The +276 G/T Single Nucleotide Polymorphism of the Adiponectin Gene Is Associated With Coronary Artery Disease in Type 2 Diabetic Patients

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OBJECTIVE — Two single nucleotide polymorphisms (SNPs) at the adiponectin locus (+45T>G and +276G>T) have been associated with low circulating adiponectin levels, insulin resistance, and type 2 diabetes. We investigated whether these genetic markers are determinants of coronary artery disease (CAD) in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS — A total of 376 consecutive type 2 diabetic patients were studied: 142 case subjects with coronary stenosis >50% or previous myocardial infarction and 234 control subjects with no symptoms, no electrocardiogram (ECG) signs of myocardial ischemia, and a normal ECG stress test (n = 189) and/or (n = 45) with coronary stenosis ≤50%.

RESULTS — No association with CAD was observed for the +45 SNP (P = 0.48). By contrast, a significant association was observed for the +276 SNP, with T/T homozygotes having a lower risk of CAD than carriers of other genotypes (adjusted odds ratio [OR] 0.13 [95% CI 0.037–0.46], P = 0.002). A similarly protective effect of the +276 T/T genotype was observed in 110 case and 45 control subjects for whom the CAD status had been determined by angiography (0.04 [0.006–0.30], P = 0.002).

Serum adiponectin, although clearly related to several features of the proatherogenic/insulin-resistant phenotype, was not different between control subjects and CAD patients (26 ± 17 vs. 25 ± 13 μg/ml).

CONCLUSIONS — In conclusion, the +276 G>T polymorphism is a determinant of CAD risk in type 2 diabetic patients. This marker may assist in the identification of diabetic individuals at especially high risk of CAD, so that preventive programs can be targeted at these subjects.

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Abbreviations: AER, albumin excretion rate; CAD, coronary artery disease; ECG, electrocardiogram; NF, nuclear factor; RIA, radioimmunoassay; SNP, single nucleotide polymorphism.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

The prevalence of coronary artery disease (CAD) is two to four times more frequent in patients with type 2 diabetes than in nondiabetic individuals, representing the leading cause of death in this population (1–3). An important role in modulating the cardiovascular risk of diabetic patients is played by comorbidities such as dyslipidemia and hypertension or by environmental risk factors such as smoking. Factors intrinsic to the vascular wall, regulating its propensity to react to the diabetic milieu with endothelial dysfunction and activation of proinflammatory mechanisms, are also believed to be major players (4). These vascular pathways are under genetic control, but the genes that are involved are mostly unknown (5–7).

Adiponectin is a circulating protein secreted by adipocytes that shares significant similarities with collagens VIII and X and complement protein C_{1q} (8,9). This adipokine has been recently shown to suppress the expression of class A scavenger receptors in macrophages, resulting in decreased uptake of oxidized LDL and reduced intracellular accumulation of cholesteryl esters (10). Adiponectin also has anti-inflammatory properties affecting the nuclear factor (NF)-κB pathway and inhibiting monocyte adhesion to aortic endothelial cells (11,12). In addition to these effects on the vasculature, this molecule is a potent enhancer of insulin action on peripheral tissues (13). Thus, adiponectin has a wide range of antiatherogenic effects. A genetic deficit of this protein might increase the risk of CAD in both the general population and type 2 diabetic patients. In support of this hypothesis is the recent finding that adiponectin homozygous knockout mice show a twofold increase in neointimal formation in response to external vascular cuff injury compared with wild-type mice (14).

Two single nucleotide polymorphisms (SNPs) at the adiponectin locus

Table 1—*Clinical characteristics of CAD cases and control patients with type 2 diabetes*

	CAD	Control	*P*
n	142	234	
Age (years)	64 ± 8	60 ± 8	**<0.001**
Men/Women	91/51	101/133	**<0.001**
Duration of diabetes (years)	15 ± 9	12 ± 6	**0.03**
Smokers	38	29	0.09
BMI (kg/m^2)	29 ± 5	30 ± 5	**0.05**
HbA_{1c} (%)	8.7 ± 2	8.4 ± 2	0.1
Insulin therapy	55	37	**0.002**
Antihypertensive therapy	84	73	**0.018**
Antidyslipidemic therapy	69	41	**<0.001**
Antiplatelet therapy	89	46	**<0.001**
Peripheral vascular disease	57	10	**<0.001**
Nephropathy	35	27	0.13
Retinopathy	62	33	**<0.001**
Neuropathy	41	25	0.1
Fibrinogen (mg/dl)	396 ± 128	351 ± 100	**<0.001**
AER (μg/min)	87 ± 187	57 ± 247	0.2

