一個亞太區域糧食與衛生安全平台：向前發展之道

多重全球危機的來臨，特別是 2008 年的氣候變遷、經濟、能源、水源、糧食及衛生等危機發生，對於那些飽嚐此類事件不良影響、應擔負相關責任或利益遭受影響的群體而言，已達應變之關鍵時刻。有鑑於此，亞太區域內致力於糧食與衛生體系工作、來自多個單位之專家學者群，於 2009 年 8 月齊聚台灣，力促建構「糧食與衛生安全網絡」，以連結該區域或區域外其他有志一同的網絡。永續健康發展包含許多面向，其中糧食與營養問題經常被忽略，然而營養失調卻可引致多種相關疾病。營養不良持續受到全球的農業研究與發展科學家的關注，並與衛生部門連結是進展之關鍵，因為農業與衛生部門的分離會對消費者的營養與健康造成負面衝擊。此外，倫理與平等亦對糧食與衛生體系造成影響。惟有當潛藏的社會不平等獲得解決，才能確保糧食與衛生安全；此倫理議題正反映出聯合國世界人權宣言之概念，亦即人權需包括可獲取足夠糧食以保障健康與福祉。糧食與衛生安全為廣泛安全議題的一環，且值得更加重視。急迫的政策建議為投入更多資源於結合糧食與衛生相關之研究，同時擬定亞太安全進程，強調全球性、人性化、衛生及糧食安全，並與傳統安全防禦系統相連結，另外加強社區及家戶之安全方案，包括提高婦女識字能力、通訊科技及創業機會。

關鍵字：人類安全、全球衛生、農業、社會決定因素、倫理、系統、網絡

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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

Jun Takezawa, Kouichi Yamada*, Akemi Morita, Naomi Aiba, Shaw Watanabe

Program of Educational Nutrition, The National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan

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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

* Corresponding author. Tel.: +81 3 3203 5723; fax: +81 3 3203 0335.
E-mail address: kouichi@nih.go.jp (K. Yamada).

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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 mM $MgCl_2$, 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)	0.212	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).

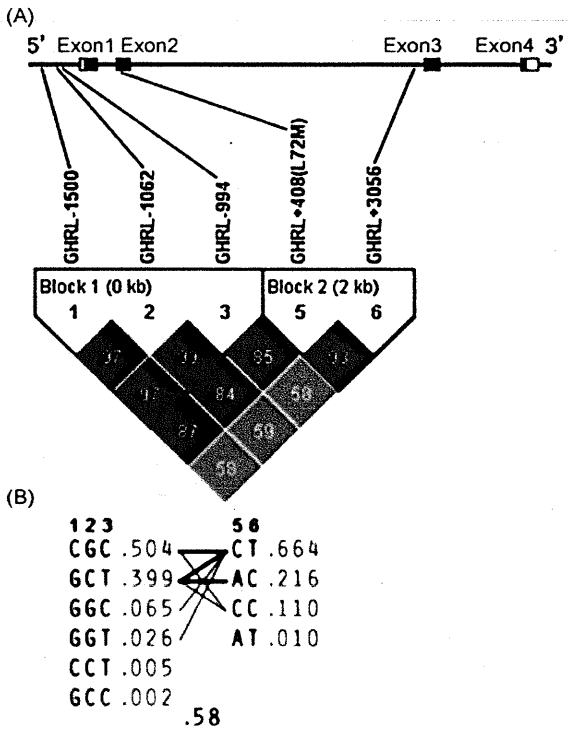

Figure 1 (A) SNP positions and LD block structure of the preproghrelin gene in SCOP. *D'* (100×) values are displayed in the squares. (B) Haplotype blocks and their population frequencies. If haplotypes in 2 blocks occur together with a frequency >10%, a thick line connects them. If >1%, a thin line 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
							$p = \mathbf{0.006}$	$p = \mathbf{0.003}$	
−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
					$p = 0.087$	$p = \mathbf{0.018}$		$p = \mathbf{0.012}$	
−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
			$p = \mathbf{0.019}$		$p = \mathbf{0.016}$	$p = \mathbf{0.009}$		$p = \mathbf{0.003}$	
−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
			$p = \mathbf{0.042}$		$p = 0.059$	$p = \mathbf{0.030}$		$p = \mathbf{0.015}$	
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
								$p = \mathbf{0.016}$	$p = \mathbf{0.024}$
+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 ≥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. ≥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	0.113/0.126	0.747
						AT	0.014	0.002/0.025	0.133
(d) BMI ≥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-

drome as well as type 2 diabetes mellitus was more prevalent among women as compared with men. Because their results were not gender-specific, it is not known whether 72Met variant was strongly associated with the above phenotypes in women.

Kuzuya et al. [13] reported a significantly higher frequency of the 72Met allele in the higher BMI (25 and more) group of middle-aged men than in the normal-weight group, suggesting that not only the gender but also the extent of obesity (obese or non-obese) should be considered.

