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ORIGINAL ARTICLE

Preproghrelin Leu72Met variant contributes to overweight in middle-aged men of a Japanese large cohort

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Objective: To investigate whether Leu72Met polymorphism of the preproghrelin gene is associated with overweight/obesity in middle-aged and older Japanese.
Design: Cross-sectional analysis.
Subjects: A total of 2238 community-dwelling middle-aged and older Japanese people (age: 40–79 years) who participated in the first wave of examinations in the National Institute for Longevity Sciences – Longitudinal Study of Aging from April 1998 to March 2000.
Measurements: The Leu72Met polymorphism of prepoghrelin gene, anthropometric variables including body weight, body mass index (BMI), waist circumference, waist-to-hip ratio and whole-fat mass and biochemical variables including serum lipid levels, fasting plasma glucose, insulin and homeostasis model assessment for insulin resistance.
Results: The frequencies of the Leu72Leu, Leu72Met and Met72Met alleles were 63.4, 32.7 and 4.0%, respectively. No differences in the genotype distributions of the Leu72Met polymorphism were found between genders or age groups, and no significant associations were observed between polymorphism and anthropometric variables in women and older men. However, middle-aged men who were 72Met allele carriers showed a higher body weight change from body weight at 18 years of age, as well as a higher waist circumference and a tendency to a higher waist-hip-ratio than noncarriers. Although there were no significant differences in the genotype distribution according to BMI in women and older men, a significantly higher frequency of the 72Met allele was found in the higher BMI group (BMI $\geqslant 25\,kg/m^2$) of middle-aged men than in the normal-weight group. No significant associations were observed between polymorphism and serum lipid, glucose or insulin levels.
Conclusions: These results suggest that the 72Met allele of the preproghrelin gene is a contributing factor for midlife weight change in men.
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Keywords: ghrelin; polymorphism; preproghrelin; lipid metabolism; glucose metabolism

Introduction

Ghrelin has been shown to be the natural ligand of the previously identified 'orphan' growth hormone secretagogue receptor.[1] Although widely expressed in many tissues, ghrelin is most abundantly produced by the stomach.[1] Ghrelin is much more than a mere natural growth hormone secretagogue, however: it has been found to have profound growth hormone-independent weight- and appetite-increasing effects.[2] Ghrelin stimulates food intake in both rodents and humans,[2,3] and is strongly involved in the regulation of energy homeostasis.[4] This suggests that derangement in the ghrelin system could play a role in obesity. In addition, ghrelin may affect carbohydrate and lipid metabolisms.[5,6]

Recently, three major polymorphisms in the human ghrelin gene were described.[7] One of these nucleotide changes, a single-base substitution C214A with Met replacing Leu at codon 72 in the preproghrelin amino-acid sequence, seems to be associated with an earlier onset of obesity,[7–9] but it has also been proposed that 72Met could provide protection against the accumulation of fat.[10] Thus, previous studies on preproghrelin genetic variants have arrived at contradictory findings as to their role in

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obesity. Additionally, most studies have had only child and adolescent subjects, whereas few studies have targeted the middle aged or elderly, or randomly sampled community-dwelling individuals.

The aim of the present study was to test whether genetic variants in the preproghrelin gene (Leu72Met) could play a role in predisposing carriers to overweight/obesity or be associated with anthropometric data, serum lipid levels and values related with glucose metabolisms in a middle-aged to elderly Japanese population.

Materials and methods

Subjects

The present study consisted of a cross-sectional analysis of 1110 women and 1128 men who participated in the first wave of examinations in the National Institute for Longevity Sciences – Longitudinal Study of Aging (NILS-LSA) from April 1998 to March 2000. The subjects of the NILS-LSA were male and female residents 40–79 years old. The population of Obu city and Higashiura town in the Aichi prefecture in central Japan was stratified by both age and gender, and randomly selected from resident registrations in cooperation with the local governments. The number of male and female participants was to be the same to test gender difference. Age at the base line is to be 40–79 years and the number of participants in each decade (1940s, 1950s, 1960s, 1970s) is to be the same. The examinations include various areas of gerontology and geriatrics such as medical examinations, anthropometry, body composition, physical functions, physical activities, psychological assessments, nutritional analysis and molecular epidemiology. The subjects will be followed up every 2 years. The details of the NILS-LSA have been described elsewhere.[11] Randomly selected men and women were invited by mail to attend an explanatory meeting. At that meeting, the procedures for each examination and the follow-up schedule were fully explained. Written, informed consent to the entire procedure was obtained from each participant. The study was approved by the Ethics Committee of the National Institute for Longevity Sciences.

