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Brief Genetics Report

Genetic Polymorphisms in Peroxisome Proliferator-Activated Receptor δ Associated With Obesity

Hyoung Doo Shin,¹ Byung Lae Park,¹ Lyoung Hyo Kim,¹ Hye Seung Jung,^{2,3} Young Min Cho,^{2,3} Min Kyong Moon,^{2,3} Young Joo Park,^{2,3} Hong Kyu Lee,^{2,3} and Kyong Soo Park^{2,3}

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors regulating the expression of genes involved in lipid and glucose metabolism. Three different PPARs, PPAR- α , - γ , and - δ , have been characterized, and they are distinguished from each other by tissue distribution and cell activation. All PPARs are, to different extents, activated by fatty acids and derivatives. Recently, it has been shown that PPAR- δ serves as a widespread regulator of fat burning, suggesting that it might be a potential target in the treatment of obesity and type 2 diabetes. In an effort to identify polymorphic markers in potential candidate genes for type 2 diabetes, we have sequenced PPAR- δ , including -1,500 bp of the 5' flanking region. Nine polymorphisms were identified in PPAR- δ : four in the intron, one in the 5' untranslated region (UTR), and four in the 3' UTR. Among identified polymorphisms, five common sites, including c.-13454G>T, c.-87T>C, c.2022 + 12G>A, c.2629T>C, and c.2806C>G, were genotyped in subjects with type 2 diabetes and normal control subjects ($n = 702$). The genetic associations with the risk of type 2 diabetes and metabolic phenotype were analyzed. No significant associations with the risk of type 2 diabetes were detected. However, several positive associations of PPAR- δ polymorphisms with fasting plasma glucose and BMI were detected in nondiabetic control subjects. The genetic information about PPAR- δ from this study would be useful for further genetic study of obesity, diabetes, and other metabolic diseases. *Diabetes* 53: 000-000, 2004

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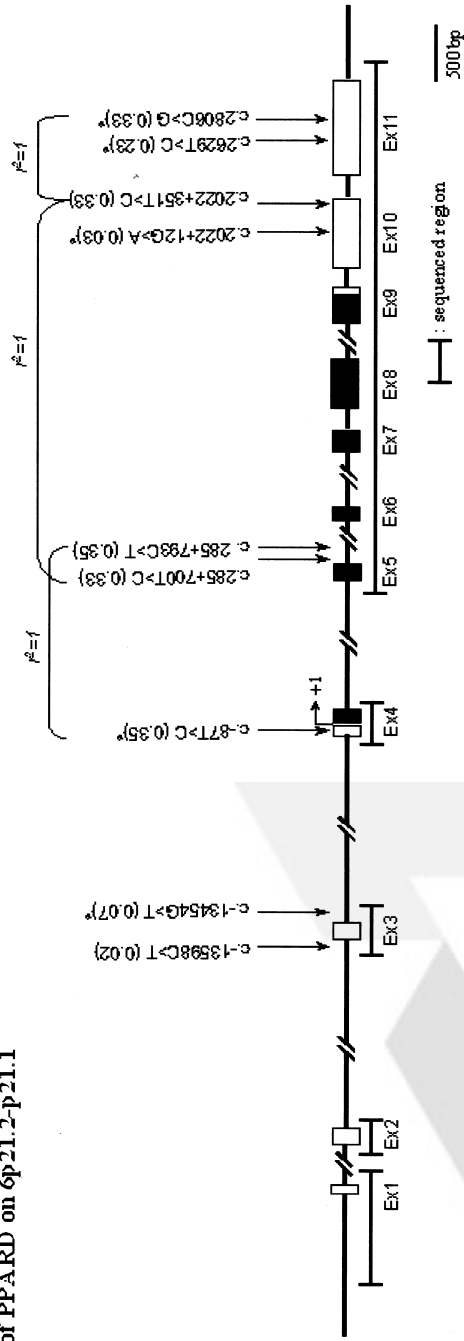
LD, linkage disequilibrium; PPAR, peroxisome proliferator-activated receptor; SNP, single nucleotide polymorphism; UTR, untranslated region.

