Associations between Two Single Nucleotide Polymorphisms of Adiponectin Gene and Coronary Artery Diseases

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Abstract. Adiponectin, an adipocyte-secreted protein, is known to have anti-atherogenic, anti-inflammatory and anti-diabetic properties and its serum levels are decreased in obesity, type 2 diabetes, and coronary artery disease. Several studies have been performed to investigate the association of genetic variations in the adiponectin with obesity, insulin resistance, and type 2 diabetes, but few studies were performed in association with coronary artery disease. Therefore we examined the associations between two single nucleotide polymorphisms (SNPs), +45T>G and +276G>T of the adiponectin gene, and coronary artery diseases (CAD). One hundred and fifty six subjects (mean age 57.4 yrs) were enrolled in which coronary angiograms were performed due to chest pain. Genotypings were done for two SNPs in the adiponectin gene by Taqman polymerase chain reaction (PCR) method. The presence of CAD was defined as a >50% reduction of coronary artery diameter. Among 156 subjects, the allele frequencies were 0.683 for G allele and 0.317 for T allele in SNP +276G>T and 0.705 for T allele and 0.295 for G allele in SNP +45T>G. Both genotypes were in compliance with Hardy-Weinberg equilibrium. No association with the presence of CAD was observed for adiponectin gene SNP276 and SNP45 ($p = 0.954$, $p = 0.843$). Also, no significant association was observed between the severity of CAD and either SNPs ($p = 0.571$, $p = 0.955$). Our study showed that SNP +276G>T and +45T>G in adiponectin gene were not associated with the presence of CAD. Further studies will be necessary to confirm the role of SNP 276G>T and 45T>G in the development of CAD.

Key words: Adiponectin, Single nucleotide polymorphism, Coronary artery disease

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IN industrialized societies, cardiovascular diseases are the leading cause of death. They develop by the interaction of diverse environmental factors and genetic factors, the major causality being atherosclerosis [1]. Adiponectin is a recently discovered adipocytokine that is specifically and highly expressed in human adi-

pose tissue, and is one of the mostly abundant protein in circulation in human body [2, 3]. Plasma adiponectin level has been reported to be decreased in the conditions of obesity and type 2 diabetes. Administration of synthetic adiponectin reduces the serum glucose level in both normal and diabetic rat models, and is thought to play an important role in insulin resistance, obesity, and diabetes in rats with obesity-induced insulin resistance [4–6]. Sakuta *et al.* have reported that plasma levels of adiponectin were associated positively with HDL-C and age and inversely with body mass index and HbA1C in Japanese men with type 2 diabetes [7]. Ouchi *et al.* have reported that in 42 patients with coro-

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nary artery diseases, serum adiponectin concentration was significantly lower than control group, and significantly low concentrations were detected in the association with the cardiovascular risk factors, for example, hypertension, obesity and type 2 diabetes [8]. In addition, in males with coronary artery diseases, subjects with hypoadiponectinemia showed 2 times higher risk for coronary artery diseases independent of the effects of other cardiovascular risk factors than those with normal serum adiponectin level [9].

In a few recent *in vitro* studies, adiponectin has been considered to inhibit the TNF- α -induced monocyte adhesion to the aortic endothelium, to suppress the expression of various adhesion molecules and the class A macrophage scavenger receptor, and to inhibit the macrophages from forming foamy cells as well as to inhibit vascular smooth muscle cell proliferation and migration, through which it has anti-inflammatory and anti-atherogenic properties [8, 10]. In animal studies, adiponectin-deficient mice exhibited severe endothelial thickening of artificially injured arteries and showed smooth muscle cell proliferation and the supplementation of adiponectin by adenovirus transfection clearly improved neointimal proliferation in this injured model, which suggest that the advanced vascular injury was the direct effect of adiponectin deficiency [11, 12].

Human adiponectin gene, referred to as *APM1*, is located in the chromosome 3q27 that is known to be the susceptibility locus for metabolic syndrome and type 2 diabetes, and is encoded by 3 exons which spans approximately 16 kb [13, 14]. It shares structural similarities with complement protein C_{1q} and the TNF family that have been known to play an important role in inflammation, immune system, and arteriosclerosis [15, 16]. In the genome-wide scan performed in Japanese and Caucasians, more than 10 single nucleotide polymorphisms were reported, and among them, the genetic polymorphism of +45 in the exon 2 and +276 in the intron 2 have been frequently referred to be in association with type 2 diabetes and obesity [17–21]. Recently, several studies have been reported in association of adiponectin SNPs with coronary artery disease, although those in Asians are scarce.