Data are means ± SD or percent. CAD: patients with either stenosis >50% in epicardial coronary artery at coronary angiography (n = 110) or with previous AMI (n = 32). Control: patients with no symptoms and resting ECG signs of ischemia and normal exercise ECG test (n = 189) and/or with coronary stenosis ≤50% at coronary angiography (n = 45). *P* values in bold are statistically significant.

(+45T>G and +276G>T) have been recently associated with low serum adiponectin levels, insulin resistance, and diabetes (15,16). One can postulate that these SNPs may also play a role in regulating the risk of CAD. To test this hypothesis in type 2 diabetic patients, we investigated the association between these SNPs and CAD in a case-control study.

RESEARCH DESIGN AND METHODS

— A total of 376 consecutive type 2 diabetic patients (defined according to World Health Organization criteria) who attended the Cardiovascular and Endocrine Department of the Scientific Institute, Casa Sollievo della Sofferenza, from January 2002 to July 2003 were enrolled in this study. Case subjects (n = 142) consisted of diabetic patients with significant CAD, defined as a >50% reduction in diameter of at least one major vessel at coronary angiography or with previous myocardial infarction. Control subjects (n = 234) were diabetic patients who had no symptoms and no electrocardiogram (ECG) signs of myocardial ischemia and a normal exercise ECG test (n = 189) and/or coronary stenosis ≤50% (n = 45) at angiography. Control subjects were type 2 diabetic patients for whom the screening of myocardial ischemia (i.e., by ECG stress test) was indicated according to the American Diabetes Association clinical practice recommendation (17) because of the presence of peripheral vascular disease and/or more than two cardiovascular risk factors. The clinical features of case and control subjects are shown in Table 1. To minimize the risk of false negatives resulting from the <100% sensitivity of the stress test, or false-positives (i.e., patients developing acute myocardial infarction [AMI] in the absence of >50% coronary lumen reduction), the association between adiponectin SNPs, serum adiponectin levels, and CAD was also analyzed in the subset of case and control subjects who underwent coronary angiography. Clinical features of this subgroup are shown in Table 2. In agreement with the Helsinki Declaration, all subjects gave informed consent to participate to this study, which was approved by the ethical committee of our institution.

All study subjects were examined between 8:00 and 9:00 A.M. after an overnight fast. Plasma glucose, lipid profile (total serum cholesterol, HDL cholesterol, and serum triglycerides), and fibrinogen were determined using commercially available enzymatic kits, as previously described (18). Serum adiponectin was measured using a commercial radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO). Intra- and interassay coefficients of variation were 2.4 and 10.2%, respectively. HbA_{1c} was determined by high-pressure liquid chromatography after removal of the labile fraction (HPLC Diamat Analyzer; Bio-Rad, Richmond, CA). The urinary albumin concentration was evaluated as the median of three timed nocturnal collections by a nephelometric method (Behring Nephelometer

Table 2—*Clinical characteristics of CAD cases and control patients with type 2 diabetes who underwent coronary angiography*

	CAD	Control	*P*
n	110	45	
Age (years)	63 ± 7	62 ± 8	0.43
Men/Women	70/40	16/29	**0.003**
Duration of diabetes (years)	15 ± 10	13 ± 6	0.3
Smokers	40	20	**0.02**
BMI (kg/m^2)	30 ± 5	31 ± 4	0.29
HbA_{1c} (%)	8.6 ± 2	8.5 ± 1	0.6
Insulin therapy	59	44	0.22
Antihypertensive therapy	83	81	0.7
Antidyslipidemic therapy	72	57	0.07
Antiplatelet therapy	90	74	**0.01**
Peripheral vascular disease	58	30	**0.003**
Nephropathy	35	26	0.4
Retinopathy	65	47	**0.049**
Neuropathy	37	39	0.9
Fibrinogen (mg/dl)	394 ± 136	369 ± 146	0.3
AER (μg/min)	90 ± 197	30 ± 47	0.07

Data are means ± SD or percent. CAD: patients with stenosis >50% in epicardial coronary artery at coronary angiography. Control: patients with stenosis ≤50% in epicardial coronary artery at coronary angiography. *P* values in bold are statistically significant.