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The peroxisome proliferator-activated receptor (PPAR) superfamily includes receptors that mediate the size and number of peroxisomes produced by cells in response to a diverse group of chemicals of both biologic and nonbiologic origin (1). PPARs have been implicated in many normal and disease-related processes, including lipid metabolism, inflammation, embryo implantation, diabetes, and cancer (2). To date, three isotypes have been identified: PPAR- α , - γ , and - δ (also known as PPAR- β and NUC-1). PPARs bind to sequence-specific DNA response elements as a heterodimer with the retinoic acid receptor (3). Unlike the PPAR- α and - γ receptors, little is known about the physiological role of the PPAR- δ isoform. PPAR- δ (MIM# 600409) was mapped to 6p21.2-p21.1 with 11 exons spanning 35 Kbp (4) and is expressed ubiquitously. It has been known as a potential downstream target of the adenomatous polyposis coli/ β -catenin/T-cell factor-4 tumor suppressor pathway (5), as well as shown to be experimentally activated by arachidonic and oleic acids and the peroxisome proliferator activator WY14643 (6). It has also been implicated in keratinocyte differentiation and wound healing and in mediating VLDL signaling of the macrophage (7-10). Recently, it was demonstrated that activation of PPAR- δ promotes fatty acid oxidation and utilization in adipocytes and skeletal muscle cells, suggesting that PPAR- δ serves as a widespread regulator of fat burning (11). Despite potential important roles in metabolic diseases, to date no genetic study of this gene has been performed in humans. In an effort to identify genetic polymorphisms in potential candidate genes for type 2 diabetes, we have sequenced the PPAR- δ gene, including the -1,500-bp 5' flanking region, and examined the association with type 2 diabetes and diabetic phenotypes in Korean patients.

Nine polymorphisms were identified in PPAR- δ : four in the intron, one in the 5' untranslated region (UTR), and four in the 3' UTR: c.-13598C>T, c.-13454G>T, c.-87T>C, c.285 + 700T>C, c.285 + 793C>T, c.2022 + 12G>A, c.2022 + 351T>C, c.2629T>C, and c.2806C>G. The locations and allele frequencies of identified polymorphic sites are shown in Fig. 1A. By pairwise linkage analysis with 24 Korean DNA samples, which were used

A. Map of PPAR-δ on 6p21.2-p21.1



B. Haplotypes of PPAR-δ

haplotype	SNPs					Frequency		
	c.-13454	c.-87	c.2022+12	c.2629	c.2806	African American	Korean	Caucasian
ht1	G	T	G	T	C	0.422	0.685	0.745
ht2	G	C	G	C	G	0.111	0.197	0.184
ht3	T	T	G	T	C	0	0.060	0
ht4	G	T	A	T	C	0	0.028	0
ht5	G	C	G	C	C	0.167	0.016	0.031
ht6	G	C	G	T	C	0.011	0.010	0.020
ht7	T	C	G	C	C	0	0.002	0
ht8	T	T	G	T	C	0	0.002	0
ht9	G	T	G	C	C	0.278	0	0.020
ht10	G	T	G	C	G	0.011	0	0

C. LD coefficients ($|D'|$ and r^2) among SNPs in PPAR-δ

SNPs	c.-13454	c.-87T	c.2022+12	c.2629	c.2806
c.-13454	-	0.857	1	0.904	1
c.-87	0.016	-	1	0.975	0.962
c.2022+12	0.002	0.008	-	1	1
c.2629	0.017	0.938	0.007	-	1
c.2806	0.019	0.870	0.007	0.914	-

FIG. 1. Gene maps and haplotypes of the PPAR-δ (PPARD) gene. A: Polymorphisms identified in PPAR-δ. Coding exons are marked by shaded blocks and 5' and 3' UTR by white blocks. *SNPs that were genotyped in the larger population. The frequencies of SNPs without larger scale genotyping were based on sequencing data ($n = 24$). The first base of transcription start site is denoted as nucleotide plus one (reference sequence of PPAR-δ mRNA: NM_006238). B: Haplotypes of PPAR-δ in Korean, Caucasian, and African-American populations. C: LD coefficients ($|D'|$ and r^2) among SNPs in PPAR-δ.