Anthropometric variables

Body weight was measured to the nearest 0.01 kg using a digital scale, height was measured to the nearest 0.1 cm using a wall-mounted stadiometer and body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference and waist-to-hip ratio (WHR) were used as the indices for body fat distribution in this study. Waist-to-hip ratio was calculated as the ratio of waist circumference measured at the mid-point between the anterior superior iliac crest and the lowest rib-to-hip circumference. Whole-body fat mass, assessed by dual-energy X-ray absorptiometry (QDR-4500; Hologic, Madison, OH, USA), was used as an index for determining body composition. The subjects' weight at 18 years of age was obtained by questionnaire. Weight change was defined as the current weight minus the weight at 18 years of age.

Biochemical assays of blood

An antecubital blood sample was drawn from each subject after an overnight fast. Serum total cholesterol, triglycerides and low-density lipoprotein cholesterol were determined enzymatically, serum high-density lipoprotein cholesterol was measured by the heparin–manganese precipitation method and fasting plasma glucose was assayed by the glucose oxidase method. Lipoprotein (a) was measured in plasma using a commercially available ELISA. Plasma insulin was measured by radioimmunoassay. The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as fasting serum insulin (μU/ml) $\times$ fasting plasma glucose (mmol/l)/22.5.[12]

Determination of preproghrelin genotypes

Genotypes were determined using a fluorescence-based allele-specific DNA primer assay system (Toyobo Tsuruga Gene Analysis, Tsuruga, Japan). The polymorphic regions (Leu72Met (C214A)) of preproghrelin were amplified by polymerase chain reaction with allele-specific sense primers labeled at the 5′-end with either fluorescein isothiocyanate (5′-CCG ACC CGG ACT TCC XTT-3′) or Texas red (5′-GTA CCG ACC CGG ACT TCC XG-3′) and with an antisense primer labeled at the 5′-end with biotin (5′-GGC TCC GCC CGG AAG ATG-3′). The reaction mixtures (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l $MgCl_2$ and 1 U of rTaq DNA polymerase (Toyobo Co., Ltd, Osaka, Japan) in polymerase buffer. The amplification protocol consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 30 s; a final extension was conducted at 68°C for 2 min. Further details are provided elsewhere.[13]

Data analysis

Quantitative data were compared among three groups by one-way analysis of variance and the Tukey–Kramer *post hoc* test, and between two groups by the unpaired Student's *t*-test. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify any significant departure from Hardy–Weinberg equilibrium. In the analyses to examine the association between genotypes and lipid or glucose metabolisms, participants who were being treated with lipid-lowering medications or oral hypoglycemic agents or insulin were excluded. Unless indicated otherwise, a *P*-value of <0.05 was considered to be statistically significant. The data were analyzed with the Statistical Analysis System (SAS), release 8.2.

Results

Genotype frequencies for the preproghrelin Leu72Met polymorphism were CC (Leu72Leu) 0.634, CA (Leu72Met) 0.327 and AA (Met72Met) 0.04. These frequencies are consistent with those expected under Hardy–Weinberg equilibrium. There were no significant differences in the genotype distributions of preproghrelin Leu72Met polymorphism between men and women, or among the different age groups (Table 1).

As shown in Table 2, although there were no differences in current body weight and body weight at 18 years of age between genotypes, middle-aged men who were 72Met allele carriers showed both a higher body weight change from body weight at 18 years of age ($P=0.013$, CC vs CA/AA) and higher waist circumference ($P=0.038$, CC vs CA/AA) than noncarriers. Among the middle-aged men in the present study, the Leu72Leu genotype was associated with the lowest BMI (trend, $P=0.049$), and the 72Met allele carriers tended to have a higher WHR ($P=0.062$, CC vs CA/AA) than subjects with the Leu72Leu genotype. However, no differences in anthropometric measurements among Leu72Met genotypes were observed in older men, or in female cohorts (Table 3).

In order to assess the association of the Leu72Met polymorphism with overweight or obesity, genotype and allele frequencies were compared among normal-weight ($BMI<25\,kg/m^2$) and overweight/obese ($BMI\geqslant25\,kg/m^2$) groups (Table 4). Although there were no significant differences in the genotype distribution according to BMI in women and older men, a significantly higher frequency of CA, AA or CA/AA was found in the higher BMI group than in the normal-weight group among middle-aged men.

No significant association was observed between the three genotypes and serum lipid, fasting glucose, insulin, HbA1c or HOMA-IR levels in men and women (Table 5). The preproghrelin Leu72Met genotypes showed similar allele frequencies in diabetic individuals and in non-diabetic controls (data not shown).