Table 3.—*Distribution of genotypes at +45 and +276 of the adiponectin gene in CAD case and control subjects*

	CAD	Control	*P*
+45 T/T	90 (69.9)	149 (68.5)	
+45 T/G	35 (26.3)	60 (26.6)	
+45 G/G	5 (3.8)	11 (5.0)	**0.48**
+276 G/G	70 (49.3)	118 (50.4)	
+276 G/T	65 (45.8)	88 (37.6)	
+276 T/T	7 (4.9)	28 (12.0)	**0.04**

Data are *n* (%). ORs are adjusted for age, sex, duration of diabetes, and smoking.

Analyser; Behring, Marburg, Germany). Peripheral vascular disease was diagnosed when lumen stenosis was >50% at ultrasound Doppler examination. The presence of retinopathy was determined by fundoscopy followed, if necessary, by fluorescein angiography. Neuropathy was determined as somatic nerve dysfunction by electromyography. Genotypes at position +45 and +276 of the adiponectin gene were determined, as previously described (15).

All statistical analyses were performed using the SPSS statistical package (version 10; SPSS, Chicago, IL). Haplotypes were inferred for each individual by maximum likelihood methods, as previously described (19). The association between CAD and genotypes was analyzed by a χ^2 test and logistic regression analysis to adjust for potential confounders. As a descriptive measure of association between genotypes and outcomes, odds ratios (ORs) were calculated along with 95% CIs. Continuous variables (expressed as mean ± SD) were compared by Student's *t* test or by univariate ANOVA when adjusted for potential confounders. A *P* value <0.05 was considered significant.

RESULTS — Clinical features of CAD cases and control subjects are shown in Table 1. Compared with type 2 diabetic control patients, individuals with CAD were older, had longer duration of diabetes, and a higher man-to-woman ratio and mean plasma fibrinogen level (Table 1). They also showed a higher frequency of peripheral vascular disease and retinopathy and were more frequently treated with insulin as well as antihypertensive, antidyslipidemic, and antiplatelet drugs (Table 1). Differences in sex, smoking, peripheral vascular disease, retinopathy, and antiplatelet therapy distribution were observed between case and control subjects in the subset who underwent angiography (Table 2).

Adiponectin polymorphisms and CAD

Case and control subjects had similar genotype distributions at position +45 (Table 3). By contrast, significant differences were observed at position +276 (P = 0.04) with T/T homozygotes being more frequent among control subjects (Table 3). Based on these results, T/T homozygotes were compared with carriers of the G allele (i.e., patients carrying the XG genotype) and showed a significantly lower risk of CAD (OR 0.30 [95% CI 0.12–0.76], P = 0.01 after adjusting for age, sex, duration of diabetes, and smoking). Since the lower CAD risk of T/T individuals remained unchanged after adding BMI to the model, this association is unlikely to be due to an effect on adiposity. T/T individuals appeared to have an even stronger protective effect when other potential confounders, including HbA_{1c}, lipid levels, systolic and diastolic blood pressure, antihypertensive and antidyslipidemic treatments, insulin therapy, and adiponectin levels were added to the model (0.13 [0.037–0.46], P = 0.002) (Table 4). A similar protective effect associated with the T/T genotype was observed when the analysis was restricted to the subset of individuals who underwent angiography (0.04 [0.006–0.30], P = 0.002) (Table 4).

The protective effect associated with the T/T genotype slightly decreased after adjusting the analysis for plasma fibrinogen and albumin excretion rate (AER) (OR 0.40 [95% CI 0.13–1.19], P = 0.1). When SNP +45 and SNP +276 were considered together, a protective effect was observed for the haplogenotypes X/X (i.e., all genotypes other than TG/TG and TG/X) (Table 5), although this was not as strong as that observed for +276 TT homozygotes and did not reach statistical significance with this sample size (0.63 [0.37–1.10], P = 0.11).

