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Polymorphisms in the Ghrelin Gene Are Associated with Serum High-Density Lipoprotein Cholesterol Level and not with Type 2 Diabetes Mellitus in Koreans

Hyung Jin Choi, Young Min Cho, Min Kyong Moon, Hye Hun Choi, Hyoung Doo Shin, Hak Chul Jang, Seong Yeon Kim, Hong Kyu Lee, and Kyong Soo Park

Department of Internal Medicine (H.J.C., Y.M.C., M.K.M., H.C.J., S.Y.K., H.K.L., K.S.P.), Seoul National University College of Medicine, Seoul 110-744, Korea; Genome Research Center for Diabetes and Endocrine Disease (Y.M.C., M.K.M., H.H.C., H.C.J., S.Y.K., H.K.L., K.S.P.), Clinical Research Institute, Seoul National University Hospital, Seoul 110-744, Korea; and Department of Genetic Epidemiology (H.D.S.), SNP Genetics, Inc., Seoul 153-803, Korea

Context: Ghrelin is known to play a role in glucose metabolism and in β-cell function. There are controversies regarding the role of *ghrelin* polymorphisms in diabetes and diabetes-related phenotypes.

Objective: The objective of this study was to examine polymorphisms of the *ghrelin* gene in a Korean cohort and investigate associations between them and susceptibility to type 2 diabetes and its related phenotypes.

Design and Patients: The *ghrelin* gene was sequenced to identify polymorphisms in 24 DNA samples. Common variants were then genotyped in 760 type 2 diabetic patients and 641 nondiabetic subjects. Genetic associations with diabetes-related phenotypes were also analyzed.

Results: Nine polymorphisms were identified, and four common polymorphisms [g.−1500C>G, g.−1062G>C, g.−994C>T, g.+408C>A (Leu72Met)] were genotyped in a larger study. The genotype distributions of these four common polymorphisms in type 2 diabetes patients were similar to those of normal nondiabetic controls. However, these four common polymorphisms were variably associated with several diabetes-related phenotypes, such as high-density lipoprotein (HDL) cholesterol, fasting plasma glucose, and homeostasis model assessment of insulin resistance. In particular, subjects harboring g.−1062C were associated with a lower serum HDL cholesterol level after adjusting for other variables ($P = 0.0004$ or 0.01 after Bonferroni correction for 24 tests).

Conclusion: The aforementioned four common polymorphisms in the *ghrelin* gene were not found to be significantly associated with susceptibility to type 2 diabetes mellitus in the Korean population. However, the common polymorphism g.−1062G>C in the promoter region of the *ghrelin* gene was found to be significantly associated with serum HDL cholesterol levels. **(*J Clin Endocrinol Metab* 91: 4657–4663, 2006)**

GHRELIN WAS ONLY recently discovered. It is a 28-amino-acid peptide and a ligand of growth hormone secretagogue receptor (1). Ghrelin is an orexigenic peptide and is predominantly produced by the stomach, and has been reported to stimulate appetite and induce body weight gain and adipogenesis (2–4). Clinically, high ghrelin levels have been suggested to cause the severe hyperphagic obesity found in Prader-Willi syndrome (5). One case report was issued on malignant gastric ghrelinoma with hyperghrelinemia accompanied by severe obesity (6). Thus, accumulating evidence suggests that ghrelin is a key regulator of body weight (3).

Ghrelin and its receptor are present in the pancreatic islets of rodents and humans (7, 8). Ghrelin was found to stimulate insulin secretion in rodents (7, 9), and in human studies, ghrelin was found to inhibit insulin secretion directly and to stimulate basal glucose levels (10). Moreover, considering the prominent secretion of ghrelin in the fasting state, it has been suggested that ghrelin integrates hormonal and metabolic responses to stabilize fasting state glucose level by directly stimulating glycogenolysis and inhibiting insulin secretion, in combination with stimulating growth hormone secretion (10). In addition, many studies have reported a clear inverse association between plasma ghrelin and fasting insulin levels. In particular, low ghrelin levels were independently associated with higher levels of fasting plasma insulin, which is an indicator of underlying insulin resistance and a characteristic of type 2 diabetes (11–13).

