of energy intake for all people (Fig.6). It may be necessary to include E-unit in the standardization and proper quality of agricultural and forestry products (JAS Law), because all consumables (food and beverages) for general consumers are subject to quality standards. This E-unit would be very useful for consumers when choosing between foods. This new energy unit and system was created in response to requests for unified and simplified of foods. This was viewed as necessary due to the diversification of food products resulting from increased imports and new foods on the one hand and rising consumer concerns about diet on the other.

CONCLUDING REMARK

The Food Safety Commission has developed a linkage between the Cabinet Office, Ministry of Health, Labour and Welfare, Ministry of Agriculture, Forestry and Fishery, and the International Organization for analysing information and scientifically assess risks. Shoku-iku (Food and dietary education throughout life) is effective to educate people. The newly established consumer agency should enable faster response to emergencies. A new food labeling system is necessary for producers, providers and consumers so that a healthier society can be formed where we would employ a new energy-unit (80 kcal) for individual energy and nutrient intake as tailor-made nutrition.

AUTHOR DISCLOSURES

Any of the authors does not have conflict with any company.

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Review

Food safety and food labeling from the viewpoint of the consumers

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由消費者的觀點來看食品安全與食品標示

在 2001 年發生狂牛症事件之後，日本民衆漸漸開始質疑食品的安全性。於是日本在內閣府下成立食品安全委員會並且在各部會之間組成一個連繫網路。新成立的消費者服務處加強對緊急事件的快速回應。內閣府與相關部會及非政府組織合作執行食品教育(食品及膳食教育)法。本文對日本食品衛生法及健康促進法做簡短的說明，並敘述功能性營養學的必要性，以研究非營養但具生物活性的物質。關於公共衛生營養，依個體需要而設計的營養已發展出一種新的食品標示，可以顯示熱量平衡及用抗氧化單位(AOU)作為水果及蔬菜的替代指標，這可使每個人更容易控制熱量攝取。

關鍵字：食品安全、特定保健食品(FOSHU)、功能性營養學、功能性食品因子(FFF)、食品標示

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Original Article

A Cross-Sectional Study on the Effects of Long Term Very Low Protein Diets in Patients with Chronic Kidney Disease: Serum and Urine DEXA and Amino Acid Profiles

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Abstract

Background: Chronic renal failure has increased among aged population in Japan. Protein-restricted diets have been successfully used to treat chronic renal failure. However, concerns over sarcopenia and other nutritional disorders have made doctors in Japan reluctant to recommend low-protein diets.

Study Design: A cross-sectional study was carried out based on dietary records, urine and blood samples and DEXA measurements to evaluate body composition.

Setting & Participants: The study was carried out at Keio University Hospital and National Institute of Health and Nutrition, Tokyo, over the 3-day period in June, 2009. Subjects were 10 CKD patients (1 male, 9 female); ten members of the patients' families (3 male, 7 female) and 11 dieticians (all female) were used as control subjects.

Factor: The CKD patients maintained a daily protein intake of less than 0.5 g/kg body weight (VLPD) for periods averaging 7 years. Members of the control group all had a daily protein intake of over 1g/kg body weight.

Outcomes: Indicators of nutritional disorders, metabolic abnormalities or changes in body composition were sought.

Measurements: Intake of various nutrients was calculated from dietary records. Blood plasma and urine content was analyzed. Body composition was measured using DEXA.

Results: The CKD subjects were found not to suffer from sarcopenia, osteoporosis, hyperkalemia, hyperphosphatemia, hyperuremia or high levels of uric acid, although slight anemia was observed. Vitamin and mineral intakes were lower than controls, but no recognizable symptoms from nutrient deficiency occurred. Urinary excretion of amino acids was different from controls.

Limitations: Results are limited by the relatively small number of test subjects, variation in time on the VLPD, and gender imbalance.

Conclusion: Results suggest that VLPD did not show any abnormality in body composition when energy requirement was fulfilled. Different amino acid metabolism would lead to cautious prescription of amino acid supplement.

KEY WORDS: CKD, low protein diet, nutrition, amino acid, DEXA

Introduction

Chronic kidney disease (CKD) has increased among aging population in Japan. It is important to save glomerular filtration rate (GFR) for Anti-Aging Medicine. Over the past 50 years, protein-restricted diets have been successfully used to treat chronic renal failure. Long-term therapy with protein- and phosphorus-restricted diets has been shown to reduce damage to the kidneys and prevent renal failure complications [1-3]. A meta-analysis of 7 randomized controlled trials with 1494 non-diabetic patients found that protein restriction decreased the incidence of composite outcomes during the trial with an odds ratio of 0.61 [4,5].

However, since the poor prognosis of very low protein diet (VLPD) was reported in the MDRD study [6-8], Japanese doctors have become reluctant to prescribe protein restriction, and regarded a protein intake of 0.8 g/kg body weight to constitute a low protein diet [9]. Protein restriction is believed to lead to nutritional disorders such as protein-calorie malnutrition, and concerns over sarcopenia and other nutritional deficiencies make doctors unwilling to treat chronic kidney disease (CKD) patients with low protein diets.

We have supported a group of patients with (CKD) aiming to avoid hemodialysis as far as possible [10]. Adhering to diets prescribed for renal failure is frequently a difficult and frustrating task for patients and their families, so we have tried over many years to develop appealing diets for the patients. The patients expressed a wish to learn more about the function and mechanism of kidney diseases; we thus arranged a course of kidney seminars on Saturday afternoons for 3 months from April 2009. The course was attended by a group of 10 CKD patients, 10 family members, and 11 dietitians; following the course, they wanted to carry out a cross sectional study of the 3 groups to ascertain the positive and negative effects of a prolonged VLPD.

The CKD patients in this study followed a VLPD on a prescription of less than 0.5 g/kg body weight for 7 years on average; they were therefore interested to ascertain their actual protein intake, metabolic state of protein and amino acids, and body composition such as bone density and lean body mass.

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Subjects and Methods

Subjects were 31 people who took part in the 2009 kidney seminar course planned by the "Adequate Protein Intake Promotion Group". Ten CKD patients (one man and nine women), 10 family members (3 men and 7 women), and 11 dietitians (all women) registered and completed the course. At the end of course a cross-sectional study of the participants was planned, and group members themselves designed the study under the guidance of authors which was approved by the Ethical Committee of the Life Science Promotion Foundation.

A profile of the CKD patients is given in *Table 1*. The average age of the 3 family men was 53.4±9.9 and that of the 7 family women was 51.3±10.3; the 11 dietitians had an average age of 36.7±15.2 years. The CKD group was prescribed a VLPD with a daily protein intake of less than 0.5 g/kg body weight, and given care by a single doctor (T.I.)every 1-3 months for periods averaging 7 years.

All participants were asked to provide dietary records and 24-hour urine collection by the urimeter^{R} over a 3 day period; on the 3rd day, 10 ml blood samples were collected and DEXA examinations performed.

Total intake of energy, water, protein, fat, carbohydrate, NaCl, Mg, Mn, Ca, P, K, ash, retinol, carotene, vitamin A, vitamin E, vitamin K, vitamin B1, folic acid, vitamin C, and amino acids was calculated from the dietary records using the FFF database [11].

Sera were used to measure total protein (TP), albumin, AST, ALT, γ-GTP, ProBNP, TG, total cholesterol, HDL cholesterol, LDL cholesterol, BUN, creatinine, BUN/Cr ratio, UA, CRP, Na, K, Cl, Ca and Mg. Whole blood was used to measure WBC, RBC, Hb, Ht, Pt, MCV, MCH, and MCHC. Blood and biochemical measurements were taken at the Serum Research Laboratory in Tokyo.

Body composition was measured in the National Institute of Health and Nutrition (Shinjuku-ku, Tokyo) using DEXA (model DPX-IQ, Lunar Radiation) with subjects in the supine position. Whole body scans provided bone, fat and lean mass composition in the head, arm, trunk and leg tissues; values were processed using a computer. DEXA measurements of muscle and fat mass in the arm and leg have been well validated against other standards [12-15].

Analysis of serum biochemistry and urine, in addition to analysis of amino acids in plasma and urine using HPLC chemical detection, was also performed by the Serum Research Laboratory, Tokyo. eGFR was calculated using an equation given by the Japanese Nephrology Group [16].

Table 1 Profile of very low protein diet group

Id	Sex	Age	Onset age	Dx of CKD	Basal Dis	LPD Period	Dialysis
8	F	55	35	40	IgA	15 yr	-
4	F	52	36	42	IgA	10 yr	3 mos
2	F	66	58	58	GN	8 yr	-
10	F	63	35	55	PK	8 yr	-
6	F	60	53	53	PK	6.5 yr	1.5 yr
5	F	50	27	45	SLE	5 yr	-
9	F	74	68	70	GN	4 yr	1 yr
7	F	45	44	44	MPO-ANCA	2 yr	-
11	F	37	-	-	PK	1 yr	-
3	M	74	49	64	GN	10 yr	-

IgA: IgA nephritis, GN; chronic glomerulonephritis,
PK; polycystic kidney, SLE; systemic lupus erythematosus,
MPO-ANCA; myeloperoxidase-antineutriphil cytoplasmic antibody related nephritis.

Statistical Analyses

All data were output in Excel format and transferred to SPSS 14.0 for statistical analysis [17]. Two-way ANOVA was performed; in the case of a significant f value, a post-hoc test using the Newman-Keuls method was performed to identify any significant differences in mean values. All data are given in the form mean ± SE; the median value is quoted where the doubled standard deviation was greater than mean. Statistical significance was set *a priori* at $p < 0.05$ for all comparisons.

Results

Profiles of the CKD patients are given in *Table 1*. Three of the patients maintained a very low protein diet (VLPD) for more than 10 years, two for 8 years, three from 4 to 6.5 years, and one each for one and two years, respectively. All of them started VLPD at CKD stage 3 (eGFR < 30 ml/min/1.73m^2). Basic diseases underlying the CKD were varied; most of the patients received anti-hypertensive drugs, anti-uremic acid, and/or anti-hyperlipidemia. Two patients took $CaCO_3$, but none used erythropoietin or other supplements.

One patient and two family members were male, and two of the eight female CKD patients had recently entered hemodialysis (hereafter "the hemodialysis group"). Six other female patients (hereafter "the LPD group"), 6 female family members ("the family group") and 11 female dietitians ("the dietitian group") completed the study and were subjected to detailed analysis. Although the number of hemodialysis patients was only two, they continued lower protein intake (0.8g/kg body weight) compared to the ordinary hemodialysis patients (1.1-1.2 g/kg), so these two were remained for the analysis.

One male patient and two male family members showed similar trends to the female groups, but were not included in the detailed analysis.

