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A functional variant in the exon 5 of *PLIN1* reduces risk of central obesity by possible regulation of lipid storage

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ABSTRACT

Purpose: Perilipin coats lipid droplets in adipocytes and steroidogenic cells. Its major role is in the regulation of intracellular lipolysis in adipocytes. Our aim was to examine the association between common variants at the *PLIN1* gene and central obesity in unrelated Chinese adults.
Methods: A case-control study was carried out on 869 patients with central obesity and 869 age- and gender-matched individuals without central obesity. Two *PLIN1* variants (rs6496589 and rs8179078) were genotyped by PCR and restriction enzyme analysis. In addition, the association of the variant with central obesity was replicated in an independent population of 629 central obesity patients and 518 controls. Finally, the relationship between rs6496589 and enhancing lipid accumulation in THP-1-derived macrophages was assessed.
Results: *PLIN1* rs6496589 allele frequencies and genotype frequencies of CG + GG in the patients' group were much lower than those in the control group. After adjustment for conventional risk factors using multiple logistical regression analysis, rs6496589G allele frequencies were significantly associated with a lower risk of central obesity (OR 0.71, 95% CI: 0.59–0.86, $P = 0.001$). These results were confirmed in an independent study. No association was found between *PLIN1* rs8179078 and central obesity. Furthermore, *in vitro* assays revealed that homozygous rs6496589G alleles presented lower lipid droplet accumulation in THP-1-derived macrophages, compared with non-carriers.
Conclusions: The functional *PLIN1* rs6496589 may influence the risk of central obesity through possible regulation of lipid storage.

1. Introduction

Obesity has become one of the major issues in public health, medicine and the economy [1]. In particular, central obesity is considered to be important because of its relation to various complications such as diabetes mellitus, dyslipidemia and hypertension. A combination of these dysfunctions is now defined as the metabolic syndrome [2], which significantly increases the risk of cardiovascular disease. Both genetic and environmental factors contribute to the development of obesity. In epidemiological studies, heritability of body weight is estimated to be approximately 70% [3,4]. Although hundreds of obesity candidate genes have been identified in different metabolic pathways, the fundamental basis of obesity resides within excessive storage of triglycerides in adipose tissue. The mechanisms that control the storage and release of triglycerides in lipid droplets are complex and poorly understood; yet, they are likely to be crucial for understanding the regulation of body weight. In this regard, perilipin may play key roles in obesity. Perilipins are a family of proteins that coat the intracellular lipid droplets. The expression of perilipin appears to be primarily in adipocytes and steroidogenic cells [5–8], and its major demonstrated role is in the regulation of intracellular lipolysis in adipocytes [9,10]. In experimental animal models, the absence of perilipin has resulted in lean phenotypes and has counterbalanced both genetic- and dietary-induced obesity in mice [11,12]. In contrast, elevated expression of perilipin has correlated with increased adiposity in humans [13]. Moreover, some studies have detected associations between *PLIN1* SNPs and obesity-related phenotypes [14]. Overall the evidence has supported the notion that *PLIN1* may be a candidate gene for human obesity. In this study, we genotyped two *PLIN1* SNPs, located in the promoter and coding region, and examined the associations between the two SNPs and the risk of central

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obesity in Chinese adults. Moreover, we studied the functions of the SNP associated with central obesity.

2. Material and methods

2.1. Study population

In the first study, a total of 869 patients, including 167 men and 702 women, diagnosed with central obesity were recruited from two local hypertension clinic services in Xinyang County, Henan Province, China, from March to May 2005. A total of 869 age- and gender-matched controls were selected from the same region and during the same period as the patients were. All the participants had not been treated with medicine for hypertension, diabetes and dyslipidemia. The patients with the following diseases were excluded: chronic lung diseases, cardiac insufficiency, renal inadequacy, valvular heart disease, chronic liver ailment and tumors. A standard questionnaire about demographic information and medical history was filled-in through in-person interviews by trained research staff.

The second case-control study comprised 629 central obesity patients and 518 controls recruited from November 2000 to November 2001 from six hypertension clinical centers (Yanzhou, Xian, Chongqing, Wuhan, Beijing and Tianjin). The inclusion and exclusion criteria were the same as that in the first study.

Both studies were approved by the local ethics committees of the collaborating hospitals. All participants reported themselves as Han nationality and provided written informed consent.

2.2. Definition of central obesity

Central obesity was defined as waist circumference $\geqslant$80 cm for women, and $\geqslant$90 cm for men according to the NCEP (National Cholesterol Education Program)-ATP (Adult Treatment Panel) III definitions [15].

