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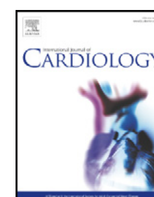

Genetic variability on adiponectin gene affects myocardial infarction risk: The role of endothelial dysfunction

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ABSTRACT

Background: Adiponectin is an adipokine with an important role in cardiovascular system conferring anti-inflammatory and anti-atherogenic effects. Two common single nucleotide polymorphisms (SNP) on adiponectin gene, rs2241766 and rs1501299, have been associated with insulin resistance and diabetes mellitus risk however their effects on cardiovascular risk remain unclear. We examined the impact of rs2241766 and rs1501299 on circulating adiponectin levels, endothelial function and cardiovascular disease risk.

Methods: We recruited in total 594 subjects; 462 patients with angiographically confirmed coronary artery disease (CAD) and 132 controls matched for age and gender. rs2241766 and rs1501299 were genotyped by polymerase chain reaction and restriction endonuclease digestion. Serum adiponectin levels were determined by enzyme-linked immunosorbent assay. Endothelial function was assessed by the flow mediated dilatation (FMD) of the brachial artery.

Results: rs2241766 had no effects on circulating adiponectin levels or FMD. In subjects without CAD, carriers of the *T/T* alleles at rs1501299 had lower adiponectin levels ($p=0.001$) and impaired endothelial function ($p<0.05$). After multivariate adjustment none of the SNPs had any effect on CAD risk. However, carriers of the *T* allele at rs1501299 were at increased myocardial infarction (MI) risk, independently of classic risk factors (OR = 2.558 [95%CI = 1.587–4.123], $p=0.0001$). The number of *T* alleles in both SNPs was strongly associated with MI history ($p=0.0001$).

Conclusions: rs1501299 polymorphism of adiponectin gene affects circulating adiponectin levels and endothelial function in subjects without CAD. Presence of the *T* variant at rs1501299 on adiponectin gene is independently associated with increased myocardial infarction risk.

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1. Introduction

Adipose tissue affects vascular function in a paracrine or endocrine manner via the release of bioactive molecules collectively called adipokines [1]. Adiponectin has been identified as an important adipokine with beneficial cardiovascular effects. In experimental studies adiponectin suppresses the expression of endothelial adhesion molecules [1] and attenuates vascular smooth muscle cell proliferation [2]. Ample evidence from further cell and animal studies have confirmed the powerful anti-atherogenic and anti-inflammatory effects of adiponectin [3].

Despite the consistent findings of experimental data, clinical studies have yielded contradictory results. Adiponectin has been associated with reduced or increased cardiovascular risk in humans, a finding that possibly highlights the complex mechanisms regulating its cardiovascular effects [4]. Moreover adiponectin synthesis in humans is dependent on underlying disease state; adiponectin expression is down-regulated in obesity, while development of heart failure is associated with a striking increase in adiponectin plasma levels [3].

The role of genetic variability of adiponectin gene (ADIPOQ) on adiponectin expression has been investigated in several studies, which have yielded though conflicting results. Two common single nucleotide polymorphisms (SNPs) of ADIPOQ have drawn the most attention, rs2241766 (NC000003.11: g.186570892T>G, NG021140.1: g.15430T>G, NM004797.3:c.45T>G, NP004788.1: p.Gly15=.) and rs1501299 (NC000003.11:g.186571123G>T, NG021140.1: g.15661G>T, NM004797.3:c.214+62G>T). These two SNPs have been associated with changes in adiponectin circulating levels, insulin resistance and the risk for developing type 2 diabetes mellitus (OMIM citation for ADIPOQ: *6054410) [5,6], but their role in endothelial dysfunction and coronary atherosclerosis development remains unclear.

In the present study we explored the impact of rs2241766 and rs1501299 on serum adiponectin levels, and we evaluated their potential

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effects on endothelial function, CAD and MI risk in coronary patients and matched controls.

2. Materials and methods

2.1. Study population

The population in this case–control study consisted of 594 unrelated Caucasians: 462 patients with coronary artery disease (CAD) and 132 control subjects, matched for age and gender. Exclusion criteria were age > 80 years, any inflammatory or infective disease, renal or liver failure, malignancy and acute coronary event during the last two months. Patients receiving nonsteroidal anti-inflammatory drugs, dietary supplements of folic acid or antioxidant vitamins were also excluded from the study. Presence of CAD was confirmed by coronary angiography, and was defined as at least one vessel disease, with lumen stenosis greater than 50%. All subjects with normal coronaries or not fulfilling the criteria for CAD diagnosis served as the control group of the study. History of myocardial infarction (MI) was defined according to the universal definition of ST elevation and non-ST elevation MI [7]. The demographic characteristics of the study population are presented in Table 1.

