9p21 locus rs10757278 is associated with advanced carotid atherosclerosis in a gender-specific manner

Ivan Zivotić¹, Tamara Djurić¹, Aleksandra Stanković¹, Ana Djordjević¹, Igor Končar^{2,3}, Lazar Davidović^{2,3}, Dragan Alavantić¹ and Maja Zivković¹

¹Laboratory for Radiobiology and Molecular Genetics, VINČA Institute of Nuclear Sciences, University of Belgrade, 11000 Belgrade, Serbia; ²Medical Faculty, University of Belgrade, 11000 Belgrade, Serbia; ³Clinic for the Vascular and Endovascular Surgery, Clinical Center of Serbia, 11000 Belgrade, Serbia

Corresponding author: Maja Zivković. Email: majaz@vinca.rs

Abstract

Single nucleotide polymorphisms from the chromosome locus 9p21 are reported to carry a risk for various cardiovascular diseases. One of the lead single nucleotide polymorphisms, rs10757278, was mostly investigated in association with coronary artery disease but rarely with carotid atherosclerosis. In this study, we aimed to analyze the association of rs10757278 A/G polymorphism with carotid plaque presence in advanced carotid atherosclerosis. The study included 803 participants, 486 patients with high-grade stenosis (>70%) who were undergoing carotid endarterectomy and 317 controls from Serbian population. Genotypes were determined using the real-time polymerase chain reaction. According to the recessive model of inheritance, GG genotype was significantly and independently associated with carotid plaque in females only (odds ratio 2.42, CI = 1.20–4.90, $P = 0.013$). Odds ratio was adjusted for age, body mass index, hypertension, TC, LDLC, HDLC and TG, and P value was corrected for multiple comparisons. Our preliminary findings suggest a gender-specific association of rs10757278 polymorphism with carotid plaque. Further studies on larger sample and in genetically and environmentally similar populations are needed.

Keywords: 9p21, gene polymorphism, rs10757278, carotid plaque, gender specific

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Introduction

Atherosclerosis is a chronic and complex vascular disorder often underlying cardiovascular and cerebrovascular events.^{1,2} The heritability of the atherosclerotic phenotypes such as intima-media thickness (IMT) and carotid plaque (CP) is over 20%.^{3,4}

The chromosome region 9p21 was previously marked as an independent risk factor associated with coronary artery disease (CAD) and myocardial infarction (MI). Same locus was associated with cranial and abdominal aneurysm, as well as with ischemic stroke, suggesting a varied impact of this region on the cardiovascular system.^{5,6} The association of 9p21 locus with carotid atherosclerosis (CA), either with IMT or plaque presence as the phenotype of interest, has been rarely explored. The results were conflicting.^{7–12} So far, there is only one study, which has proved an association of 9p21 polymorphism rs1333049 with severe CA.¹³

The 9p21 polymorphism rs10757278 (c. 22114477A > G) that is in total linkage disequilibrium (LD) with rs1333049 was the only one independently associated with CAD after the adjustment for the effect of other significantly associated

single nucleotide polymorphisms (SNPs) from the 9p21 region.¹⁴ It is located near the gene for CDKN2B-AS1 (ANRIL) and in the functional enhancer, which can regulate the expression of downstream positioned genes.^{15,16} Harismendy *et al.*¹⁶ showed that the G allele of the SNP rs10757278 undermines the binding site for transcription factor STAT1. Functional studies demonstrated that rs10757278 influences the expression of the INK4/ARF locus genes (ANRIL, CDKN2B, CDKN2A, ARF),^{17,18} where the GG genotype was associated with the lowest expression level.¹⁷ Also, the rs10757278 genotypes were linked to differential expression of ANRIL splice variants, MTAP, and CDKN2A genes in CP tissue.¹⁹ Atherosclerosis development and progression may be affected by differential expression of ANRIL splice variants that could interfere with gene expression in many processes, such as proliferation, remodeling of extracellular matrix, and response to inflammation.²⁰

The aim of this study was to analyze the possible association of 9p21 rs10757278 polymorphism with CP presence in patients with advanced atherosclerosis. We have conducted the study in the group of patients with the

stenosis greater than 70%, who were undergoing carotid endarterectomy.