2.3. Genotype determination

Two single nucleotide polymorphisms (SNPs) at the *PLIN1* locus were genotyped: *PLIN1* rs8179078A/G (promoter), and *PLIN1* rs6496589C/G (exon 5), which is expected to cause an amino acid substitution (Ala194Pro). Criteria for the selection of these polymorphisms include: (a) according to HapMap, they are all tag SNPs in Chinese individuals, (b) the minor allele frequency is $\geqslant$5%, and (c) SNPs in the coding region or promoter region are preferred to those in introns.

The two SNPs were genotyped by PCR-restriction fragment length polymorphism. The PCR and SNP genotyping conditions are shown in Table 1. Reactions were performed with ABI 9700 (96 wells format; Applied Biosystems, Foster City, State of California, USA). The products were separated using a 3% agarose gel. Primers were designed with Oligo 6.0 (Molecular Biology Insights, Inc.). Reproducibility of genotyping for each variant was confirmed by bidirectional sequencing of 96 randomly selected samples with a DNA sequencer (ABI 3730, Perkin Elmer, Foster City, State of California USA). The reproducibility was 100%.

2.4. Plasmid construction

The plasmid carrying rs6496589C was purchased from FulenGen (Guang Zhou, Guang Dong Provence, China), which contained the open reading frame of *PLIN*, and the plasmid carrying rs6496589G was constructed by using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) following the kit protocol without modifications.

2.5. Cell culture and transfection

Human monocytic THP-1 cells (American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI-1640 medium containing 25 mmol/L HEPES buffer and 10% heat-inactivated FBS (Fetal bovine serum). Three days before transfection, cells were seeded in 12-well culture dishes at a density of 4×10^4 cells/well. Differentiation of THP-1 monocytes to macrophages occurred in the presence of 160 nM phorbol 12-myristate 13-acetate (Sigma, Saint Quentin, France) for 72 h [16]. The cells were then transfected with an empty plasmid or a plasmid carrying either the wild type (CC) or the rs6496589 variant (GG), using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.6. Oil Red O staining

For lipid staining, normal and transfected THP-1-derived macrophages were washed twice with PBS and fixed for 10 min with 4% paraformaldehyde in PBS (pH 7.4). Cells were then stained with Oil Red O (0.3% in 60% isopropanol), followed by extensive washes with 70% alcohol for 5 s to remove background staining. Finally, the cells were rinsed in distilled water. The images of the stained cells were captured with a microscope (Leitz DMRB). Image-Pro Plus version 6.0 (Media Cybernetics, Inc. USA) was used to determine relative lipid accumulation in cells, as indicated by Oil Red O-positive area.

2.7. Statistical analysis

A chi-square test was used to test for qualitative variables, genotype/allele frequencies, and for the Hardy–Weinberg equilibrium of the polymorphisms. Differences in quantitative variables between groups were analyzed by Student *t*-test. Because the levels of plasma triglycerides (TG) were highly skewed, the Mann–Whitney U test was used to examine the differences in this variable between groups. The associations between each SNP and central obesity were detected using multiple regression analysis. The covariates selected for the logistic regression models included the following conventional risk factors: age, sex, cigarette smoking and alcohol abuse. All statistics were performed with the SPSS 13.0 package. Quantitative analysis of lipid-stained lesions was performed using Image-Pro plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The integrated optical density (IOD) was then calculated for the red fat. IOD values are presented as mean ± SD. A value of $P < 0.05$ was regarded as significant (2 tailed).

Table 1
Description of *PLIN1* SNPs, primers, PCR conditions and restriction enzymes.

SNP	Primers	PCR (bp)	Ann. temp.	RE	Size (bp) of wt allele	Size (bp) of mut allele
rs8179078	F: CTCCTGCTTCAGCCTCCAGAGTATC R: ACAGGGTCACAATGGGTGCCTATA	450	66	HpaII	450	232 + 218
rs6496589	F: AGCCACCTCCTGCTGATTCCC R: GCAAGACCACATGCCTAACACCC	379	58	PstI	285 + 94	379

F: forward, R: reverse, wt allele: wild type (common) allele, mut allele: mutant (rare) allele, Ann. temp.: annealing, temperature, RE: restriction enzyme.

3. Results

3.1. Case-control association study

The clinical characteristics of patients and controls are shown in Tables 2 and 3, respectively. The genotype frequencies of variant *PLIN1* rs8179078 were in accordance with the Hardy–Weinberg equilibrium ($P > 0.05$) in both cases and controls in the first study. No association was found between *PLIN1* rs8179078 and central obesity in the first study ($P > 0.05$) (Tables 4 and 5).

