Original Article

Association of Genetic Variation of the Adiponectin gene with Body Fat Distribution and Carotid Atherosclerosis in Japanese Obese Subjects

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***Aim*: The aim of this study was to investigate the effect of SNP45 of the adiponectin gene on body fat distribution and carotid atherosclerosis in Japanese obese subjects.**
***Methods*: A total of 64 obese subjects were investigated. Genotypes of SNP45 were assayed by polymerase chain reaction-restriction fragment length polymorphism. Visceral fat area (VFA) and subcutaneous fat area (SFA) were measured using computed tomography. The progression of atherosclerosis was evaluated by plaque score (PS) of carotid artery using B-mode ultrasonography.**
***Results*: Men carrying the G allele of SNP45 showed higher VFA (172.8 ± 50.8 vs. 147.1 ± 58.7, $p = 0.005$), lower SFA (209.9 ± 101.8 vs. 273.4 ± 142.2, $p = 0.007$), higher VFA/SFA (V/S) ratio (1.00 ± 0.46 vs. 0.60 ± 0.26, $p < 0.001$) and higher PS (9.5 ± 3.7 vs. 6.8 ± 4.2, $p = 0.012$) than those with TT genotype. Multivariate analysis showed that SNP45 was an independent determinant of V/S ratio and PS in men. In subgroup analysis, PS tended to be associated with V/S ratio only in the carrier of 45G allele.**
***Conclusion*: These results suggest that the G allele could be a risk factor of metabolic syndrome and the development of atherosclerosis in Japanese obese subjects.**

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Key words; Visceral obesity, Plaque score, Polymorphism, PCR-RFLP

Introduction

Adiponectin is a 244-amino acid protein synthesized and secreted exclusively by adipose tissue[1, 2] and plays an important role in the regulation of energy homeostasis and insulin sensitivity[3-5]. Adiponectin also has anti-atherogenic effects. This protein has been shown to suppress the expression of class A scavenger receptors in macrophages, affect the nuclear factor (NF)-κB pathway and inhibit monocyte adhesion to aortic endothelial cells[6-8].

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Genetic variations in the human adiponectin gene, especially two single nucleotide polymorphisms (SNPs) (+45T>G and +276G>T), have been reported to be associated with obesity, insulin resistance[9], type 2 diabetes[10, 11], and coronary artery disease[12]. Hara *et al.* reported that these two SNPs were associated with insulin resistance, indicating the pathogenesis of type 2 diabetes[11]. The mechanism underlying insulin resistance in type 2 diabetes is not fully understood, but many studies in nondiabetic populations have addressed the importance of upper body fat distribution. However, the association between these SNPs and body fat distribution has not been investigated. Based on these previous findings, it has recently been reported that the G allele of SNP45 was associated with susceptibility to coronary artery disease independent of conventional risk factors[12]. Although, the mechanism is not clear, we hypothesized that SNP45 could modify body

fat distribution and lead to more accumulation of visceral adipose tissue, resulting in metabolic abnormalities and the development of atherosclerosis in the process of increasing adipose tissue. To determine the validity of this hypothesis, we investigated the association of SNP45 with (1) various clinical and metabolic parameters, (2) body fat distribution, and (3) the progression of atherosclerosis using the plaque score of the carotid artery and maximum IMT in a group of Japanese obese patients.

Material and Methods

Subjects

Sixty-four Japanese obese subjects (40 men and 24 women, aged 54.2 ± 16.6 years, BMI 30.3 ± 5.3 kg/m^2), receiving medical checkups in our institute from 2002 to 2004, were recruited for this study. These included 49 patients with type 2 diabetes, among whom 24 were treated with oral hypoglycemic agents, 13 with insulin, and 12 with diet alone. Subjects with other endocrine diseases or significant renal or hepatic disease were excluded.

Obesity was defined as a body mass index (BMI) ≧25 kg/m^2, based on the criteria of the Japan Society for the Study of Obesity[13]. Diabetes mellitus was diagnosed according to World Health Organization criteria[14] and/or receiving treatment for diabetes mellitus. Informed consent was obtained from all subjects. This study was approved by the Ethics Committee of Kanazawa University.

Screening of Mutations in the Adiponectin gene

Genomic DNA was extracted from peripheral blood leukocytes using the standard procedure. Genotypes were determined at position 45 relative to the translation start site (corresponding to GenBank AB012163S1, 2, 3) by PCR, followed by allele-specific hybridization.