Recently, Zavarella et al. [24] reported on the protective effect of ghrelin in insulin resistance by the 72Met allele in a Caucasian (Italian) population consisting of obese and normal weight subjects; the Met/Met minor homozygotes had lower values of triglycerides, fasting insulin, HOMA-IR, and a higher total ghrelin level. We could not observe such effects in Leu72Met. They also reported that the T/T minor homozygotes of −604C>T had decreasing values of fasting insulin and HOMA-IR. We have not yet analysed −604T>C (rs27647): however, if LD is maintained between −604T>C and −994C>T, our results in the −994 minor homozygotes are not in agreement with theirs. Preproghrelin gene polymorphism may affect insulin metabolism differently according to the race and gender of the subjects or extent of obesity (obese or non-obese).

We are particularly interested in the relationship existing among the SNPs, plasma ghrelin concentration, and susceptibility to diabetes or obesity. Unfortunately, the plasma ghrelin was not measured in the present study. Ando and his colleague [25] reported on the association of higher acylated ghrelin (active form) concentration and body weight, BMI, fat area, waist circumference, skinfold thickness, and a lower HDL cholesterol in Japanese women with the +3056C allele. They stated that the 72Met carriers also had a higher acylated ghrelin but to a lesser extent than the +3056C carriers. Some groups reported a higher ghrelin concentration in the 72Met carriers [24]. These results predict that a higher ghrelin predisposes to obesity in the subjects with the −1500G - −1062C - −994T haplotype as well. Above mentioned Choi et al. [11] used biochemical experiments and found that a transcription factor, myoD, preferentially binds to the region with −1062C, although they described that the region with −1062G had a 1.7-fold higher promoter activity of preproghrelin than that with −1062C.

About the reason why preproghrelin gene SNP may relate to diabetes in obese men, we suppose that higher ghrelin affects serum glucose homeostasis via androgens. Dezaki et al. described that besides effects on appetites and energy homeostasis, ghrelin is also involved in regulating insulin release and glucose metabolism: ghrelin inhibits insulin release [26]. As shown in Table 4, male +3056C/C homozygotes indeed had lower insulin and C-peptide levels on an average, though these were not significant.

Haploview enables the study of LD among SNPs and the estimation of frequencies of the analysed haplotype. Our *D'* values of the LD are mostly in agreement with those of Ando et al. [12] or of Choi et al. [11]. We observed 2 haplotype blocks in this region: block 1, −1500C>G - −1062G>C - −994C>T and block 2: Leu72Met - +3056T>C. These SNPs shows a strong LD in each haploblocks. This is probably because not only the coding-rich region but also the promoter region also has functional importance.

In conclusion, the +3056C/C minor homozygotes were associated with predisposition to diabetes mellitus in obese men. In obese women, the −1500G - −1062C - −994T haplotype was correlated with visceral adiposity, and the 72Met - +3056C haplotype was associated with susceptibility to obesity via aberrant fat metabolism (reduction of serum cholesterol). Thus, polymorphisms of the preproghrelin gene are suggested to be closely related to diabetes and obesity in obese Japanese. Further studies are required to evaluate these findings and to elucidate the underlying biological mechanisms.

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Research Article

Rev1, Rev3, or Rev7 siRNA Abolishes Ultraviolet Light-Induced Translesion Replication in HeLa Cells: A Comprehensive Study Using Alkaline Sucrose Density Gradient Sedimentation

Jun Takezawa,[1] Yukio Ishimi,[2] Naomi Aiba,[1] and Kouichi Yamada[1]

[1] *Division of Genetic Biochemistry, The National Institute of Health and Nutrition, Shinjuku-ku, Tokyo 162-8636, Japan*
[2] *Faculty of Science, Ibaraki University, Mito-shi, Ibaraki-ken 310-8512, Japan*

Correspondence should be addressed to Kouichi Yamada, kouichi@nih.go.jp

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When a replicative DNA polymerase stalls upon encountering a lesion on the template strand, it is relieved by other low-processivity polymerase(s), which insert nucleotide(s) opposite the lesion, extend by a few nucleotides, and dissociate from the 3′-OH. The replicative polymerase then resumes DNA synthesis. This process, termed translesion replication (TLS) or replicative bypass, may involve at least five different polymerases in mammals, although the participating polymerases and their roles have not been entirely characterized. Using siRNAs originally designed and an alkaline sucrose density gradient sedimentation technique, we verified the involvement of several polymerases in ultraviolet (UV) light-induced TLS in HeLa cells. First, siRNAs to Rev3 or Rev7 largely abolished UV-TLS, suggesting that these 2 gene products, which comprise Polζ, play a main role in mutagenic TLS. Second, Rev1-targeted siRNA also abrogated UV-TLS, indicating that Rev1 is also indispensable to mutagenic TLS. Third, Polη-targeted siRNA also prevented TLS to a greater extent than our expectations. Forth, although siRNA to Polι had no detectable effect, that to Polκ delayed UV-TLS. To our knowledge, this is the first study reporting apparent evidence for the participation of Polκ in UV-TLS.

1. Introduction

Multiple systems have evolved to manage the genomic photoproducts generated by harmful UV light. One such system is nucleotide excision repair (NER), which eliminates photoproducts from DNA strands by dual incision on both sides of a damaged base. The NER system cannot, however, remove all UV-damaged bases. When a replicative DNA polymerase stalls upon encountering a residual photoproduct on the template strand, it is relieved by other low-processivity polymerase(s), which incorporate nucleotide(s) opposite the lesion, extend by a few nucleotides and dissociate from the 3′-OH. The replicative polymerase then resumes DNA synthesis. This process, termed translesion replication (TLS) or replicative bypass (reviewed in [1]), is also one of the subtle systems that have evolved for the management of genomic photoproducts.