Methods

Study population

The study had enrolled 803 (486 patients and 317 controls) unrelated Caucasians with European ancestry from the population of Serbia. All the participants had the same ethnicity (Serbian). Patients were selected among subjects who had scheduled carotid endarterectomy in the Clinic for Vascular and Endovascular Surgery, Clinical Center of Serbia, Belgrade, Serbia, in the period of 2008–2010. The inclusion criterion was the CP presence in internal carotid artery (ICA) and/or common carotid artery (CCA). Extensive medical data were collected, including chronic smoking and alcohol consumption, diabetes mellitus, diagnoses of hypertension, coronary heart, and peripheral artery disease (PAD), as well as drug therapy treatments (oral hypoglycemic drugs, insulin, antihypertensive drugs). Subjects who had a glucose level greater than 7 mmol/L or were receiving insulin/hypoglycemic therapy were considered to have diabetes mellitus. Subjects with previous MI or coronarographically evaluated stable angina pectoris were considered to have CAD. Criteria for PAD were ankle brachial index below 0.90; for hypertension, a blood pressure > 140/90 mm Hg or current antihypertensive therapy. Patients with previous carotid endarterectomy (possible restenosis), carotid aneurysm, kinking, chronic inflammatory diseases, renal failure, tumors, or autoimmune disease were excluded from the study.

Control subjects were collected at Occupational Medical Center in the Institute of Nuclear Sciences VINČA, Belgrade, Serbia. All of them underwent clinical, ultrasound, and electrocardiogram examination. Exclusion criteria for the control subjects were the same as for the patients with the addition of CA presence. The local Ethics Committees of the participating medical centers approved the study. All the participants have signed informed consent for participation in the study.

Biochemical analysis

All the measurements of biochemical parameters were performed by standard laboratory procedures.

Ultrasound assessment of the carotid arteries

High-resolution B-mode ultrasound Acuson AntaresTM system (Siemens, Munich, Germany) was used for ultrasound evaluation of the bilateral carotid arteries. Degree of artery stenosis has been evaluated according to the North American Symptomatic Carotid Endarterectomy Trial method (NASCET).²¹ ICA/CCA ratio and the highest peak systolic and end-diastolic velocity were recorded. All the patients enrolled in this study had > 70% stenosis (NASCET).²¹ Criteria for defining the CA were the presence of atherosclerotic plaques in the bifurcation, internal, and/or common carotid artery. CP was defined as local thickening relative to the surrounding segments, characterized by

protrusion into the lumen, and/or localized roughness with an increased echogenicity. The intraclass correlation coefficients for interrater and intrarater reliability of ultrasound measurements were 0.916 and 0.968, respectively.

Genetic analysis

Genomic DNA was isolated from the whole-blood samples containing ethylenediaminetetraacetic acid using the commercial BloodPrep[®] DNA Chemistry isolation kit (Applied Biosystems, Forester City, California, USA) on the ABI PRISM[™] 6100 Nucleic Acid PrepStation (Applied Biosystems, Forester City, California, USA), or by the proteinase K/phenol extraction method.²² Rs10757278 alleles were detected via real-time polymerase chain reaction (PCR) (AB 7500, Applied Biosystems, Foster City, California, USA) (120 ng of DNA per reaction was used) using the TaqMan[®] SNP Genotyping Assay (C_11841860_10), created and tested by Applied Biosystems (Applied Biosystems, Forester City, California, USA). We performed PCR in a 25- μ L volume containing 12.5 μ L of TaqMan[®] Universal PCR Master Mix with 0.625 μ L of 40 $\times$ TaqMan[®] genotyping assay, which contains fluorescent oligonucleotide probes (FAM and VIC) and flanking primers probes were marked with a reporter and a quencher die at the 5' and 3' ends (respectively) and specifically bind to the sequence containing the base change, in the sequence to be amplified. Allele calling was done with quality value of >95% using SDS software (v1.4.0) (Applied Biosystems, Foster City, California, USA). The samples that did not pass the allele quality value >95% were excluded from the study and further analysis (0.02%). Genotyping validation was performed by an independent researcher on randomly chosen 92 samples, and the concordance of genotypes was 100%.

Statistical analysis

Software package Statistica version 8 (StatSoft Inc, 2007) was used for performing statistical analysis. Hardy-Weinberg equilibrium as well as the differences in allele frequencies and genotype frequencies distribution between cases and controls were calculated by Chi-square (χ^2) test. The possible association of rs10757278 polymorphism with CP occurrence was analyzed according to dominant, recessive, and additive inheritance models. The strength of association between genotypes and CP presence expressed as crude and adjusted odds ratios (OR) with their 95% confidence interval (CI) was analyzed using univariate and multivariate logistic regression analyzes. Genotype by sex interaction was calculated using Generalized linear logit model. Values of normally distributed continuous variables between tested groups were compared by unpaired t-test or analysis of variance (ANOVA), while values of skewed continuous variables have been compared by the nonparametric Mann-Whitney U test or Kruskal-Wallis ANOVA. Values of continuous variables that are presented in the tables are expressed as mean $\pm$ standard deviation (SD). We used Bonferroni correction for multiple comparisons. Statistically significant *P* value for the analysis of one polymorphism using three chi-square tests was set as $P \leq 0.016$.