TABLE 1
Clinical characteristics of type 2 diabetic patients and normal control subjects

Features	Patients	Control Subjects
Sex (M/F)	218/235	74/175
Age (years)	57.1 ± 10.7	65.5 ± 5.0
BMI (kg/m ²)	24.5 ± 2.8	23.6 ± 3.1
Waist-to-hip ratio	0.90 ± 0.05	0.88 ± 0.06
Systolic blood pressure (mmHg)	131.2 ± 20.5	139.2 ± 20.5
Diastolic blood pressure (mmHg)	79.5 ± 11.7	83.3 ± 12.7
HOMA-IR	4.99 ± 3.87	1.97 ± 0.88
Total cholesterol (mmol/l)	5.15 ± 0.98	5.17 ± 0.85
Triglyceride (mg/dl)	2.2 ± 0.2	2.1 ± 0.2
HDL cholesterol (mmol/l)	1.23 ± 0.29	1.15 ± 0.28
Fasting blood glucose (mmol/l)	8.66 ± 2.74	5.26 ± 0.51
HbA _{1c} (%)	8.20 ± 1.85	5.13 ± 0.50

Data are means ± SE. HOMA-IR, homeostasis model assessment of insulin resistance.

for direct sequencing, we have found that two sets of single nucleotide polymorphisms (SNPs) (c.-87T>C: c.285 + 793C>T and c.285 + 700T>C: c.2022 + 351T>C: c.2806C>G) were in absolute linkage disequilibrium (LD) ($D' = 1$ and $r^2 = 1$), and five sets of SNPs were in complete LD ($D' = 1$ and $r^2 \neq 1$) (Fig. 1C).

Among identified polymorphisms, five common sites (c.-13454G>T, c.-87T>C, c.2022 + 12G>A, c.2629T>C, and c.2806C>G) were selected for larger scale genotyping based on LDs, frequencies, and haplotype tagging status. Ten haplotypes were constructed in three ethnic groups by five SNPs, and significant differences were observed among the three ethnic groups (Fig. 1B). The minor allele frequencies of genotyped polymorphisms were 0.067 (c.-13454G>T), 0.229(c.-87T>C), 0.026(c.2022 + 12G>A), 0.227(c.2629T>C), and 0.211 (c.2806C>G) in the Korean population ($n = 690$). (See Fig. 1A for allele

frequencies of SNPs that were not genotyped in the full set.) Genotype distributions of all loci were in Hardy-Weinberg equilibrium ($P > 0.05$). Among four common haplotypes (frequency >0.025) identified in the Korean population, only ht1 was used for further analysis because c.-13454G>T, c.2022 + 12G>A, and c.2806C>G were almost tagging ht3 (99.6%), ht4 (100%), and ht2 (100%), respectively (Fig. 1B).

Associations of PPAR- δ polymorphisms with the risk of type 2 diabetes and diabetic phenotypes were analyzed using logistic and multiple regressions. Allelic frequency differences between type 2 diabetic patients and nondiabetic subjects were analyzed (adjusting for age and sex) for the risk of type 2 diabetes. The clinical characteristics of subjects are shown in Table 1. Only nondiabetic subjects were used for association analyses of diabetic phenotypes because treatment for type 2 diabetes may affect those values.

No significant associations were detected with the risk of type 2 diabetes among patients and normal control subjects (Table 2). Genetic associations with several metabolic diabetic phenotypes, including lipid levels (triglyceride, total cholesterol, and HDL cholesterol), waist-to-hip ratio, BMI, and fasting plasma glucose, were analyzed. Although no significant associations of PPAR- δ polymorphisms with triglyceride, total cholesterol, HDL cholesterol, and waist-to-hip ratio were detected (supplemental table available online at <http://diabetes.diabetesjournals.org>), several positive associations of PPAR- δ polymorphisms with fasting plasma glucose and BMI were revealed in nondiabetic control subjects (Table 3). Analysis results of other phenotypes are available in the supplemental table. In initial single SNP analyses, c.-87T>C, c.2629T>C, and c.2806C>G showed weak associations with fasting plasma glucose ($P = 0.05$ – 0.01). Similarly, c.2022 + 12G>A, c.2629T>C, and c.2806C>G also showed

TABLE 2
Logistic analysis of PPAR- δ polymorphisms with the risk of type 2 diabetes among patients and normal control subjects