UV-C (100–290 nm wavelength) induces 2 main photoproducts [2]: the more frequent cyclobutane pyrimidine dimer (CPD) and the several-fold lower pyrimidine-pyrimidone (6-4) photoproduct ((6-4)PP). *cis-syn* CPD, a predominant form of the multiple configurations, contains 2 adjacent pyrimidines that are covalently linked in parallel. Although the frequency of CPD varies with nucleotide composition, a ratio of T-T to C-T to T-C to C-C of 68 : 13 : 16 : 3 is obtained from UV-irradiated plasmid DNA. Cytosines within CPD are unstable, and are deaminated to uracil or 5-methylcytosine, and further deaminated to thymine [3]. The helical distortion caused by CPD is so inconspicuous that almost half of the lesions remain unrepaired by NER, even 6 hours after UV irradiation in the case of CHO cells [2].

The (6-4)PPs from T-C, C-C, and, less frequently, T-T sequences are detected in UV-irradiated DNA whereas that of C-T are not. In (6-4)PP, linkage between C-6 of one pyrimidine and C-4 of the adjacent pyrimidine cause the 2 bases to be in nearly perpendicular position. Consequently, formation of this lesion causes a major distortion in the double helix. NER preferentially removes (6-4)PP more

rapidly than it removes CPD from the genome in human and rodent cells [4].

At least 5 mammalian DNA polymerases are suggested to be implicated in UV-induced TLS: Pols η, ι, ζ, κ, and Rev1, all of which belong to the Y family except for Polζ (B family) (reviewed in [1, 5, 6]). However, the participating polymerases and their roles have not been entirely characterized.

Patients with the autosomal recessive disorder, xeroderma pigmentosum variant (XP-V), have a predisposition to skin cancer, and XP-V cells demonstrate hypermutability after UV irradiation (reviewed in [7]). The defective gene in XP-V encodes Polη, which was first purified from a HeLa cell extract as an activity that complements TLS defect in XP-V cell extract [8]. Human Polη catalysed DNA synthesis past TT-CPD very efficiently and in a relatively accurate manner, as demonstrated by the lesion-bypass assay [7, 9]. When template DNA contained a (6-4)TT-PP, Polη incorporated one (random) nucleotide opposite the first thymine and another nucleotide opposite the second thymine of the lesion, but rarely continued across the lesion [7, 9].

Human Polη was also identified via a search for the homolog of yeast *Saccharomyces cerevisiae Rad30* gene, which encodes an error-free bypass protein [10]. Various XP-V causative mutations have been found in the Polη gene, *hRAD30A*, of XP-V patients [10, 11].

Polι (*RAD30B*) is the other mammalian homolog of yeast Polη, isolated by a similar approach [12]. In contrast to Polη, Polι is less efficient and less accurate [13].

Polκ was obtained by cloning of a human homolog of the *E. coli dinB* gene, encoding DNA Pol IV [14]. Polκ was reported to be unable to bypass either CPD or (6-4)PP [15, 16].

Originally, Rev1, 3, and 7 were cloned from *S. cerevisiae* isolates, in which the frequency of UV-induced reversion from *cyc1* mutations was reduced [17]. Human and mouse homologs (*Rev1, 3, 7* gene) were later isolated [18–20]. Polζ is a comprex of the *Rev3* and *Rev7* gene products, which act as the catalytic and regulatory subunit, respectively. Yeast Polζ is shown to be responsible for 98% of UV-induced base substitutions and 90% of frameshift mutations, in addition to spontaneous mutations [21]. Nonetheless, yeast Polζ itself was revealed to be too faithful to incorporate opposite CPD. Instead, it can efficiently extend from a matched or mismatched 3′-end (reviewed in [5, 6]). Human or mouse Polζ is assumed to have similar enzymatic properties to that of yeast, because several lines of antisense RNA expression or siRNA knockdown experiments in human or mouse cells have proven that Polζ is involved in mutagenic TLS [22–24].

Yeast and human Rev1 encode highly specialized DNA polymerases that preferentially insert a C residue opposite an abasic site in the template. This deoxycytidyl (dC) transferase activity is, however, unlikely to be required for UV-TLS, judging by observation in a yeast *rev1-1* mutant strain [25], which retains much of its dC transferase activity, but has a missense mutation (G193R) in the N-terminal BRCT domain. Rev1 protein also contains ubiquitin-binding motifs (UBMs) that interact with monoubiquitinated PCNA (a DNA polymerase sliding clamp) [26]. In the downstream C-terminal region, Rev1 interacts not only with Rev7 but also with other bypass-polymerases [27], suggesting that Rev1 acts as a mediator and physical bridges between PCNA and Polζ.

Following DNA damage, such as that caused by UV and MMS, monoubiquitins are conjugated to PCNA arrested at the lesion-site by RAD6/RAD18 and recruit bypass-polymerases [28, 29]. In addition to Rev1, Pols η, ι, and κ possess UBMs and physically interact with PCNA [1, 5, 6]. Stalled replicative Polδ is replaced, in turn, with one of these bypass polymerases bound on the PCNA by yet unknown "polymerase switching" mechanisms (reviewed in [1, 30]).

