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CDKN2B gene expression is affected by 9p21.3 rs10757278 in CAD patients, six months after the MI

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ABSTRACT

Background: Chromosomal region 9p21.3 is most robustly associated with coronary artery disease (CAD) in western European populations. However, heterogeneity in CAD phenotypes leads to uncertainty whether 9p21.3 is associated with stable and/or acute clinical presentations of CAD. 9p21.3 is rich in regulatory elements, but the underlying mechanisms of its actions in CAD remain unclear. We investigate the association of 9p21.3 two haplotype blocks lead variants (rs10757278 and rs518394) with first-ever non-fatal myocardial infarction (MI) in CAD patients and their association with *CDKN2B* mRNA expression in peripheral blood mononuclear cells 6 months after the event.

Methods: We included CAD patients with sustained first MI (n = 523) and controls (n = 583). Gene expression was assessed in 72 patients 6 months after MI and 43 healthy controls. TaqMan® technology was used for the gene expression and genotyping analysis.

Results: *CDKN2B* mRNA was significantly lower in MI patients compared with the controls (p = 0.002) and in patients carrying the rs10757278 G risk allele versus AA homozygotes (p = 0.012) 6 months after the event. While we confirmed the association of rs10757278 with *CDKN2B* expression in MI patients, we failed to find an association between the investigated variants and MI or disease burden.

Conclusions: We suggest a dysregulation of gene expression in the 9p21.3 region six months after acute MI, which is affected by a genetic variant in patients. The rs10757278 rare allele is one factor that might lead to prolonged risk for proatherogenic complications.

1. Introduction

Independent genome-wide association studies have pinpointed variants from the 9p21.3 gene desert region as being strong and independent markers for coronary artery disease (CAD) [1–4] and myocardial infarction (MI) [1,3]. A recent meta-analysis favored the association of 9p21.3 with a larger atherosclerosis burden rather than prevalent MI [5]. The heterogeneity of CAD phenotypes should be taken into account in future research. We investigated two 9p21.3 variants—rs10757278 and rs518394—in a homogeneous CAD phenotype. Variant rs10757278, adjacent to the tumor-suppressor genes *CDKN2A* ($p16^{INK4a}$, $p14^{ARF}$) and *CDKN2B* ($p15^{INK4b}$), represents the lead single nucleotide polymorphism of the haplotype block (block 1) and has been associated with CADand/or MI. It is the most commonly investigated 9p21.3 variant other than its proxy rs1333049 [1,3,6]. The less-commonly investigated variant rs518394 (100 kb telomeric from rs10757278) tags the other haplotype block (block 2), and it has been associated with nominal risk for CAD [2] and with MI in patients with pre-existing CAD [6].

The chromosome 9p21.3 region is rich in enhancers and surrounded by tumor-suppressor genes. It has been proposed that variants from 9p21.3 could modulate proximal genes expression. Harismendy et al. suggested that rs10757278 resides within an enhancer and disrupts the binding of transcription factor STAT1, thereby affecting the expression of *CDKN2B* and *ANRIL*, a long non-coding RNA in the INK4 locus [7]. Several studies have investigated the association of 9p21.3 variants

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from the haplotype block 1 with the expression of the *CDKN2A* and *CDKN2B* genes and recovered conflicting results [8–13]. Only one study thus far has tested the association of rs518394 (block 2) with the expression of genes in the 9p21.3 locus (*CDKN2A*, *CDKN2B*, *MTAP*, *ARF*, *ANRIL*) in peripheral blood leukocytes [10]. However, this study only focused on healthy individuals. The literature data suggest that MI changes the transcriptome in a time-dependent manner [14,15]. We accordingly investigated the association of variants with gene expression 6 months after the presentation of an acute event in order to minimize the interference of MI and variants' effect on gene expression.

Knowledge about the role of 9p21.3 variants in CAD has been gleaned mostly from studies performed with western and northern European populations [1–5]. We aimed to contribute knowledge about a potential association of 9p21.3 variants, rs10757278 and rs518394, with first non-fatal MI in CAD patients from Serbia (i.e., population from southeastern Europe). Moreover, we hypothesized that the 9p21.3 variants rs10757278 and rs518394 could be associated with altered *CDKN2B* mRNA expression in the peripheral blood mononuclear cells (PBMC) of patients and that altered gene expression may be identified 6 months after the first MI.