Locus	Genotype	Patients	Control Subjects	Codominant		Dominant		Recessive	
				OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
c.-13454G>T	G	403 (87.0)	161 (89.4)						
	GT	56 (12.1)	19 (10.6)	2.12 (1.02–4.4)	0.07	1.93 (0.91–4.1)	0.09	—	—
	T	4 (0.86)	0 (0)						
c.-87T>C	T	269 (58.4)	108 (60.3)						
	CT	171 (37.1)	65 (36.3)	0.94 (0.61–1.46)	0.79	0.95 (0.57–1.56)	0.82	0.86 (0.23–3.2)	0.83
	C	21 (4.6)	6 (3.4)						
c.2022+12G>A	G	434 (94.1)	172 (96.6)						
	AG	27 (5.9)	6 (3.4)	1.3 (0.39–4.3)	0.67	1.3 (0.39–4.3)	0.67	—	—
	A	0 (0)	0 (0)						
c.2629T>C	T	271 (58.7)	109 (60.6)						
	CT	171 (37.0)	65 (36.1)	0.94 (0.61–1.45)	0.78	0.94 (0.57–1.55)	0.80	0.88 (0.24–3.25)	0.84
	C	20 (4.3)	6 (3.3)						
c.2806C>G	C	285 (61.7)	113 (62.8)						
	CG	158 (34.2)	62 (34.4)	0.95 (0.61–1.48)	0.81	0.93 (0.56–1.54)	0.77	1.04 (0.26–4.21)	0.95
	G	19 (4.1)	5 (2.8)						
ht1	ht1/ht1	209 (45.5)	89 (50.0)						
	-ht1	200 (43.6)	76 (42.7)	0.84 (0.58–1.23)	0.37	0.55 (0.23–1.3)	0.17	0.92 (0.56–1.5)	0.72
	-/-	50 (10.9)	13 (7.3)						

Genotype distributions, odd ratio (OR) (95% CI), and *P* values for logistic analyses of three alternative models (codominant, dominant, and recessive), controlling age and sex as covariables, are shown. Duration of diabetes (years) was additionally added in the model as a covariable for analysis of complications. Haplotypes and their frequencies were inferred using the algorithm developed by Stephens et al. (16). Missing genotype data were omitted for exact haplotype construction.

TABLE 3

Regression analyses for age, BMI (for fasting blood glucose), and sex-adjusted diabetes phenotypes with PPAR- δ polymorphisms among nondiabetic subjects

Phenotype	Locus	C/C*	C/R*	R/R*	P		
					Codominant	Dominant	Recessive
Fasting plasma glucose	c.-13454G>T	154 (5.26 \pm 0.48)	18 (5.32 \pm 0.75)	0	0.56	0.56	—
	c.-87T>C	103 (5.21 \pm 0.52)	62 (5.35 \pm 0.48)	6 (5.32 \pm 0.63)	0.04	0.04	0.49
	c.2022+12G>A	165 (5.27 \pm 0.52)	5 (5.10 \pm 0.38)	0	0.87	0.87	—
	c.2629T>C	104 (5.22 \pm 0.52)	62 (5.35 \pm 0.48)	6 (5.32 \pm 0.63)	0.05	0.04	0.51
	c.2806C>G	107 (5.22 \pm 0.52)	60 (5.35 \pm 0.47)	5 (5.32 \pm 0.70)	0.04	0.04	0.46
	ht1	12 (5.23 \pm 0.53)	73 (5.36 \pm 0.55)	85 (5.18 \pm 0.46)	0.03	0.71	0.01
BMI	c.-13454G>T	160 (23.7 \pm 3.1)	19 (22.9 \pm 3.1)	0	0.30	0.30	—
	c.-87T>C	107 (23.8 \pm 3.0)	65 (23.6 \pm 3.2)	6 (21.7 \pm 2.8)	0.10	0.21	0.10
	c.2022+12G>A	171 (23.8 \pm 3.1)	6 (21.2 \pm 2.3)	0	0.03	0.03	—
	c.2629T>C	108 (23.9 \pm 3.0)	65 (23.5 \pm 3.3)	6 (21.7 \pm 2.8)	0.05	0.10	0.10
	c.2806C>G	112 (23.9 \pm 3.0)	62 (23.5 \pm 3.3)	5 (20.9 \pm 2.5)	0.04	0.12	0.04
	ht1	13 (21.8 \pm 2.1)	76 (23.5 \pm 3.4)	88 (24.1 \pm 2.9)	0.005	0.02	0.02