DNA fragments containing SNP45 (372 bp) were amplified by PCR from genomic DNA using primers 5'-GCAGCTCCTAGAAGTAGACTCTGCTG-3' and 5'-GGAGGTCTGTGATGAAAGAGGCC-3'. PCR products were incubated at 25℃ for 2 hours using *SmaI* (New England BioLabs Inc. UK). Digested products were separated by size on 3% agarose gel with ethidium bromide staining. The DNA segment from the G/G homozygote of SNP45 was digested into 209 and 163 bp fragments.

Laboratory Measurements

BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Waist circumference at the umbilical level was measured in the exhalation phase of respiration while standing.

Venous blood samples were obtained after a 12-hour overnight fast. Serum total cholesterol (TC) and triglyceride (TG) were determined by enzymatic methods, and high-density lipoprotein cholesterol (HDL-C) levels were measured by a polyanion-polymer/detergent method. Serum immunoreactive insulin (IRI) was measured by enzyme-linked immunosorbent assay, blood glucose with the glucose oxidase method, and HbA_{1c} by high-pressure liquid chromatography. The insulin resistance index was calculated based on homeostasis model assessment (HOMA) [fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5][15]. Plasma adiponectin levels were measured with an enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co., Tokushima, Japan), and leptin was measured by radioimmunoassay.

Body Fat Distribution

All subjects underwent computed tomography (CT) at the umbilical level to measure the cross-sectional abdominal subcutaneous fat area (SFA) and visceral fat area (VFA) using Fat Scan (N2 System Corp, Osaka, Japan)[16]. The VFA/SFA ratio was calculated as visceral fat area divided by subcutaneous fat area.

Determination of Plaque Score and Max IMT

A high resolution B-mode ultrasonography unit (SS-A 370A; Toshiba; Tokyo) with a 7.5 MHz transducer was used to determine the plaque score of the carotid artery[17]. Carotid Intima-Media Thickness (IMT) was measured at each common carotid, carotid bulb, and internal carotid artery.

The maximum IMT (Max-IMT) was defined as the highest IMT value at any location in the near and far walls of the carotid arteries, including atheromatous plaques on both sides. We defined a plaque, focal IMT thickening, as an area where IMT ≧ 1.1 mm, and calculated the plaque score by totaling the maximum thickness of all plaques on the near and far walls of vessels in the scanned area[17].

Statistical Analysis

All data are shown as the mean ± SD. A chi-square test was used to confirm that the genotype frequency was in Hardy-Weinberg equilibrium and to compare differences. Continuous variables were compared by ANOVA after being adjusted for age, BMI, and sex. Univariate and stepwise regression analyses were employed to examine the association between the plaque score and clinical parameters. All statistical analyses were conducted with StatView 5.0 for Macintosh

Table 1. Genotype distribution and allele frequencies for the adiponectin gene SNP45

	SNP45 genotypes			Allele frequencie	
	T/T	T/G	G/G	T	G
n (%)	34 (53.1)	25 (39.1)	5 (7.8)	0.72	0.28
male (n = 40)	23 (57.5)	13 (32.5)	4 (10.0)	0.74	0.26
female (n = 24)	11 (45.8)	12 (50.0)	1 (4.2)	0.71	0.29

Table 2. Clinical characteristics according to adiponectin genotypes at position 45

	T/T	T/G + G/G	*P*
n (%)	34 (53.1%)	30 (46.9%)	
M/F	23/11	17/13	0.36
Age (years)	53 ± 15	56 ± 18	0.39
Type 2 diabetes (%)	73.5	73.3	0.98
BMI (kg/m^2)	30.7 ± 6.3	29.4 ± 3.6	0.56
Waist (cm)	103.3 ± 14.2	103.2 ± 13.0	0.37
HOMA-R	3.6 ± 2.3	3.3 ± 1.9	0.80
HbA_{1c} (%)	7.1 ± 1.9	6.7 ± 1.6	0.32
Total cholesterol (mg/dL)	211 ± 35	204 ± 43	0.44
Triglycerides (mg/dL)	152 ± 104	151 ± 82	0.80
HDL cholesterol (mg/dL)	46 ± 10	45 ± 13	0.35
Adiponectin (μg/mL)	5.5 ± 2.3	6.8 ± 4.5	0.26
Leptin (ng/mL)	10.8 ± 6.4	14.1 ± 12.3	0.07
Systolic blood pressure (mmHg)	131 ± 19	135 ± 20	0.34
Diastolic blood pressure (mmHg)	79 ± 11	79 ± 14	0.65
Subcutaneous fat area (cm^2)	275.4 ± 127.5	246.5 ± 94.6	0.32
Visceral fat area (cm^2)	140.5 ± 56.6	151.1 ± 51.0	0.06
V/S ratio	0.56 ± 0.24	0.76 ± 0.45	0.009
Max IMT	1.89 ± 0.81	2.21 ± 0.91	0.27
Plaque score	6.1 ± 4.1	9.7 ± 3.9	$<$0.001