Discussion

We observed that the frequency of the 72Met allele of the present cohort was 36.6%. It has been demonstrated that

Table 1 Distribution of Leu72Met genotype of preproghrelin gene of the subjects

	n	*CC*		*CA*		*AA*		*CA/AA*	
		n	%	n	%	n	%	n	%
Total	2228	1412	63.4	728	32.7	88	4.0	816	36.6
Men*†	1121	709	63.3	371	33.1	41	3.7	412	36.8
Women	1107	703	63.5	357	32.3	47	4.3	404	36.5
Age (years)‡§									
40–49	562	364	64.8	177	31.5	21	3.7	198	35.2
50–59	556	357	64.2	177	31.8	22	4.0	199	35.8
60–69	560	359	64.1	180	32.1	21	3.8	201	35.9
70–79	550	332	60.4	194	35.3	24	4.4	218	39.6

*CC, CA, AA, men vs women, $\chi^2=0.6159$, $P=0.7350$; †CC, CA/AA, men vs women, $\chi^2=0.0160$, $P=0.8995$; ‡CC, CA, AA, age groups, $\chi^2=2.9716$, $P=0.8124$; §CC, CA/AA, age groups, $\chi^2=2.9149$, $P=0.4049$.

Table 2 Anthropometric variable of men according to age group and Leu72Met polymorphism of preproghrelin gene

	Middle aged (n = *563*)								*Older* (n = *556*)							
	CC		*CA*		*AA*		*CA/AA*		*CC*		*CA*		*AA*		*CA/AA*	
	Mean	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*
Weight (kg)	64.6	0.5	65.6	0.7	67.5	2.0	65.8	0.6	59.7	0.5	59.1	0.6	57.2	1.9	58.9	0.6
Weight at 18 years (kg)	56.9	0.4	56.4	0.5	57.2	1.5	56.5	0.5	55.3	0.4	54.5	0.5	55.4	1.5	54.6	0.5
Weight change from 18 years (kg)	7.7	0.4	9.2	0.6	10.3	1.7	9.3	0.5*	4.6	0.5	4.9	0.7	1.8	2.1	4.6	0.7
Height (cm)	167.0	0.3	167.2	0.4	166.3	1.4	167.2	0.4	162.0	0.3	161.9	0.4	161.7	1.2	161.9	0.4
BMI (kg/m^2)	23.1	0.1	23.4	0.2	24.4	0.6	23.5	0.2†	22.7	0.2	22.5	0.2	21.9	0.6	22.4	0.2
Waist circumference (cm)	82.2	0.4	83.4	0.6	84.9	1.8	83.6	0.6‡	82.4	0.5	82.1	0.6	80.5	1.9	81.9	0.6
Hip circumference (cm)	92.2	0.3	92.7	0.4	93.4	1.1	92.8	0.3	90.0	0.3	89.6	0.4	88.7	1.1	89.5	0.3
Waist-hip-ratio	0.891	0.003	0.899	0.004	0.907	0.012	0.899	0.004§	0.913	0.003	0.914	0.005	0.904	0.014	0.913	0.004
Fat mass (kg)	20.6	0.2	21.2	0.3	20.8	1.0	21.2	0.3	21.9	0.2	22.0	0.3	22.1	0.9	22.0	0.3

Except for *, †, ‡ and §, no significant trends and differences were detected among three groups (CC, CA and AA) and between two groups (CC and CA/AA). *$P=0.013$ (CC vs CA/AA); †$P=0.049$ (trend); ‡$P=0.038$ (CC vs CA/AA); §$P=0.062$ (CC vs CA/AA). Analysis of variance and the Tukey–Kramer *post hoc* test or the unpaired Student's *t*-test between two groups. BMI = Body mass index.

Table 3 Anthropometric variable of women according to age group and Leu72Met polymorphism of preproghrelin gene