Dietary intake

Dietary intakes, calculated from the 3-day dietary records, are summarized in *Table 2* into 6 groups including the males. Daily intake was 2046±8 kcal for male and 1782±340 kcal for female patients. On average, female intake was 32.7 kcal/kg body weight. Daily protein intake by the LPD group was 0.39±0.08 g/kg body weight, compared to 0.55±0.1 g in the hemodialysis group. The three control groups (male and female family members, and dieticians) took more than 1g/kg of protein daily. Intakes of lipid soluble retinol, carotene, vitamin A, water soluble vitamin K, folic acid, and vitamin C by the VLPD group were less than half that of family and dietitians, but no patients showed clinical manifestation of associated vitamin deficiencies. Intakes of P, K, Ca, and Mg by VLPD were also less than one third that of family and dietitians. NaCl, K and P intakes were kept in the low range within allowable limits.

Protein intake calculated from 24 hr urinary urea excretion using the equation of Kopple and Mitch [2,18], was found to be significantly correlated with actual intake ($r=0.6$, $p<0.01$), but dietary amino acid showed no correlation with either plasma or urinary amino acids (data not shown).

Table 2 Dietary nutrient intake/body weight by 3 groups (average of 3 day dietary record)

		VLPD (m)		Family (m)		VLPD (f)		Dialysis (f)		Family (f)		Dietitian	
		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
Body weight	(kg)	54.0	0.0	66.5	7.2	54.3	4.0	52.1	6.1	48.8	3.8	50.1	3.4
Energy	(kcal/kg/day)	37.9	0.1	32.6	9.8	31.7	5.0	33.5	2.8	33.1	4.5	32.8	6.9
Protein	**(g/kg/day)**	**0.41**	**0.01**	**1.09**	**0.25**	**0.39**	**0.08**	**0.55**	**0.10**	**1.17**	**0.26**	**1.25**	**0.28**
NaCl	(g/day)	2.0	0.3	3.2	1.2	**2.1**	0.5	2.1	0.3	2.9	0.8	4.3	1.5
Vit. B1	(ug/day)	0.20	0.05	0.44	0.11	**0.21**	0.09	0.19	0.05	0.45	0.14	0.55	0.21
Folic acid	(ug/day)	64	7	173	83	**77**	20	94	12	213	73	231	120
Vit. C	(mg/day)	29	4	53	22	**26**	8	36	10	78	35	65	28
Vit. A	(mg/day)	228	70	414	207	**283**	151	272	115	633	280	885	1156
Carotene	(ug/day)	781	9	1896	1157	**1072**	551	1451	293	2923	1530	2558	1477
Vit. K	(mg/day)	60	3	142	81	**91**	67	91	37	160	87	141	115
Ca	(mg/day)	79	6	226	114	**96**	50	105	38	302	121	370	199
Mg	(mg/day)	34	2	129	35	**48**	16	52	12	141	44	167	73
K	(mg/day)	454	29	1342	497	**616**	113	650	100	1521	383	1428	567
P	(mg/day)	168	12	527	134	**227**	39	265	14	570	126	595	209
Mn	(mg/day)	0.3	0.1	1.5	0.5	**0.4**	0.2	0.3	0.1	1.9	0.7	1.8	0.6

Laboratory date

Laboratory data of the CKD group showed moderate anemia, low hematocrit, and higher MCV and MCH compared to the family and dietitian groups (*Table 3*).

The patients' group showed significantly higher values for γ-GTP, creatinine (Cr), BUN/Cr ratio, uric acid (UA) and triacylglycerol (TG) and lower HDL cholesterol.

ProBNT was remarkably high in one case of IgA glomerulonephritis, and was over 200 mg/dl in 7 out of 9 CKD patients, but less than 100 mg/dl in the control groups (36.9±20.8). K was slightly higher in CKD group, and plasma Ca level did not vary significantly from controls.

Dual-Energy X-ray Absorptiometry (DEXA)

Body composition by DEXA showed no significant differences between the LPD and healthy groups. Lean body mass and bone density values were similar across 4 groups (*Table 4*).

eGFR and urnary findings

eGFR of the LPD group was 27.6±22.0 ml/min (median=20.0), while those of the family and dietitian groups were 83.3±14.8 and 91.5±12.6 ml/min/1.73m^2, respectively (*Table 5*) [15]. Median values of the latter two groups were over 90 ml/min/1.73m^2.

Daily cumulative urinary albumin and β$_2$-microglobulin values were significantly high in the CKD group. pH was high in the hemodialysis group, but the median value was similar to that of the dietitian group.

Amino acid profile

Amino acid profiles in both serum and 24-hour urine on the 3rd day are given in *Table 6*. The high plasma urea in CKD patients resulted in significantly high citrullin in the urea cycle. Arg did not vary among the 4 groups, but ornithin was high in hemodialysis group. Urinary excretion of these amino acids in the urea cycle was less in CKD patients, and very low in the dialysis group. Tyr and Thr showed similar trends in dialysis patients.

In general, hydrophobic amino acids were excreted less in CKD patients. Although the amino acid with the highest excretion was Gly, it was less in urine of CKD patients with accumulation in the blood. Lower urinary amino acid excretion was related to the low plasma levels of Ala, Tyr, Leu, Ile, Glu etc. in the LPD group. Plasma levels of some amino acids such as Thr, Phe, Ser, Asp, Pro, etc., were not excreted into the urine and remained on a par with healthy controls. Urinary Lys was lower in CKD patients, while plasma Lys level remained the same.

In the methionin cycle, only Cys and taurin appeared in urine, with some accumulation in plasma. Homocystine and cystathionin were not present in either plasma or urine in VLPD group.

There was no significant change in Glu or Gln, but there was an absence of alpha-adipinate in the urine of CKD patients. His was only present in the plasma of all groups, but its metabolites 3-methyl histidine and carnosin (beta-alanyl-L-histidine) appeared in urine, being less in CKD patients with higher plasma concentration.

Metabolites such as beta-amino-isobutyrate, beta-alanin and hydroxyprolin were also higher in the plasma of the CKD group. In the hemodialysis group, excretion of branched chain amino acids such as Val, Leu and Ile were markedly higher in urine but lower in plasma.

Table 3 Blood and serum biochemistry among 4 female groups

		VLPD		Hemodialysis		Family		Dietitian		
		mean	sd	mean	sd	mean	sd	mean	sd	*p*
WBC	(/ul)	5283	1187	5100	283	5825	1256	4708	1469	
RBC	(x10⁴/ul)	**374**	37	**391**	43	**425**	37	**441**	32	*0.006*
Hb	(g/dl)	11.6	1.4	12.3	1.3	12.8	1.4	12.6	1.5	
Ht	(%)	36.2	3.3	38.6	3.2	40.1	4.2	39.6	4.4	
Pt	(x10⁴/ul)	21.1	4.1	15.9	5.2	19.8	6.0	23.4	4.9	
MCV	(fl)	96.8	0.6	98.9	2.8	94.2	3.5	89.8	8.1	
MCH	(pg)	30.8	1.1	31.6	0.2	30.0	1.3	28.5	3.0	
MCHC	(%)	31.9	1.2	31.9	0.7	31.9	0.9	31.7	0.7	
TP	(g/dl)	7.1	0.3	7.5	0.3	7.3	0.2	7.4	0.3	
Alb	(g/dl)	4.3	0.2	4.4	0.0	4.3	0.3	4.3	0.3	
AST	(IU/l)	20.0	6.4	20.0	7.1	23.8	3.8	20.2	8.3	
ALT	(IU/l)	13.5	4.0	13.5	7.8	17.8	5.1	15.4	6.6	
γ-GTP	(IU/l)	30	14	36	38	23	13	16	10	
ProBNT	(pg/ml)	**257**	128	**10780**	13746	**47**	37	**37**	23	*0.010*
TG	(mg/dl)	**167**	142	72	17	71	32	42	18	*0.012*
Chol	(mg/dl)	184	36	154	12	203	37	189	38	
HDL	(mg/dl)	**52**	13	54	4	71	10	72	12	*0.007*
LDL	(mg/dl)	102.2	28.4	76.5	14.8	109.5	26.0	101.3	33.6	
BUN	(mg/dl)	**17.7**	8.7	**24.7**	1.1	**12.3**	1.7	**13.7**	3.6	*0.010*
Creatinin	(mg/dl)	**3.0**	3.0	**8.7**	0.7	**0.6**	0.2	**0.6**	0.1	*0.000*
BUN/Cr ratio		**8.6**	4.5	**2.8**	0.1	**21.1**	3.4	**22.6**	5.6	*0.000*
UA	(mg/dl)	**5.3**	1.3	**4.9**	0.6	**4.9**	0.9	**3.8**	0.7	*0.012*
CRP	(mg/dl)	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.0	
Na	(mEq/l)	141.3	2.5	137.5	4.9	126.0	45.3	141.3	1.1	
K	(mEq/l)	**4.6**	0.4	5.3	0.3	4.2	0.6	4.2	0.3	*0.012*
Cl	(mEq/l)	105.7	3.4	99.0	5.7	104.3	2.5	103.6	1.9	*0.041*
Ca	(mg/dl)	8.8	0.5	9.7	0.2	9.0	0.3	8.9	0.3	*0.026*
Mg	(mg/dl)	2.2	0.2	2.6	0.1	2.2	0.1	2.4	0.2	

p: Kruskal Wallis test

Table 4 DEXA data of 3 groups

		VLPD n=8		Family n=6		Dietitian n=11	
		mean	sd	mean	sd	mean	sd
Age	(yrs)	47.7	19.5	52.7	10.5	36.7	15.2
Height	(cm)	159.8	4.1	154.9	3.7	158.8	5.3
Body weight	(kg)	54.2	5.3	50.3	6.3	51.3	4.3
Whole body total_BMD		0.986	0.125	1.013	0.126	1.050	0.100
Thoracic spine BMD		0.752	0.139	0.752	0.118	0.842	0.122
Lumber spine_BMD		0.913	0.155	0.960	0.134	1.008	0.170
Pelvis_BMD		1.019	0.149	1.076	0.127	1.071	0.138
Whole body total_FAT		15205	3126	15618	3221	13669	3224
Whole body total_LEAN		39702	4310	35762	4285	38517	2718
l-arm_LEAN		1699	215	1484	264	1697	167
r-arm_LEAN		1778	236	1605	237	1790	163
l-leg_LEAN		6277	749	5848	818	6171	563
r-leg_LEAN		6270	793	5715	732	6158	703

LPD; low protein diet, BMD; bone mineral density, LEAN; lean body mass

There is no difference between LPD-patient and dialyzer, so two groups were combined.