Table 1 Main characteristics of controls and patients with carotid plaque (CP) overall and divided by gender

Overall	Controls, n = 317	Patients, n = 486	P
Age, years	59.1 ± 14.3	66.1 ± 8.0	<0.01
Gender F/M, %	45.1/54.9	40.7/59.3	ns
BMI, kg/m ²	25.1 ± 3.7	26.6 ± 3.3	<0.01
TC, mmol/L	5.61 ± 1.30	5.74 ± 1.27	ns
LDLC, mmol/L	3.31 ± 1.23	3.69 ± 1.08	<0.01
HDLC, mmol/L	1.47 ± 0.86	1.21 ± 0.35	<0.01
TG, mmol/L	1.58 ± 1.09	1.89 ± 1.26	<0.01
Hypertension, %	34.4	86.1	<0.01
Smokers, %	55.0	72.3	<0.01
Diabetes, %	0.0	22.3	N/A
Females	Controls, n = 143	Patients, n = 198	P
Age, years	56.7 ± 14.8	65.0 ± 7.9	<0.01
BMI, kg/m ²	24.2 ± 4.1	27.3 ± 3.3	<0.01
TC, mmol/L	5.69 ± 1.42	5.95 ± 1.18	0.024
LDLC, mmol/L	3.34 ± 1.34	3.82 ± 1.05	<0.01
HDLC, mmol/L	1.55 ± 0.52	1.3 ± 0.37	<0.01
TG, mmol/L	1.40 ± 0.90	1.83 ± 0.84	<0.01
Hypertension, %	25.4	82.9	<0.01
Smokers, %	50.8	64.5	ns
Diabetes, %	0.0	23.7	N/A
Males	Controls, n = 174	Patients, n = 288	P
Age, years	61.0 ± 13.5	66.7 ± 8.0	<0.01
BMI, kg/m ²	26.1 ± 2.9	26.1 ± 3.2	ns
TC, mmol/L	5.53 ± 1.78	5.60 ± 1.30	ns
LDLC, mmol/L	3.27 ± 1.09	3.59 ± 1.09	0.01
HDLC, mmol/L	1.39 ± 1.11	1.15 ± 0.31	<0.01
TG, mmol/L	1.78 ± 1.25	1.92 ± 1.47	0.04
Hypertension, %	42.4	88.3	<0.01
Smokers, %	59.4	77.4	<0.01
Diabetes, %	0.0	21.3	N/A

Note: Values are mean ± SD for age, BMI, TC, LDLC, HDLC, TG; Mann-Whitney *U* test was used to compare values between controls and patients with CP for continuous variables that have skewed distribution; Chi-square test was used for categorical variables; *P* values < 0.05 were considered statistically significant.
Ns: non-significant; N/A: not applicable.

The statistical power of the study was calculated using the Power and Sample Size Calculation software (v3.0.43).²³

Results

Description of population

Relevant parameters of study groups are presented in Table 1. Several parameters were significantly different between patient and control groups. Patients were older, with a higher proportion of smokers and hypertensive subjects, higher body mass index (BMI), low-density lipoprotein cholesterol (LDLC), and triglyceride (TG) levels, and lower high-density lipoprotein cholesterol (HDLC) levels.

Polymorphism rs10757278 in controls and patients with CP

All tested groups were in Hardy-Weinberg equilibrium. We have not found significant differences of allele and

genotype frequency distributions between CP patients and controls overall and in the group of males. In the group of females, we have found a significantly higher frequency of G allele among CP patients compared to controls with an OR of 1.40 (CI = 1.03–1.90, *P* = 0.03). After correction for multiple comparisons, this result was no longer statistically significant (*P* cut-off value ≤ 0.016). We have analyzed the possible association of the rs10757278 polymorphism with the CP in females, according to dominant, recessive, and additive inheritance models. Statistically significant difference was found only according to the G allele recessive model. The frequency of the GG genotype was significantly higher in female patients compared to female controls (*P* = 0.015) (Table 2). Female patients carrying the GG genotype had an OR of 1.83 for CP presence compared to AA + AG carriers (crude OR = 1.83, CI = 1.12–2.97, *P* = 0.015). Study power for this association was 50% at the significance level of *P* < 0.016. After adjustment for