Several genetic studies have addressed the role of *ghrelin* polymorphisms. Some of these studies resulted in the identification of various polymorphisms in the *ghrelin* gene and found that they are associated with obesity and obesity-related phenotypes (14–18), whereas others have demonstrated a positive genetic association between various *ghrelin* polymorphisms and type 2 diabetes or diabetes-related phenotypes (13, 17, 19). It has also been reported that *ghrelin* polymorphisms are positively associated with hypertension (13, 19), and a recent study suggested that mutations in the *ghrelin* gene may confer a risk of metabolic syndrome (20). However, other studies have reported negative associations (21–23). Therefore, at present, there is much debate regarding the roles of *ghrelin* polymorphisms in obesity, diabetes, and

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Abbreviations: BMI, Body mass index; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LD, linkage disequilibrium; OR, odds ratio; SNP, single nucleotide polymorphism.

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metabolic syndrome. Discordant findings are probably explained by different ethnic and environmental factors and by our lack of understanding of the underlying molecular complexities. In this study, we examined the association between common polymorphisms of the *ghrelin* gene and type 2 diabetes and with diabetes-related phenotypes in the Korean population.

Subjects and Methods

Subjects

For initial sequencing, 24 Koreans were randomly selected from unrelated local residents without any history of familial diseases. Given 24 samples (48 chromosomes), one can expect to identify more than 90% of polymorphisms with a frequency greater than 0.05 (24). A total of 760 unrelated patients with type 2 diabetes registered at the Diabetes Clinic of Seoul National University Hospital (age: 59 ± 10 yr, 354 men, 406 women) and 641 nondiabetic control subjects (age: 65 ± 4 yr, 287 men, 354 women) were studied. All subjects enrolled in this study were of Korean ethnicity. Type 2 diabetes was diagnosed using World Health Organization criteria (25). Subjects positive for glutamic acid decarboxylase antibodies were excluded. Nondiabetic control subjects were selected according to the following criteria: 60 yr old or older, no history of diabetes, no first-degree relatives with diabetes, a fasting plasma glucose concentration of less than 110 mg/dl and a glycosylated hemoglobin value of less than 5.8%.

The Institutional Review Board of the Clinical Research Institute in Seoul National University Hospital approved the study protocol, and informed consent for genetic analysis was obtained from each subject. All study subjects were examined in the morning after an overnight fast. Height, weight, waist and hip circumferences, and blood pressure were measured. Blood samples were drawn after overnight fasting for biochemical measurements [fasting plasma glucose, fasting plasma insulin, glycosylated hemoglobin, total serum cholesterol, serum triglyceride, and serum high-density lipoprotein (HDL) cholesterol] and DNA analysis. For the measurement of total serum cholesterol, serum triglyceride, and serum HDL cholesterol, 200 FR system (Toshiba Medical Co., Tokyo, Japan) was used.

Identification of polymorphisms of the human ghrelin gene

We sequenced all exons and exon-intron boundaries, including the promoter region (~1.5 kb), to identify polymorphisms in the 24 samples using an ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA). The eight primer sets used for the amplification and sequencing analysis were designed based on GenBank registered sequences (reference genome sequence: NT_007933.14 released on March 2, 2006). Information regarding these primers is available on www.snp-genetics.com. Sequence variants were verified using chromatograms.

Genotyping with fluorescence polarization detection

For genotyping polymorphic sites, amplifying primers and probes were designed for TaqMan (26). Primer Express (Applied Biosystems) was used to design both the PCR primers and the MGB TaqMan probes. One allelic probe was labeled with 6-carboxyfluorescein dye and the other with fluorescent VIC dye. PCRs were run in TaqMan Universal Master mix without uracil DNA glycosylase (Applied Biosystems) at PCR primer concentrations of 900 nM and a TaqMan MGB-probe concentration of 200 nM. Reactions were performed in 384-well format in a total reaction volume of 5 μl using 20 ng of genomic DNA. The plates then were placed in a thermal cycler (PE 9700, Applied Biosystems) and heated at 50 C for 2 min and then 95 C for 10 min and then subjected to 40 amplification cycles (95 C for 15 sec; 60 C for 1 min). The TaqMan assay plates were then transferred to a Prism 7900HT instrument (Applied Biosystems), and fluorescence intensities of each well were measured. Fluorescence data files from each plate were analyzed using automated software (SDS 2.1).