Therefore, we investigated whether the two SNPs of adiponectin gene, that is, +276G>T in intron 2 and +45T>G in exon 2, are associated with the presence of coronary artery diseases in Korean subjects who underwent coronary angiograms.

Materials and Methods

Subjects and Measurements

156 subjects were enrolled in which coronary angiograms were performed due to chest pain in Kangbuk Samsung Hospital from April to August, 2003 (97 males, 59 females, mean age 57.40 ± 11.15 years). The patients with acute infectious diseases, chronic kidney disease (creatinine ≥ 2.0 mg/dL), osteoporosis, malignant tumor, and other medical diseases were excluded from the study population. Written informed consent was obtained from each participant and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Height, weight, waist circumference and systolic and diastolic blood pressures were measured in duplicate and the results were averaged. Weight and height were measured in Kg and cm, respectively, down to two decimal points. The body mass index (BMI) was calculated by dividing the weight (Kg) with the square of height (m).

After 12 hours of fasting, blood sampling was done. Fasting blood glucose, total cholesterol, triglyceride, HDL-C, and LDL-C were measured from the samples and the hexokinase method was used to measure blood glucose levels and enzymatic calorimetric test was used to measure the total cholesterol and triglyceride levels. The selective inhibition method was used to measure the level of HDL-C and the homogeneous enzymatic calorimetric test was used to measure the level of LDL-C.

Coronary artery angiography was performed in all patients. Significant stenosis was defined as the internal diameter decreased by more than 50%. Patients were grouped according to the number of significantly stenotic vessels into normal, 1-vessel, 2-vessel and 3-vessel diseased groups.

Genotyping of adiponectin polymorphisms of +276G>T in intron 2 and +45T>G in exon 2 by real-time polymerase chain reaction (PCR)

The buffy coat was obtained from blood samples, refrigerated at -70°C , and the genomic DNA was then extracted using Takara DNA Purification kits. The genotyping of the T45G polymorphism in exon 2 and the G276T in intron 2 of the adiponectin gene was per-

formed by employing an allelic discrimination assay and by using the TaqMan probe. The detector used in this experiment was an ABI Prism 7200 sequence detection platform (Perkin Elmer, USA). The primers and probes used were as follows.

1) T45G

forward primer:

5'-CAC ATG TGG ATT CCA GGG C-3'

reverse primer:

5'-CCC TTG GGC AGG GGA A-3'

FAM-labeled probe:

5'FAM-CCG GTC ATG ACC AGG3'

VIC-labeled probe:

5'VIC-TGC CCG GGC ATG A3'

2) G276T

forward primer:

5'-TTC TCC CTG TGT CTA GGC CTT AGT T-3'

reverse primer:

5'-CTT TCA TCA CAG ACC TCC TAC ACT GA-3'

FAM-labeled probe:

5'FAM-TGA ATG CCT TCA TAT AG3'

VIC-labeled probe:

5'VIC-ATA ATG AAT GAC TTC ATA TAG TT3'

Statistical method

Statistical analysis was performed using the SPSS for Windows version 11.0. All results were presented as the mean $\pm$ standard deviation (SD). The comparison of mean values between different coronary artery disease severity group was analyzed by Student's *t*-test. The test for Hardy-Weinberg equilibrium was

performed using the χ^2 -test. The association of the genotypes of the adiponectin gene with the presence or absence of coronary artery diseases in each group was analyzed by χ^2 -test, and as a statistically significant level, *p* value less than 0.05 was used.

Results

General characteristics of the subjects

The general characteristics of the study subjects are presented in Table 1. In 63 cases (65.6%) out of total 97 cases of males and 25 cases (42.4%) out of 59 female cases, coronary artery stenosis was detected by coronary artery angiogram, which showed significantly higher prevalence of coronary artery stenosis in males ($p = 0.005$). Subjects with coronary artery was significantly older than those with normal coronary artery (60.44 ± 10.18 vs 53.40 ± 11.18 , $p < 0.001$), and high-density lipoprotein cholesterol level was significantly lower in subjects with coronary stenosis than those with normal coronary artery disease (48.50 ± 12.29 vs 54.34 ± 12.30 , $p = 0.004$). In regard to total cholesterol, low-density lipoprotein cholesterol, triglyceride, body mass index, and fasting serum glucose, significant differences between two groups were not detected (Table 1). In the entire study population, patients with fasting serum glucose over 126 mg/dL were 34 cases out of 156 cases, the cases with over 126 mg/dL who showed coronary artery stenosis were 28 patients, the group showing normal coronary arteries was