p: Kruskal Wallis test

Table 5 Urinary profile of 4 female groups

		VLPD (f)		Dialysis		Family (f)		Dietitian		
		mean	sd	mean	sd	mean	sd	mean	sd	*p*
eGFR3f	(ml/min/1.73m²)	**27.6**	22.0	**4.3**	0.2	**83.3**	14.8	**91.5**	12.6	*0.000*
Urinary volume	(ml/day)	2013.3	763.4	766.7	230.9	1462.5	649.8	1180.8	513.2	*0.022*
β2microglobulin	(g/day)	**9.9**	7.3	**20.8**	9.2	**0.1**	0.0	**0.1**	0.0	*0.000*
Albumin	(mg/day)	**677.4**	627.5	**712.0**	866.9	**19.1**	6.5	**10.7**	5.7	*0.001*
Urea N	(mg/day)	**3447.7**	3087.5	**630.0**	508.6	**5584.5**	1677.8	**6975.6**	1754.4	*0.001*
Creatinin	(g/day)	1.11	0.48	0.35	0.35	0.77	0.16	0.87	0.26	*0.066*
pH		5.96	0.42	6.97	0.68	5.96	0.35	6.24	0.48	*0.019*
Specific gravity		1.01	0.00	1.01	0.00	1.01	0.01	1.02	0.01	*0.029*

p: Kruskal Wallis test

Table 6 Plasma and urinary concentration of amino acids by group

	VLPD		Dialysis		Family		Dietitian			
	blood	urine	blood	urine	blood	urine	blood	urine	*p plasma*	*p urine*
Urea	**4893**	**75.4**	**8315**	**1.3**	4038	179.7	4497	223.8		*0.000*
Ornithin	**138**	**11.8**	**177**	**0.0**	**164**	**26.0**	**122**	**32.3**	*0.025*	
Citrulin	**52**	**4.8**	**107**	**0.0**	**31**	**11.0**	**28**	**11.6**	*0.009*	
Arg	**88**	27.4	83	15.3	73	54.4	87	86.2		
p_ethanolamin	3.3	54.7	1.7	**18.4**	3.2	68.8	4.4	69.9	*0.027*	
Asp	54	**4.9**	58	26.3	53	29.3	47	25.9		
Gly	**417**	1040.2	400	1911.3	346	1480.8	366	1947.6	*0.009*	
Ala	424	108.4	388	264.2	490	134.2	415	170.8		
Phe	92	0.0	98	**77.5**	95	0.0	87	19.5		
Tyr	**56**	15.5	35	33.1	68	16.7	67	36.6	*0.041*	*0.096*
Thr	**120**	**0.0**	**122**	**28.1**	172	6.7	165	10.3	*0.015*	
Lys	201	**4.9**	178	**8.4**	225	21.3	199	19.5		
oh_Lys	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
Val	**202**	8.5	**169**	**177.7**	246	3.7	215	10.1	*0.033*	
Ile	61	3.6	**52**	**63.1**	70	8.2	63	10.9		*0.096*
Leu	125	8.0	**110**	**158.8**	143	10.1	135	13.4		
Try	34	0.0	18	0.0	51	0.0	46	0.0	*0.000*	
Met	26	0.0	23	0.0	26	0.0	32	0.0	0.027	
Homocystin	0	0.0	0	0.0	0	0.0	0	304.4		
Ser	190	0.0	193	0.0	220	0.0	208	0.0		
Cystathionin	**0**	0.0	0	0.0	0	6.6	0	7.7	*0.024*	
Cys	**44**	5.8	51	0.0	35	7.3	33	13.5	*0.009*	
Taurin	206	809.0	157	**115.4**	184	1266.0	215	817.6		
Asp	73	0.0	70	0.0	88	0.0	80	21.6		
Glu	200	27.8	227	90.6	241	51.7	205	41.6		
Gln	559	11.7	535	**0.0**	528	14.6	510	19.2		
Sarcosin	0	0.0	0	0.0	0	0.0	0	0.0		
α_adipin	0	**0.0**	0	**0.0**	0	42.7	0	32.7		*0.000*
Pro	153	0.0	154	0.0	150	0.0	136	0.0		
oh_Pro	**10**	0.0	**36**	0.0	0	0.0	0	0.0	*0.027*	
α_lact	**7**	0.0	3	0.0	20	0.0	15	0.0	*0.010*	
OH_butyrate	0	0.0	0	0.0	0	0.0	0	0.0		
β_aminoisobutyrate	**6**	**378.2**	13	131.9	0	104.4	0	190.0	*0.016*	
m_ethanolamin	12	0.0	11	0.0	13	0.0	12	0.0		
His	88	0.0	84	0.0	101	0.0	97	0.0		
3_methyl His	**11**	**20.3**	**25**	**42.3**	0	361.5	0	534.0	*0.005*	
1_methyl_His	10	187.7	**31**	**80.0**	7	217.8	7	185.3		
Carnosin	0	**124.8**	0	**191.9**	0	353.0	0	517.9		
β_alanin	0	23.2	8	**0.0**	0	12.2	0	18.3		
Anserin	0	0.0	0	0.0	0	0.0	0	0.0		

Plasma concentration is mmole/dl, and urinary concentration is mmole/day.
Values are median.
p: Kruskal Wallis test

Discussion

Low protein diet is considered to be most effective to save the function of GFR, which tends to decrease according to aging. However, degree of protein restriction is still in debate.

Menon *et al.* [19] recently reported an increased risk of death from very low protein diets (VLPD) based on a 7-year follow-up after the MDRD study. Their study B group took 0.28 g/kg body weight protein and amino-keto acid supplements (about 0.28 g/kg body weight). They listed several reasons for the increased risk of death. We evaluated their study and considered that the notably low energy intake (30 kcal/kg body weight was ordered, but 25 kcal/kg at midterm and 22 kcal/kg at the end) may be the cause of malnutrition and resulting death. Actual protein intake of control and VLPD groups during the MDRD trial was 0.73 g/kg and 0.66 g/kg, respectively; hence, protein deficiency should not occur if the energy intake is sufficient.

In the present study, the CKD group maintained a VLPD (less than 0.4 g/kg body weight) for 7 years on average, and eGFR appeared to remain at the low level over many years. Three patients began hemodialysis, but have since maintained a low protein diet, compensating for the protein estimated to be lost in dialysis. They opted for only two weekly sessions of hemodialysis instead of the normal three sessions. One patient with a polycystic kidney started restriction of dietary protein early in their course of PKD, which seemed to slow disease progression [12].

Good awareness by patients and families about the importance of protein restriction and sufficient calorie intake ensured a high level of compliance to the low protein diet. Energy requirements were calculated in the form [body weight x 0.4] energy units, where one energy unit is defined as the energy needed to melt 1 kg of ice (80 kcal) [20,21]. Instruction in cooking methods using low-protein foodstuffs, particularly low-protein rice (containing 1/25th of the protein in ordinary rice) and bread and noodles made from the low protein rice powder, was found to be helpful [22]. Japanese people consume on average 60 g of protein per day according to the National Dietary Survey [23], about 30 g of which is derived from rice, so substitution of low protein rice can easily be used to reduce the protein intake. The amino acid score of rice is 85, higher than bread made from wheat powder which has a score of 45-50. Rice and soy-based foods form the bulk of the Japanese diet, making it simple to achieve a lower protein intake than in Western countries. Suitable amounts of protein-containing foods, such as meat, fish and eggs, can be used to make the diet more acceptable.

Long term VLPD at 0.4 g/kg body weight was found not to cause sarcopenia or osteoporosis in our CKD patients. They led normal daily lives, and did not have a lean body mass different from control groups. Only slight anemia occurred. CKD patients usually suffer from hyperkalemia, hyperphosphatemia, hyperuremia, high uric acid etc., but values remained within normal levels for the VLPD. Vitamin and mineral intakes were lower than controls, but no recognizable symptoms from nutrient deficiency occurred. The values were far less than the corresponding dietary reference intakes (DRIs), indicating the importance of tailor-made nutrition based on the individual non-deficient level. Common deficiencies in CKD patients are said to include 1,25 dihydroxycholecalciferol, vitamin B_6, folic acid, vitamin C, iron and possibly carnitine and zinc [18]. These values are often compared with the corresponding DRIs, although the adequate or recommended doses are typically set high to avoid deficiencies in these nutrients. Minimum requirements have not been proposed yet. Tailor-made nutrition based upon individual requirements is thought to be necessary for CKD patients, whether or not they require supplements.

Another factor proposed by Menon *et al.* [19] in their long term follow-up study of MDRD was toxicity of amino acid-ketoacid supplements. Amino acid supplement was often used for VLPD [24-26].

Comparison of free amino acid profiles of plasma and urine showed interesting findings. In general, excretion of most amino acids in urine was lower, with some completely absent. It is not determined whether this was due to VLPD, or to changes in secretion from the glomeruli and/or increased reabsorption from the renal tubules.

High citrulin concentration in both plasma and urine may reflect increased production of ammonia inside the body and lowered ability to excrete urea. Citrulin appears to be a rate-limiting amino acid in the urea cycle; hence, ornithin supplementation as in MDRD study should increase overloading of the cycle. Increased urine excretion of BCAA and a corresponding decreased plasma level is also noteworthy, suggesting increased activity of alpha-keto valerinate dehydrogenase, deficiency of which is known to cause maple syrup urine disease [27].

Decreased histidine in both plasma and urine, increased methyl derivatives and absence of detectable carnosin (β-alanyl-L-histidine) and anserine (β-alanyl N(p) methyl-L-histidine) demonstrates changes in histidine metabolism by CKD. Carnosine is a dipeptide of the amino acids β-alanine and histidine, with a number of antioxidant properties that may be beneficial [28]. β-alanine is formed *in vivo* from the degradation of carnosine; its supplementation has been shown to increase carnosine concentration in muscles, decrease fatigue in athletes and increase total muscular work output. It is a component of the naturally occurring peptides carnosine and anserine, and also of pantothenic acid (vitamin B_5) which itself is a component of coenzyme A. It was only present in plasma of hemodialytic patients.

Ethanolamine, low in hemodialytic patients, is the second-most-abundant head group in membrane phospholipids. Monoethanolamine is produced by reacting ethylene oxide with aqueous ammonia. Aminoisobutyric acid is a strong helix inducer in peptides; 2-aminoisobutyric acid may be synthesized from acetone cyanohydrin, by reaction with ammonia followed by hydrolysis [29]. An increase in aminoisobutyric acid was observed in both urine and plasma of CKD patients.

Further study is necessary to clarify the metabolic changes of amino acids in CKD at critical levels of protein restriction. Many changes are thought to occur inside the body, so administration of amino acid supplements should be more carefully considered.

In addition to the increased creatinine, proBNT was very high among the patients. It is believed to be a risk factor for cardiac disease [30], but it is necessary to clarify whether proBNT is a biomarker for kidney damage, like β_2 microglobulin.

