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RESEARCH ARTICLE

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Contribution of adiponectin polymorphisms to the risk of coronary artery disease in a North-African Tunisian population

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Background: Adiponectin, an adipocyte-derived protein, is known to play a key role in the processes leading to atherosclerosis and coronary artery disease (CAD) through its anti-atherogenic, anti-inflammatory, antioxidative, and anti-apoptotic properties. In the current study, we have studied the association of two single nucleotide polymorphisms (SNPs) +45 T>G (rs2241766) and +276 G>T (rs1501299) of the adiponectin gene with coronary artery disease (CAD) on an Arab/North-African population from Tunisia.

Methods: Subjects comprised 277 patients with angiographically demonstrated CAD and 269 age- and gender-matched control subjects. The adiponectin genotypes were performed by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP). The contribution of adiponectin variants to CAD was analyzed by haplotype and regression analysis.

Results: Adiponectin +45T>G and +276G>T genotypic and allelic distributions did not show a significant difference between cases and controls. Similarly, no association with CAD was observed for the haplotype analysis. Assuming dominant model of transmission for both polymorphisms and after adjustment of a number of traditional risk factors for CAD, logistic regression analysis showed an association of SNP +45 T>G with increased risk of developing CAD [adjusted OR (95% CI) = 2.59 (1.17-5.70); P = .01]. However, SNP + 276 G>T is associated with decreased risk of developing CAD [adjusted OR (95% CI) = 0.47 (0.22-0.97); P = .04].

Conclusion: There is no allelic or genotypic association of +45 T>G and +276 G>T of the adiponectin gene with CAD in the Tunisian population.

KEYWORDS

adiponectin, coronary artery disease, genetic association, haplotype, single nucleotide polymorphism

1 | INTRODUCTION

Adiponectin is one of several adipocytokines secreted especially by the adipocytes[1,2] which circulates at high concentration in the blood (3-30 μg/mL) as three oligomeric complexes.[3,4] Adiponectin consists of 247 amino acids with a molecular weight of about 30 kDa, consisting of a signal peptide at the N-terminus, a collagenous domain, and a globular domain at the C-terminus.[5]

Adiponectin has been implicated in a wide spectrum of biological pathways with anti-inflammatory, anti-oxidative, anti-apoptotic, and anti-atherogenic effects[6-8] that are linked to coronary artery disease[9,10] or cardiovascular disease.[11,12] Adiponectin reduces the

expression of the cellular adhesion molecules in the endothelium, suppresses the accumulation of lipids and inhibits the transformation of the macrophages into foamy cells, as well as the proliferation of the smooth muscle cells, a succession of phenomena encountered in the initiation and evolution of atherosclerotic lesions.[13] Furthermore, the degree of plasma adiponectin reduction is expected to correlate with the extension, the volume and the complexity of coronary atherosclerotic lesions.[14-16]

The relationship between several genetic variants of the adiponectin gene and the development of CAD or cardiovascular disease has been investigated previously in several populations. Two of the most commonly studied SNPs are a silent T to G substitution in exon 2 (+45T>G) and a G to T substitution in intron 2 (+276G>T). However, inconsistent findings on the association of these two SNPs, either independently or as a haplotype, have been reported.[17,18] These two SNPs have been repeatedly found to correlate with CAD: SNP +45T>G[19-22] or +276G>T,[23–25] albeit with some controversial study that report no association with CAD and related complications[20,26] which could be due to a difference in ethnic populations. Consequently, data regarding the association between adiponectin polymorphisms and CAD need further investigation because of the conflicting reported results.

The present case–control study aimed to examine the association of SNPs +45 and +276 in the ADIPOQ gene with coronary artery disease in 277 Tunisian patients with angiographically confirmed CAD, and in 269 age- and gender-matched controls from whom informations regarding most known risk factors for CAD were obtained.

2 | MATERIALS AND METHODS

2.1 | Study population

The participants in this study were genetically unrelated Tunisian subjects. The sample included 277 patients with CAD (mean age ± SD: 65.26 ± 9.86) who were admitted for coronary angiography at the cardiology service of Rabta Hospital or cardiology service of Gafsa Hospital. All CAD patients had ≥50% stenosis in the left main coronary artery or multiple significant stenosis (≥70%) in more than one coronary artery and was documented by coronary angiography, prior cardiac bypass surgery, or acute coronary syndrome. Diagnosis of MI was confirmed as per WHO criteria[27] which were based on diagnosis of chest pain and clinical symptoms, elevation in cardiac enzymes or ECG changes.