2. Material and methods

2.1. Study population

The study included 523 CAD patients with first non-fatal MI and 583 healthy control subjects from Serbia. All of the participants were unrelated Caucasians of European descent from Serbia. The method of recruiting and including patients in the study is presented in a flow chart (Supplemental Fig. S1). The study group consisted of 337 patients recruited at the Cardiology Clinic, Clinical Center of Serbia, Belgrade, Serbia from February through November 2013 and 186 patients recruited at the Coronary Care Unit in the Department of Cardiology, University Clinical Center “Zvezdara”, Belgrade, Serbia from December 2011 through September 2013. All of the patients were consecutively admitted with symptoms of MI [16] and were referred for primary percutaneous coronary intervention (PCI). The inclusion criterion was assessed stenosis > 50% in at least one coronary artery. The exclusion criteria for all patients were as follows: an age over 70 years, a history of previous MI, significant rhythm disturbancies, previous pace-maker or cardioverter-defibrilator implantation, tumors, chronic inflammatory diseases, autoimmune disease, or renal failure. The severity of disease was categorized by the number of diseased artheries as 1 vessel disease (VD), 2 VD or 3 VD. Demographic characteristics, previous use of medications, co-morbidities, risk factors and Killip class were obtained upon admission. Of the patients recruited at the Coronary Care Unit in the Department of Cardiology, University Clinical Center “Zvezdara”, Belgrade, Serbia 167 were prospectively monitored during the 6-month follow-up period. For the gene expression study, we consecutively recruited 80 patients at the 6 month check up. Seventy-three of them, who were successfully followed up for 6 months, agreed to participate in the study.

Heart failure (HF) in the monitored patients was diagnosed 6 months after MI according to the European Society of Cardiology Guidelines for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 [17]. HF was classified as falling into different categories (NYHA I–IV) according to a patient's symptoms [18].

Control subjects were consequtively recruited from the individuals undergoing annual medical check–up at the Occupational Medical Center VINČA, Institute of Nuclear Sciences in Belgrade, Serbia, during the period February 2011–December 2016. All controls underwent clinical, ultrasound and ECG examination and those with no evidence of cerebrovascular or cardiovascular diseases, chronic inflammatory diseases, diabetes mellitus type 2 (DMT II) and renal failure were included in the study. From June–December 2016, 44 consecutive participants with complete demographic and biochemical data agreed to provide additional blood samples for the gene expression study.

Hypertension was defined as a systolic blood pressure ≥140 mmHg, a diastolic blood pressure ≥90 mmHg, or current treatment with antihypertensive drugs. Subjects with a fasting glucose level of ≥7.0 mmol/L, or taking insulin or oral hypoglycemic drugs were characterized to have DMT II.

The study was approved by the ethics committees of medical centers involved and each participant gave written informed consent to participate in the study.

2.2. Laboratory analysis

All biochemical analyses were performed in the hospital laboratory following standard laboratory procedures upon admission to the hospital.

2.3. Genetic analysis

Peripheral blood samples for genomic DNA extraction were collected from patients within 5 days after MI. Genomic DNA was extracted by phenol–chloroform extraction method using standard procedures. Genetic variants rs10757278 and rs518394 were detected by quantitative real-time PCR (AB 7500, Applied Biosystems, Foster City, CA, US), using pre–designed TaqMan® SNP Genotyping Assays C_11841860_10 and C__2618035_10 respectfully, purchased from and tested by Applied Biosystems (Applied Biosystems, Forester City, CA, US). Each PCR reaction contained 120 ng of DNA. Fifty samples were randomly selected and genotyped a second time by another investigator. Results in the repeated genotyping were 100% concordant with the results of the original genotyping.

2.4. Quantitative real-time reverse transcriptase-PCR

Peripheral blood samples for RNA extraction were collected from 44 control subjects and 73 MI patients, which were incuded in the follow-up. None of the included patients sustained recurrent MI until the blood samples collection, 6 months after the first MI. PBMC from 3 ml of each peripheral blood sample were separated with lymphocyte separation medium (PAA, GE Healthcare, Pasching, Austria). RNA was extracted using TRIZOL reagent (Life Technologies, Carlsbad, CA, US) in compliance with the manufacturer's instructions. The quantity and structural integrity of RNA was evaluated by Agilent RNA 6000 Nano Kit on Agilent 2100 Bioanalyzer. A total of 72 mRNA samples from patients and 43 from controls had satisfying quality and were converted to cDNA. One microgram of RNA was treated with DNAse I (Fermentas, Vilnius, Lithuania) and reverse transcription was performed using a First strand cDNA synthesis kit with oligo–dT18 primers (Fermentas, Vilnius, Lithuania) in compliance with manufacturer's protocol. The real-time quantitative PCR was performed using ABI Real-time 7500 system (Applied Biosystems, Foster City, CA, US). All samples were run in duplicate. Relative *CDKN2B* mRNA expression was detected using commercial TaqMan® gene expression assay Hs00793225_m1 (Applied Biosystems, Foster City, CA, US). The detection of the internal reference control, *peptidylprolyl isomerase A* (*PPIA*), was performed using the commercial TaqMan® gene expression assay Hs99999904_m1 (Applied Biosystems, Foster City, CA, US).