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation.
Abbreviations: BMI = body mass index, HOMA-R = homeostasis model assessment of insulin resistance
* *P* values adjusted for age, sex, and body mass index.

(Abacus Concepts, Berkeley, CA). A *P* value of less than 0.05 was considered statistically significant. In stepwise analysis, an F value greater than 4 was significant.

Results

Genotypes and Allele Distribution of SNP45 of the Adiponectin gene

The genotype and allele frequencies of study subjects are shown in **Table 1**. Genotype distributions were in Hardy-Weinberg equilibrium at both loci, with T being the major allele. The frequency of the T allele of SNP45 was 72%, and the frequencies of the T/T genotype, T/G genotype, and G/G genotype were 53.1%, 39.1%, and 7.8%, respectively.

Clinical and Metabolic Characteristics of this Study According to SNP45 of the Adiponectin gene

Table 2 shows a comparison of clinical characteristics and body composition according to adiponectin genotypes. Subjects were divided into 45T/T homozygote and those carrying the G allele (45T/G and 45G/G).

No differences in sex, age, or the proportion with diabetes were observed between any groups. Plasma leptin levels tended to be higher in carriers of the 45G allele (10.8 ± 6.4 vs. 14.1 ± 12.3, P = 0.07). Other variables (HbA_{1c}, plasma lipid, and plasma adiponectin levels) did not differ between these genotypes.

Table 3. Clinical characteristics according to gender

	men	women	*P*
n	40	24	
Age (years)	51 ± 16	60 ± 14	0.02
BMI (kg/m^2)	30.7 ± 6.3	29.4 ± 3.6	0.56
Waist (cm)	102.7 ± 13.7	104.2 ± 13.5	0.03
HOMA-R	3.6 ± 2.4	3.1 ± 1.3	0.76
HbA_{1c} (%)	6.7 ± 1.9	7.4 ± 1.5	0.14
Total cholesterol (mg/dL)	204 ± 40	216 ± 36	0.15
Triglycerides (mg/dL)	166 ± 108	132 ± 59	0.37
HDL cholesterol (mg/dL)	42 ± 9	53 ± 12	0.02
Adiponectin (μg/mL)	5.2 ± 2.5	7.6 ± 4.4	0.03
Leptin (ng/mL)	9.6 ± 9.2	16.8 ± 9.2	0.03
Systolic blood pressure (mmHg)	132 ± 19	135 ± 19	0.27
Diastolic blood pressure (mmHg)	80 ± 13	77 ± 9	0.65
Subcutaneous fat area (cm^2)	246.4 ± 129.1	287.6 ± 76.3	< 0.001
Visceral fat area (cm^2)	158.0 ± 56.3	124.5 ± 43.1	0.002
V/S ratio	0.77 ± 0.41	0.47 ± 0.17	< 0.001
Max IMT	2.09 ± 1.00	1.96 ± 0.61	0.18
Plaque score	8.1 ± 4.1	7.9 ± 5.0	0.41

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation.
Abbreviations: BMI = body mass index, HOMA-R = homeostasis model assessment of insulin resistance
* *P* values adjusted for age and body mass index.