2.2. Study protocol

The participants underwent assessment of endothelial function as well as blood sampling for genotyping and determination of serum adiponectin levels. Venous blood samples were centrifuged at 2000 *g* at 4 °C for 15 min, and serum was collected and stored at −80 °C until assayed. The study complied with the Declaration of Helsinki and was approved by the local institutional ethics committee. An informed written consent was obtained from all participants.

2.3. DNA extraction and genotyping

Genomic DNA was extracted from 2 mL of whole blood using standard methods (QIAamp® DNA Blood kit (Qiagen)). Genotyping was performed by using appropriate primers to amplify a part of ADIPOQ gene by polymerase chain reaction (PCR) followed by restriction endonuclease digestion and visualization of the digested fragments by ethidium bromide staining and under UV light on a 3% agarose gel. For the detection of rs2241766, we used the following flanking intronic primers: sense 5′-TGGACGGAGTCCTTTGTAGG-3′ and antisense 5′-TCATCCTTGGAAGACCAACC-3′, followed by *SmaI* digestion (R0141L, New England BioLabs Inc., 240 County Road, Ipswich, MA 01938–2723, U.S.), while for detection of rs1501299: sense 5′-GGCCTCTTTCATCACAGACC-3′, antisense 5′-AGATGCAGCAAAGC CAAAGT-3′ and digestion with *BsmI* (R0134L, New England BioLabs Inc.). In order to overcome the possible limitation of using a single restriction site for both endonucleases in the respective PCR fragment, optimal conditions were employed to maximize digestion efficiency, by using an extended digestion period and an enzyme concentration of 5units/μg DNA in NEBuffer 4 (B7004S, New England BioLabs Inc.). A genotyped sample carrying the digestion sequence was used as the positive control, while digestion master mix plus nuclease free H_2O served as the negative control in every experiment.

Table 1
Demographic characteristics of the study population.

	CAD patients	Controls	p-value
n (%)	462	132	
Gender (M/F)	395 (85.5)/67 (14.5)	109 (82.6)/23 (17.4)	0.409
Age (years)	64.9 ± 0.4	63.4 ± 0.7	0.086
Arterial Hypertension, n(%)	309 (66.9)	94 (71.2)	0.348
Hypercholesterolemia, n(%)	310 (67.1)	49 (37.1)	0.001
Diabetes mellitus, n(%)	134 (29.0)	13 (9.8)	0.001
Family history, n(%)	200 (43.3)	39 (29.5)	0.005
Smoking			0.005
no, n(%)	174 (37.7)	70 (53.0)	
ex, n(%)	151 (32.2)	36 (27.3)	
current, n(%)	137 (29.7)	26 (19.7)	
Myocardial infarction, n(%)	146 (39.4)	0 (0.0)	–
BMI (kg/m^2)	27.89 ± 0.27	27.83 ± 0.38	0.893
Total cholesterol (mg/dL)	175.4 ± 2.4	203.9 ± 4.3	0.001
LDL-C (mg/dL)	115.1 ± 2.5	124.8 ± 4.0	0.034
HDL-C (mg/dL)	38.9 ± 0.7	46.6 ± 1.1	0.001
Triglycerides (mg/dL)	136.1 ± 4.6	133.3 ± 7.1	0.744
SBP (mmHg)	125.6 ± 1.6	120.5 ± 2.9	0.165
DBP (mmHg)	73.7 ± 1.0	72.4 ± 2.3	0.589
Treatment			
Aspirin, %	80.7	6.5	0.001
Clopidogrel, %	41.6	19.4	0.020
Statins, %	78.9	31.5	0.001
ACEi. %	52.0	34.0	0.001
ARBs, %	14.9	17.6	0.546
β-blockers, %	72.6	34.3	0.001
CCBs, %	28.6	32.4	0.471
Diuretics, %	25.2	32.0	0.386

Abbreviations; ACEi: angiotensin converting enzyme inhibitors; ARBs: angiotensin receptor blockers; BMI: body mass index; CAD: coronary artery disease; CCBs: calcium channel blockers; DBP: diastolic blood pressure; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; n: number of participants; SBP: systolic blood pressure; continuous variables are expressed as means ± SEM; adiponectin levels are expressed as median [25th–75th percentile].