Translesion replication is typically detected with an alkaline sucrose density gradient centrifugation (ASDG) technique. Pulse-labelled replication products are smaller in UV-irradiated XP-V cells than in unirradiated cells; however, on prolonged incubation, the replication products in the irradiated cells eventually attain a high molecular weight similar to that in unirradiated cells. This conversion is interpreted that DNA synthesis is temporarily retarded by UV photoproducts, and then continues beyond the lesion, leaving a gap that is subsequently sealed [31]. The initial size of the newly synthesized DNA is approximately equal to the average distance between lesions in the template strands [32]. This means the gaps in the newly synthesized DNA are opposite the photoproducts [33]. Therefore, sealing of the gaps, by translesion or other postreplication repair mechanisms, can be observed by monitoring the molecular weight of labelled DNA.

Using a modified ASDG technique [34], we precisely detected the elongation of pulse-labelled replication products in the irradiated XP-V cells, showing that UV-TLS is delayed in the cells, but not completely abolished [35]. The marginal TLS is markedly prevented by caffeine at millimolar concentrations, as Lehmann et al. pointed out [31], and by proteasome inhibitors as well (unpublished results). In contrast, these agents do not retard UV-TLS in normal diploid cells. To know more about the inefficient polymerase(s) *in vivo*, we added specific DNA polymerase inhibitors. Butylphenyldeoxyguanosine (BuPGdR) inhibited TLS in XP-V cells [35], suggesting that Polζ may be involved in this Polη-independent bypass.

We recently reported that caffeine or proteasome inhibitors inhibit UV-TLS also in human cancer cells [36], and that, similar to XP-V cells, UV-TLS was much slower than in normal cells. These results suggested that UV bypass in cancer cells is predominantly of the Polη-independent type. Therefore, we expected that Polζ plays a major part in UV-TLS in cancer cells. Although Polη exists in normal quantity in these cells, it was supposed to be inactivated by some reasons. We explored these hypotheses here.

2. Materials and Methods

2.1. Design of siRNAs. All siRNAs duplexes were synthesized by JBioS (Japan) (Table 1). We designed the sequences for siRNA according to the JBioS guide (http://www.JBioS.co.jp/RNAiselect.htm). Selection of target sequence for each siRNA proceeds as follows: first, an AAG or AAC sequence is found at least 75 nucleotides (nt) downstream from the start codon; the AAG (or AAC) and the following 18 nt sequence

Table 1: siRNAs used.

siRNA	Sense strand [a],[b]	Antisense strand [a],[b]	Prevention of UV-TLS (result)
siRev3-A	gcuuuacaugagauacaaaTT	uuuguaucucauguaaagcTT	significant
siRev3-B	gacaguuuucagucaagauTT	aucuugacugaaaacugucTT	significant
siRev3-C	gagguaugauccugauauuTT	aauaucaggaucauaccucTT	significant
siRev3-D	guauugacuuaugucggauTT	auccgacauaagucaauacTT	significant
siRev3cont-A (6nt mismatches)	g***g****u*uugag***u****a*augu***a****c*gauTT	auc***g****u*acau***u****a*cucaa***a****c*cTT	no effect
siRev3cont-B (4nt mismatches)	gg*a*uugag***u****u*augu***a****c*gauTT	auc***g****u*acauaa*c*ucaau*c*cTT	no effect
siRev3cont-C (2nt mismatches)	guauugag***u****u*auguc*c*gauTT	auc*g*gacauaa*c*ucaauacTT	no effect
siRev7-A	gauccaggucaucaaggauTT	auccuugaugaccuggaucTT	partial
siRev7-B	gaugcagcuuuacguggaaTT	uuccacguaaagcugcaucTT	significant
siRev7-C	cacugucugucucaaauacTT	guauuugagacagacagugTT	significant
siRev7cont-A	gaugcag*g*uuuacguc*g*aaTT	uuc*g*acguaaaccugcaucTT	no effect
siRev1-A	cacauauuauugccacaaaTT	uuuguggcaauaauaugugTT	significant
siRev1-B	gaagauugaaacggaaaauTT	auuuuccguuucaaucuucTT	significant
siRev1-C	ccuucagacugcaauuuuaTT	uaaaauugcagucugaaggTT	significant
siRev1-D	gugugaauugacugaguuuTT	aaacucagucaauucacacTT	significant
siRev1cont-E	ccuucacac*c*gcaa*c*guuaTT	uaa*c*guugc*g*gugugaaggTT	little to no effect
siPolη-4	uaaaccuugugcaguuguaTT	uacaacugcacaagguuuaTT	partial
siPolη-A	gaaguuauguccagaucuuTT	aagaucuggacauaacuucTT	significant
siPolη-B	gcuucgcuuucaucucuuaTT	uaagagaugaaagcgaagcTT	significant
siPolηcont-A	uaaacc*u*g*g*ugcagu*c*guaTT	uac*g*acugcac*c*agguuuaTT	no effect
siPolι-A	gccucauacagugagauuaTT	uaaucucacuguaugaggcTT	no effect
siPolι-5	aaguguccacaguugguauTT	auaccaacuguggacacuuTT	no effect
siPolκ-A	gagaaaauuaacaaaauuaTT	uaauuuuguuaauuuucucTT	partial
siPolκ-B	gaauaaaccaaauggacaaTT	uuguccauuugguuuauucTT	partial
siPolκ-C	cuguuaccauuaaguugaaTT	uucaacuuaaugguaacagTT	considerable
siPolκcont-B	gagaaaa*g*uaacaa*g*cuuaTT	uaa*g*cuuguua*c*uuuucucTT	little to no effect

[a]Small letters mean ribonucleotides, and large letters mean deoxyribonucleotides.
[b]Mismatched bases are illustrated in bold and italic letters.