Conflicts of interest

We affirm there to be no conflicts of interest with any company in relation to this study.

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ORIGINAL ARTICLE

Preproghrelin gene polymorphisms in obese Japanese: Association with diabetes mellitus in men and with metabolic syndrome parameters in women

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Summary Preproghrelin gene polymorphisms (SNPs) are possible predisposing factors to obesity and metabolic syndrome. We analysed SNPs in obese Japanese individuals and studied the correlation with diabetes and metabolic syndrome. We recruited 235 subjects (BMI > 28.3) from individuals undergoing periodic medical check-up at Saku Central Hospital. Their SNPs were genotyped using PCR-RFLP method. Frequencies of 5 SNPs in the preproghrelin gene −1500C>G (rs3755777), 1062G>C (rs26311), 994C>T (rs26312), Leu72Met (+408C>A) (rs696217), and +3056T>C (rs2075356) were compared with healthy individuals (data from HapMap Project or Asian population studies). Associations between these SNPs and clinical parameters were investigated. The phenotypes evidently differed between men and women. In men, higher fasting glucose and HbA1c values were observed in the +3056C/C minor homozygotes without leptin or insulin accumulation. The +408C-+3056C haplotype was more frequent in the diabetic subgroup, in which diagnosis was based on fasting glucose, 75gOGTT, and HbA1c values, than normal subgroup. In contrast, in women, a significant correlation was observed between fat metabolism and obesity. The −1062C/C minor homozygotes had higher values of C-peptide, insulin, total and visceral fat area, waist circumference and BMI. The 72Met/Met minor homozygotes showed reduced leptin, total, HDL and LDL cholesterol concentrations and increased value of visceral fat area. Further, in the other SNPs, the minor homozygotes showed a similar trend, and the heterozygotes had intermediate values. Preproghrelin gene polymorphisms in obese Japanese may be predisposing

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factors to diabetes mellitus in men and to obesity via aberrant fat metabolism in women.

Introduction

In order to study how to overcome obesity, diabetes mellitus and metabolic syndrome, we recruited 235 subjects with a body mass index (BMI) >28.3 (upper quintile) from examinees who appeared for periodic medical check-up in the Human Doc at Saku Central Hospital (Nagano, Japan). They were asked to participate in the weight loss intervention program named the Saku Control Obesity Program (SCOP). The details of the SCOP were described in elsewhere [1,2]. At the baseline study, we investigated the association among the SNPs, BMI, and other clinical parameters measured at the start point. We had previously reported their 10 SNPs in metabolic syndrome-related genes. Although many population studies have reported on the associations of BMI and other clinical parameters with the SNPs in UCP1/2/3 or β2AR/β3AR genes, few of these associations were confirmed in the obese subjects in the SCOP [3]. In the present study, we reported the effect of preproghrelin gene polymorphisms on the diabetes and obesity.

The human preproghrelin (GHRL) gene is located at locus 3p25–26 and consists of 4 exons and 3 introns. The gene is expressed as a prohormone, preproghrelin, which generates ghrelin and obestatin after posttranslational processing (for review, see NCBI OMIM 605353; www.ncbi.nlm.nih.gov/sites/entrez). O-n-octanoylation at serine-3 is essential for ghrelin activity. Mature ghrelin is secreted from the stomach and stimulates the pituitary to release the growth hormone and also up-regulates eating appetite via the hypothalamus. (Obestatin has the opposite effect; treatment of rats with obestatin suppressed food intake and decreased body-weight gain [4].) Ghrelin is suggested to play an important role in regulating energy balance, insulin signalling, and control of serum glucose concentration. Indeed, administration of ghrelin caused weight gain by reducing fat utilization in mice and rats [5]. Accordingly, dysfunction or insufficient generation of ghrelin may cause growth incompetence. Conversely, overexpression may result in obesity or metabolic syndrome.

Relationships between preproghrelin gene SNPs and susceptibility to metabolic syndrome have been intensively investigated. Leu72Met is the most frequently studied SNP of the gene, which is located in the inter-region of mature ghrelin and obestatin (neighboring the putative convertase cleavage site of obestatin) [4], thereby causing incompetence of the processing due to the polymorphism, possibly affecting the active ghrelin and/or obestatin concentration. However, conflicting results are reported. Ukkola et al. [6] first found this SNP in obese subjects, and reported that the age at onset of self-reported weight problems tended to be lower among 72Met carriers. Korbonits et al. [7] noted that children carrying the 72Met allele had a significantly higher BMI as compared to those carrying only the wild type allele. Hinney et al. [8] also identified this variant but at a similar frequency in both extremely obese children and adolescents and normal weight students. Comprehensive studies examining and comparing the maximum number of SNPs and parameters possible are further required for explaining these disagreements.

Here, we conducted the genotyping of 5 SNPs in the preproghrelin gene −1500C>G, −1062G>C, −994C>T, Leu72Met (+408C>A), and +3056T>C in SCOP. Their frequencies were compared with those of healthy people that were published in the HapMap Project or those analysed in East Asians by other research groups. Additionally, the associations between these SNPs and the clinical parameters related to diabetes mellitus or metabolic syndrome were studied.

HapMap is a public database of common gene variations (human genome) maintained by The International HapMap Consortium [9]. The map includes information on more than 1 million SNPs obtained in 269 DNA samples from 4 populations: Yoruba in Ibadan, Nigeria; Utah, USA; Beijing, China; and Tokyo, Japan.

Subjects and methods

Subjects

Japanese obese subjects aged 40–64 years with a BMI greater than 28.3 were selected from examinees undergoing a medical check-up at the Saku Central Hospital. They were asked to partici-

pate in the intervention program for weight loss named the Saku Control Obesity Program (SCOP); they were divided into two groups and received different instructions on food intake and daily exercise to evaluate the outcome with single nucleotide polymorphisms (SNPs) [1,2]. The participants underwent an anthropometric and clinical examination (height, weight, body fat percentage, waist circumference, visceral fat area, and biochemical markers of blood and urine) (Table 1) and were assessed for present illness, physical activity and dietary habits at the start of this program.

The ethics committees of The National Institute of Health and Nutrition and the Saku Central Hospital approved this investigation. All the participants gave their written informed consent before the start of this program.

Medical examination and measurements

The height (cm) and weight (kg) of the subjects were measured using an automatic scale (Tanita, BF-220, Tokyo, Japan). The percentage body fat was evaluated by the bioelectric impedance method using the same scale. Visceral and subcutaneous fat areas were assessed by a computed tomography scan at the level of the umbilicus, with the subjects in the supine position, and calculated using commercially available software (Fat Scan; N2 System Corp., Osaka, Japan).

Adipocytokines (i.e. leptin, TNF-α, adiponectin, and free fatty acid), C-peptide, and insulin concentrations were examined using the laboratory testing services provided by SRL Inc. (Tokyo, Japan). Leptin (ng/mL) was measured by a radioimmunoassay (Human Leptin RIA Kit, LINCO Research, St. Charles, MO, USA) with a sensitivity of 0.5 ng/mL. TNF-α (pg/mL) was measured by an enzyme-linked immunoassay (ELISA; Quantikine TNF-α HS Immunoassay Kit, R&D Systems Inc., Minneapolis, MN, USA) with a sensitivity of 0.12 pg/mL. The high-molecular-weight form adiponectin (μg/mL) was determined using ELISA (Fujirebio Inc., Tokyo, Japan) with a detection limit of 0.18 μg/mL. Free fatty acid (mequiv./L) was determined using an enzymatic assay (NEFA-SS 'Eiken', Eiken Chemical Co. Ltd., Tokyo, Japan) with a sensitivity of 0.005 mequiv./L. C-peptide (ng/mL) was measured by a chemiluminescent enzyme immunoassay (Lumipulse Presto C-peptide, Fujirebio Inc.) with a minimal detection limit of 0.1 ng/mL. Insulin (μIU/mL) was measured by a chemiluminescent

Table 1 Basic characteristics of the subjects in SCOP.

	All (n = 223)	Male (n = 115)	Female (n = 118)	p-Value*
Age (years)	53.9 ± 6.5	53.3 ± 6.6	54.5 ± 6.4	0.171
Height (cm)	161.8 ± 8.7	168.4 ± 5.8	155.3 ± 5.5	<0.001
Weight (kg)	80.7 ± 12.1	86.5 ± 11.8	75.1 ± 9.6	<0.001
BMI (kg/m^2)	30.78 ± 3.36	30.44 ± 3.55	31.10 ± 3.13	0.134
Body fat percentage (%)	34.97 ± 7.66	29.04 ± 4.44	40.69 ± 5.43	<0.001
Waist circumference (cm)	102.6 ± 8.6	101.5 ± 8.7	103.7 ± 8.4	0.052
Total fat area (cm^2)	441.9 ± 124.4	414.4 ± 133.2	468.7 ± 109.2	<0.001
Subcutaneous fat area (cm^2)	297.3 ± 104.4	255.2 ± 102.9	338.3 ± 88.7	<0.001
Visceral fat area (cm^2)	144.6 ± 52.5	159.1 ± 54.3	130.4 ± 46.7	<0.001
Visceral fat ratio (%)	33.3 ± 10.2	38.8 ± 9.0	27.9 ± 8.3	<0.001
Total cholesterol (mg/dL)	210.3 ± 34.8	204.2 ± 28.0	216.3 ± 39.6	0.007
HDL cholesterol (mg/dL)	52.87 ± 11.10	49.80 ± 9.68	55.86 ± 11.61	<0.001
LDL cholesterol (mg/dL)	125.7 ± 31.8	120.4 ± 28.6	130.8 ± 34.0	0.012
Triacylglycerol (mg/dL)	161.5 ± 101.9	174.9 ± 120.3	148.4 ± 78.3	0.047
HbA1c (%)	5.85 ± 1.06	5.81 ± 0.97	5.89 ± 1.14	0.556
Fasting glucose (mg/dL)	112.0 ± 25.7	111.7 ± 24.9	112.3 ± 26.6	0.875
HOMA-IR	3.3 ± 2.5	3.4 ± 3.1	3.1 ± 1.9	0.380
Free fatty acids (mequiv./L)	0.54 ± 0.20	0.51 ± 0.18	0.57 ± 0.21	0.017
Leptin (ng/mL)	14.83 ± 11.02	8.21 ± 5.63	21.34 ± 11.16	<0.001
Tumor necrosis factor-α (pg/mL)	1.25 ± 0.47	1.29 ± 0.50	1.22 ± 0.43	0.263
Adiponectin (μg/mL)	4.14 ± 2.84	2.79 ± 1.77	5.45 ± 3.06	<0.001
C-peptide (ng/mL)	2.70 ± 1.11	2.86 ± 1.25	2.55 ± 0.92	0.034
Insulin (μIU/mL)	11.62 ± 8.20	12.11 ± 10.01	11.14 ± 5.92	0.373
Creatinin (mg/dL)	0.77 ± 0.17	0.87 ± 0.13	0.67 ± 0.13	<0.001

Values are mean ± SD.