EMSA

Nuclear proteins were prepared from C2C12 mouse myoblast cell line. Double-stranded oligonucleotides for the ghrelin gene containing g.−1062C (TCTGCTCAACCGGCAGGTGGGACTCCT) were prepared using the Klenow fragment of DNA polymerase (Ambion, Austin, TX). The [α-^{32}P]ATP-labeled probe (25,000 cpm) was incubated for 15 min at room temperature with nuclear proteins (10 μg) in 10 mmol/liter HEPES, pH 7.9, 50 mmol/liter KCl, 0.1 mmol/liter EDTA, 0.25 mmol/liter dithiothreitol, 0.1 mg/ml poly(dIdC), 0.01% Nonidet P-40, and 10% glycerol. Antibody against myoD (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with nuclear proteins for 15 min on ice before the addition of the labeled probe. Competitor oligonucleotides were added at 100- or 200-fold molar excesses. The sequence of competitor g.−1062G is TCTGCTCAACCGGGAGGTGGGACTCCT. Reaction mixtures were loaded on 6% acrylamide gel and subjected to electrophoresis. Complexes were visualized by exposure to x-ray films (Kodak, Rochester, NY).

Transient transfection and reporter assay

C2C12 cells were cultured in DMEM supplemented with 10% FBS. The cells were transfected in 12-well plates using Lipofectamine Plus (Invitrogen, Carlsbad, CA). The cells were transfected with 0.6 μg of the reporter plasmids and 0.15 μg of pCMV-β-galactosidase. The cells were harvested 40 h after transfection, and luciferase assays were performed using the luciferase assay system (Promega, Madison, WI). A DNA fragment representing the region between the −1600 and −834 bp of the ghrelin gene was inserted into the region upstream of the luciferase coding sequences of the pGL2-basic vector. Each construct containing g.−1062G or g.−1062C is referred to as ghrelin (−1062G) Luc or ghrelin (−1062C) Luc, respectively. One of the reporter constructs, pGL2-basic, ghrelin (−1062G) Luc or ghrelin (−1062C) Luc, was transfected into C2C12 myoblast cells. The β-galactosidase activity was measured to normalize any variations in the transfection efficiency.

Statistical analyses

All data were analyzed using SPSS for Windows (SPSS Inc., Chicago, IL). Variables not normally distributed were logarithmically transformed before statistical analysis. The χ^2 test was used to determine whether individual polymorphisms were in Hardy-Weinberg equilibrium. Logistic regression analysis was used to calculate odds ratios (ORs), 95% confidence intervals, and corresponding *P* values, after controlling for age, sex, and body mass index (BMI) as covariates. Genotypes were given 0, 1, and 2 as codes in the additive model. In the additive model, OR was expressed per number of rare alleles. Multiple regressions adjusted for age, sex, BMI, fasting plasma glucose, fasting plasma insulin, triglyceride, and HDL cholesterol were used for association analyses with diabetes-related phenotypes. We examined Lewontin's D′ (D′) and the linkage disequilibrium (LD) coefficient r^2 between all pairs of biallelic loci (27). Haplotype associations were also estimated using the Haplo.Score program (www.biostat.wustl.edu/genetics/geneticssoft), which tests associations between haplotypes and a wide variety of traits, including binary, ordinal, quantitative, and Poisson (28). This software provides several haplotype-specific tests for determining associations, allows adjustment for nongenetic covariates, and computes simulation *P* values, based on the assumption that subjects are unrelated and that haplotypes are ambiguous due to unknown linkage phases of the genetic markers. *P* values of <0.05 were considered statistically significant. Additional statistic analyses using Bonferroni correction were applied to adjust for multiple comparisons.

Results

Identification of polymorphisms in the ghrelin gene

By sequencing all exons, including exon-intron boundaries and promoter region, nine single nucleotide polymorphisms (SNPs) were identified in the *ghrelin* gene (Fig. 1). Seven were located in the promoter region: g.−1500C>G (rs3755777), g.−1385C>T (novel), g.−1062G>C (rs26311), g.−994C>T (rs26312), g.−887C>T (rs4684678), g.−678C>T (novel), and

A Map of *ghrelin* on chromosome 3p26-p25

B Haplotypes in *ghrelin*

Hap.	*g.-1500C>G*	*g.-1062G>C*	*g.-994C>T*	*g.+408C>A*	Freq.
ht1	C	G	C	C	0.496
ht2	G	C	T	C	0.189
ht3	G	C	T	A	0.165
ht4	G	G	C	C	0.100
Others	-	-	-	-	0.050

C LDs among *ghrelin* SNPs

		|D'|			
	SNPs	*g.-1500C>G*	*g.-1062G>C*	*g.-994C>T*	*g.+408C>A*
r^2	*g.-1500C>G*	-	0.978	0.984	0.916
	g.-1062G>C	0.578	-	0.939	0.905
	g.-994C>T	0.605	0.852	-	0.897
	g.+408C>A	0.182	0.294	0.279	-