(N18) are picked up. Ideally, the 2 nt following the N18 are TT, TN, NT, or AA. GC content of N18 is recommended to be about 40% to 50%. If GC content is less than 30%, or greater than 70%, avoid the sequence. The N18 had better be AT-rich in the 3′ half and especially at the 3′ end (2 nt).

2.2. HeLa Cell Culture and siRNA Treatment. HeLa cells were maintained in monolayers in Dulbecco's modified Eagle's medium (D'MEM) supplemented with 10% fetal calf serum (FCS) ("normal" medium), trypsinized and seeded into culture dishes (2×10^5 cells/ϕ 60 mm dish). About 6–8 hours later, the cells were treated with micelles of siRNA and OligofectAMINE (Invitrogen), formed according to the manufacturer's protocol, except Opti-MEM was replaced with D'MEM. The siRNA concentration used in RT-PCR analysis and western blot analysis was 5 nM.

2.3. UV Irradiation and Translesion Replication. Forty hours after siRNA addition, HeLa cells were exposed to UV light (10 J/m^2) from a germicidal lamp (Toshiba GL15) at 0.6 J/m^2 per s. After 30 minutes in culture, the medium was changed to labeling medium consisting of D'MEM supplemented with 10% FCS and 10 μCi/mL of [*U*-^{14}C]thymidine (Moravek MC267, 470 mCi/mmol). UV-irradiated cells were pulse-labelled for 1 hour, while nonirradiated cells were labelled for 30 minutes. The medium was changed to normal medium, and the cells were chased for 5 hours. These cells were harvested by trypsinisation and examined by ASDG [34].

2.4. Alkaline Sucrose Density Gradient Centrifugation (ASDG). Cells (about 1×10^5 in 50 μL PBS) were gently layered onto 50 μL of 1% sucrose in PBS, which was overlaid on 100 μL of lysis solution (0.6 M KOH, 2.0 M KCl, 10 mM EDTA, and 1% *N*-lauroylsarcosine), which was placed on top of a 4.35 mL alkaline 5–20% sucrose gradient (0.3 M KOH, 2.0 M KCl, 1 mM EDTA, and 0.1% *N*-lauroylsarcosine) with 0.4 mL of alkaline 80% sucrose as a cushion at the bottom. The gradient was centrifuged at 6,000 rpm ($4,320 \times$ g) for 15.6 hours at 15°C in a Beckman SW 50.1 rotor. The gradient was fractionated onto 30 circles of no. 17 paper

(Whatman). The paper circles were dried, immersed in cold 5% trichloroacetic acid for 10 minutes, washed 3 times with ethanol and once with acetone, and dried; radioactivity was then measured. As a molecular weight marker, [^{14}C]-labelled T4 DNA phage particles were placed on the lysis layer and sedimented in a parallel run. The approximate fragment length of each fraction was estimated on the basis of the position of the T4 DNA marker and that of *E. coli* DNA and adjusted by sucrose density curve [34]. Average fragment length (in megabase(Mb)) of each profile is shown in Figures as fragment length of the median fraction [35]. (Median fraction is the middle fraction that separates the higher half of the profile from the lower half.)

2.5. RT-PCR. Total RNA was isolated using an RNeasy spin column (QIAGEN). One μg of total RNA was treated with DNase I (Invitrogen), reverse-transcribed using SuperScript II (Invitrogen) with random hexamers or PrimeScript II (TaKaRa) with oligo(dT) primers, followed by treatment with *E. coli* RNase H (Invitrogen). The PCR mixture contained cDNA templates, dNTPs, rTaq Pol (TOYOBO), anti-Taq (TOYOBO), and appropriate primers. Primers to assess knockdown of each gene were as follows: Rev3, 5′-GGAACGTCAACAGGAGCAAC-3′ and 5′-GGAGCAAATCCAACACCTGC-3′; Rev7, 5′-TGCTGTCCATCAGCTCAGAC-3′ and 5′-AGAGCACTTGGAATCAGGGC-3′; Rev1, 5′-CTCCTGCAGAGAAACCCCTG-3′ and 5′-ACAAGCACTTATGGCACAGCT-3′; Polη, 5′-CCCAGGCAACTACCCAAAAC-3′ and 5′-GGGCTCAGTTCCTGTACTTTG-3′; Polι, 5′-ATGATCAAGTGTTGCCCACAC-3′ and 5′-ACATGACCCGACACAGTCAC-3′; Polκ, 5′-AGACAAGAATACCGCCAGCC-3′ and 5′-AGGAAGGATTATTGCACTTGCC-3′; GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. All PCR reactions were carried out for 27 cycles, with the exception of GAPDH (25 cycles). PCR products were subjected to a MultiNA microtip electrophoresis DNA/RNA analyzer (Shimadzu Biotech).