Table 1. General characteristics of CAD cases and control patients

	Total	CAD	Control	P
Age (years)	57.40 $\pm$ 11.15	60.44 $\pm$ 10.18	53.40 $\pm$ 11.18	<0.001
Gender, M/F	97/59	63/25	34/34	0.005
BMI (kg/m ²)	25.34 $\pm$ 2.88	25.43 $\pm$ 2.74	25.23 $\pm$ 3.05	0.679
DM	34 (21.8%)	28 (31.8%)	6 (8.8%)	0.001
IFG	54 (34.6%)	31 (57.4%)	23 (42.6%)	0.855
FPG (mg/dL)	116.26 $\pm$ 43.53	123.39 $\pm$ 48.84	107.03 $\pm$ 33.67	0.015
T-chol (mg/dL)	200.91 $\pm$ 47.42	200.40 $\pm$ 51.33	201.58 $\pm$ 42.10	0.878
TG (mg/dL)	180.17 $\pm$ 181.85	188.91 $\pm$ 186.63	168.16 $\pm$ 175.81	0.489
HDL-chol (mg/dL)	105.20 $\pm$ 51.01	48.50 $\pm$ 12.30	54.34 $\pm$ 12.30	0.004
LDL-chol (mg/dL)	112.41 $\pm$ 34.54	112.96 $\pm$ 34.71	111.67 $\pm$ 34.55	0.819

Data are means $\pm$ SD. BMI, body mass index; DM, diabetes mellitus; IFG, impaired fasting glucose; FPG, fasting plasma glucose; T-chol, total cholesterol; TG, triglyceride; HDL-chol, high-density lipoprotein cholesterol; LDL-chol, low-density lipoprotein cholesterol

6 patients, and a statistically significant difference was observed (31.8% vs 8.8%, $p = 0.001$).

Relation of adiponectin gene polymorphisms and presence of coronary artery diseases

In total 156 study subjects, in regard to the frequencies of the G276T polymorphism in intron 2 of the adiponectin gene, GG was 44.2% (69 cases), GT was 48.1% (75 cases), and TT was 7.7% (12 cases). The allele frequency was 0.683 for the G allele and 0.317 for the T allele, which were in compliance with Hardy-Weinberg equilibrium ($p = 0.39$). In addition, regarding the frequencies of the T45G genotype of adiponectin exon 2, TT was 48.1% (75 cases), TG was 44.9% (70 cases), and GG was 7.1% (11 cases). The frequency of T allele was 0.705 and the frequency of G allele was 0.295, which also was in compliance with Hardy-Weinberg equilibrium ($p = 0.61$).

Between the group with and without coronary artery diseases, the frequency of the genotype of SNP45 was not different (Table 2, $p = 0.843$). After adjustment for other confounding CAD risk factors such as age, gender and HDL-C, there was no significant association

between the frequency of the genotype SNP45 and CAD ($p = 0.675$). Similarly, concerning the frequency of SNP276 genotype, no significant difference between two groups was detected (Table 2, $p = 0.954$). Also, no significant association was detected after adjustment for age, gender and HDL-C ($p = 0.880$). Regarding the frequency of SNP 45 and SNP276 genotype according to the number of coronary arteries with stenosis, no significant difference was detected (Table 3, $p = 0.995$, $p = 0.571$).

Discussion

Among approximately 10 adiponectin gene polymorphisms reported until now, +45T>G of exon 2 and +276G>T of intron 2 were associated with the increased risk of insulin resistance and type 2 diabetes in particular [17–20]. Numerous previous studies conferred the anti-inflammatory and anti-atherosclerotic effects of adiponectin, and strong genetic associations were reported between adiponectin and cardiovascular risk factors [8, 10, 11, 22, 23].

Several studies have been performed in regard to the association of the adiponectin polymorphisms with coronary artery diseases. Ohashi *et al.* examined the association of the adiponectin gene polymorphisms with the incidence of coronary artery diseases in 383 Japanese patients with confirmed coronary artery diseases by cardiovascular angiogram and compared the results with 368 controls [24]. Three SNPs were investigated, that is, I164T, SNP94, and SNP276, and they found that SNP276 and SNP94 had no associations with CAD. But the frequency of I164T was significantly higher in subjects with coronary artery disease. In addition, the serum adiponectin levels were significantly lower in individuals with the I164T mutation