FIG. 1. Gene map and haplotypes of the *ghrelin* gene identified in 24 Korean DNA samples. A, Polymorphisms identified in *ghrelin* gene. Coding exons are marked with *shaded blocks*, and 5′ and 3′ untranslated regions are marked with *white blocks*. *Asterisks* indicate SNPs that were genotyped in the larger population (n = 1401). The frequencies of SNPs without larger-scale genotyping are based on sequencing data (n = 24). The first base in the transcription start site is denoted as nucleotide +1. B, Haplotypes of *ghrelin* gene in Koreans. "Others" category contains rare haplotypes: GGTC, GCCC, CGCA, GGCA, CCTC, and CCCC. C, LD coefficients (D′ and r^2) among SNPs in ghrelin.

g.−604A>G (rs27647). One was located in exon 2: g.+408C>A encoding Leu72Met (rs696217), and one SNP was located in an exon-intron boundary: g+3615G>T (rs35683). Of these identified polymorphisms, four polymorphic sites [g.−1500C>G, g.−1062G>C, g.−994C>T, g.+408C>A (Leu72Met)] were selected for larger-scale genotyping based on location (SNPs in the exons and the promoter region were preferred), LD (only one SNP, if there are absolute LDs), frequency (>0.10), and haplotype tagging status. The genotype distributions of these four loci in the *ghrelin* gene were in Hardy-Weinberg equilibrium ($P > 0.05$).

Association with type 2 diabetes

We analyzed for associations between each genotype and susceptibility to type 2 diabetes by logistic regression analyses, after adjusting for age, sex, and BMI. The genotype distributions of these four SNPs [g.−1500C>G, g.−1062G>C, g.−994C>T, g.+408C>A (Leu72Met)] among the type 2 diabetic patients were similar to those among control subjects (Table 1). Four common haplotypes (frequency > 0.10) were found to include these four SNPs; they accounted for 95.1% of the observed haplotypes. The distributions of these four haplotypes among the type 2 diabetic patients were similar to those of the control subjects (Table 2).

Association with diabetes-related phenotypes

Because treatment for diabetes might affect diabetes-related phenotypes, only control subject data were used for association analyses with diabetes-related phenotypes. Associations were determined by multiple linear regression with or without adjusting for multiple covariates (age, sex, BMI, fasting plasma glucose, fasting plasma insulin, triglyceride, and HDL cholesterol) (Table 3). Fasting plasma glucose was found to be associated with g.−1500G ($P = 0.002$), g.−1062C ($P = 0.007$), and g.−994T ($P = 0.005$); homeostasis model assessment of insulin resistance (HOMA-IR) was associated with g.−1062C ($P = 0.04$); HDL cholesterol was associated with g.−1500G ($P = 0.003$), g.−1062C ($P = 0.00003$), g.−994T ($P = 0.0005$), and g.408A ($P = 0.01$) after adjusting for age, sex, and BMI. Additional multiple regressions adjusted for multiple variables showed that these associations regarding fasting plasma glucose and HDL cholesterol were independent of all other factors. However, after applying the Bonferroni correction for multiple comparisons correction and adjusting for multiple variables, only the association between the g.−1062G>C polymorphism and HDL cholesterol remained significant ($P = 0.01$ after Bonferroni correction for 24 tests). Additional analyses on haplotypes produced similar results regarding HDL cholesterol and insulin resistance (Table 4).

TABLE 1. Logistic analysis of *ghrelin* polymorphisms with respect to the risk of type 2 diabetes among patients and normal subjects

Loci	Genotype	Frequency		Additive model	
		Patients	Controls	OR (95% CI)	*P*
g.−1500C>G	CC	199 (26.2%)	167 (26.1%)		
	CG	377 (49.6%)	316 (49.3%)	1.02 (0.87–1.20)	0.778
	GG	184 (24.2%)	158 (24.6%)		
g.−1062G>C	GG	306 (40.4%)	257 (40.2%)		
	CG	349 (46.0%)	289 (45.2%)	1.00 (0.85–1.18)	0.984
	CC	103 (13.6%)	94 (14.7%)		
g.−994C>T	CC	300 (39.5%)	235 (36.8%)		
	CT	352 (46.3%)	306 (47.9%)	0.94 (0.80–1.11)	0.492
	TT	108 (14.2%)	98 (15.3%)		
g.+408C>A	CC	518 (68.3%)	429 (67.5%)		
	AC	215 (28.4%)	185 (29.1%)	0.96 (0.78–1.19)	0.727
	AA	25 (3.3%)	22 (3.5%)		

Genotype distributions, OR (95% confidence interval), and *P* values were obtained by logistic analyses using an additive model after controlling for age, sex, and BMI as covariates. *P* values were not adjusted for multiple comparisons.