We investigated the association of rs6496589 with central obesity in two case-control studies. The distribution of variant rs6496589 is shown in Tables 4 and 6 for the two populations, and conformed to Hardy–Weinberg equilibrium (HWE) in both cases and controls.

Table 2
Baseline characteristics of central obesity patients and controls in the first study.

	Controls (n = 869)	Central obesity (n = 869)	P
Sex (m/f)	179/690	167/702	0.471
Age, y	57.2 ± 9.2	57.8 ± 8.0	0.312
Waist circumference, cm	74.1 ± 5.5	94.5 ± 6.8	<0.001
BMI, kg/m^2	22.6 ± 2.3	28.9 ± 3.1	<0.001
SBP, mmHg	146 ± 31	166 ± 25	<0.001
DBP, mmHg	88 ± 14	99 ± 13	<0.001
T-Cho, mmol/L	5.20 ± 1.06	5.78 ± 1.23	<0.001
Ln (TG), mmol/L	2.35 ± 0.40	3.07 ± 0.52	<0.001
Glu, mmol/L	5.01 ± 1.50	6.13 ± 2.11	<0.001
HDL-C, mmol/L	1.66 ± 0.34	1.40 ± 0.30	<0.001
LDL-C, mmol/L	2.81 ± 0.82	3.32 ± 0.93	<0.001
DM history	0.016	0.087	<0.001
Hypertension history	0.471	0.794	<0.001
CHD history	0.045	0.122	<0.001
Stroke history	0.055	0.093	<0.001
Smoke (never/former/current)	761/22/86	795/26/48	0.002
Drink (never/former/current)	769/12/88	741/29/99	0.026

Age, waist circumference, body mass index (BMI), systolic (SBP) and diastolic (DBP) blood pressure, glucose (Glu), HDL-C, LDL-C and TC values are presented as mean ± SD; TG values are given as Ln (TG), and other values as percentage (n/N).

Table 3
Baseline characteristics of central obesity patients and controls in the second study.

	Controls (n = 518)	Central obesity (n = 629)	P
Sex (m/f)	278/240	295/334	0.023
Age, y	60.6 ± 8.5	60.1 ± 8.4	0.366
Waist circumference, cm	77.2 ± 6.89	93.44 ± 8.36	<0.001
BMI, kg/m^2	22.55 ± 2.76	26.08 ± 3.11	<0.001
SBP, mmHg	148.41 ± 20.24	148.89 ± 19.85	0.694
DBP, mmHg	88.58 ± 11.43	88.98 ± 11.7	0.561
T-Cho, mmol/L	4.83 ± 1.1	5.07 ± 0.99	<0.001
Ln (TG), mmol/L	1.83 ± 1.3	2.16 ± 1.36	<0.001
Glu, mmol/L	6.31 ± 2.61	6.72 ± 2.66	0.100
HDL-C, mmol/L	0.98 ± 0.32	0.95 ± 0.29	0.187
LDL-C, mmol/L	2.82 ± 1.12	3.11 ± 1.24	0.003
DM history	0.095	0.127	0.082
Hypertension history	0.701	0.789	<0.001
CHD history	0.129	0.194	0.003
Stroke history	0.064	0.057	<0.646
Smoke (never/former/current)	318/78/122	406/104/119	0.154
Drink (never/former/current)	358/59/101	438/72/119	0.970

Age, waist circumference, body mass index (BMI), systolic (SBP) and diastolic (DBP) blood pressure, glucose (Glu), HDL-C, LDL-C and TC values are presented as mean ± SD; TG values are given as Ln (TG), and other values as percentage (n/N).

Table 4
Genotype and allele frequencies of *PLIN1* polymorphisms in cases and controls in the first study.

SNP	Genotypes and alleles	Controls (n = 869)	Central obesity (n = 869)
rs6496589	CC, n (%)	441 (50.7)	505 (58.1)
	CG, n (%)	343 (39.5)	309 (35.6)
	GG, n (%)	85 (9.8)	55 (6.3)
	P value		0.002
	CC, n (%)	441 (50.7)	505 (58.1)
	GG + CG, n (%)	428 (49.3)	364 (41.9)
	P value		0.002
	C, n (%)	1225 (70.5)	1319 (0.759)
	G, n (%)	513 (29.5)	419 (0.241)
	P value		<0.001
rs8179078	AA, n (%)	700 (80.6)	687 (79.1)
	AG, n (%)	158 (18.2)	165 (19.0)
	GG, n (%)	11 (1.3)	17 (2.0)
	P value		0.459
	A, n (%)	1558 (89.6)	1539 (88.6)
	G, n (%)	180 (10.4)	199 (11.4)
	P value		0.301

Table 5
Association between *PLIN1* SNPs genotype and risk of central obesity before and after adjustment for conventional risk factors in the first study.