Table 4. Body fat distribution and PS according to adiponectin genotypes at position 45 in men and women

	men			women		
	T/T	T/G + G/G	*P*	T/T	T/G + G/G	*P*
VFA	147.1 ± 58.7	172.8 ± 50.8	0.005	126.7 ± 52.0	122.7 ± 36.1	0.763
SFA	273.4 ± 142.2	209.9 ± 101.8	0.007	279.8 ± 95.6	294.3 ± 58.8	0.091
V/S ratio	0.60 ± 0.26	1.00 ± 0.46	< 0.001	0.49 ± 0.18	0.44 ± 0.16	0.262
PS	6.8 ± 4.2	9.5 ± 3.7	0.012	4.4 ± 4.0	10.8 ± 3.8	0.135

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation.
Abbreviations: VFA = visceral fat area, SFA = subcutaneous fat area

Relationship between Genotypes and Body Fat Distribution

When we considered the VFA/SFA ratio as a marker of body fat distribution, it was significantly higher in carriers of the 45G allele (0.76 ± 0.45 vs. 0.56 ± 0.24, $P = 0.009$), whereas there were no associations between SFA and SNP45. VFA tended to be higher in carriers of the 45G allele than TT homozygote (151.1 ± 51.0 vs. 140.5 ± 56.6 cm^2, $P = 0.06$). Neither BMI nor waist circumference significantly differed between the two groups. Since there were sex differences in body fat distribution in this study (**Table 3**: men vs. women; VFA: 158.0 ± 56.3 vs. 124.5 ± 43.1, $P = 0.002$; SFA: 246.4 ± 129.1 vs. 287.6 ± 76.3, $P < 0.001$; V/S ratio: 0.773 ± 0.410 vs. 0.470 ± 0.176, $P < 0.001$), we performed separate analyses of the association between SNP45 and body fat distribution by sex (**Table 4**, **Fig. 1**). In men, SNP45 was associated with the VFA, SFA, and V/S ratio, whereas in women SNP45 was not associated with body fat distribution.

Furthermore, to evaluate the contribution of SNP45 to the V/S ratio in men, stepwise regression analysis was used (**Table 5**). Selected variables were age, BMI, SNP45, and adiponectin. The data showed that age, SNP45, and plasma adiponectin levels were independent determinants of the V/S ratio in men ($R^2 = 0.588$, $P < 0.0001$).

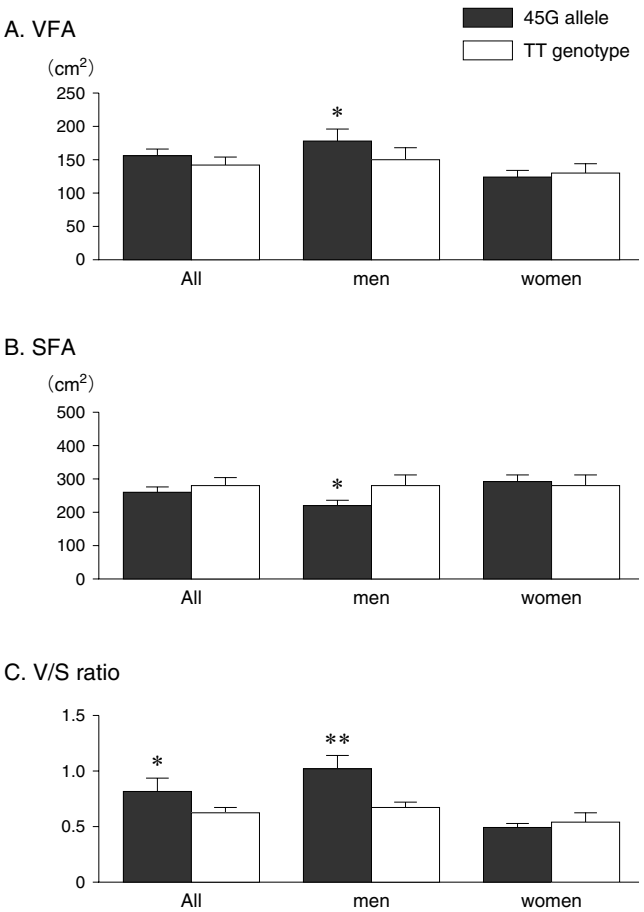

Fig. 1. Effect of SNP45 of the adiponectin gene on body fat distribution in all subjects, men and women

A. Effect of SNP45 on visceral fat area (VFA)
B. Effect of SNP45 on subcutaneous fat area (SFA)
C. Effect of SNP45 on VFA/SFA (V/F) ratio
Data are the means ± SE. $^{*}P < 0.05$, $^{**}P < 0.001$