* p-Values are given by Student's t-test between male and female.

enzyme immunoassay (Lumipulse Presto Insulin, Fujirebio Inc.) with a minimal detection limit of 0.3 μIU/mL. Other biochemical markers were examined in the clinical laboratory of the Saku Central Hospital by Hitachi Automated Analyser (Hitachi).

Genotyping and statistical analysis

DNA was purified from the subjects' blood using QIAamp DNA blood 96 Kit (Qiagen). All the SNPs were genotyped by PCR followed by digestion with a restriction enzyme [PCR-RFLP (restriction fragment length polymorphism) method]. Fragments (80–200 bp) containing the objective SNP were amplified in a 20 μL reaction mixture, including 0.2 mM 4dNTPs, 1.25 units rTaq DNA polymerase (TOYOBO), genomic DNA, and 12.5 pmol of each primer (with/without 1 or 2 bases mismatch). PCR was conducted as follows: 10 min at 94 °C for initial denaturation, 35 cycles of 2 min at 94 °C, 2 min at 52 °C or 61 °C, 1 min at 72 °C, and 10 min at 72 °C as final extension. The PCR product was digested with each restriction enzyme (NEB) and subjected to electrophoresis in a Spreadex EL300 gel (Elchrom Scientific) at 55 °C or a MultiNA microtip electrophoresis DNA/RNA analyser (Shimadzu Biotech) (Table 2).

The database was created in Microsoft Excel file format and converted to SPSS. Statistical analysis was performed by SPSS ver14.0.1 (SPSS Japan, Inc., Tokyo, Japan). Linkage disequilibrium (LD) and haplotype block were predicted using Haploview (www.broad.mit.edu/haploview/haploview) based on an accelerated expectation-maximization algorithm similar to the partition-ligation method [10]. Using the software, the haplotype frequencies in case/control data were also estimated, and their associations were computed.

Results

Comparison of preproghrelin SNPs frequencies between men and women, with healthy Asian populations

We measured the frequencies of 5 SNPs in the preproghrelin gene in SCOP, −1500C>G (rs3755777), −1062G>C (rs26311), −994C>T (rs26312) (these 3 locate in the promoter), Leu72Met (+408C>A) (rs696217) (2nd exon), and +3056T>C (rs2075356) (2nd intron). Also, Arg51Gln (+346 G>A, rs34911341) (2nd exon) and +3083T>C (rs35682) (2nd intron) were analysed (Table 2). The frequencies of the

Table 2 Genotyping of Preproghrelin genes by PCR-RFLP.

SNP	dbSNP	PCR primer F (5′ to 3′)	PCR primer R (5′ to 3′)	Anneal temp.	PCR buffer[a]	Restriction enzyme	Generated frag. (bp)
−1500C>G	rs3755777	CCCAGTTGGATGAAGCACTC	TCATACCAGGCCCATAGGAC	52	A	Sau96 I	91, 12 → 41 + 50, 12(G)
−1062G>C	rs26311	GCCACTGGCTGAAGTTATCC	CTGTTGCTGCTCTGGCCACT	52	A	Nci I	128, 30 → 67 + 61, 30(G)
−994C>T	rs26312	GCCACTGGCTGAAGTTATCC	CTGTTGCTGCTCTGGCCACT	52	A	HpyCH4 III	158 → 139 + 19(C)
Leu72Met(C>A)	rs696217	GTCGAAGAAGCCACCAGCC	AGGTACCGACCCGGACTTAC	52	B	HpyCH4 III	116 → 20 + 96(C)
Arg51Gln(G>A)	rs34911341	GTCGAAGAAGCCACCAGCC	AGGTACCGACCCGGACTTAC	52	B	Sac I	116 → 37 + 79(G)
+3056T>C	rs2075356	GAGAATGCTGGGCAGACCC	GATAAAAGCTGTGGTGCCCAC	61	B	Hpy188 I	122 → 69 + 53(C)
+3083A>G	rs35682	GAGAATGCTGGGCAGACCC	GATAAAAGCTGTGGTGCCCAC	61	B	Ale I	122 → 99 + 23(G)

[a] PCR buffer A: 50 mM Tris–HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.7 m/M $MgCl_2$, 10% DMSO. PCR buffer B: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$.

Table 3 Genotype frequencies of preproghrelin gene.

SNP	Cohort (population)	Genotype (%)						
		n	Major homo	Hetero	Minor homo	MAF	HWP	*p*-Value
−1500C>G	SCOP	233	61 (26.2)	115 (49.4)	57 (24.5)	0.491	0.85	
	Male	115	30 (26.1)	51 (44.3)	34 (29.6)	0.517	0.23	0.168**
	Female	118	31 (26.3)	64 (54.2)	23 (19.5)	0.466	0.33	
	HapMap-JPT(ss68852548)	45	11 (24.4)	20 (44.4)	14 (31.1)	0.533	0.47	0.643*
	Choi et al. (Korean) [11]	641	167 (26.1)	316 (49.3)	158 (24.6)	0.493	0.73	0.998*
−1062G>C	SCOP	233	84 (36.1)	109 (46.8)	40 (17.2)	0.406	0.65	
	Male	115	41 (35.7)	49 (42.6)	25 (21.7)	0.430	0.16	0.164**
	Female	118	43 (36.4)	60 (50.8)	15 (12.7)	0.381	0.40	
	HapMap-JPT(ss68852547)	45	19 (42.2)	21 (46.7)	5(11.1)	0.344	0.82	0.537*
	Choi et al. (Korean) [11]	640	257 (40.2)	289 (45.2)	94 (14.7)	0.373	0.39	0.468*
−994C>T	SCOP	233	78 (33.5)	110 (47.2)	45 (19.3)	0.429	0.58	
	Male	115	39 (33.9)	50 (43.5)	26 (22.6)	0.443	0.20	0.375**
	Female	118	39 (33.1)	60 (50.8)	19 (16.1)	0.415	0.61	
	JBIC-allele(ss4941811)	454	Not applicable			0.427		0.900*
	Choi et al. (Korean) [11]	639	235 (36.8)	306 (47.9)	98 (15.3)	0.393	0.92	0.336*
Leu72Met (+408C>A)	SCOP	223	143 (64.1)	75 (33.6)	15 (6.7)	0.225	0.23	
	Male	115	74 (64.3)	33 (28.7)	8 (7.0)	0.213	0.12	0.526**
	Female	118	69 (58.5)	42 (35.6)	7 (5.9)	0.237	0.86	
	HapMap-JPT(ss68852544)	45	28 (62.2)	17 (37.8)	0 (0.0)	0.189	0.12	0.196*
	Ando et al. (Japanese female) [12]	300	205 (68.3)	84 (28.0)	11 (3.7)	0.177	0.52	0.149*
	Kuzuya et al. (Japanese male) [13]	2228	1412 (63.4)	728 (32.7)	88 (3.9)	0.203	0.63	0.195*
	Tang et al. (Chinese) [14]	323	195 (60.4)	112 (34.7)	16 (5.0)	0.223	0.99	0.668*
	Zou et al. (Chinese) [15]	125	77 (61.6)	43 (34.4)	5 (4.0)	0.217	0.74	0.611*
	Choi et al. (Korean) [11]	636	429 (67.5)	185 (29.1)	22 (3.5)	0.180	0.71	0.080*
	Kim et al. (Korean) [16]	80	54 (67.5)	23 (28.8)	3 (3.8)	0.181	0.78	0.515*
+3056T>C	SCOP	233	111 (47.6)	92 (39.5)	30 (12.9)	0.326	0.12	
	Male	115	58 (50.4)	43 (37.4)	14 (12.2)	0.309	0.18	0.701**
	Female	118	53 (44.9)	49 (41.5)	16 (13.6)	0.343	0.39	
	HapMap-JPT(ss44387483)	45	22 (48.9)	19 (42.2)	4 (8.9)	0.300	0.97	0.751*
	Ando et al. (Japanese female) [12]	300	162 (54.0)	112 (37.3)	26 (8.7)	0.273	0.30	0.182*

MAF = minor allele frequency, HWP = Hardy Weinberg plot, JBIC = Japanese Biological Informatics Consortium's data.

* *p*-Values are given by the chi-square test between SCOP and another cohort.

** *p*-Values are given by the chi-square test between male and female.

latter 2 were very low (minor allele frequency <0.05): therefore, further analysis was not performed. First, the 5 SNP frequencies between men and women in SCOP were compared (Table 3), but no specific differences were observed. We also compared the SNP frequencies in SCOP with those of healthy people published in the HapMap Project or those analysed in East Asians by other research groups (Table 3) [11–16]: no statistically significant differences were observed among these frequencies. HapMap also introduces these SNP frequencies in Han Chinese healthy people in Beijing (www.hapmap.org/cgi-perl/gbrowse/), and these are very similar to HapMap-JPT and to the results in SCOP, suggesting that the Japanese, Chinese, and Koreans are not quite genetically diverse in the preproghrelin gene polymorphism.

Associations between preproghrelin SNPs and anthropometric or clinical parameters

The phenotypes between men and women were rather different. In men, although the leptin and insulin were not elevated (Table 4), higher concentrations of fasting glucose and HbA1c were observed in the +3056C/C minor homozygotes (Table 5). Additionally, 72Met/Met minor homozygotes also showed the trend of higher concentrations of fasting glucose and HbA1c (Table 5). In these homozygotes, however, the other parameters were not so different.

In contrast, women showed a significant correlation not with diabetes but with fat metabolism and obesity. The −1062C/C minor homozygotes had higher values of C-peptide, insulin (Table 4), total and visceral fat area, waist circumference, and BMI (Table 6). Additionally, 72Met/Met minor homozygotes showed reduced concentrations of leptin (Table 4), total, HDL and LDL cholesterol (Table 5), and increased of visceral fat area (Table 6). Also, in the other SNPs, minor homozygotes showed a similar trend as above and heterozygotes had the intermediate values.

After Bonferroni adjustment for multiple comparisons, almost all of the associations mentioned above remained significant between the major and minor homozygotes. In comparison with the total cholesterol concentration and visceral fat ratio in women, the 72Leu/Met heterozygotes were also significant against Met/Met minor homozygotes (Tables 5 and 6). Similarly, the −1500C/G and −1062G/C heterozygotes have significant values of visceral fat area against their minor homozygotes (Table 6).