PLIN SNP	Before adjustment		After adjustment	
	OR (95% CI)	P	OR (95% CI)	P
rs8179078A/G				
AA	1.00		1.00	
AG	1.06 (0.84–1.36)	0.615	1.48 (0.67–3.27)	0.336
GG	1.58 (0.73–3.39)	0.245	0.90 (0.71–1.16)	0.422
rs6496589C/G				
CC	1.00		1.00	
CG + GG	0.74 (0.62–0.90)	0.002	0.71 (0.59–0.86)	0.001

Conventional risk factors include age, sex, cigarette smoking and alcohol use.

Table 6
Genotype and allele frequencies of *PLIN1* polymorphisms in cases and controls in the second study.

SNP	Genotypes and alleles	Controls (n = 518)	Central obesity (n = 629)
rs6496589	CC, n (%)	257 (49.6)	367 (58.3)
	CG, n (%)	221 (42.7)	219 (34.8)
	GG, n (%)	40 (7.7)	43 (6.8)
	P value		0.012
	CC, n (%)	257 (49.6)	367 (58.3)
	GG + CG, n (%)	261 (50.4)	262 (41.7)
	P value		0.003
	C, n (%)	735 (70.9)	953 (75.8)
	G, n (%)	301 (29.1)	305 (24.2)
	P value		0.009

In the first study, the frequency of the CG + GG genotype was significantly lower in patients with central obesity than in the controls [6.3 versus 9.8%, $P = 0.002$ (crude OR 0.74, 95% CI 0.62–0.90]. After adjustment for conventional vascular risk factors, including age, sex, cigarette smoking and alcohol use, using unconditional logistic regression analysis, the CG + GG genotype resulted in a significantly reduced risk of central obesity [OR, 0.71 (95% CI, 0.59–0.86), $P = 0.001$] (Tables 4 and 5). The possible association was then repeated for the second independent case-control study. The frequency of the CG + GG genotype was significantly lower in patients with central obesity than in the controls [6.8 versus 7.7%, $P = 0.012$ (crude OR 0.70, 95% CI 0.56–0.89)]. After adjustment for

Table 7
Association between *PLIN1* SNPs genotype and risk of central obesity before and after adjustment for conventional risk factors in the second study.

PLIN SNP	Before adjustment		After adjustment	
	OR (95% CI)	*P*	OR (95% CI)	*P*
CC	1.00		1.00	
CG + GG	0.70 (0.56–0.89)	0.002	0.72 (0.57–0.92)	0.007

Conventional risk factors include age, sex, cigarette smoking and alcohol use.

conventional vascular risk factors using unconditional logistic regression analysis, the CG + GG genotype conferred a 28% lower risk of central obesity [OR, 0.72 (95% CI, 0.57–0.92), $P = 0.007$] (Tables 6 and 7).

3.2. Regulatory effect of PLIN1 rs6496589 on lipid accumulation

To investigate the effect of the rs6496589 variant on lipid accumulation, we constructed a vector carrying rs6496589G or rs6496589C. Equal amounts of the vectors were transfected. The THP-1-derived macrophages transfected with plasmids carrying the GG genotype of rs6496589 exhibited 28.7% reduction in lipid droplet accumulation compared with that of plasmids carrying the CC genotype (Fig. 1).