The control group consists of 269 age- and gender-matched healthy subjects (mean age ± SD: 64.5 ± 9.98), from the same geographical area who had no clinical evidence of CAD: no history of typical angina pectoris, no abnormal Q wave or ST-T changes on electrocardiography, or multiple significant stenosis (≥70%) in more than one coronary artery.

All subjects who agreed to participate in the study were evaluated on the basis of a standard questionnaire that provided details on socio-demographic characteristics completed by information on some CAD risk factors such as age, smoking habits, the presence of diabetes mellitus, hypertension, or body mass index. BMI was calculated as the ratio of weight in kilograms divided by the square of height in meters. Blood pressure was measured twice, using mercury sphygmomanometer with participants in the sitting position following a 5-minute rest; the mean of two readings measured 1 minute apart was adopted. Hypertension was determined as BP readings of 140/90 mm Hg or higher, and/or use of antihypertensive medications. Diabetes was diagnosed according to fasting blood glucose (WHO criteria ≥ 7 mmol/L), and/or the use of glucose-lowering drugs/oral hypoglycemics and insulin). Serum cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides were measured by routine methods using enzymatic colorimetric assays. Written informed consent was obtained from all subjects. The study was approved by the hospital Ethic Committee of Gafsa.

2.2 | Gene analysis

Peripheral blood was collected, separated within 1 hour, and the samples were kept at −20°C until analysis. Total genomic DNA was extracted from blood leukocytes-rich interphase layer of EDTA anticoagulated by the proteinase K/salting-out method[28] and was dissolved in nuclease-free water and stored at 4°C pending assay.

+45 T>G and +276 G>T genotype analysis was performed by polymerase chain reaction-restriction fragment length polymorphism analysis using *SmaI* and *BsmI* digestion, respectively.[29] The primer sequences for +45T>G were: forward, 5′- gCA gCT CCT AgA AgT AgA CTC TgC Tg -3′, and reverse, 5′- gCA ggT CTg TgA TgA AAg Agg CC -3′. Amplification of the +45T>G polymorphism resulted in a 372-bp DNA product. Undigested fragments with *SmaI* indicate the presence of the wild genotype; heterozygous TG were at 372, 219, and 153 bp; and appearance of two bands at 219 and 153 bp length represented the GG genotypes of CAD patients and controls groups.

Genotyping of the SNP +276 G>T of the adiponectin gene was performed using a mismatched oligonucleotide approach. A 456-bp fragment length was amplified with primers 5′-CTG AGA TGG ACG GAG TC TTT-3′ (forward) and 5′-CCA AAT CAC TTC AGG TTG CTT-3′ (reverse), containing a G instead of a T nucleotide in intron 2 of the adiponectin gene, thereby introducing an artificial *BsmI* restriction site in the presence of the mutant allele which results in the digestion of the 456-pb amplicon into 374-bp and 82-bp fragments. Digested fragments for both studied polymorphisms were separated by electrophoresis on 2.5% ethidium bromide-staining agarose gels and were visualized by UV transillumination.

2.3 | Statistical analysis

All statistical analyses were performed using the SPSS for windows version 20.0 software (SPSS, Inc., Chicago IL, USA). Allele frequencies were calculated using the gene-counting method, and both polymorphisms were tested for Hardy-Weinberg's equilibrium using the chi-squared test. Differences in characteristics between CAD patients and control groups were analyzed using the χ^2 test or Student's *t* test. Baseline characteristics were expressed

as mean ± standard deviation, and categorical variables were presented as total number (percentage). For all analysis, results were expressed as *P*-value, odds ratio (OR), and 95% confidence intervals (CI). Statistical significance level was set at $P < .05$.

Genotypic associations to CAD presence for dominant, additive, and recessive genetic models were tested by calculating a logistic regression statistic and corresponding *P* value using the program SNPstats (http://bioinfo.iconcologia.net/snpstats/start.htm). The results are expressed as *P* value (two-tailed), odds ratio (OR), and 95% confidence intervals (CI).

Haplotype analysis was performed using a maximum-likelihood method,[30] in which the haplotypes frequencies were expressed as OR (95% CI) for a binary phenotype by comparison with the most frequent haplotype (http://genecanvas.ecgene.net). The significance level was set at $P < .05$.