	Middle aged (n = *553*)								*Older* (n = *552*)							
	CC		*CA*		*AA*		*CA/AA*		*CC*		*CA*		*AA*		*CA/AA*	
	Mean	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*
Weight (kg)	53.9	0.4	53.9	0.6	54.4	1.6	54.0	0.6	50.8	0.4	50.9	0.6	52.4	1.7	51.1	0.6
Weight at 18 years (kg)	48.8	0.3	49.1	0.4	49.2	1.2	49.1	0.4	47.9	0.4	47.7	0.5	49.2	1.4	47.8	0.5
Weight change from 18 years (kg)	5.2	0.4	4.8	0.6	5.2	1.6	4.9	0.5	3.0	0.5	3.2	0.7	3.1	1.9	3.2	0.7
Height (cm)	154.0	0.3	154.1	0.4	154.4	1.0	154.1	0.3	148.5	0.3	148.6	0.4	147.9	1.2	148.5	0.4
BMI (kg/m^2)	22.7	0.2	22.7	0.2	22.8	0.7	22.7	0.2	23.0	0.2	23.0	0.2	24.1	0.7	23.2	0.2
Waist circumference (cm)	73.5	0.5	73.4	0.6	73.4	1.7	73.4	0.6	76.4	0.5	77.5	0.7	77.8	2.0	77.5	0.7
Hip circumference (cm)	91.5	0.3	91.5	0.4	90.9	1.1	91.4	0.4	89.8	0.3	89.9	0.4	90.8	1.2	90.0	0.4
Waist-hip-ratio	0.802	0.003	0.801	0.005	0.806	0.012	0.802	0.004	0.849	0.004	0.860	0.005	0.855	0.014	0.860	0.005
Fat mass (kg)	30.7	0.3	30.3	0.4	30.5	1.0	30.3	0.3	32.3	0.3	32.7	0.4	33.3	1.1	32.7	0.4

No significant trends and differences were detected among three groups (CC, CA and AA) and between two groups (CC and CA/AA). Analysis of variance and the Tukey–Kramer *post hoc* test or the unpaired Student's *t*-test between two groups. BMI = Body mass index.

Table 4 Distribution of Let72Met genotype of preproghrelin gene

	n	*CC*		*CA*		*AA*		*CA/AA*		P	P*
		n	%	n	%	n	%	n	%		
All age groups											
Men											
BMI < 25 kg/m^2	854	546	63.9	280	32.8	28	3.3	308	36.1	0.411	0.393
BMI ⩾ 25 kg/m^2	267	163	61.1	91	34.1	13	4.9	104	39.0		
Women											
BMI < 25 kg/m^2	866	558	64.4	273	31.5	35	4.0	308	35.6	0.454	0.224
BMI ⩾ 25 kg/m^2	241	145	60.2	84	34.9	12	5.0	96	39.8		
Middle ages (younger than 60 years)											
Men											
BMI < 25 kg/m^2	413	280	67.8	123	29.8	10	2.4	133	32.2	0.032	0.036
BMI ⩾ 25 kg/m^2	151	88	58.3	54	35.8	9	6.0	63	41.7		
Women											
BMI < 25 kg/m^2	446	288	64.6	139	31.2	19	4.3	158	35.4	0.694	0.395
BMI ⩾ 25 kg/m^2	108	65	60.2	38	35.2	5	4.6	43	39.8		
Older (60 years or older)											
Men											
BMI < 25 kg/m^2	441	266	60.3	157	35.6	18	4.1	175	39.7	0.692	0.394
BMI ⩾ 25 kg/m^2	116	75	64.7	37	31.9	4	3.5	41	35.3		
Women											
BMI < 25 kg/m^2	420	270	64.3	134	31.9	16	3.8	150	35.7	0.604	0.389
BMI ⩾ 25 kg/m^2	133	80	60.2	46	34.6	7	5.3	53	39.9		

P-value by the χ^2 analysis among groups CC, CA and AA. *P**-value by the χ^2 analysis between groups CC and CA/AA. BMI = Body mass index.

the frequency of the 72Met allele of the preproghrelin gene is approximately 8% in the Caucasian population and approximately 2% in the black population in three different cohorts.[10] Compared with these previous studies, the frequency of the 72Met allele in our Japanese cohort was much higher than that observed in Caucasian or African populations, probably reflecting genetic/ethnic heterogeneity.

The Leu72Met polymorphism of preproghrelin was previously found in a group of obese French children and adolescents.[9] In this case, a significant association was observed between the 72Met allele and earlier age of onset of obesity. Additionally, obese Italian children and adolescents with the preproghrelin 72Met allele have also been reported to become obese earlier than homozygous patients for the wild Leu72 allele, even though 72Met allelic frequency was similar between obese and control groups.[8] These findings were not confirmed, however, in a group of extremely obese German children.[14] In addition, one report