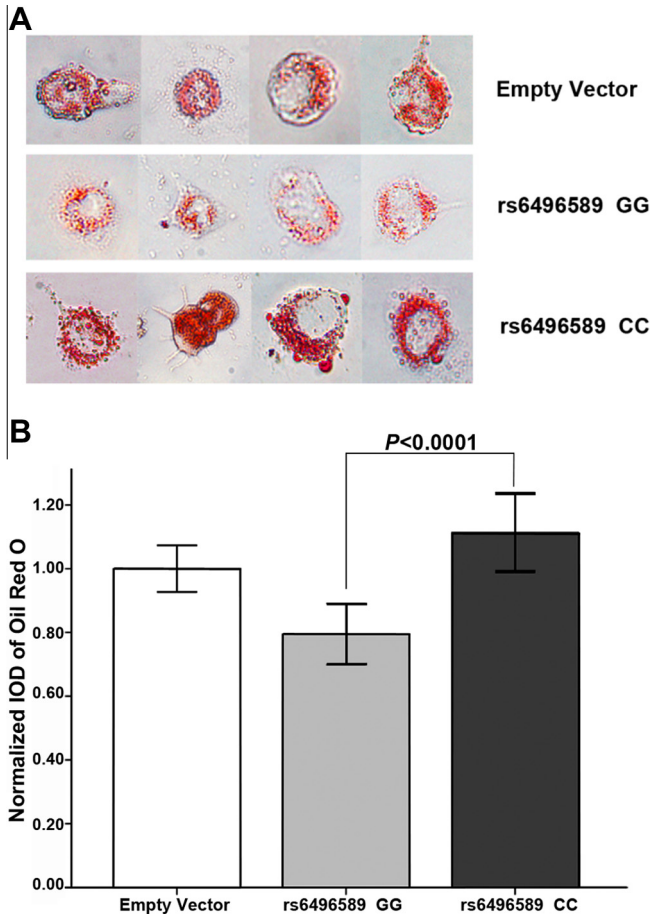

Fig. 1. (A) Lipid droplets stained with Oil Red O in THP-1-derived macrophages (40 × magnification) transfected with the indicated vectors. (B) Fifty randomly selected cells were stained with Oil Red O and quantified using Image-Pro plus version 6.0 (Media Cybernetics, Inc.). The integrated optical density (IOD) was then calculated for the red fat. IOD values are presented as mean ± SD.

4. Discussion

Experimental evidence has shown that perilipin plays a major role in central obesity. In the present report, we found that the *PLIN1* rs6496589G was associated with lower waist circumference. Accordingly, logistic regression analysis results revealed an independent association between the *PLIN1* rs6496589G and lower waist circumference. On the other hand, *in vitro* assays demonstrated that homozygous rs6496589G alleles presented lower lipid droplet accumulation in THP-1-derived macrophages, compared with non-carriers. Taken together, these findings indicate that the functional *PLIN1* polymorphism is associated with a waist circumference phenotype.

Population stratification might lead to a spurious association. However, in the present study, all subjects were of Han nationality, and the distribution of the genotypes was in agreement with the Hardy–Weinberg equilibrium in both patients and controls in the two studies.

First reported in the early 1990s, perilipin has been emerging as a key regulator of lipolysis in adipocytes and body fat accumulation [10–13]. The function of perilipins is to prevent lipolysis in basal conditions, favoring fat deposition. Animals lacking perilipin were lean, resistant to diet-induced or genetic obesity, and had peripheral insulin resistance. In human studies, the common variations in the *PLIN* gene have been associated with several risk factors for diabetes, including obesity, weight gain, insulin resistance and hypertension [17–19]. It has been documented that perilipin transcription in central adipose tissue may differ from that in other adipose tissue depots, and that central obesity may substantially affect the expression of perilipin [13,20–21].

In recent years, many studies have analyzed the effect of *PLIN* polymorphisms on obesity in different ethnic populations. Both positive and negative results were detected [19,22–28].

Additionally, diet strongly modifies the risk of obesity. Examination of dietary modulations of *PLIN* associations has generated fruitful results [29,30].

However, the association between *PLIN1* rs6496589 and central obesity has not been analyzed. *PLIN1* rs6496589 has been previously reported by Jang et al. [31] in association with fasting plasma free fatty acid (FFA) changes following a modest weight loss in overweight-obese subjects. GG subjects at SNP *rs*6496589C/G experienced significantly higher reduction in FFA compared with CC carriers before and after adjustment for age, gender and BMI. Thus, the greater reduction in FFA levels may partially result from an associated impairment of the perilipin inhibitory actions in PKA-mediated lipolysis in response to calorie restriction. Because FFAs may reflect increased lipolysis accompanying weight loss, GG subjects at SNP *rs*6496589C/G experienced greater rates of lipolysis. This is in accordance with our results, showing that GG at SNP *rs*6496589C/G was associated with lower waist circumference.

In conclusion, our results support the hypothesis that *PLIN1* is a modifier gene of central obesity. Furthermore, variant rs6496589 can be used to predict central obesity risk in the Chinese population.

Conflicts of interest

The authors declared no conflict of interest.

Acknowledgments

Weihua Song, Hui Yu, Rutai Hui and Jingzhou Chen conceived and carried out experiments. Yahui Lin, Yinhui Zhang and Yan Song carried out experiments. Kai Sun analyzed the data. All authors were involved in writing the paper and approved it for submission.

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