3 | RESULTS

The clinical characteristics of the 277 CAD patients and 269 control subjects are presented in Table 1. The two groups were matched for age ($P = .38$) and gender ($P = .69$). Significantly higher percentage of hypertensive, diabetic, and smoker individuals were seen in cases ($P < .001$). Elevated total cholesterol ($P < .001$), low-density lipoprotein ($P < .001$), and triglycerides ($P < .005$) were seen in cases compared with control subjects and mean serum HDL was lower ($P < .001$) in patients compared to controls. Mean systolic BP and diastolic BP ($P < .005$) was higher in patients group.

The distribution of 45 T>G and 276 G>T alleles and genotypes was comparable between the healthy and the patient groups. The genotype distributions of these SNPs obeyed Hardy Weinberg equilibrium in the control group. The genotypic distributions were comparable between cases and controls either for +45 T>G ($P = .72$) or +276 G>T ($P = .92$) (Table 2). Minor allele frequencies of +45G was 0.21 in CAD patients and 0.19 in healthy controls ($P = .41$). Similarly, +276T allele frequency was 0.31 in CAD patients and 0.32 in healthy controls ($P = .78$).

The subsequent univariate analysis, carried out under assumption of dominant, recessive or additive model of transmission, showed no significant difference at any model between the two groups of the study. After adjustments of a number of traditional risk factors for CAD (hypertension, diabetes, age, sex, smoking, BMI, triglyceride, and cholesterol), an increased risk in the dominant model comparing subjects carrying the TT to those carrying the TG+GG genotypes for SNP +45 T>G has been reported [adjusted OR (95% CI) = 2.59 (1.17-5.70); $P = .01$]. However, a decreased risk for CAD for SNP +276G>T in the dominant model was observed [adjusted OR (95% CI) = 0.47 (0.22-0.97); $P = .04$] (Table 3).

Haplotype distribution (Table 4) taking +45T/+276G haplotype as reference (OR = 1.00) showed that none of the adiponectin haplotypes was associated with CAD in our study ($P = .89$). Double mutant haplotype +45G/+276T was present at very low frequencies either in controls (0.03) or patients (0.04).

4 | DISCUSSION

Previous studies have examined the relationship between adiponectin gene polymorphisms and coronary artery disease in different populations including Europeans, Asians, and Americans. Although studies on the association between SNPs at +45 (rs2241766) and +276 (rs1501299) loci of adiponectin gene and

TABLE 1 Characteristics of study participants

Clinical characteristics	controls (n = 269)	Cases (n = 277)	*P*
Gender M/F n (%)	198/71 (73.6/26.4)	208/69 (75.1/24.9)	.69[b]
Mean age ± SD (y)	64.5 ± 9.98	65.26 ± 9.86	.38[a]
Mean BMI (kg/m^2)	25.76 ± 1.45	27.25 ± 1.93	<.001 [a]
Hypertension; n(%)	47/222 (17.5/82.5)	109/168 (39.4/60.6)	<.001[b]
Systolic BP (mm Hg; ±SD)	12.82 ± 1.74	14.54 ± 2.75	<.005[a]
Diastolic BP (mm Hg; ±SD)	7.43 ± 0.9	7.86 ± 0.94	<.05[a]
Diabetes n(%)	55/214 (20.4/79.6)	148/129 (53.4/46.6)	<.001[b]
Smoking n(%)	55/214 (20.4/796)	119/158 (43/57)	<.001[b]
Urea (mmol/L)	5.3 ± 1.23	5.7 ± 1.77	<.001[a]
Uric acid (mmol/L)	288.83 ± 71.63	316 ± 68.82	<.001[a]
Creatinine phosphokinase (mmol/L)	90.77 ± 23.26	101.81 ± 40.22	<.001[a]
Total cholesterol (mmol/L)	4.59 ± 1.03	5.11 ± 1.3	<.001[a]
Triglycerides (mmol/L)	1.42 ± 0.76	1.66 ± 0.75	<.005[a]
HDL-cholesterol (mmol/L)	1.61 ± 0.6	1.03 ± 0.26	<.001[a]
LDL-cholesterol (mmol/L)	1.97 ± 1	3.35 ± 1.02	<.001[a]

[a]2-tailed Student's *t* test (continuous variables).
[b]Pearson's chi-square test (categorical variables).