Table 2 Association of rs10757278 genotypes with carotid plaque presence overall and divided by gender

9p21 rs10757278	Overall			Males			Females		
	Controls, n (%) n = 317	Patients, n (%) n = 486	<i>P</i> (χ^2)	Controls, n (%) n = 174	Patients, n (%) n = 288	<i>P</i> (χ^2)	Controls, n (%) n = 143	Patients, n (%) n = 198	<i>P</i> (χ^2)
AA + AG	227 (71.61)	332 (68.31)	0.32	117 (67.24)	204 (70.83)	0.41	110 (76.92)	128 (64.65)	0.015 ^a
GG	90 (28.39)	154 (31.69)		57 (32.76)	84 (29.17)		33 (23.08)	70 (35.35)	
Alleles A/G	0.47/0.53	0.46/0.54	0.71	0.45/0.55	0.48/0.52	0.27	0.50/0.50	0.42/0.58	0.03

^a*P* value was corrected for multiple testing and $P \leq 0.016$ was considered statistically significant.

Table 3 Odds ratios (ORs) of 9p21 rs10757278 polymorphism and the risk factors included in multivariate regression model for the carotid plaque presence in females

Factors	β	$\pm$ SE (β)	OR ^a ($\pm$ 95% CI)	<i>P</i>
AA + AG vs. GG (rs10757278)	0.89	0.36	2.42 (1.20–4.90)	0.013 ^b
Age, years	0.08	0.01	1.08 (1.06–1.12)	<0.01
BMI, kg/m ²	0.29	0.15	1.34 (1.00–1.80)	0.04
TC, mmol/L	0.16	0.10	1.17 (0.97–1.42)	ns
LDL, mmol/L	0.07	0.16	1.07 (0.79–1.46)	ns
HDL, mmol/L	−0.83	0.49	0.44 (0.16–1.15)	ns
TG, mmol/L	0.11	0.21	1.12 (0.74–1.69)	ns
Hypertension	2.12	0.31	8.32 (4.51–15.33)	<0.01

OR: odds ratio; CI: confidence interval; β -beta; SE (β): standard error of beta; ns: non-significant.

^aAdjusted ORs for all the factors included in the multivariate logistic regression model.

^b*P* value was corrected for multiple testing and $P \leq 0.016$ was considered statistically significant.

risk factors that were significantly different between groups (age, BMI, hypertensive status, total cholesterol (TC), HDLC, LDLC, and TG) calculated OR for CP presence was even higher and remained significant (adjusted OR 2.42, CI = 1.20–4.90, $P = 0.013$) (Table 3). Additionally, we analyzed genotype by sex interaction for the rs10757278 genotypes and have got that the rs10757278 GG genotype by sex interaction effect was significant for the CP presence (interaction $P = 0.016$).

Association of rs10757278 genotypes with risk factors in patients with CP

We have analyzed possible associations of the biochemical parameters commonly associated with higher risk for atherosclerosis, according to rs10757278 genotypes among the CP patients. We have not found significant associations of rs10757278 genotypes either with lipid status parameters (TC, HDLC, TG, apolipoprotein AI, Apo AII, apolipoprotein B, lipoprotein(a)) or inflammatory parameters (C-reactive protein, interleukin-6, white blood cells, fibrinogen) (data not shown). In addition, there was no association of rs10757278 with hypertension, diabetes mellitus, or BMI.

Discussion

In the current study, the rs10757278 rare allele homozygote appeared as the significant risk factor for CP presence only in females. We have not found significant association of 9p21 polymorphism with hypertension, BMI, and other

biochemical risk factors, which is in accordance with the results of other studies.^{10,24,25} Among CP patients, there was no significant difference according to type 2 diabetes mellitus.