Identification of transcription factor binding to the region around g.−1062C of the ghrelin gene

MatInspector analysis (29) provided several candidate transcription factors that prefer g.−1062C to g.−1062G as their binding site, such as c-Myb, MyoD/E47 dimer, and MyoD/E12 dimer. To determine whether there is a specific nuclear protein binding on g.−1062C, we did an EMSA using oligonucleotides containing g.−1062C as probes. As shown in Fig. 2A, cold oligonucleotides containing g.−1062C (self competitor) but not g.−1062G competed well with the probe for the binding of the transcription factors. As shown in Fig. 2B, a DNA-protein complex (*band 1*) was supershifted upon the addition of an antibody against myoD. Therefore, we concluded that myoD specifically bind to the g.−1062C region of the *ghrelin* gene.

Transactivation of ghrelin promoter activity

To investigate the effect of the g.−1062G>C polymorphism on the ghrelin promoter activities, a proximal region of the ghrelin promoter (from −1600 to −834) was linked to the luciferase reporter gene of the pGL-2 basic vector. The recombinant vectors were transfected into C2C12 cells. As shown in Fig. 3, the promoter activity of ghrelin (−1062G) Luc was 1.7-fold higher than that of ghrelin (−1062C) Luc.

Discussion

The present study shows that the genotype distributions and allele frequencies of the four common *ghrelin* gene SNPs found among type 2 diabetic patients were similar to those of control subjects. Further statistical analysis using PHASE software also showed no association between *ghrelin* polymorphisms and susceptibility to type 2 diabetes. Our present findings do not support the hypothesis that *ghrelin* gene polymorphisms impact the risk of type 2 diabetes. This finding is in accordance with previous results (16, 22, 23). To our knowledge, the study by Poykko *et al.* (19) is the only genetic association study that shows a positive association between *ghrelin* polymorphism and the risk of type 2 diabetes. This previous study reported that the *ghrelin* polymorphism 51Gln (allele frequency 0.022) increased the risk of type 2 diabetes. However, this rare mutation [g.+346G>A (Arg51Gln)] was not detected in the present study, probably due to the design of this study.

Polymorphism g.−1500C>G, g.−1062G>C, and g.−994C>T had high r^2 values (0.578~0.852, Fig. 1C); therefore, the significant associations between these polymorphisms and the phenotypes (lower HDL cholesterol, higher fasting plasma glucose and higher HOMA-IR) might be due to the g.−1062G>C polymorphism, which showed the strongest evidence of association among these three polymorphisms.

Subjects harboring the rare allele g.−1062C had higher fasting plasma glucose levels, higher HOMA-IR values, and lower HDL cholesterol levels. Fasting plasma insulin and triglyceride and BMI trended to be higher in the subjects harboring g.−1062C (Table 3). These data indicate that subjects harboring the rare allele g.−1062C manifest a subset of phenotypes tending toward obesity and metabolic syndrome (low HDL, high insulin resistance, high triglyceride, and high BMI). Because ghrelin has a major role in energy balance and obesity, a significant change in the production rate or in the action of ghrelin could induce obesity and, in turn, alter many components of obesity. In this regard, the present study is in accordance with a recent study (20), which sug-

TABLE 2. Association between haplotypes of *ghrelin* polymorphisms and the risk of type 2 diabetes

Haplotype	Locus				No. (% Frequency)		*P*
	g.−1500	g.−1062	g.−994	g.408	Diabetes	Control	
ht1	C	G	C	C	764 (50.3)	634 (49.5)	0.982
ht2	G	C	T	C	283 (18.6)	238 (18.6)	0.853
ht3	G	C	T	A	249 (16.4)	223 (17.4)	0.618
ht4	G	G	C	C	149 (9.8)	125 (9.8)	0.731

Each haplotype with a frequency of more than 0.05 is shown. *P* values of haplotype associations were calculated using the Haplo.Score algorithm developed by Schaid *et al.* (28), after controlling for age, sex, and BMI as covariates. *P* values were not corrected for multiple comparisons.