TABLE 2 Adiponectin +45T>G and +276G>T genotypic and allelic distributions

	Genotype frequency %			P^a	Allele frequency %		P^a
SNP	TT	TG	GG		T	G	
+45T>G Controls[b]	182 (67.7%)	70 (26%)	17 (6.3%)	.72	81%	19%	.41
Patients[b]	181 (65.3%)	74 (26.7%)	22 (8%)		79%	21%	
	GG	GT	TT		G	T	
+276 G>T Controls[b]	138(51.3)	88(32.7)	43(16.0)	.92	67.65	32.35	.78
Patients[b]	143(51.6)	93(33.6%)	41(14.8)		68.4	31.6	

[a]Pearson's χ^2 test.
[b]Number (% of total).

TABLE 3 Risk of CAD according to +45T>G and +276 G>T polymorphisms in cases and controls

		Unadjusted		Adjusted	
	Genotype	OR (95% CI)	*P*	OR (95% CI)	*P*
+276 G>T (rs1501299)					
Additive model GG vs GT,TT	GG vs GT	0.9 (0.53-1.51)	.90	1.04 (0.37-2.88)	.90
	GG vs TT	1.1 (0.66-1.86)	.69	0.96 (0.34-2.66)	.93
Dominant model (GG vs GT+TT)	GG vs GT + TT	1.01 (0.72-1.41)	.94	0.47 (0.22-0.97)	.04
Recessive model (GG+GT vs TT)	GG + GT vs TT	1.09 (0.68-1.74)	.7	0.64 (0.25-1.60)	.34
+45 T>G (rs2241766)					
Additive model (TTvs TG, GG)	TT vs TG	1.22 (0.60-2.49)	.57	2.21 (0.48-10.16)	.30
	TT vs GG	0.81 (0.40-1.66)	.72	1.38 (0.33-5.76)	.3
Dominant TT vs TG+GG	TT vs TG + GG	0.9 (0.63-1.28)	.56	2.59 (1.17-5.70)	.01
Recessive TT+TG vs GG	TT+TG vs GG	0.78 (0.40-1.50)	.46	0.96 (0.24-3.89)	.96

Unadjusted univariate logistic regression analysis.
Adjusted multivariate logistic regression analysis after adjustment for confounders.
Confounders used for this analysis: hypertension, diabetes, age, sex, smoking, BMI, triglyceride, and cholesterol.

TABLE 4 Estimation of the main haplotype frequencies

Haplotypes		Frequency Controls	Frequency Cases	OR (95% CI)
+45T	+276G	0.518	0.512	1[a]
+45G	+276G	0.157	0.171	1.08 [0.81-1.45]
+45G	+276T	0.035	0.041	1.17 [0.59-2.32]
+45T	+276T	0.287	0.274	0.98 [0.91-1.05]

Haplotype frequencies determined by the maximum-likelihood method; *P* = .89. OR, odds ratio; CI, confidence interval.
[a]Haplotype used as reference.

many disorders such as type 2 diabetes and obesity are documented,[31–33] findings on their association with coronary artery disease are rather inconclusive and inconsistent.[19,21,24–26,34,35] The aim of the present case–control study was to evaluate the association between these two single nucleotide polymorphisms with the risk of developing CAD on an Arab/North-African population.

In our study, similar distributions were observed between patients and controls either for genotype or allelic frequencies for both SNPs +45T>G and +276G>T, so that our data failed to demonstrate any association with development of coronary artery disease in the Tunisian population. Lack of association of the SNP +276 with CAD in our Study is in agreement with some other reports. Ohashi et al[36] reported that no association has been observed for this SNP and incidence of coronary artery disease in 383 Japanese patients with documented coronary heart disease and 368 healthy controls. Tong et al[37] recently reported that in 1110 subjects with or without CAD in type 2 diabetes (560 CAD patient and 550 control subjects), polymorphism + 276 G>T showed no significant association with coronary artery disease incidence with an odds ratio being 0.83 (0.6-1.03). Similarly, Pischon et al[34] found in two parallel

nested case–control studies no association of five common SNPs in the adiponectin gene including +45T>G and +276G>T with the development of coronary artery disease among white men (Health Professionals Follow-up Study) and women (Nurses' Health Study). Moreover, Hou et al[38] in a recent meta analysis including thirty-five articles with a total of 28 947 participants showed no significant association between +276 G>T polymorphism and CAD for the different studied populations, except for Caucasians when recessive model analysis was performed. Whereas Filippi et al[23] in a previous cohort of 595 Caucasians from Italy (325 with CAD and 270 matched controls) observed a significant association ($P < .001$) between the SNP +276G>T and coronary heart disease. In addition, a previous meta-analysis found that the +276G>T polymorphism was associated with a decreased susceptibility to CAD among peoples with T2DM background.[39]