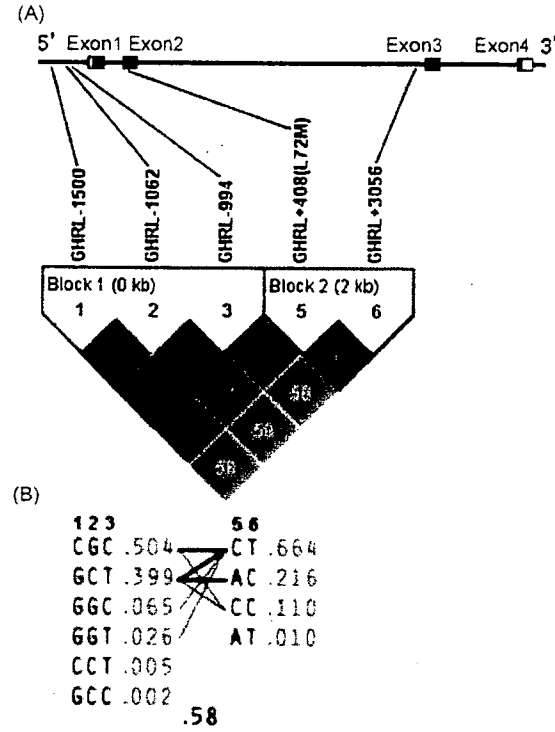

Figure 1 (A) SNP positions and LD block structure of the preproghrelin gene in SCOP. *D'* (100×) values are displayed in the squares. (B) Haplotype blocks and their population frequencies. If haplotypes in 2 blocks occur together with a frequency >10%, a thick line connects them. If >1%, a thin line does so. Beneath these lines, a recombination rate between the 2 blocks estimated using Haploview was shown.

Haploview analysis of haplotype frequencies in case/control subgroups to detect causative loci

To know about the causative loci of the above abnormalities, we depicted the LD map beforehand, using Haploview (Fig. 1A). In this region, two haplotype blocks were found: block 1, −1500-−1062-−994 and block 2: Leu72Met-+3056. Haplotype block 1 expands in the promoter region and block 2, in the exon–intron region. Fig. 1B shows the haplotypes and their estimated frequencies.

Next, we divided the 115 male subjects into 2 subgroups of diabetic and non-diabetic and estimated the frequencies of each haplotype in the subgroups, using Haploview (Table 7a). Based on the criterion of The Japan Diabetes Society, the diabetic subgroup comprised 59 subjects diagnosed with diabetes mellitus and under medication for diabetes, with a fasting glucose value >126 mg/dL, 2 h 75 g oral glucose tolerance test (OGTT) value >200 mg/dL, or HbA1c value >6.5%. The +408C-

Table 4 Preproghrelin genotypes and plasma biochemical parameters.

SNP	Genotype	*n*	Free fatty acids	Leptin	TNF-α	Adiponectin	C-peptide	Insulin	Creatinin
Male									
−1500C/G	C/C	30	0.46 ± 0.16	9.58 ± 7.95	1.44 ± 0.58	2.72 ± 1.58	2.88 ± 1.27	12.00 ± 9.20	0.90 ± 0.14
	C/G	51	0.50 ± 0.19	7.56 ± 3.88	1.26 ± 0.48	2.92 ± 1.91	2.99 ± 1.31	12.98 ± 11.44	0.87 ± 0.13
	G/G	34	0.55 ± 0.20	7.96 ± 5.36	1.19 ± 0.45	2.66 ± 1.76	2.62 ± 1.15	10.90 ± 8.41	0.85 ± 0.12
−1062G/C	G/G	41	0.47 ± 0.15	9.09 ± 7.33	1.40 ± 0.59	2.61 ± 1.49	2.74 ± 1.20	11.63 ± 8.50	0.89 ± 0.14
	G/C	49	0.51 ± 0.19	7.62 ± 3.82	1.21 ± 0.44	2.99 ± 1.93	3.05 ± 1.31	12.85 ± 11.41	0.87 ± 0.13
	C/C	25	0.56 ± 0.21	7.91 ± 5.46	1.25 ± 0.48	2.68 ± 1.91	2.67 ± 1.23	11.44 ± 9.66	0.84 ± 0.12
−994C/T	C/C	39	0.47 ± 0.15	9.26 ± 7.46	1.43 ± 0.58	2.64 ± 1.52	2.77 ± 1.21	11.72 ± 8.66	0.89 ± 0.14
	C/T	50	0.51 ± 0.19	7.37 ± 3.62	1.21 ± 0.44	2.91 ± 1.91	3.00 ± 1.31	13.00 ± 11.42	0.87 ± 0.13
	T/T	26	0.55 ± 0.21	8.23 ± 5.57	1.23 ± 0.48	2.77 ± 1.89	2.70 ± 1.22	11.00 ± 9.18	0.86 ± 0.12
Leu72Met	Leu/Leu	74	0.48 ± 0.16	8.21 ± 6.15	1.35 ± 0.55	2.57 ± 1.44	2.91 ± 1.37	12.39 ± 11.20	0.88 ± 0.14
	Leu/Met	33	0.53 ± 0.21	8.22 ± 4.84	1.15 ± 0.43	3.09 ± 2.12	2.81 ± 1.12	11.67 ± 8.00	0.86 ± 0.12
	Met/Met	8	0.63 ± 0.23	8.13 ± 3.87	1.25 ± 0.28	3.53 ± 2.65	2.57 ± 0.48	11.32 ± 5.43	0.84 ± 0.12
			$p = 0.084$						
+3056T/C	T/T	58	0.47 ± 0.16	8.40 ± 6.59	**1.40** ± 0.54	2.70 ± 1.55	2.90 ± 1.33	12.22 ± 11.27	0.89 ± 0.13
	T/C	43	0.52 ± 0.19	8.06 ± 4.85	**1.17** ± 0.48	2.82 ± 1.97	2.95 ± 1.29	12.77 ± 9.52	0.86 ± 0.13
	C/C	14	0.59 ± 0.23	7.88 ± 3.36	**1.17** ± 0.28	3.04 ± 2.08	2.38 ± 0.55	9.63 ± 4.55	0.86 ± 0.12
			$p = 0.080$		$p = \mathbf{0.049}$				
Female									
−1500C/G	C/C	31	0.57 ± 0.16	19.42 ± 7.84	1.17 ± 0.39	6.01 ± 3.20	2.21 ± 0.70	**9.13** ± 4.28	0.67 ± 0.10
	C/G	64	0.56 ± 0.22	23.19 ± 12.71	1.23 ± 0.45	5.28 ± 3.12	2.64 ± 0.75	**11.45** ± 4.66	0.66 ± 0.14
	G/G	23	0.58 ± 0.27	18.87 ± 9.82	1.25 ± 0.43	5.16 ± 2.73	2.72 ± 1.43	**13.04** ± 9.43	0.68 ± 0.13
							$p = 0.060$	$p = \mathbf{0.046}$	
−1062G/C	G/G	43	0.57 ± 0.16	18.63 ± 7.47	1.17 ± 0.37	5.89 ± 3.07	**2.22** ± 0.67	**9.13** ± 4.05	0.66 ± 0.10
	G/C	60	0.57 ± 0.23	23.37 ± 13.02	1.26 ± 0.47	5.29 ± 3.12	**2.66** ± 0.75	**11.66** ± 4.56	0.66 ± 0.14
	C/C	15	0.57 ± 0.29	21.15 ± 11.07	1.17 ± 0.46	4.82 ± 2.81	**3.02** ± 1.66	**14.90** ± 11.27	0.71 ± 0.15
							$p = 0.006$	$p = 0.003$	
−994C/T	C/C	39	0.58 ± 0.16	**18.30** ± 7.65	1.16 ± 0.38	5.96 ± 3.05	**2.23** ± 0.70	**9.20** ± 4.22	0.66 ± 0.11
	C/T	60	0.56 ± 0.22	**23.97** ± 12.69	1.26 ± 0.46	5.23 ± 3.17	**2.67** ± 0.74	**11.54** ± 4.60	0.66 ± 0.14
	T/T	19	0.59 ± 0.29	**19.41** ± 10.66	1.19 ± 0.44	5.07 ± 2.74	**2.81** ± 1.54	**13.89** ± 10.17	0.69 ± 0.14
				$p = \mathbf{0.033}$			$p = \mathbf{0.028}$	$p = \mathbf{0.013}$	
Leu72Met	Leu/Leu	69	0.59 ± 0.21	**20.18** ± 9.64	1.24 ± 0.44	5.98 ± 3.18	2.39 ± 0.76	10.30 ± 5.06	0.66 ± 0.12
	Leu/Met	42	0.53 ± 0.22	**24.30** ± 12.98	1.15 ± 0.43	4.75 ± 2.71	2.81 ± 1.09	12.54 ± 6.88	0.67 ± 0.14
	Met/Met	7	0.53 ± 0.19	**14.91** ± 9.74	1.33 ± 0.35	4.49 ± 3.21	2.53 ± 1.04	10.98 ± 6.87	0.70 ± 0.15
				$p = \mathbf{0.048}$		$p = 0.082$	$p = 0.065$		
+3056T/C	T/T	53	0.57 ± 0.19	20.89 ± 10.10	1.23 ± 0.40	5.98 ± 3.38	2.41 ± 0.77	10.41 ± 5.26	0.67 ± 0.13
	T/C	49	0.57 ± 0.24	20.48 ± 9.95	1.18 ± 0.46	5.10 ± 2.82	2.69 ± 1.10	11.85 ± 6.37	0.65 ± 0.11
	C/C	16	0.55 ± 0.23	25.47 ± 16.68	1.27 ± 0.43	4.80 ± 2.50	2.56 ± 0.79	11.37 ± 6.61	0.68 ± 0.18

p-Values are given by ANOVA, and $p < 0.05$ are bold.

Table 5 Preproghrelin genotypes and lipidemia- or diabetes- related parameters.