Table 5—*Distribution of adiponectin +45/+276 haplogenotypes*

	CAD	Control	*P*
n	130	220	
TG/TG	40 (30.8)	64 (29.1)	
TG/X	64 (49.2)	97 (44.1)	
TT/TG	43 (33.1)	59 (26.8)	
TG/GG	21 (16.1)	38 (17.3)	
X/X	26 (20.0)	59 (26.8)	0.35*
TT/TT	7 (5.4)	26 (11.8)	
TT/GG	14 (10.8)	22 (10.0)	
GG/GG	3 (23)	11 (5.0)	
GG/GT	2 (1.5)	0 (0.0)	

Data are *n* (%). *TG/TG versus TG/X versus X/X (2 degrees of freedom).

Table 4—*Distribution of adiponectin X/G and T/T genotypes at +276 SNP*

	CAD	Control	OR (95% CI)	*P*
All patients				
n	142	234		
+276 X/G	135 (95.1)	206 (88)		
+276 T/T	7 (49)	28 (12)	0.13 (0.037–0.46)	**0.002**
Patients who underwent coronary angiography				
n	10	45		
+276 X/G	104 (94.5)	37 (82.2)		
+276 T/T	6 (55)	8 (17.8)	0.04 (0.006–0.30)	**0.002**

Data are *n* (%) unless otherwise indicated. X/G patients: patients carrying the G allele (i.e., either T/G or G/G genotype). ORs are adjusted for age, sex, duration of diabetes, smoking, BMI, HbA_{1c} lipid levels, systolic and diastolic blood pressure, antihypertensive, antidyslipidemic, and insulin therapy, and adiponectin levels.

Serum adiponectin levels and CAD

Serum adiponectin levels were similar in case and control subjects in both the whole sample (25 ± 13 vs. 26 ± 17 μg/ml, after adjusting for age, sex, and BMI, P = 0.9) (Table 6) and the subgroup of patients who underwent coronary angiography (25 ± 13 vs. 28 ± 16 μg/ml, P = 0.7). When the case and control subjects were considered together, no difference in serum adiponectin levels were observed in +276 T/T compared with +276 X/G patients (Table 6). Although not associated with either CAD or the

Table 6—*Serum adiponectin levels (μg/ml) according to several potential determinants*

			P	*P**	*P*†	*P*‡
Sex (men versus women)	23 ± 14	28 ± 16	**<0.01**	—	**<0.01**	—
CAD status (case versus control subjects)	25 ± 13	26 ± 17	0.25	0.57	0.45	0.90
Adiponectin +276 genotype (X/G versus T/T)	26 ± 15	29 ± 21	0.23	0.25	0.30	0.38
BMI status (≥30 versus <30 kg/m^2)	25 ± 14	28 ± 17	0.11	**0.04**	—	—
Antidiabetic therapy (insulin/OHA versus diet)	25 ± 15	31 ± 17	**0.01**	**0.01**	**0.02**	**<0.01**
Antihypertensive therapy (no versus yes)	22 ± 12	27 ± 16	**0.04**	**0.05**	**0.03**	**0.04**
Triglycerides (above versus below the median value)	23 ± 14	29 ± 17	**<0.001**	**<0.001**	**<0.001**	**<0.001**
HDL cholesterol (below versus above the median value)	23 ± 15	29 ± 16	**<0.001**	**<0.01**	**<0.001**	**<0.01**

Data are means ± SD. *Adjusted for age; †adjusted for age and BMI; ‡adjusted for age, sex, and BMI. X/G patients: patients carrying the G allele (i.e., either T/G or G/G genotype). *P* values in bold are statistically significant. OHA, oral hypoglycemic agent.

+276 T/T genotype, serum adiponectin levels were significantly associated with several features related to a proatherogenic/insulin-resistant phenotype including BMI, treatment of hyperglycemia, and lipid profile (Table 6).

CONCLUSIONS — The novel finding of our case-control study is that type 2 diabetic patients who are homozygous for the T allele at position +276 of the adiponectin gene are at lower risk of CAD than carriers of the G allele. This protective effect is especially strong when T/T homozygotes are compared with G/T heterozygotes and less visible when they are compared with G/G homozygotes. A possible explanation for this phenomenon is that homozygosis for the +276 G allele induces such an acceleration on the atherosclerosis evolution that the case subjects are depleted of carriers of this genotype due to early thrombotic complications and an increased rate of fatal ischemic events (i.e., survivor bias effect). In support of this hypothesis is the finding that when cases who had suffered an AMI ($n = 70$, 28 G/G, 39 G/T, and 3 T/T) were stratified according to their median age (64 years), G/G carriers were more frequent in the younger subgroup (OR 7.7 [95% CI 1.6–36], $P = 0.009$ after adjusting for sex, duration of diabetes, and smoking), suggesting that G/G patients may die earlier after surviving an acute event.