Genotype and haplotype distributions, means \pm SD of each value, and *P* values of codominant models for regression analyses are shown. *C/C, C/R, and R/R represent homozygotes for common allele and heterozygotes and homozygotes for rare allele, respectively. Haplotypes and their frequencies were inferred using the algorithm developed by Stephens et al. (16). Missing genotype data were omitted for exact haplotype construction.

marginal associations with BMI ($P = 0.05$ – 0.03). By further haplotype analysis, more significant associations were detected with fasting plasma glucose and BMI, e.g., individuals ($n = 85$) who were homozygous for the most common haplotype 1 ([G-T-G-T-C], abbreviated as ht1, frequency = 0.69; Fig. 1B) showed significantly decreased fasting plasma glucose level (5.19 ± 0.46 mmol/l) compared with that of others (–/– or –/ht1) (5.34 ± 0.55 mmol/l) ($P = 0.01$). Similarly, ht1 showed more reliable and gene dose-dependent association with BMI, e.g., lowest BMI (21 ± 2.18 kg/m²) in individuals who were not carrying ht1 ($n = 13$), intermediate BMI (23.5 ± 3.4 kg/m²) in heterozygous individuals for ht1 (–/ht1) ($n = 76$), and highest BMI (24.1 ± 2.94 kg/m²) in homozygous people (ht1/ht1) ($n = 88$) ($P = 0.005$).

The present study provides, for the first time, information about genetic polymorphisms in PPAR- δ and positive associations of those polymorphisms with fasting plasma glucose and BMI in the Korean population. We screened the PPAR- δ gene extensively for polymorphic markers and found that the SNPs/haplotype in PPAR- δ were associated with the level of fasting plasma glucose and BMI.

Since identification and cloning ~ 10 years ago, PPARs, as nuclear hormone receptors, have been implicated in numerous biologic pathways. PPAR- α is expressed in the liver, heart, muscle, and kidney, where it regulates fatty acid catabolism. PPAR- γ is highly enriched in adipocyte and macrophage and is involved in adipocyte differentiation, lipid storage, and insulin sensitivity. PPAR- δ is expressed ubiquitously with a less defined function. It has been implicated in keratinocyte differentiation and wound healing and in mediating VLDL signaling of the macrophage (7–10). Recently, it has been demonstrated that activation of PPAR- δ promotes fatty acid oxidation and utilization in adipocytes and skeletal muscle cells, which suggests that PPAR- δ might serve as a widespread regulator of fat burning (11).

In our study, we found that the most common PPAR- δ haplotype ht1 showed gene dose-dependent association

with BMI in nondiabetic control subjects. Considering PPAR- δ as a widespread regulator of fat burning, the association between PPAR- δ polymorphism and obesity is very intriguing. PPAR- δ polymorphisms were also associated with fasting plasma glucose in nondiabetic control subjects but were not associated with insulin resistance or the risk of type 2 diabetes. It is interesting that those who are ht1/ht1 homozygotes had lower fasting glucose levels despite a higher degree of obesity than other subjects. A similar phenomenon was observed in PPAR- γ mutation (12).

The effects of PPAR- δ genetic polymorphisms on the level of fasting plasma glucose and BMI were not dramatic in the present study. Therefore, it may be argued that Bonferroni correction should be applied to the *P* values obtained. If Bonferroni correction were strictly adopted, associated *P* values could not retain the significances (the threshold of significance would be 0.003 [six polymorphisms and three phenotypes analyzed]). However, although there is a chance of type 1 error due to multiple comparisons, considering that the comparisons were not totally independent of each other due to tight LDs among SNPs/haplotype and related phenotypes and that 14 positive signals ($P \leq 0.05$) of 20 *P* values (Table 3) were detected in a single gene, the significance of associations might be acceptable. Further biological and/or functional evidence would be needed to confirm the suggestive association of PPAR- δ polymorphisms with obesity in this study. And although no significant associations ($P < 0.05$) with risk of type 2 diabetes were detected, it would be worthy to follow-up the weak signal (odds ratio = 2.12–1.93, $P = 0.07$ – 0.09) of rare SNP (c.-13454G>T, minor allele frequency = 0.07) in a larger diabetic cohort due to the relatively small sample size in this study, especially in normal control subjects ($n = 249$).

