ^bAdjusted for age, sex and BMI in ANCOVA. ^cAdjusted for age, sex, BMI and alcohol consumption in ANCOVA. In pairwise comparisons the difference occurs: ^abetween C/C and C/T genotypes ($P = 0.011$ and $P = 0.032$ with Bonferroni correction), ^bbetween C/C and C/T genotypes ($P = 0.012$ and $P = 0.037$ with Bonferroni correction), ^cbetween G/G and G/A genotypes ($P = 0.014$ and $P = 0.042$ with Bonferroni correction) and ^dbetween G/A and A/A genotypes ($P = 0.021$ and $P =$ not significant (ns) after Bonferroni correction).

Considering the role of ghrelin in hypsomatotrophism of human obesity, no causative relationship could be demonstrated in a recent study (29). This and other earlier observations provide evidence that the effects of ghrelin on GH secretion and energy homeostasis may be distinct. Our results suggest that the five ghrelin receptor polymorphisms found in this study have no evident association with IGF-I levels. However, two SNPs were found to be associated with glucose metabolism-related parameters: AUCIN values and IGFBP-1 plasma levels.

Insulin is a powerful downregulator of IGFBP-1 synthesis, and IGFBP-1 levels have been shown to be elevated in type 1 diabetes (30). However, the situation in type 2 diabetes and insulin resistance may be different. The increased AUCIN values observed in the 171C/C subjects in the current study indicate impairment of insulin action, although the GHS-R1a 171C > T polymorphism was not associated with any other insulin metabolism parameters. Low IGFBP-1 levels have been associated with obesity and with cardiovascular risk factors in insulin resistance syndromes (31). The latter association is in accordance with our observations in the present study, where 171C/C homozygotes showed both high AUCIN values and low IGFBP-1 levels. These subjects may, therefore, have the lowest insulin sensitivity for an unknown reason. One hypothesis could be that the different expression levels of GHS-R1a might have an effect on the regulation of insulin metabolism by affecting ghrelin signalling. For example, the SNP 171C > T could be in linkage disequilibrium with a mutation that is able to alter the level of ghrelin receptor expression. A mutation of this kind could be present in, for example, promoter or intron areas that were not screened in this study. Another theory could be that SNP might be able to change a binding site for transcription factors and, in this way, may also lead to altered expression levels of the protein product. There is evidence that decreased expression levels of GHS-R1a lead to decreased GH and IGF-I values in transgenic female mice (20); however, the relationship between altered GHS-R1a expression levels and IGFBP-1 levels has yet to be elucidated.

The role of ghrelin in insulin secretion is controversial as ghrelin has been shown both to lower (32) and to raise (33) insulin levels. Insulin has been shown to downregulate IGFBP-1 synthesis and, probably for this reason, IGFBP-1 levels are altered in different nutritional states (34). These acute and long-term states of malnutrition are also characterised by elevated ghrelin levels (18, 28). These findings together with our results raise a question: is it possible that ghrelin levels might play a role in the regulation of IGFBP-1 levels?

Considering the results of SNP 477G > A, significant differences were seen only between heterozygotes and either wild-type or variant homozygotes. Heterozygotes

had lower VLDL cholesterol levels and higher HDL cholesterol levels than did the variant 477A/A homozygotes. This result could be interpreted to indicate that heterozygosity at this nucleotide is beneficial in terms of the VLDL/HDL ratio. Anyhow, the statistical significance was lost when some adjustments were made, which further suggests that this polymorphism does not play an independent role in plasma lipid metabolism. The heterozygotes had significantly lower AUCIN values than did the wild-type 477G/G homozygotes. However, no significant associations of this SNP with IGFBP-1 were observed.

The three other polymorphisms were not associated with any of the parameters studied, a result which was predictable due to their low allele frequencies. Actually, in our population, SNPs 60C > T and 447C > G have too low an allelic frequency (allelic frequency <1%) to fulfil the criteria for polymorphism.

It must be mentioned that, in theory, there is always a risk for false positive signals when sequencing PCR products. We increased the reliability of the method by sequencing the exons in both directions. Also, the detected SNPs were not located close to the primers. Therefore, our genotyping results can be regarded as being quite reliable. Another thing that must be remembered is that the sample size for each genotype is not very large in any of our five SNPs. If we had analysed a larger number of subjects, the differences would probably have been clearer. However, our results provide useful information for the genes and pathways that should be studied further.

It is important to point out that the role of endogenous ghrelin in the regulation of GH secretion in a physiological situation is unknown, and that the ghrelin/GHS-R1a interaction at the molecular level has not yet been fully clarified. GHS-R1a has been demonstrated to be a highly conserved gene throughout evolution (35), and no mutations with an effect on the protein structure have been described. It is likely that this receptor has an essential or even vital function in organisms, and our result of no amino acid-changing mutations in 192 patients is therefore not very surprising.

In conclusion, our study was the first to describe a genomic screening of the GHS-R1a gene in a population. We found five SNPs, but no amino acid-changing mutations in the protein-coding area. The SNPs were not associated with IGF-I levels. Instead, SNP 171C > T was associated with IGFBP-1 levels and AUCIN values and SNP 477G > A was associated with AUCIN values and VLDL and HDL levels. The data suggest that genetic variations in the protein coding area of the ghrelin receptor GHS-R1a gene are not the main regulators of IGF-I levels. However, the variants may be associated with the regulation of insulin metabolism and IGFBP-1 levels.

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