It is unlikely that the association that we report here is a false-positive due to chance or to phenotype misclassification. The protective effect present in T/T versus X/G patients is dramatic (87–90% risk reduction), the *P* values for the ORs are robust ($P = 0.002$), and the results obtained in the whole study were confirmed in the subgroup who underwent coronary angiography. In addition, the well-established role of adiponectin in human (20) and animal (14,21) models of CAD and the previous association of the +276 SNP with proatherogenic phenotypes make our results biologically plausible.

We confirm the previously described association of low circulating adiponectin with features of the proatherogenic/insulin-resistant phenotype (15). However, we could not confirm the association with CAD that was previously reported in the Japanese (20). Our sample size has 80% power to identify a significant ($P = 0.05$) difference of 5 μg/ml in adiponectin levels, a difference whose magnitude is in the same range as that previously reported when comparing groups with different adiponectin serum levels (15,22). Therefore, although possible, a lack of power does not seem to be a major cause of our negative findings. Differences in the genetic background of the two populations (i.e., Japanese versus Caucasian populations) and/or recruitment modalities (i.e., men from the general population in the previous study [20] compared with diabetic patients [49% women] in our present report) may well explain the different results obtained. In fact, in diabetic patients adiponectin levels are likely to be altered, independently of CAD, because of hyperglycemia/insulin resistance (22,23). In addition, in our sample, the multiple ongoing treatments (particularly the antihypertensive therapy with renin-angiotensin system blockade agents) that are known to modulate serum adiponectin concentrations (24) and their different distribution between case and control subjects (Table 1) may have overridden differences in adiponectin levels between the two groups of patients.

In a previous study, we described an association between variability at the adiponectin locus and several traits of the insulin resistance syndrome (15). The strongest association was with a haplotype defined by SNPs +45 and +276 but was also visible for SNP +276 considered by itself (15). In contrast, in the present study, the association with CAD primarily concerned SNP +276, with a weaker effect observed for the +45/+276 haplotype. It is possible that the same genetic effect, related to a causal variant in linkage disequilibrium with SNP +276 and the +45/+276 haplotype, underlies both associations, with differences between the two studies being due to random variations in the marker preferentially associated with the phenotype. Alternatively, the associations with CAD and insulin resistance could be unrelated, with one due to a variant in strong linkage disequilibrium with the +276 and the other to a variant associated with the +45/+276 haplotype system.

The biology underlying the association between adiponectin genotype and reduced risk of CAD is currently unknown. Serum adiponectin levels are quite similar in +276 TT subjects compared with carriers of other genotypes. However, current levels of serum adiponectin might not be good indicators of the levels in the pre-diabetic state when atherosclerotic lesions start to develop. In addition, serum levels do not necessarily reflect adiponectin concentrations in the subendothelial space, which is where the targets for the antiatherogenic effect of this cytokine are located (14,21,25). Whether adiponectin concentration at the subendothelial level is different across different genotype groups is a possibility

that deserves further specifically designed studies.

Whatever the mechanism underlying the genotype-phenotype association that we report, it is unlikely to be directly related to the +276 SNP, which is placed in an intronic region and has no apparent biological function. Rather, it seems more likely that this SNP is a marker in linkage disequilibrium, with other sequence differences(s) having biological effects. This is also suggested by the observation that it is the T allele that is associated with protection from the insulin resistance/atherosclerotic phenotype in our population from southeast Italy, whereas it is the G allele that is protective in other populations (26). In conclusion, in our sample of type 2 diabetic patients from the Gargano region, the +276 T/G polymorphism of the adiponectin gene is associated with CAD. If confirmed in other populations, this genetic marker may help the identification of type 2 diabetic patients who are at increased risk for CAD so that preventive programs could be specifically targeted at these subjects early in the course of the disease.

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