SNP	Genotype	*n*	Total-cho.	HDL-cho.	LDL-cho.	Triacylglycerol	HbA1c	Fasting Glc.	HOMA-IR
Male									
−1500C/G	C/C	30	198.7 ± 30.8	47.27 ± 9.40	117.8 ± 30.4	184.7 ± 185.6	5.61 ± 0.87	107.8 ± 21.1	3.25 ± 2.72
	C/G	51	209.0 ± 25.3	49.24 ± 9.27	122.5 ± 29.5	186.5 ± 89.5	5.84 ± 0.83	113.5 ± 26.1	3.62 ± 3.11
	G/G	34	201.7 ± 28.7	52.88 ± 9.98	119.5 ± 26.1	148.9 ± 81.2	5.95 ± 1.22	112.6 ± 26.4	3.25 ± 3.32
				$p = 0.057$					
−1062G/C	G/G	41	202.6 ± 28.6	48.10 ± 9.71	122.8 ± 27.1	158.7 ± 90.5	5.62 ± 0.76	107.5 ± 19.1	3.12 ± 2.47
	G/C	49	208.2 ± 28.5	51.29 ± 10.49	120.7 ± 31.1	181.2 ± 93.0	5.92 ± 0.92	114.9 ± 26.1	3.62 ± 3.11
	C/C	25	198.8 ± 25.6	49.68 ± 7.66	115.9 ± 26.2	189.0 ± 192.6	5.90 ± 1.32	112.4 ± 30.3	3.47 ± 3.83
−994C/T	C/C	39	202.2 ± 29.2	48.26 ± 9.87	122.1 ± 27.6	159.6 ± 92.6	5.66 ± 0.76	107.9 ± 19.5	3.15 ± 2.52
	C/T	50	206.6 ± 27.8	50.10 ± 10.21	120.1 ± 30.4	181.7 ± 91.5	5.87 ± 0.88	114.6 ± 25.9	3.65 ± 3.10
	T/T	26	202.5 ± 26.9	51.54 ± 8.25	118.4 ± 27.4	184.7 ± 189.8	5.94 ± 1.36	112.0 ± 29.8	3.34 ± 3.73
Leu72Met	Leu/Leu	74	200.7 ± 26.6	49.20 ± 9.54	118.2 ± 25.6	166.7 ± 93.1	5.68 ± 0.77	108.8 ± 19.2	3.43 ± 3.37
	Leu/Met	33	212.6 ± 29.5	51.64 ± 9.91	125.8 ± 33.8	192.5 ± 173.8	5.95 ± 0.98	115.2 ± 29.0	3.26 ± 2.16
	Met/Met	8	201.6 ± 29.9	47.75 ± 10.18	118.4 ± 32.5	177.6 ± 72.2	6.46 ± 2.00	125.1 ± 44.9	3.84 ± 3.51
							$p = 0.061$		
+3056T/C	T/T	58	202.0 ± 29.2	48.40 ± 9.71	122.4 ± 27.5	156.1 ± 79.4	5.58 ± 0.65	**106.8** ± 16.0	3.31 ± 3.19
	T/C	43	207.1 ± 27.2	50.51 ± 8.32	119.6 ± 29.2	187.0 ± 106.4	5.93 ± 0.95	**113.3** ± 27.3	3.62 ± 3.03
	C/C	14	204.2 ± 26.1	53.43 ± 12.65	114.5 ± 32.2	215.8 ± 242.6	6.37 ± 1.70	**127.6** ± 38.7	3.20 ± 2.71
							$p = 0.013$	$p = 0.016$	
Female									
−1500C/G	C/C	31	222.5 ± 38.1	59.81 ± 12.72	135.3 ± 33.1	137.0 ± 84.4	5.67 ± 0.68	112.1 ± 26.2	2.67 ± 2.04
	C/G	64	215.2 ± 40.6	54.55 ± 10.24	131.8 ± 33.9	144.3 ± 71.3	6.00 ± 1.34	113.8 ± 28.4	3.18 ± 1.36
	G/G	23	211.2 ± 39.5	54.17 ± 12.89	122.1 ± 35.3	175.3 ± 85.7	5.90 ± 1.02	108.3 ± 22.2	3.55 ± 2.66
				$p = 0.086$					
−1062G/C	G/G	43	223.8 ± 43.0	58.81 ± 12.25	137.1 ± 35.3	139.3 ± 89.4	5.74 ± 0.71	111.2 ± 24.6	**2.62** ± 1.81
	G/C	60	214.4 ± 35.7	54.20 ± 10.56	130.1 ± 31.3	150.6 ± 71.0	6.02 ± 1.39	114.5 ± 29.3	**3.25** ± 1.33
	C/C	15	202.9 ± 42.5	54.00 ± 12.81	115.9 ± 37.7	165.7 ± 73.8	5.82 ± 1.06	106.7 ± 20.4	**4.03** ± 3.17
									$p = 0.030$
−994C/T	C/C	39	223.4 ± 42.8	59.18 ± 12.46	137.2 ± 35.5	134.8 ± 78.3	5.76 ± 0.73	112.2 ± 25.5	2.67 ± 1.89
	C/T	60	214.9 ± 37.5	53.58 ± 10.05	130.8 ± 32.3	153.1 ± 81.4	5.97 ± 1.36	112.9 ± 28.4	3.19 ± 1.35
	T/T	19	206.4 ± 38.8	56.21 ± 13.26	117.9 ± 34.3	161.5 ± 67.1	5.95 ± 1.10	110.4 ± 23.9	3.83 ± 2.85
				$p = 0.062$					$p = 0.080$
Leu72Met	Leu/Leu	69	**223.2** ± 39.0	**58.06** ± 11.47	**137.5** ± 32.6	138.4 ± 70.9	5.81 ± 0.98	111.4 ± 27.2	2.89 ± 1.84
	Leu/Met	42	**211.0** ± 38.4	**52.88** ± 10.87	**124.9** ± 33.5	165.9 ± 92.4	5.99 ± 1.33	115.0 ± 26.1	3.54 ± 1.93
	Met/Met	7	**181.1** ± 33.2	**52.00** ± 13.95	**101.0** ± 32.8	141.9 ± 28.2	6.13 ± 1.52	104.6 ± 24.4	2.79 ± 1.60
			$p = 0.014$	$p = 0.048$	$p = 0.008$				
+3056T/C	T/T	53	**224.5** ± 40.6	**59.30** ± 12.03	137.5 ± 34.4	138.2 ± 76.1	5.73 ± 0.71	110.9 ± 24.3	2.96 ± 2.02
	T/C	49	**214.0** ± 34.1	**52.51** ± 10.18	128.6 ± 29.9	164.8 ± 86.2	6.03 ± 1.37	113.0 ± 26.9	3.25 ± 1.65
	C/C	16	**196.6** ± 46.1	**54.69** ± 11.76	115.5 ± 40.3	132.3 ± 49.2	6.03 ± 1.48	114.8 ± 33.7	3.24 ± 2.08
			$p = 0.040$	$p = 0.011$	$p = 0.062$				

p-Values are given by ANOVA, and $p < 0.05$ are bold.

Table 6 Preproghrelin genotypes and obesity-related parameters.

SNP	Genotype	*n*	BMI	Body fat (%)	Waist circumf.	Total fat area	Subcutaneous f.a.	Visceral f.a.	Visceral fat (%)
Male									
−1500C/G	C/C	30	30.53 ± 4.04	28.83 ± 5.00	101.6 ± 9.6	414.3 ± 144.3	255.3 ± 105.3	159.0 ± 62.5	38.70 ± 9.54
	C/G	51	29.81 ± 2.04	29.05 ± 4.11	100.4 ± 6.3	392.1 ± 89.5	237.2 ± 63.9	154.9 ± 47.1	39.52 ± 7.84
	G/G	34	31.31 ± 4.64	29.21 ± 4.50	103.1 ± 10.8	447.9 ± 170.0	282.3 ± 139.3	165.6 ± 57.8	37.98 ± 10.24
−1062G/C	G/G	41	30.31 ± 3.59	28.31 ± 4.57	101.3 ± 8.6	410.7 ± 130.1	253.4 ± 93.5	157.2 ± 59.2	38.47 ± 9.00
	G/C	49	30.22 ± 2.93	29.52 ± 4.58	101.1 ± 7.5	407.4 ± 120.8	248.5 ± 84.1	158.9 ± 58.6	38.91 ± 9.09
	G/C	25	31.09 ± 4.55	29.34 ± 3.91	102.8 ± 11.1	434.0 ± 162.1	271.4 ± 145.8	162.6 ± 36.0	39.37 ± 9.13
−994C/T	C/C	39	30.41 ± 3.65	28.46 ± 4.62	101.6 ± 8.8	412.8 ± 132.5	253.2 ± 95.6	159.6 ± 59.6	38.91 ± 9.01
	C/T	50	30.10 ± 2.90	29.36 ± 4.59	100.7 ± 7.4	403.0 ± 118.9	247.4 ± 83.2	155.6 ± 57.9	38.51 ± 9.15
	T/T	26	31.15 ± 4.48	29.32 ± 3.90	103.0 ± 10.9	438.5 ± 160.0	273.4 ± 142.8	165.1 ± 38.0	39.41 ± 8.98
Leu72Met	Leu/Leu	74	30.39 ± 3.42	28.90 ± 4.59	101.1 ± 8.2	410.3 ± 130.5	251.4 ± 91.5	158.9 ± 59.0	38.85 ± 8.81
	Leu/Met	33	30.28 ± 3.97	29.38 ± 4.09	101.8 ± 9.8	415.6 ± 148.9	258.1 ± 131.4	157.6 ± 47.7	38.98 ± 9.83
	Met/Met	8	31.58 ± 3.07	28.95 ± 4.89	104.0 ± 9.3	446.6 ± 91.7	279.1 ± 73.9	167.5 ± 36.9	38.29 ± 8.06
+3056T/C	T/T	58	30.37 ± 3.63	28.88 ± 4.92	101.5 ± 8.7	411.1 ± 141.7	254.3 ± 100.7	156.8 ± 59.0	38.52 ± 8.75
	T/C	43	30.57 ± 3.74	29.24 ± 3.82	101.7 ± 9.1	419.0 ± 136.6	254.2 ± 117.2	164.9 ± 51.0	40.01 ± 9.13
	C/C	14	30.36 ± 2.73	29.11 ± 4.33	101.1 ± 8.0	413.6 ± 84.5	262.4 ± 63.8	151.1 ± 45.1	36.62 ± 9.71
Female									
−1500C/G	C/C	31	30.45 ± 2.71	40.20 ± 5.32	102.3 ± 7.9	**453.1 ± 101.1**	330.9 ± 87.7	**122.2 ± 44.7**	27.08 ± 9.07
	C/G	64	31.14 ± 3.19	40.34 ± 4.84	103.2 ± 7.9	**455.6 ± 95.1**	330.5 ± 77.3	**125.1 ± 45.3**	27.41 ± 7.99
	G/G	23	31.87 ± 3.44	42.33 ± 6.90	107.1 ± 9.5	**526.0 ± 138.7**	370.0 ± 113.9	**156.0 ± 46.5**	30.17 ± 8.02
					$p = 0.087$	$p = 0.018$		$p = 0.012$	
−1062G/C	G/G	43	**30.11 ± 2.55**	39.90 ± 5.09	**101.3 ± 7.7**	**439.4 ± 94.2**	321.8 ± 79.2	**117.6 ± 42.0**	26.73 ± 8.33
	G/C	60	**31.49 ± 3.10**	40.75 ± 4.82	**104.3 ± 7.7**	**472.2 ± 98.1**	341.2 ± 80.5	**131.0 ± 46.2**	27.78 ± 8.02
	C/C	15	**32.40 ± 4.09**	42.73 ± 8.01	**108.1 ± 10.8**	**538.4 ± 156.4**	373.8 ± 132.1	**164.6 ± 47.3**	31.44 ± 8.86
			$p = 0.019$		$p = 0.016$	$p = 0.009$		$p = 0.003$	
−994C/T	C/C	39	**30.09 ± 2.67**	39.60 ± 5.18	101.4 ± 7.8	**438.5 ± 97.8**	321.3 ± 82.0	**117.2 ± 43.2**	26.70 ± 8.54
	C/T	60	**31.52 ± 3.08**	41.06 ± 4.82	104.3 ± 7.8	**472.6 ± 98.1**	341.4 ± 79.5	**131.2 ± 46.3**	27.77 ± 7.94
	T/T	19	**31.86 ± 3.79**	41.78 ± 7.38	106.6 ± 10.1	**518.0 ± 145.4**	363.4 ± 122.1	**154.6 ± 47.5**	30.55 ± 8.72
			$p = 0.042$		$p = 0.059$	$p = 0.030$		$p = 0.015$	
Leu72Met	Leu/Leu	69	30.69 ± 2.91	40.34 ± 5.27	102.6 ± 8.0	458.4 ± 106.3	334.1 ± 87.7	**124.3 ± 40.8**	**27.22 ± 7.52**
	Leu/Met	42	31.83 ± 3.39	41.27 ± 5.63	105.0 ± 8.9	480.0 ± 112.5	347.4 ± 90.2	**132.5 ± 54.1**	**27.55 ± 9.27**
	Met/Met	7	30.83 ± 3.30	40.71 ± 6.30	106.7 ± 7.5	502.1 ± 120.2	325.2 ± 98.8	**176.9 ± 27.3**	**36.08 ± 5.52**
								$p = 0.016$	$p = 0.024$
+3056T/C	T/T	53	30.63 ± 2.99	40.60 ± 5.50	103.2 ± 8.1	466.4 ± 111.1	339.0 ± 92.6	127.4 ± 39.9	27.51 ± 7.35
	T/C	49	31.35 ± 3.20	40.96 ± 5.57	103.2 ± 8.9	462.0 ± 105.9	334.6 ± 86.6	127.4 ± 50.4	27.51 ± 9.25
	C/C	16	31.93 ± 3.36	40.18 ± 5.04	106.8 ± 7.2	496.8 ± 115.0	347.4 ± 86.7	149.3 ± 54.2	30.12 ± 8.34