2.6. Western Blotting. Cells were rinsed with PBS, lysed with 1 × SDS-PAGE loading buffer and collected by scraping. Cell lysate was boiled for 10 minutes, sonicated for 30 seconds and centrifuged at 20,000 × g for 10 minutes. The supernatant was used for western blotting after measuring protein content by Bradford method. The supernatant (60 μg protein) was separated on 5–20% (Rev7, Rev1 and Polι) or 3–10% (the rest) SDS-polyacrylamide gels (Wako) and transferred onto Immobilon-P^{SQ} membranes (Millipore). The membranes were incubated with antibodies to bypass-polymerases (Rev1, Santa Cruz Biotechnology sc-48806; Rev7, BD Transduction Laboratories 612266; Polη, Santa Cruz Biotechnology sc-17770; Polι, Abnova H00011201-M01; Polκ, a kind gift from Dr. Tomoo Ogi, Nagasaki University), and then with horseradish peroxidase-conjugated secondary antibodies (anti mouse IgG, DAKO PO-0447; anti-rabit IgG, DAKO PO-0448). Antibodies were diluted with CanGetSignal (TOYOBO) to enhance immunoreactivity. The signals were developed with ECL-Plus (GE Healthcare), and blots were stripped for reprobing with anti α-tubulin antibody (Sigma T5168).

3. Results

3.1. Effect of siRNAs to Rev3 or Rev7 on HeLa UV-TLS. We selected 4 target sites from the human Rev3 mRNA sequence (NCBI locus: NM_002912), according to the JBioS guide. All the Rev3 siRNAs (Table 1) effectively knocked down expression of *Rev3* (Figure 1(a)) and, at 5 nM, abolished UV-TLS in HeLa cells (Figure 1(b)). Replication products immediately after UV irradiation were sedimented as a sharp peak, illustrated by a thin line in Figure 1(b), slightly larger in size than the T4 phage DNA marker. When only Oligofectamine-treated cells were chased in normal medium for 5 hours, the products joined to form larger DNA with lengths in the order of megabases (Mb), illustrated by a thick line. In Rev3 siRNA-transfected cells, the products remain in smaller size, as depicted by a thin line with open marks, demonstrating these siRNAs prevent UV-TLS (Figure 1(b)).

We assessed how many mismatched nucleotides (nt) are necessary at minimum for the negative control siRNA (Figure 1(c)) and found that siRev3cont-C, designed from siRev3-D with 2 nt mismatches, did not prevent UV-TLS, indicating that these Rev3 siRNAs degrade Rev3 mRNA with high specificity. The dose-response profile of siRev3-D, shows that only 1 nM siRNA sufficiently inhibited UV-TLS (Figure 1(d)). The siRev3-D siRNA had no effect on normal replication (Figure 1(e)).

We selected 3 target sites from the human Rev7 mRNA sequence (NM_006341) (Table 1), and found that all siRNAs effectively reduced *Rev7* expression (Figure 2(a)); 5 nM siRev7-B or siRev7-C completely abolished UV-TLS (siRev7-A elicited only partial prevention) (Figure 2(b)). The siRev7cont-A, designed from siRev7-B with 2 nt mismatches, had no effect (ibid). The dose-response profile of siRev7-B shows that 1 nM siRNA was sufficient to inhibit UV-TLS (Figure 2(c)); Rev7 siRNAs had no effect on normal replication (Figure 2(d)).

3.2. SiRNAs to Rev1 Significantly Abrogated UV-TLS. For knockdown of *Rev1* expression, we selected 4 sites from the human Rev1 mRNA sequence (NM_016316) (Table 1) (Figure 3(a)). The siRNAs (5 nM) targeted to these sites abolished UV-TLS in HeLa cells (Figure 3(b)). The siRev1cont-E, designed from siRev1-C with 4 nt mismatches, had little to no effect. The dose-response profile of siRev1-C shows that 1 nM of the siRNA was enough to inhibit UV-TLS (Figure 3(c)); siRev1-C or siRev1-D siRNA had no effect on normal replication (Figure 3(d)).

3.3. SiRNAs to Polη Prevented UV-TLS to a Great Extent. First, we tested the siRNA described by Choi and Pfeifer [37], siPolη-4, which partially inhibited UV-TLS (Figure 4(a)). The negative control, siPolηcont-A, was designed from siPolη-4 with 2 nt mismatches, had no effect (ibid). We added 2 Polη siRNAs of new design (human Polη mRNA sequence: NM_006502) (Table 1), and these siRNAs also effectively