TABLE 3. Regression analyses for diabetes related phenotypes *vs. ghrelin* polymorphisms among nondiabetic control subjects

Phenotype	Locus	Without variants (C/C)[a]	With variants (C/R + R/R)[a]	*P*		
				Not adjusted	Adjusted for age, sex, and BMI	Adjusted for multiple variables
BMI (kg/m^2)	*g.−1500C>G*	23.48 ± 3.27	23.56 ± 3.02	0.7919		0.8819
	g.−1062G>C	23.35 ± 3.14	23.67 ± 3.05	0.1946		0.9446
	g.−994C>T	23.36 ± 3.17	23.65 ± 3.04	0.2537		0.9441
	g.+408C>A	23.40 ± 3.03	23.79 ± 3.18	0.1421		0.5780
Fasting plasma glucose (mg/dl)	*g.−1500C>G*	87.6 ± 8.7	90.0 ± 8.8	**0.0019**	**0.0021**	**0.0134**
	g.−1062G>C	88.2 ± 8.7	90.2 ± 8.8	**0.0066**	**0.0069**	0.0523
	g.−994C>T	88.1 ± 8.5	90.1 ± 8.9	**0.0048**	**0.0046**	**0.0358**
	g.+408C>A	89.2 ± 8.7	89.7 ± 9.1	0.5575	0.7162	0.9842
Fasting plasma insulin (μU/ml)	*g.−1500C>G*	6.4 (1.1–68.8)	7.3 (0.8–67.6)	0.1170	0.1702	0.4469
	g.−1062G>C	6.5 (1.1–68.8)	7.4 (0.8–67.6)	**0.0260**	0.0967	0.3599
	g.−994C>T	6.4 (1.1–68.8)	7.4 (0.8–67.6)	**0.0471**	0.1592	0.4963
	g.+408C>A	6.75 (0.8–68.8)	7.4 (1.6–67.6)	0.2972	0.5910	0.7558
HOMA-IR	*g.−1500C>G*	1.42 (0.22–15.28)	1.61 (0.18–15.51)	**0.0452**	0.0658	0.0871
	g.−1062G>C	1.43 (0.22–15.28)	1.66 (0.18–15.51)	**0.0103**	**0.0395**	0.3849
	g.−994C>T	1.42 (0.22–15.28)	1.64 (0.18–15.51)	**0.0191**	0.0681	0.3420
	g.+408C>A	1.51 (0.18–15.28)	1.64 (0.33–15.51)	0.2808	0.5703	0.9652
Triglyceride (mg/dl)	*g.−1500C>G*	111.5 (34–294)	116 (33–530)	0.6221	0.8094	0.5818
	g.−1062G>C	108 (34–447)	117.5 (33–530)	0.1533	0.3382	0.9338
	g.−994C>T	108 (34–447)	117 (33–530)	0.1632	0.3316	0.9337
	g.+408C>A	114 (34–447)	115 (33–530)	0.4768	0.8874	0.3965
HDL cholesterol (mg/dl)	*g.−1500C>G*	47 (26–92)	45 (20–97)	**0.0020**	**0.0027**	**0.0085**
	g.−1062G>C	48 (20–92)	44 (21–97)	**<0.0001**	**<0.0001**	**0.0004**
	g.−994C>T	47 (20–92)	44 (21–97)	**0.0004**	**0.0005**	**0.0043**
	g.+408C>A	46 (20–92)	44 (22–97)	**0.0042**	**0.0112**	**0.0073**

[a] C/C, C/R, and R/R represent homozygotes for common alleles, heterozygotes, and homozygotes for rare alleles, respectively. In case of normal distribution, means ± SD of values are shown, otherwise median (range) values are shown. *P* values with various adjustments are shown [before adjustment; after adjustment for age, sex, and BMI; after adjustment for multiple variables (age, sex, BMI, fasting plasma glucose, fasting plasma insulin, triglyceride, and HDL cholesterol; for each analysis, the dependent variable itself was not included as covariates)]. Fasting plasma insulin, HOMA-IR, triglyceride, and HDL cholesterol were log-transformed before analyses. *P* values were not adjusted for multiple comparisons. Only the association between HDL cholesterol and g.−1062G>C was significant after applying the Bonferroni correction. *P* values of <0.05 are *bold*.