The finding that the silent mutation +45 T>G (GGT→GGG, Gly15Gly) at exon 2 was not associated with coronary artery disease in our study was previously reported in several studies. Qi et al,[20] in a survey including 879 diabetic Americans male from the Health Professional Follow-up, failed to find any significant associations between SNP +45 and cardiovascular risk. Similarly, the same authors did not find any significant association between this SNP and the risk of cardiovascular disease in 989 female diabetic Americans from the Nurses' Health Study. Moreover, Bacci et al[19] failed to demonstrate any significant difference in genotype distribution at position +45 between healthy controls and CAD patients in a total of 376 Italian individuals with type 2 diabetes. Likewise, Pischon et al[34] study does not support the hypothesis that the common SNP +45T>G play an important role in the development of CAD among men (Health Professionals Follow-up Study) and women (Nurses' Health Study). Our finding was also in accordance with a study published by Jung et al. This study failed to demonstrate any significant association between this polymorphism and the presence of CAD in their survey of 156 Korean subjects.[26] Similar to our results, a published meta-analysis consisting of 4303 subjects reported that adiponectin +45T > G polymorphism yielded no significant overall associations with CAD.[40] However, these finding has not been confirmed by others studies. Lacquement et al[21] in a cross-sectional study from France and Switzerland have reported that in 162 Caucasian patients with angiographically diagnosed CAD and type 2 diabetes, polymorphism +45T>G showed a significant association with increased risk of CAD, with an OR 1.9 ($P = .0036$). Moreover, Chang et al[35] demonstrate that the minor allele +45G was associated with a lower risk of CAD (OR = 0.76, $P = .001$) in a retrospective study including 600 CAD patients and in 718 healthy controls. Sabouri et al[41] conclude that the presence of the G allele at the position +45 of the adiponectin gene may be associated with the risk of CAD in the Iranian population. Similar to these results, a recently published meta analysis involving 28 case–control studies, with 12 378 CVD cases and 19 368 controls, reported that the G allele of rs2241766 was found to be associated with an increased risk of CVD (random-effects OR = 1.22, 95% CI 1.08-1.39, $P = .002$)[18]. Hou et al[38] demonstrate that subgroup analyses based on ethnic group illuminated that +45T>G polymorphism is significantly associated with CAD risk among Arabians, with an overall ORs (95% CIs) of 1.65 (1.12-2.42) for allelic frequency. However, in the same meta analysis, only three populations showed no association with CAD from the eight studied Arab populations including 2306 Arabians.[42-44]

Concerning the association of the haplotypes comprised of the two common SNPs +45T>G and +276G>T, we did not found a significant association between any haplotype and CAD. Our findings are in agreement with some other studies. Pischon T[34] found no significant difference in the frequency of haplotypes defined by the 5 adiponectin SNPs studied. Furthermore, in the same study, haplotype analysis revealed no effect on the development of CHD among white men and woman when they used the +45T>G and +276G>T SNPs only to define the haplotypes. Our results were in disagreement with two Iranien case–control studies. First, Esteghamati et al[45] on a study of 114 type 2 diabetic subjects with CAD, and 127 type 2 diabetic patients without CAD revealed that two haplotypes 45T-276T and 45G-276T were associated with a decreased risk of CAD [adjusted OR = 0.47 (95% CI: 0.32–0.94); $P = .03$ and adjusted OR = 0.33 (95% CI: 0.13–0.83); $P = .02$, respectively]. Second, Mohammadzadeh et al[44] found that the haplotype consisting of mutant allele of SNP +45 and wild allele of SNP +276 was more prevalent in CAD cases compared to the controls (OR = 0.37, 95% CI = 0.16-0.86, $P = .022$).

In conclusion, the strength of this case–control study lie in being performed in an ethnically homogeneous North-African Tunisian population, which increase the validity of the statistical analysis, and in the inclusion of haplotype and regression analysis, in which contribution to CAD pathogenesis was demonstrated in the dominant model of transmission after controlling for a number of conventional risk factors. However, some limitations should be noted. First, we did not measure serum adiponectin levels and thus could not perform genotype–phenotype correlation. Second, our study was limited to assess only the role of two polymorphisms of adiponectin gene, so that additional SNPs should be involved to confirm, or alternatively rule out the pathophysiological role of these variants in CAD occurrence. Finally, larger numbers of Tunisians cases and controls or on other populations of similar ethnic origin need to be included in later study.

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