p-Values are given by ANOVA, and $p < 0.05$ are bold.

Table 7 Association check of preproghrelin gene polymorphisms and haplotype.

SNP	Allele	Freq.	Freq. in case/control	*p*-Value	Block	Haplotype	Freq.	Freq. in case/control	*p*-Value
(a) Diabetes mellitus in male (case = 59/control = 56)									
GHRL – 1500	G	0.517	0.576/0.455	0.067	Block 1	CGC	0.473	0.423/0.526	0.118
GHRL – 1062	C	0.430	0.492/0.366	0.055		GCT	0.417	**0.482**/0.348	**0.038**
GHRL – 994	T	0.443	0.492/0.393	0.132		GGC	0.079	0.077/0.081	0.907
GHRL + 408 (L72M)	A	0.213	0.237/0.188	0.357		GGT	0.017	0.009/0.027	0.291
GHRL + 3056	C	0.309	**0.390**/0.223	**0.006**	Block 2	CT	0.687	0.610/**0.767**	**0.010**
						AC	0.208	0.237/0.178	0.272
						CC	0.100	**0.153**/0.045	**0.007**
(b) Visceral fat area $\geq$100 cm^2 in female (case = 84/control = 34)									
GHRL – 1500	G	0.466	0.506/0.368	0.054	Block 1	CGC	0.534	0.494/0.632	0.054
GHRL – 1062	C	0.381	0.423/0.279	**0.040**		GCT	0.381	**0.423**/0.279	**0.040**
GHRL – 994	T	0.415	**0.458**/0.309	**0.035**		GGC	0.051	0.048/0.059	0.723
GHRL + 408 (L72M)	A	0.237	0.256/0.191	0.289		GGT	0.034	0.036/0.029	0.809
GHRL + 3056	T	0.657	0.661/0.647	0.841	Block 2	CT	0.643	0.647/0.631	0.814
						AC	0.223	0.242/0.175	0.260
						CC	0.120	0.097/0.178	0.083
						AT	0.014	0.014/0.016	0.880
(c) Total-cho. $\geq$220 mg/dL in female (case = 54/control = 64)									
GHRL – 1500	C	0.534	0.537/0.531	0.929	Block 1	CGC	0.534	0.537/0.531	0.929
GHRL – 1062	G	0.619	0.648/0.594	0.391		GCT	0.381	0.352/0.406	0.391
GHRL – 994	C	0.585	0.602/0.570	0.624		GGC	0.051	0.065/0.039	0.370
GHRL + 408 (L72M)	C	0.763	**0.824**/0.711	**0.042**		GGT	0.034	0.046/0.023	0.334
GHRL + 3056	T	0.657	0.713/0.609	0.095	Block 2	CT	0.643	**0.711**/0.584	**0.043**
						AC	0.223	0.174/0.264	0.099
						CC	0.120	0.113/0.120	0.717
						AT	0.014	0.002/0.025	0.133
(d) BMI $\geq$30 kg/m^2 in female (case = 67/control = 52)									
GHRL – 1500	G	0.466	**0.538**/0.375	**0.013**	Block 1	CGC	0.534	0.462/**0.625**	**0.013**
GHRL – 1062	C	0.381	**0.462**/0.279	**0.004**		GCT	0.381	**0.462**/0.279	**0.004**
GHRL – 994	T	0.415	**0.492**/0.317	**0.007**		GGC	0.051	0.045/0.058	0.671
GHRL + 408 (L72M)	A	0.237	0.273/0.192	0.149		GGT	0.034	0.030/0.038	0.731
GHRL + 3056	C	0.343	**0.409**/0.260	**0.016**	Block 2	CT	0.643	**0.582**/**0.720**	**0.028**
						AC	0.223	0.263/0.172	0.094
						CC	0.120	0.146/0.088	0.174
						AT	0.014	0.009/0.020	0.475

p-Values are given by the chi-square test between case and control, and $p < 0.05$ are bold.

+3056C haplotype was more frequent in the diabetic subgroup (case) than in the non-diabetic subgroup (control) (Table 7a). Simultaneously, the +408C - +3056T haplotype was more frequent in the non-diabetic subgroup, suggesting that the causative locus is located around not 72Met but +3056C.

Similarly, 118 female subjects were grouped into the subgroup of higher visceral fat area (100 cm^2 and more) and the normal one. A visceral fat area $\geq$100 cm^2 is the criterion for the metabolic syndrome, indicated by The Japanese Society of Internal Medicine. The higher visceral fat area subgroup (84 subjects) had the −1500G - −1062C - −994T haplotype more frequently (Table 7b). Particularly, the −1062C and −994T were highly significant (Table 7b).

The female subjects were also divided into two subgroups of higher total cholesterol ($\geq$220 mg/dL) and normal concentration. A value $\geq$220 mg/dL for total cholesterol is the criterion for lipidemia, according to The Japan Atherosclerosis Society. A higher total cholesterol subgroup (54 subjects) had a higher ratio of the +408C - +3056T haplotype than the lower subgroup (Table 7c).

Furthermore, the female subjects were grouped into a higher BMI (30 kg/m^2 and more) subgroup and relatively lower BMI subgroup. The higher BMI subgroup (67 subjects) had the −1500G - −1062C - −994T haplotype more frequently (Table 7d). Conversely, the +408C - +3056T haplotype was more frequent in a subgroup with a relatively lower BMI (52 subjects) (Table 7d), implying that there are more than 2 loci relevant in adiposity. Thus, the susceptibility to obesity is a mixed feature of the above-mentioned higher visceral fat area subgroup with the lower total cholesterol subgroup.

Discussion

Phenotypes of the preproghrelin gene SNPs are particularly complex, since this study revealed them to be quite different between men and women. In obese men, the +3056C/C minor homozygotes were demonstrated to be susceptible to diabetes mellitus, which is not accompanied by insulin or leptin accumulation.

In contrast, in obese women, the correlation of preproghrelin polymorphism with diabetes may be insignificant, i.e., although the −1062C/C minor homozygotes (those of −1500C>G and −994C>T also) had higher concentrations of insulin and C-peptide, their fasting glucose and HbA1c values were normal. Instead, these preproghrelin polymorphisms may contribute to the development of obesity via aberrant fat storage; female subjects with a −1500G - −1062C - −994T haplotype had a higher BMI and a tendency of fat mass accumulation, particularly visceral. This is notable because generally, women tend to accumulate subcutaneous rather than visceral fat mass. Likewise, in SCOP, females had a lower visceral fat area (130.4 cm^2 on an average) than males (159.1 cm^2) (Table 1). To the contrary, females with the above haplotype tended to have a higher visceral fat area comparable to male average. (This is because estrogen influences body fat distribution; the estradiol concentration is inversely associated with visceral fat accumulation in menstruating females [17]; and body-fat distribution shifts toward the upper part of the body (viscera) after menopause [18].)

Additionally, preproghrelin polymorphism also affected serum cholesterol homeostasis: female subjects with the 72Met allele showed lower serum concentrations of total, HDL and LDL cholesterol. Thus, reduced cholesterol are probably linked to the accumulation of body fat: however, interestingly, the causative loci may be distinct; lower cholesterols, 72Met; accumulation of visceral fat, −1062C and −994T.

This study suggested that the contribution of the preproghrelin gene SNPs to metabolic syndrome should be estimated separately in women and men. This is a reasonable proposition because androgens regulate ghrelin secretion or catabolism in a gender-specific manner; androgens and ghrelin have similar effect in males but opposite effects in females [19]. Conversely, ghrelin may down-regulate the concentrations of testosterone [20] or estrogen: Misra and his group reported that adolescent athletes, who are possibly in the negative energy balance state, showed high ghrelin and decreased estradiol concentrations [21,22].

Choi et al. identified the 9 SNPs in the promoter region and compared their frequencies in type 2 diabetic patients with non-diabetic controls in a Korean population [11]. They found the association of the −1062C allele with lower HDL cholesterol, higher fasting glucose, and higher homeostasis model assessment of insulin resistance (HOMA-IR) values. Their results were similar to ours. Possibly, gender-specific estimation may uncover the contribution of these SNPs to diabetes or obesity.

In the study examining the risk of metabolic syndrome in an Amish population, a Caucasian cohort of European descent, Steinle et al. [23] found the association of the 72Met variant with increased prevalence of metabolic syndrome and higher fasting glucose, lower HDL cholesterol, and higher triglyceride concentrations. Their results partly agree with our data. In the cohort, metabolic syn-