Table 5. Stepwise regression analysis for determinant of V/S ratio in men

Factor	β	F-value
Age	0.017	35.763
SNP45*	0.340	24.945
Adiponectin	−0.073	13.688

$R^2 = 0.588$
*TT genotype=0, TG genotype=1, GG genotype=2

Table 6. Stepwise regression analysis for determinant of PS in men

Factor	β	F-value
Age	0.143	13.362
SNP45*	2.509	7.563

$R^2 = 0.372$
*TT genotype=0, TG genotype=1, GG genotype=2

Table 7. Correlation of PS to V/S ratio according to the genotype in men and women

	equation	r	p
men			
all	y=4.05x+4.71	0.39	0.02
G allele	y=3.22x+6.17	0.38	0.14
TT genotype	y=2.91x+4.93	0.17	0.49
women			
all	y=4.67x+5.85	0.17	0.47
G allele	y=12.00x+5.41	0.60	0.06
TT genotype	y=−2.57x+5.59	0.13	0.75

Relationship between Genotypes and Plaque Score of Carotid Artery

We investigated the effect of SNP45 on the plaque score and max IMT of carotid arteries. Carriers of the G allele had significantly greater PS than TT genotype after adjusting for age, sex, and BMI (10.0 ± 3.7 vs. 6.4 ± 4.2, $P < 0.001$).

As shown in **Table 4**, SNP45 was associated with PS in men, and PS of the G allele tended to be higher than the TT genotype in women. To analyze the independent contribution of SNP45 to PS in men, stepwise regression analysis was applied (**Table 6**). Selected variables were age, BMI, V/S ratio, adiponectin, TC, HDL-C, systolic BP, and SNP45. The data showed that age and SNP45 were independent determinant of PS in men ($R^2 = 0.372$, $P = 0.0007$).

Relationship between V/S Ratio and Plaque Score of Carotid Artery

To examine the effect of the V/S ratio on PS, we performed univariate analysis (**Table 7**).

There was a significant positive correlation between the V/S ratio and PS in men ($r = 0.39$, $P = 0.02$), whereas in women that correlation were not statistically significant ($r = 0.17$, $P = 0.47$).

Next, to investigate the impact of SNP45 on the association between the V/S ratio and PS, we subdivided into two groups according to the genotype of SNP45 in men and women. The V/S ratio tended to be associated with PS in the G allele in both men and women (men: $r = 0.38$, $p = 0.14$; women: $r = 0.60$, $p = 0.06$, respectively). In contrast, in subjects with the TT genotype, there was no relationship between the V/S ratio and PS.

Discussion

Our study had three major findings in Japanese obese subjects. First, SNP45 in the adiponectin gene was associated with body fat distribution. Second, SNP45 was associated with the development of carotid atherosclerosis. Moreover, SNP45 had an impact on the effect of visceral obesity for the progression of atherosclerosis. Third, there was a gender difference in the effect of SNP45.

First, we demonstrated that the G allele had higher VFA, lower SFA, and a significantly higher V/S ratio compared to the TT genotype in men. Multivariate regression analysis showed that SNP45 was an independent determinant of the V/S ratio. These results indicated that the G allele of SNP45 is a risky genotype of visceral adiposity, resulting in metabolic syndrome. To our knowledge, this is the first study to demonstrate the association of SNP45 with body fat distribution. Some reports have shown that SNP45 contributes to obesity, insulin resistance, or dyslipidemia[10, 18, 19]. In contrast, Ukkola *et al.* reported that SNP45 was found in equal frequency among obese and non-obese Swedish subjects[20]. In French Caucasians, the 45G allele frequency was similar in morbidly obese adults and control subjects[21]. The inconsistency between these reports suggested that SNP45 could not be associated simply with weight or prevalence of obesity, but might contribute to body fat distribution in the process of becoming obese. Since visceral adipose tissue is widely believed to play a key role in the pathogenesis of metabolic abnormalities, the G allele of SNP45 could be an independent risk factor for metabolic syndrome.

Second, another important finding of the present study was the significant association between SNP45 and carotid artery PS in men. A similar trend was observed in women. Multivariate regression analysis showed that SNP45 was an independent determinant of PS. These findings suggest that SNP45 may affect the development of carotid atherosclerosis not only by modulating visceral obesity but also by other pathways.

To the best of our knowledge, PS tends to be associated with the V/S ratio only in the G allele in both men and women. In a previous study, we described a strong association between the V/S ratio and carotid artery PS in Japanese males with metabolic syndrome[22], but patients with the TT genotype were protected from the atherogenic effect of visceral obesity. We hypothesized that visceral obesity might exaggerate the dysregulation of adiponectin properties of the G allele. The mechanism was unclear, but this hypothesis needs confirmation by expression studies.