Table 2. Distribution of genotypes at +45G>T and +276T>G of the adiponectin gene in CAD case and control subjects

n		Control subject	CAD patients	P value
		68	88	
SNP 276, n (%)	GG	31 (45.6%)	38 (43.2%)	0.954
	GT	32 (47.1%)	43 (48.9%)	
	TT	5 (7.4%)	7 (8.0%)	
SNP 45, n (%)	TT	34 (50.0%)	41 (46.6%)	0.843
	TG	30 (44.1%)	40 (45.5%)	
	GG	4 (5.9%)	7 (8.0%)	

Table 3. Distribution of genotypes at +45G>T and +276T>G of the adiponectin gene among different groups according to the severity of coronary artery disease

n		Number of vessels involved				P
		Normal (68)	One (49)	Two (23)	Three (16)	
SNP 276, n (%)	GG	31 (45.6%)	25 (51.0%)	9 (39.1%)	4 (25.0%)	0.571
	GT	32 (47.1%)	21 (42.9%)	11 (47.8%)	11 (68.8%)	
	TT	5 (7.4%)	3 (6.1%)	3 (13.0%)	1 (6.3%)	
SNP 45, n (%)	TT	34 (50.0%)	21 (42.9%)	12 (52.2%)	8 (50.0%)	0.955
	TG	30 (44.1%)	23 (46.9%)	10 (43.5%)	7 (43.8%)	
	GG	4 (5.9%)	5 (10.2%)	1 (4.3%)	1 (6.3%)	

than control group. Lacquemant *et al.* have reported that in 162 Caucasian subjects with type 2 diabetes, SNP+45 showed significant association with increased coronary artery disease risk whereas SNP+276 failed to show any association [25]. On the other hand, Filippi *et al.* recently reported that in 325 coronary artery disease patients and 270 controls, SNP+276 showed significant association with coronary artery disease, with odds ratio for coronary artery disease being up to 4.99 [26]. Bacci *et al.* have reported that in 376 Caucasian type 2 diabetes patients, SNP+276 showed significant association with coronary artery diseases, which association was independent of the serum adiponectin levels [27]. In American diabetic males, Qi *et al.* reported the protective effect of SNP+276 for coronary artery disease with odds ratio for coronary artery disease being 0.38 after adjustment of other confounding variables [28].

In our study, no significant associations were observed between SNP+45 and SNP+276 with the presence of coronary artery diseases. The reason for the discrepancies with the previous studies could be explained by the small sample size. Furthermore, this was the second association study next to the study by Ohashi *et al.*, performed in Asian population analyzing the effect of adiponectin polymorphisms (I164T, SNP94, SNP276) on coronary artery disease incidence [24]. As referred above, there was no significant associations between SNP+276 and coronary artery disease in Japanese population, which is the same Asian population as our Korean population. As the allelic frequencies and the genetic effects of adiponectin gene are considered to be significantly different between Japanese and Caucasians, these discrepancies could be partly explained by ethnic differences, which needs further research in larger numbers of study subjects [29, 30].

Our study has several limitations. The first limitation is that as serum adiponectin level was not measured, hence the functional significance of adiponectin polymorphism cannot be assessed. However, in the previous studies, contradictory results were reported on

the association of the adiponectin polymorphism with serum adiponectin levels. In the studies by Bacci *et al.* and Qi *et al.*, serum adiponectin did not show association with genetic difference, and in other studies performed in Japanese, SNP276 showed significant association with plasma adiponectin levels only in the obese subgroup [27, 28, 31]. In addition, it is not so clear how serum adiponectin levels might reflect the adiponectin concentration in tissue level, such as, in the subendothelial space where the targets for the anti-atherogenic effect of adiponectin are located [32–34]. Therefore, to reflect the difference of adiponectin concentration among genotypes, more specifically designed studies are required *in vitro*. The second limitation is that the number of study population was too small to have statistically significant power as to the association of the adiponectin polymorphism with coronary artery diseases. We have estimated the power of our study to detect a difference, should it exist, between different adiponectin polymorphisms and the presence of coronary artery disease. In the sample size of 156 subjects, a hazard ratio of 1.69 would have been detectable with 90% power and 95% confidence limits [35]. Therefore we believe that our study has reasonable power and it is unlikely that we have missed any meaningful association because of insufficient numbers. However, this study could have meaning as the first association study of adiponectin polymorphism with coronary artery disease in Koreans, and the second study in Asian population.

In summary, no significant association was observed between adiponectin polymorphisms, +45T>G and +276G>T, and coronary artery disease in Korean subjects. Further studies are warranted in the future, with larger study populations in various ethnic groups.

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