The rs10757278, which is in complete LD with rs1333049, was denoted as one of the lead SNPs in a haplotype block of 9p21, spanning a region of approximately 50 kb. Either rs10757278 or rs1333049 was associated with CAD and MI and became the most replicated markers of these phenotypes.^{14,25–28} It was proven that beside susceptibility for CAD and MI, rs10757278 showed association with severity of CAD and its duration.²⁹ It was suggested that in the analysis of 9p21 locus the precise phenotype definition is relevant to clearly distinct acute disease MI from stable CAD without MI, which was differently associated with 9p21.³⁰ Although it was shown that association of 9p21 is not restricted to CAD, only few studies investigated it so far in CA.¹³ It appears that 9p21 region is less important for subclinical atherosclerosis, but rather for the promotion of plaque formation.^{8,9,11–13,31} Thus, we saw the rationale to investigate 9p21 rs10757278 with CP presence in patients undergoing carotid endarterectomy. According to our knowledge, this is the first study of 9p21 with advanced carotid stenosis >70% and the first one conducted in South-Eastern European population. We did not find the association with CP presence in the patient group overall, but the significant association of the rare allele G and its homozygote, GG, with CP was found in females. The association of G allele with CP was not significant after

Bonferroni correction. The gender-specific association of 9p21 rs1333049 with the CP occurrence, using IMT as a measure of atherosclerosis, was found among stroke and MI free Chinese Han males.⁹ Another study in Chinese Han population showed as well the gender-specific association of the rs1333049 rare allele homozygote with high grade coronary stenosis (>50% in one vessel at least), but in females.³² This result is similar to our finding, although we analyzed carotid stenosis.

Previously, the 9p21 was significantly associated with premature CAD. For males, it was defined as <55 or 60 years and for females as <65 to 70 years.³² Here, in the age range of females with CP falls into the range for premature atherosclerosis according to previous criteria. Thus, the significant association of rs10757278 with a CP presence that we found in females might be explained as association with premature atherosclerosis in women. In male patients, whose mean age was similar to females (66.7 ± 8.0 years), the frequency of rs10757278 rare homozygote was lower compared to female patients (29.17% vs. 35.35%). The certain number of male patients with rare homozygote might have died as the consequence of the acute event before they could enter the study. Acute CAD and ischemic stroke ensues about a decade earlier and are more common in men compared to women, independently of age.³³ It might be that after the development of atherosclerosis the unfavorable effect of 9p21 risk genotype may drive to acute events, thus increasing mortality and reducing this genotype representation in patients.

Up to date, most of the studies that investigated 9p21 locus in CAD used one of the following genetic markers rs1333049, rs10757278, rs2383206, rs10757274 that are in strong LD. The tight LD between multiple SNPs in a haplotype block containing rs10757278 was shown in one of the first genome-wide association study (GWAS) which detected the association of 9p21 with CAD.²⁷ Thus, either rs1333049 or rs10757278 was used as tag SNPs for the entire commonly reported haplotype core block of the 9p21 locus for association studies in CAD.^{14,34,35} According to recent meta-analysis on CAD and CHD, there was no evidence of heterogeneity for effect of either SNP, and effect size was similar for SNPs from this hap-block.^{34,36} As we already described, in this study, we chose to analyze the SNP with proposed functional role.¹⁷ Nevertheless, the single SNP analysis is always the limitation and the use of additional SNPs may improve the definition of the risk haplotype of 9p21 locus. The major limitation of this study is the moderate statistical power for the reported association (50%). Therefore, we assume our results preliminary and further validation in a larger patient group or replication in genetically similar population is necessary before the final conclusion about the gender-specific association could be made. Another limitation is the absence of diabetes mellitus in the control group. It was suggested that certain SNPs located in 9p21 showed association with type 2 diabetes mellitus, the atherosclerosis risk factor, but those SNPs were not in the strong LD with SNPs associated with CAD or ankle brachial index.³⁷

Nevertheless, the 9p21 region interethnic risk allele prevalence as well as a gender-specific influence, the role

of other variants and environmental factors are still needed to be resolved,^{34,38} especially for those populations that were not included in GWASs. The most recent study suggested that although independent of classical cardiovascular risk factors the 9p21 locus exerted a context-dependent association with CAD.³⁹ In parallel, the functional basis of this chromosomal region in atherosclerosis should be addressed because 9p21 is one of the main potential genetic risk factors that may be used in new therapy approaches.

In conclusion, our preliminary findings suggest a gender-specific association of rs10757278 polymorphism with CP. Replication and validation of these results are essential in genetically and environmentally similar population.

Authors' contribution: All authors contributed to conception and design, analysis and data interpretation and final approval of the paper; ZI and DJA conducted the experiments; AD and AS obtained funding; ZM designed the study; ZI, DJT and ZM analyzed the results and wrote the manuscript, AS performed the critical reading and revised the manuscript; DJT, KI, DL collected the data.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL STANDARDS

The local Ethics Committee approved the study. The written informed consent was obtained from all participants prior to inclusion in the study.

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