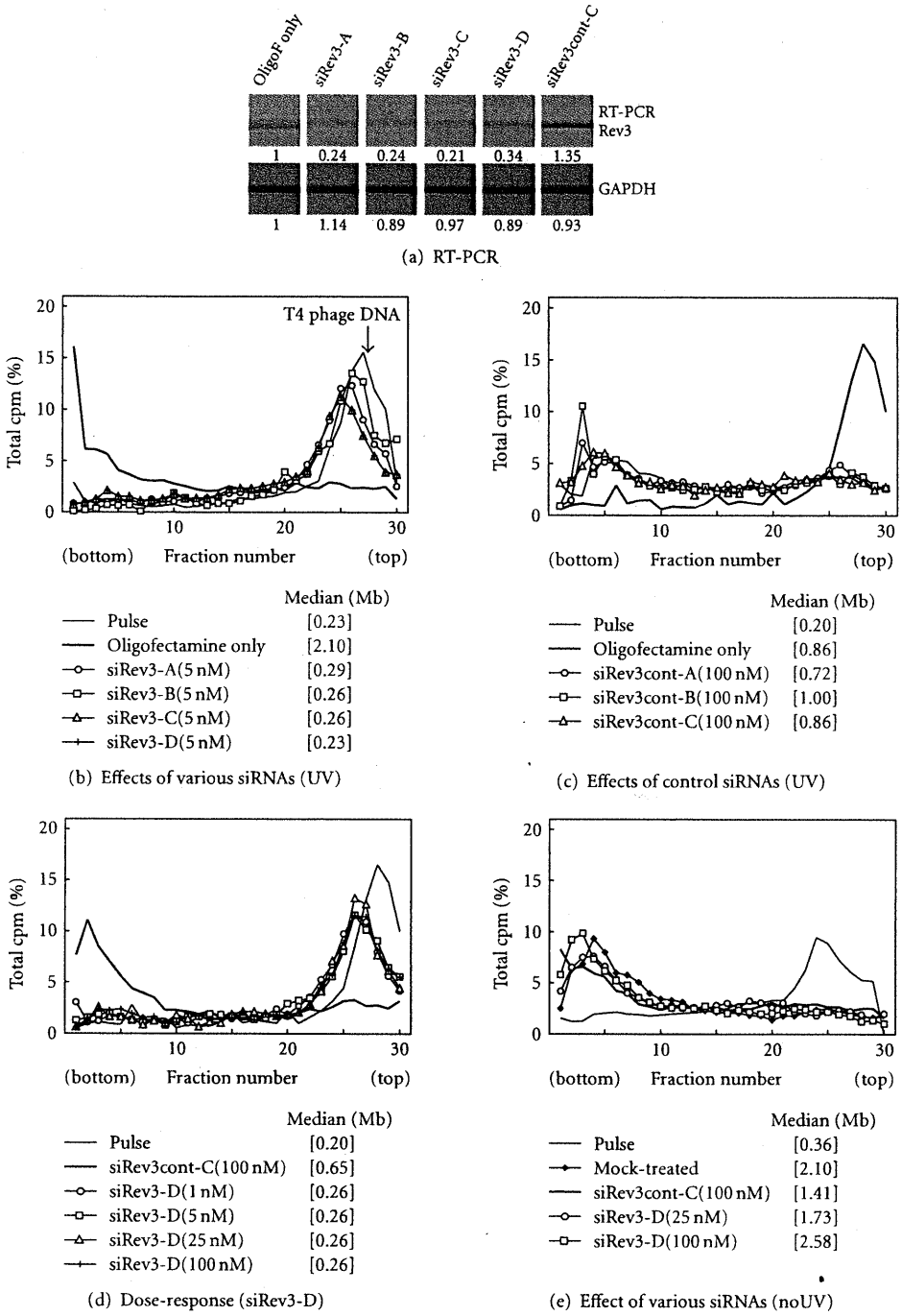

FIGURE 1: Efficient knockdown by Rev3 siRNAs and their effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on Rev3 expression (RT-PCR analysis); (b) Effects of various Rev3 siRNAs; (c) Effects of Rev3 control siRNAs; (d) Dose-response of siRev3-D; (e) Effect of various Rev3 siRNAs (no UV control). Twenty-four hours after Rev3 siRNA transfection, total RNA was isolated and Rev3 RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after Rev3 siRNA transfection, cells were UV-irradiated (10 J/m^2), incubated in normal medium for 30 minutes, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 1 hour, washed twice with PBS, and incubated for 5 hours at 37°C in normal medium (b, c, d). siRev3cont-A, 6 nt mismatches; siRev3cont-B, 4 nt mismatches; siRev3cont-C, 2 nt mismatches (c). Forty hours after Rev3 siRNA transfection, cells were not UV-irradiated, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 30 minutes, washed twice with PBS, and incubated at 37°C in normal medium for 1 hour (e). Some of these profiles overlap (c, d, e). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately 5.5 × 10^7 Da/single strand). Labelled *E.coli* DNA (approximately 4 Mb) sedimented near the bottom (fractions 3–6) (4). Average fragment length (in Mb) of each profile is shown in square brackets as fragment length of the median fraction.