Third, in this study the degree of the effect of SNP45 on body fat distribution and PS was different between men and women. Adipose tissue is sexually dimorphic in humans, with gender-specific differences in body fat distribution[23, 24]. Gonadal steroids are the major mediator of sex dimorphism of body composition in adults[25, 26]. Estrogen regulates both the metabolism and location of adipose tissue and plays a role in adipogenesis, adipose deposition, lipogenesis, lipolysis, and adipocyte proliferation[27]. Furthermore, in recent studies, Clegg *et al.* reported that gonadal steroids mediate body fat distribution and interact with the integrated adiposity messages conveyed to the brain[28]. Taken together with previous studies, our findings suggest that estrogen may interact with the adiponectin gene in adipocyte and modulate the effect of SNP45.

In addition, estrogen is known to have a cardioprotective effect. *In vivo* evidence suggests that the effect of estrogen on adhesion molecules is mediated by the inhibition of nuclear factor (NF)-κB DNA binding[29, 30]. As adiponectin has been shown to suppress the expression of class A scavenger receptors in macrophages, to affect the NF-κB pathway and to inhibit monocyte adhesion to aortic endothelial cells[6-8], atherogenic properties of the G allele may be suppressed by the effect of estrogen. Estrogen could interact with SNP45 and modulate the atherogenic function of adiponectin, but further large studies are needed to confirm the mechanism of gender-specific differences in the effect of SNP45.

The mechanistic relationship between SNP45 and both body fat distribution and the progression of atherosclerosis is unclear. SNP45 is located in exon 2 of the adiponectin gene and does not cause an amino acid change (GGT to GGG, Gly15Gly). One possibility is that SNP45 may have linkage disequilibrium with other undiscovered SNPs of the adiponectin gene having an effect on adiponectin expression, secretion, structure, or action. Another possibility is that SNP45 located in exon 2 is relatively close to the exon-intron boundary which may affect splicing machinery and effect adiponectin expression. The G allele of SNP45 may act through decreased adiponectin expression, which may cause increased visceral adipose tissue. Indeed, in Japanese type 2 diabetes, SNP45 is reported to be associated with reduced adiponectin levels[11]. Similar findings have been shown in an other study[31]. Furthermore, recent studies have reported various adiponectin functions as an adipocyte differentiation factor, helping to maintain equilibrium adipocyte size, as an autocrine/paracrine factor in adipose tissue and as a participating factor in the regulation of adipocyte metabolism and adipose tissue mass. In 3T3-L1 preadipocytes,

adiponectin overexpression accelerates cell proliferation and differentiation, while in mature adipocytes, autocrine adiponectin increases glucose uptake and lipid accumulation[32]. Transgenic overexpression of adiponectin in the physiological range induced morbid obesity without insulin resistance in ob/ob mice[21]. These reports indicated that hyperadiponectinemia may induce simple obesity with more subcutaneous fat accumulation, while decreased adiponectin levels may induce visceral obesity. Interestingly, the present study showed that hypoadiponectinemia was the third independent determinant of the V/S ratio. Due to these previous findings combined with our present study, the G allele might be genetically determined to have hypoadiponectinemia, contributing to the progression of visceral obesity. In contrast, the TT genotype might favor the accumulation of subcutaneous adipose tissue through hyperadiponectinemia, preventing insulin resistance, and eventually metabolic syndrome.

Adiponectin exists largely as low molecular weight (LMW) hexamers and high molecular weight (HMW) multimers[32, 33]. Recent article showed that the ratio of HMW to total adiponectin was responsible for metabolic effects[34]. Another study showed that HMW adiponectin was an important factor in metabolic syndrome[35]. Therefore, the alternative possibility of the atherogenic effect of SNP45 is that the proportion of HMW adiponectin might decrease in the G allele of SNP45, leading to atherosclerosis. As we measured total adiponectin and did not assess multimeric forms of adiponectin, further study is needed.

In conclusion, we demonstrated that SNP45 was associated with body fat distribution and PS of carotid arteries. The TT genotype is a protective genotype from metabolic syndrome and atherosclerosis progression in Japanese obese subjects. The mechanism by which SNP45 affects body fat distribution and the development of atherosclerosis has not been clarified at present. Further investigations will be needed to elucidate the functional mechanism of this polymorphism.

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