Table 5 Metabolic variables and Leu72Met polymorphism of preproghrelin gene

	Men								*Women*							
	n	*CC*		*CA*		*AA*		*CA/AA*		n	*CC*		*CA*		*AA*		*CA/AA*	
		Mean	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*		*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*
Total cholesterol (mM)[a]	1044	5.48	0.03	5.49	0.05	5.42	0.14	5.48	0.04	996	5.83	0.03	5.92	0.05	5.83	0.14	5.91	0.05
Triglyceride (mM)[a]	1027	1.48	0.04	1.53	0.06	1.32	0.16	1.51	0.10	977	1.20	0.03	1.23	0.04	1.21	0.10	1.23	0.04
HDL-C (mM)[a]	1044	1.49	0.01	1.48	0.02	1.49	0.06	1.48	0.02	996	1.71	0.02	1.71	0.02	1.71	0.06	1.71	0.02
LDL-C (mM)[a]	1035	3.40	0.03	3.42	0.05	3.36	0.13	3.42	0.04	980	3.57	0.03	3.63	0.05	3.62	0.14	3.63	0.05
Lipoprotein (a) (mM)[a]	1034	0.39	0.02	0.37	0.03	0.35	0.07	0.37	0.02	980	0.40	0.02	0.46	0.03	0.33	0.07	0.44	0.02
Glucose (mM)[b]	1049	5.71	0.04	5.74	0.05	5.91	0.15	5.75	0.05	1051	5.51	0.03	5.52	0.05	5.20	0.13	5.49	0.04
Insulin (μU/ml)[b]	1048	8.28	0.22	8.21	0.31	7.63	0.91	8.15	0.29	1050	8.23	0.19	8.57	0.27	8.02	0.74	8.51	0.25
HbA1c (%)[b]	1064	5.21	0.02	5.26	0.03	5.41	0.10	5.28	0.03	1071	5.16	0.02	5.15	0.03	5.06	0.07	5.14	0.02
HOMA-IR[b]	1048	2.20	0.08	2.13	0.11	2.06	0.33	2.13	0.11	1050	2.07	0.06	2.20	0.09	1.88	0.26	2.16	0.09

[a]Analysis of subjects who were not under lipid treatment. Adjusted for age. [b]Analysis of subjects who were not on oral hypoglycemic agents or insulin. Adjusted for age. No significant differences were observed in any metabolic values among three different genotypes (CC, CA and AA) or between CC and CA/AA. Analysis of variance and the Tukey–Kramer *post hoc* test or the unpaired Student's *t*-test between two groups. Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; LDL, low-density lipoprotein.

suggests that preproghrelin 72Met carrier status may be protective against fat accumulation.[10] A limited number of observations have been made on the relationship between preproghrelin Leu72Met polymorphism and overweight/obesity in middle-aged subjects, and no report has been published to date on older subjects. In a Swedish middle-aged female obese cohort, no difference of 72Met allele frequency was observed between obese subjects and controls.[7] However, the self-reported age of onset of weight problems tended to be lower among 72Met allele carrier obese subjects than among those without this allele.

In the present study, we observed a significant effect of the preproghrelin variant on overweight/obesity only in middle-aged men, as the 72Met allele was more commonly observed among overweight/obese middle-aged men. We also demonstrated that body weight change from weight at 18 years of age is associated with Leu72Met variants, given that middle-aged men with the 72Met allele had a greater body weight change than Leu72 homologous subjects. Similar trends were also observed for BMI, waist circumference and WHR in middle-aged men, but not in older men or in women when our population was subdivided into three subgroups according to preproghrelin genotype. Consequently, the 72Met allele may contribute to body weight change from adolescence to middle age in men but not in women. We observed the absence of the effect of Leu72Met genotypes on the anthropometric measurements in older man. Although we do not know the exact reasons, the effects of aging or environmental influences may overcome the genetic influence on the anthropometric measurements. The limitation of our study is that the weight at 18 years was recalled by the participants in the present study, as the documented measurements of weight at 18 years of age were not available. Although several studies have observed that adults are able to recall their earlier weights fairly accurately,[15] it is possible that the reported weight might not be accurate or under-reported. In fact, it has been reported that overweight women underestimated their earlier weights and that lean men overestimated their earlier weight.[16]

Based on recent studies, it appears that ghrelin may play a role in the glucose and lipid metabolisms. However, only limited data are currently available with regard to the effect of ghrelin polymorphism on these metabolisms. It has been reported that Leu72Met polymorphism is associated with triglyceride or lipoprotein (a) levels.[10,17] In the present study, however, we observed no association between serum lipid levels, fasting glucose, insulin, HbA1c or HOMA-IR levels and preproghrelin Leu72Met genotypes.

In the present study of a community-dwelling Japanese middle-aged to elderly cohort, we demonstrated that the 72Met allele of the preproghrelin gene is a contributing factor for midlife weight change in men but not in women or elderly men. However, Leu72Met polymorphism was not found to be associated with the metabolic variables studied.

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