2.4. Measurement of adiponectin serum levels

Serum adiponectin levels were measured by a commercially available enzyme-linked immunosorbent assay kit (BioVendor Laboratory Medicine, Inc., Czech Republic, Europe), with an intra-assay and inter-assay coefficient of variation of 4.9% and 6.7% respectively.

2.5. Assessment of endothelial function

Endothelial function was evaluated by the flow mediated dilatation (FMD) of the right brachial artery using high-resolution ultrasound, as previously described [8]. Briefly, all measurements were performed in the morning in a dark quiet room under constant temperature of 22 °C to 25 °C. All subjects abstained from alcohol for 24 h and from food, tobacco, and caffeine-containing drinks for at least 12 h before each vascular study. Before measurements were started, subjects were rested in a supine position for 15 min. A sphygmomanometer cuff was placed in the middle of the forearm and inflated 50 mmHg above systolic blood pressure for 5 min. FMD was determined as the percent change of the baseline brachial artery diameter 60 s post cuff deflation. Automated edge-detecting software was used for assessing brachial artery diameter changes (Medical Imaging Applications LLC, U.S.A.)

2.6. Statistical analysis

Power calculations were based on previously published genotype frequencies for the two SNPs. Assuming a frequency of 25% for the *G* variant of rs2241766 and a SD = 0.18 for log(adiponectin), with 36 *T/T* homozygotes and 12 carriers of the *G* variant (*G/G* + *G/T*) we would be able to detect a 25% difference in log(adiponectin) levels between the genotype groups with power = 90% and $\alpha = 0.05$. Similarly, assuming a frequency of 10% for *T/T* homozygotes of rs1501229 and a SD = 0.18 for log(adiponectin), we would need 108 carriers of the *G* variant (*G/G* + *G/T*) and only 12 *T/T* homozygotes to detect a 25% difference in log(adiponectin) levels between the genotype groups with power = 90% and $\alpha = 0.05$. Power calculations for assessing the effects of SNPs on MI risk were based on the assumption that 25% of the subjects undergoing coronary angiography would have a history of MI. For multivariate analysis, our power calculations indicated that a study population of 588 subjects would be required to detect an odds ratio for MI 2.0 for *T/T vs. G/T* + *G/G* of rs2241766 with power 90% and $\alpha = 0.05$; similarly, a study population of at least 375 subjects would be required to detect an odds ratio for MI 2.0 for *G/T* + *T/T vs. G/G* of rs1501299 with power 90% and $\alpha = 0.05$. The effects of polymorphisms on studied parameters were examined using both a dominant and a recessive genetic model. Continuous variables were tested for normal distribution by using Kolmogorov–Smirnov test. Normally distributed variables are presented as mean ± SEM, while non-normally distributed data were log-transformed for analysis and are presented in the non-logarithmic format as median (25th–75th percentiles). Comparisons of continuous variables between patients and controls or between the genotypes, were performed by unpaired Student's t-test. Comparisons of categorical variables between the groups were performed by using Chi-square test and Fisher's exact test where appropriate. Hardy–Weinberg's equilibrium was tested by using Chi-square test. Linkage disequilibrium (LD) between the two SNPs was tested by using MIDAS software [9]. Odds ratios were calculated using Cochran's and Mantel–Haenszel statistics, and were adjusted for clinical cofounders as reported. All reported p-values are based on two-sided tests and compared to a significance level of 5%. SPSS version 15.0 (SPSS Inc, U.S.A.) software was used for all the statistical calculations.

3. Results

Our results indicated that the two SNPs were not in LD and the genotypes' distribution did not deviate from Hardy–Weinberg equilibrium (for rs2241766: $\chi^2 = 0.15$, p = NS and for rs1501299: $\chi^2 = 1.75$, p = NS). In the overall study population minor allele frequency (MAF) for rs2241766 was 0.123, while for rs1501299 MAF was 0.298, similar to what has been previously reported [10]. The genotypes' distribution in coronary patients and controls is presented in Table 2.

3.1. Effects of rs2241766 and rs1501299 on adiponectin levels

There was a trend towards reduced serum adiponectin levels in patients with CAD (9.80[6.84–16.17] μg/mL) compared to control subjects (14.75[8.92–34.04] μg/mL), which did not though reach statistical

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