In summary, we have identified nine polymorphic sites in the PPAR- δ gene, and 10 haplotypes were constructed with five genotyped common polymorphisms. Statistical analyses revealed that SNPs and ht1 [G-T-G-T-C] in the

AQ: B

PPAR- δ gene are associated with fasting plasma glucose and BMI. We suggest that polymorphisms in the PPAR- δ gene might play important roles in controlling glucose level and/or BMI. The polymorphism/association information identified in this study would be useful for further genetic studies of other metabolic diseases and pharmacogenomic study for treatment agents.

RESEARCH DESIGN AND METHODS

A total of 453 unrelated patients with type 2 diabetes and 249 unrelated nondiabetic control subjects were recruited. Nondiabetic control subjects were recruited from an unselected population undergoing a routine health checkup at Seoul National University Hospital. Inclusion criteria were as follows: ≥ 60 years of age, no past history of diagnosis of diabetes, no diabetes in first-degree relatives, fasting plasma glucose level < 6.1 mmol/l, and HbA_{1c} $< 5.8\%$. The diabetic subjects were randomly recruited from patients attending the outpatient clinic of Seoul National University Hospital. Diabetes was diagnosed based on the guideline of American Diabetes Association criteria (13). Subjects with positive GAD antibodies were excluded. All subjects enrolled in this study were of Korean ethnicity. The study protocol was approved by the Institutional Review Board of the Clinical Research Institute at Seoul National University Hospital. Informed consent was obtained from all subjects before drawing blood. All study subjects were examined in the morning after an overnight fast. Height, weight, circumferences of waist and hip, and blood pressure were measured. The maximum body weight before the development of diabetes was obtained by taking histories. Blood samples were drawn for biochemical measurements (fasting plasma glucose, postprandial 2-h glucose, fasting plasma insulin, HbA_{1c}, total cholesterol, triglyceride, and HDL cholesterol) and DNA extraction. The insulin resistance index (homeostasis model assessment of insulin resistance) was calculated as fasting serum insulin (pmol/l) \times fasting plasma glucose (mmol/l)/135 (14). The clinical characteristics of subjects are shown in Table 1.

Sequencing analysis of the human PPAR- δ gene. We have sequenced all exons, including exon-intron boundaries and the promoter region (~ 1.5 kb), to discover SNPs in 24 Korean DNA samples using the ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA). Twenty-four primer sets for the amplification and sequencing analysis were designed based on GenBank sequences (reference sequence of PPAR- δ mRNA: NM_006238). Information regarding primers is available in the supplemental table. Sequence variants were verified by chromatograms. For comparison of allele frequencies of PPAR- δ SNPs, which were identified in Korean population, with other major ethnic groups, we also genotyped 50 Caucasian and 50 African-American DNAs obtained from the Human Genetic Cell Repository (<http://locus.umd.edu/nigms/>).

Genotyping by single-base extension and electrophoresis. For genotyping of five common polymorphic sites in our type 2 diabetic and normal control groups, amplifying and extension primers were designed for single-base extension (15). Primer extension reactions were performed with the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems). Information regarding the primers is available in the supplemental table. To clean up the primer extension reaction, 1 unit SAP was added to the reaction mixture, and the mixture was incubated at 37°C for 1 h, followed by 15 min at 72°C for enzyme inactivation. The DNA samples, containing extension products, and Genescan 120 Liz size standard solution were added to Hi-Di formamide (Applied Biosystems) according to the recommendation of the manufacturer. The mixture was incubated at 95°C for 5 min, followed by 5 min on ice, and then electrophoresis was performed using the ABI Prism 3100 Genetic Analyzer. The results were analyzed using ABI Prism GeneScan and Genotyper (Applied Biosystems).

Statistics. χ^2 tests were used to determine whether individual variants were in equilibrium at each locus in the population (Hardy-Weinberg equilibrium). We examined widely used measures of linkage disequilibrium between all pairs of biallelic loci, Lewontin's D' (D') (16) and r^2 . Haplotypes and their

frequencies were inferred using the algorithm developed by Stephens et al. (17). Logistic regression analyses were used for calculating odds ratios (95% CI) and corresponding P values, controlling for the covariables age, BMI, and sex. Multiple regression analyses were performed for fasting plasma glucose and BMI, controlling for the covariables age and sex.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

1

A—Au: Table 1: Are data corrected denoted as means \pm SE?

B—Au: Please note that original ref. 17 was switched to 12 to reflect order cited in text and subsequent refs. renumbered.

C—Au: If appropriate, please expand SAP.