TABLE 4. Association between haplotypes of *ghrelin* polymorphisms and diabetes-related phenotypes among nondiabetic control subjects

Phenotype	Locus	*P*	
		Adjusted for age and sex	Adjusted for age, sex, and BMI
BMI (kg/m^2)	*ht1 (CGCC)*	0.50	
	ht2 (GCTC)	0.36	
	ht3 (GCTA)	0.49	
	ht4 (GGCC)	0.07	
Fasting plasma glucose (mg/dl)	*ht1 (CGCC)*	0.07	0.07
	ht2 (GCTC)	0.07	0.06
	ht3 (GCTA)	0.92	0.90
	ht4 (GGCC)	0.32	0.28
Fasting plasma insulin (μU/ml)	*ht1 (CGCC)*	0.26	0.20
	ht2 (GCTC)	0.26	0.13
	ht3 (GCTA)	0.80	0.97
	ht4 (GGCC)	0.59	0.29
HOMA-IR	*ht1 (CGCC)*	0.13	0.09
	ht2 (GCTC)	0.11	**0.05**
	ht3 (GCTA)	0.98	0.75
	ht4 (GGCC)	0.42	0.19
Triglyceride (mg/dl)	*ht1 (CGCC)*	0.52	0.47
	ht2 (GCTC)	0.64	0.53
	ht3 (GCTA)	0.65	0.78
	ht4 (GGCC)	0.23	0.36
HDL cholesterol (mg/dl)	*ht1 (CGCC)*	**0.002**	**0.002**
	ht2 (GCTC)	**0.02**	**0.02**
	ht3 (GCTA)	**0.01**	**0.01**
	ht4 (GGCC)	0.64	0.65

Each haplotype with a frequency of more than 0.05 is shown. *P* values of haplotype associations were calculated using the Haplo.Score algorithm developed by Schaid *et al.* (28), controlling for age, sex, and BMI as covariates. *P* values were not corrected for multiple comparisons. *P* values of <0.05 are *bold*.

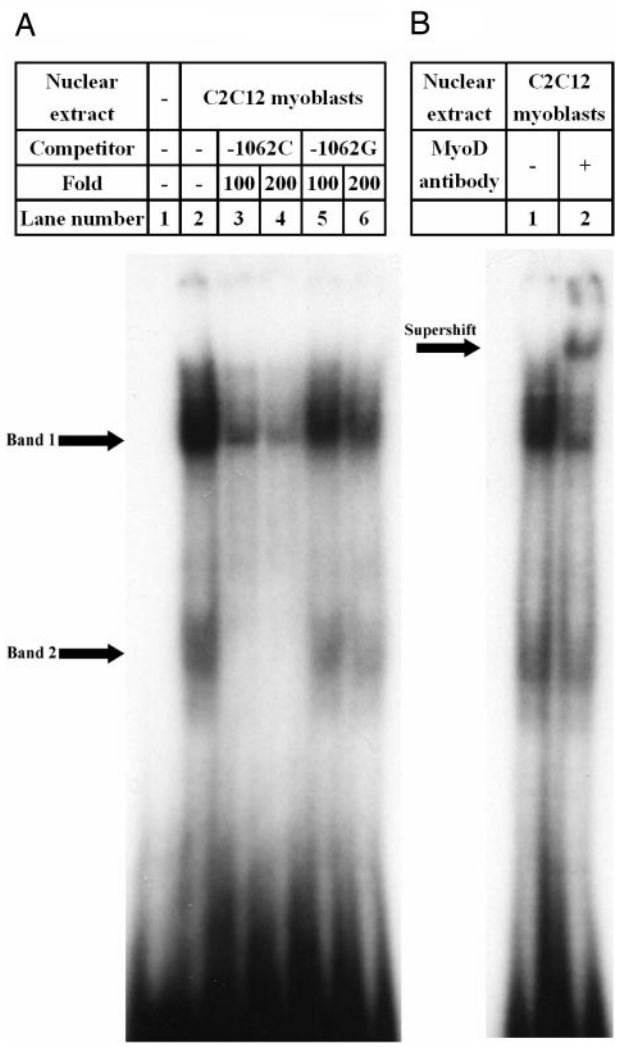

FIG. 2. MyoD specifically binds to the region around g.−1062C of the *ghrelin* gene. A, EMSA with competitors. Double-stranded oligonucleotides representing the region from g.−1075 to g.−1049 bp of the *ghrelin* gene were radiolabeled with [α-^{32}P]dATP and incubated without nuclear extracts (lane 1) or with nuclear extracts (lanes 2–6). For competition assay, unlabeled oligonucleotides containing g.−1062C (lanes 3 and 4) or g.−1062G (lanes 5 and 6) in 100- or 200-fold molar excesses were used. B, EMSA with antibody against myoD. Nuclear extracts were incubated with antibody against myoD (lane 2) for 15 min before the addition of labeled probes. Supershifted bands are indicated.