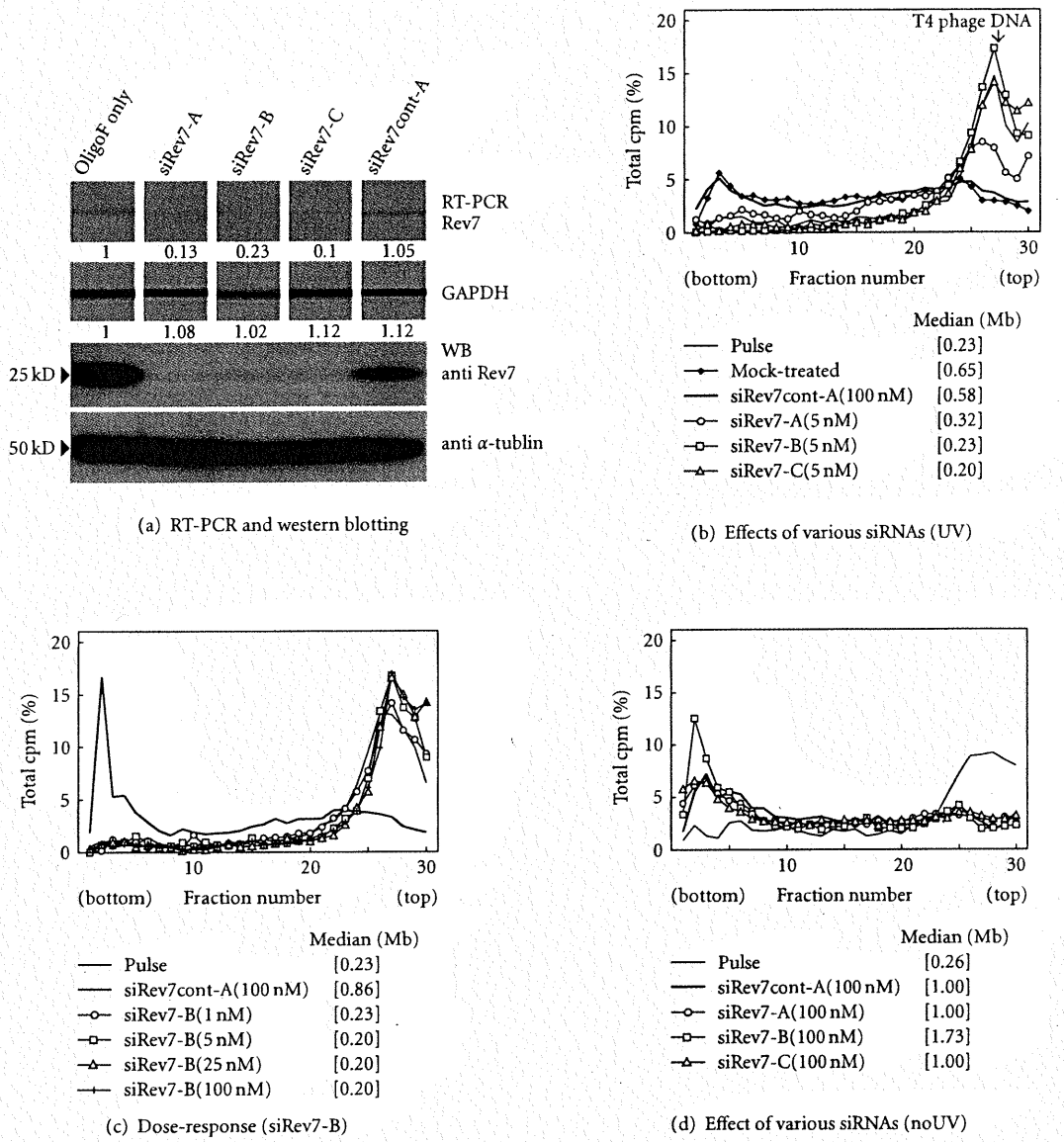

FIGURE 2: Efficient knockdown by Rev7 siRNAs and their effects on UV-induced TLS in HeLa cells (ASDG profiles of replication products). (a) Efficiency of knockdown on Rev7 expression (RT-PCR analysis and western blot analysis); (b) Effects of various Rev7 siRNAs; (c) Dose-response of siRev7-B; (d) Effect of various Rev7 siRNAs (no UV control). Twenty-four hours after Rev7 siRNA transfection, total RNA was isolated and Rev7 RNA was quantified by RT-PCR. Results were shown in MultiNA gel images and the expression level was presented under the panel (a). Forty hours after Rev7 siRNA transfection, whole cell extracts were prepared and Rev7 protein was quantified by western blot analysis (a). Forty hours after Rev7 siRNA transfection, cells were UV-irradiated (10 J/m^2), incubated in normal medium for 30 minutes, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 1 hour, washed twice with PBS, and incubated for 5 hours at 37°C in normal medium (b, c). Forty hours after Rev7 siRNA transfection, cells were not UV-irradiated, pulse-labelled with 10 μCi/mL of [^{14}C]thymidine for 30 minutes, washed twice with PBS, and incubated at 37°C in normal medium for 1 hour (d). Some of these profiles overlap (c, d). Sedimentation is from right to left. The arrow indicates the position of T4 phage DNA (166 kb, i.e., approximately 5.5 × 10^7 Da/single strand). Average fragment length (in Mb) of each profile is shown in square brackets.

knocked down Polη expression (Figure 4(b)). These latter siRNAs at 5 nM abolished UV-TLS to a greater extent than siPolη-4 (Figure 4(c)). The Polη siRNAs had no effect on normal replication (Figure 4(d)).

3.4. Polκ siRNAs Delayed UV-TLS, While siRNAs to Polι Did Not. Although Polι siRNAs efficiently prevented Polι expression (Figure 5(a)), we could not detect these effects on ASDG profiles (Figure 5(b)). The siPolι-5 was reported by