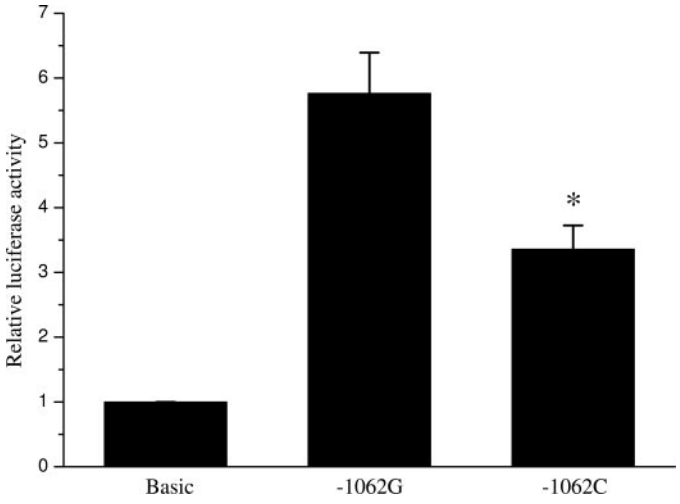

FIG. 3. Effect of *ghrelin* gene polymorphism g.−1062G>C on the ghrelin promoter activity. Luciferase reporter assay using C2C12 cells. Luciferase activity of empty pGL2-basic vector (basic) was set to one, and the other activities were expressed relative to this measure. *Bars* indicate means ± SE of mean from seven independent experiments. *, $P < 0.01$ compared with the value of ghrelin (−1062G) Luc.

gested that a common polymorphism in *ghrelin* gene has a substantial influence over development and progression of obesity, and that it has a deleterious effect on various components of metabolic syndrome.

To determine whether there is a specific nuclear protein binding on g.−1062C, we did an EMSA using oligonucleotides containing g.−1062C as probes. We found that there are nuclear proteins which bind to g.−1062C with much higher affinity than to g.−1062G. MatInspector analysis (www.genomatix.de/products/MatInspector/index.html) (29) provided several candidate transcription factors that prefer g.−1062C to g.−1062G as their binding site, such as c-Myb, MyoD/E47 dimer, and MyoD/E12 dimer. We could identify that one of the nuclear proteins binding to g.−1062C is transcription factor myoD by showing a supershifted band after addition of myoD antibody. To determine whether these specific nuclear protein bindings on g.−1062C would have influences over transcription efficiency of ghrelin gene, we performed promoter assays using C2C12 myoblast cell, which is well known to produce endogenous myoD transcription factors. And the promoter activity of ghrelin (−1062G) Luc was 1.7-fold higher than that of ghrelin (−1062C) Luc. These results suggest that the g.−1062C>G polymorphism is a functional variation that affects transcription efficiency by modifying transcription factor binding affinity.

In the present study, the association between HDL cholesterol levels and the *ghrelin* g.−1062G>C polymorphisms was found to be most significant, and was independent of all other factors, *i.e.* age, sex, BMI, fasting plasma glucose, fasting plasma insulin, and triglyceride. Moreover, this finding concurs with previous studies that found a positive correlation between plasma ghrelin concentrations and plasma HDL cholesterol concentrations (11, 13). Another study showed that an immobilized form of ghrelin specifically binds a species of HDL (30), which suggests a specific interaction between ghrelin and HDL cholesterol, and the possibility that HDL particles act as circulating transporters of ghrelin (11). Further studies are needed to explore the specific association between ghrelin and HDL cholesterol.

Due to the possibility of a type I error (false positive) resulting from the multiple comparison analyses, we applied the Bonferroni correction for multiple comparisons (*P* values multiplied by 24, corresponding to four SNPs and six phenotypes). After Bonferroni correction, most of the associations were not considered significant anymore. However, because these comparisons were not totally independent of each other due to tight LDs among these four SNPs and close association between these six phenotypes, the Bonferroni correction method may be too strict (or conservative) and

may introduce the risk of type II errors (false negative) (31). Nevertheless, even after applying the Bonferroni correction for multiple comparisons, the association between HDL cholesterol and polymorphisms g.−1062G>C remained significant ($P = 0.01$ after adjustment for multiple variables and Bonferroni correction).

In conclusion, our findings suggest that common polymorphisms in the promoter region of the *ghrelin* gene are associated with HDL cholesterol levels. However, no association was found between these polymorphisms and susceptibility to type 2 diabetes mellitus in the Korean population.

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Address all correspondence and requests for reprints to: Kyong Soo Park, M.D., Ph.D., Department of Internal Medicine, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-744, Korea. E-mail: kspark@snu.ac.kr.

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