2.5. Statistical analysis

Statistical analysis was performed using software package Statistica Version 8 (StatSoft Inc., 2007). Differences in allele frequencies and genotype distribution between the cases and controls as well as deviations from Hardy–Weinberg equilibrium were estimated using chi–square (χ^2) test. Normality of distribution of continuous variables was tested using Shapiro-Wilk test. The mean values of normally distributed continuous variables between the sample groups were

compared using unpaired *t*–Test, while the values of skewed continuous variables were analyzed using nonparametric Mann–Whitney *U* test. Values of continuous variables are expressed as mean ± standard deviation (SD). Strength of the association between genotypes and MI and/or severity of the disease was analyzed using logistic regression analysis and was presented as OR with 95% confidence interval (CI). The p value < 0.05 was considered statistically significant. The REST software, which incorporates a pairwise randomization and bootstrapping technique, was used for analysis of *CDKN2B* mRNA relative expression [19]. It provides 95% confidence intervals for the mRNA level ratios presented as mean factor (mean fold) induction with standard errors (SE) [19]. Since the level of *CDKN2B* mRNA expression showed skewed/non-Gaussian distribution the Spearman's correlation test was used for the analysis of correlation of gene expression with continuous variables.

3. Results

3.1. Description of the participants

The clinical characteristics of the control subjects and MI patients included in the study are listed in Table 1. Compared with the controls, the patients were older, more likely to be male, and characterized by a higher body mass index (BMI), low-density lipoprotein cholesterol (LDLC) and triglycerides (Tg). They also had higher number of hypertensives and smokers, as well as lower level of high–density lipoprotein cholesterol (HDLC). Total cholesterol (TC) level was not different between the groups (Table 1).

3.2. The association of variants rs10757278 and rs518394 with first non-fatal MI and disease severity

The genotype and allele frequencies of both the rs10757278 and rs518394 variants are listed in Table 2. Genotype frequencies were in Hardy-Weinberg equilibrium. We did not find a significant difference in genotype or allele frequencies of rs10757278 between the controls and MI patients (p = 0.36 for one copy of G allele and p = 0.12 for two copies of G allele; Table 2). In terms of rs518394, we did not find a significant difference in genotype frequencies between the controls and MI patients according to the additive model of inheritance (p = 0.07 for one copy of C allele and p = 0.06 for two copies of C allele). However, the frequency of C allele was significantly lower in the patients compared with the controls (p = 0.04, OR = 0.83, 95% CI = 0.70–0.99; Table 2). Table 3 lists data pertaining to the significant protective effect of rs518394 according to the dominant model of inheritance (GG vs. GC + CC) with nominal significance (p = 0.03, OR = 0.76 and 95% CI = 0.59–0.97). After adjusting for common risk factors, the nominal significance did not persist (p = 0.08; Table 3).

We did not find an association of rs10757278 with disease severity among patients with first, non-fatal MI. Assesed according to the recessive model of inheritance, the rs518394 CC genotype (adjusted for rs10757278 effect) was associated with disease severity with a nominal significance of 0.05 (OR = 0.55, 95% CI = 0.30–1.02). This significance did not persist after adjusting for risk factors (p = 0.16, OR = 0.63, 95% CI = 0.35–1.15).

Table 1
Main characteristics of the controls and the patients with first non-fatal MI.

Overall	Controls, n = 583	Patients, n = 523	p
Age, years	50.59 ± 13.51	58.45 ± 11.25	< 0.001[a]
Gender, males (%)	48.03	70.37	< 0.001[b]
BMI (kg/m^2)	25.12 ± 3.75	27.27 ± 4.01	< 0.001[a]
TC (mmol/L)	5.54 ± 1.26	5.58 ± 1.15	ns[a]
LDLC (mmol/L)	3.41 ± 1.16	3.64 ± 1.03	< 0.001[a]
HDLC (mmol/L)	1.52 ± 0.73	1.1 ± 0.33	< 0.001[a]
Tg (mmol/L)	1.48 ± 1.01	1.88 ± 1.24	< 0.001[a]
Hypertension (%)	30.53	65.20	< 0.001[b]
Smokers (%)	52.98	69.21	< 0.001[b]

Values are mean ± SD for age, BMI, TC, HDLC, LDLC, Tg.
[a] P-Mann-Whitney *U* test was used to compare values between controls and patients for continuous variables that have skewed distribution.
[b] P-Pearson's Chi-square test was used for categorical variables; DMT II - diabetes mellitus type 2; *p* values < 0.05 were considered statistically significant; ns-non significant; N/A-not applicable.

3.3. Relative CDKN2B mRNA expression in controls and patients 6 months after the first non-fatal MI

CDKN2B relative expression was significantly lower in MI patients (n = 72) compared with controls (n = 43) for a mean factor of 0.65, SE = 0.278–1.556, p = 0.002 (Fig. 1). The clinical characteristics of the tested subgroups are listed in Table 4. Hypertension and smoking were underrepresented in this subgroup of MI patients compared with the entire MI patient group and the percentage of DMT II was increased. We tested the possible infuence of these parameters on *CDKN2B* relative expression. None of them were associated with *CDKN2B* relative expression (data not shown). We found a significant positive correlation between the age of patients and *CDKN2B* mRNA (r = 0.25). However, the patients were significantly older than the controls and the age was not significantly associated with rs10757278 genotypes in the group of patients (ANOVA p = 0.7). The tested groups were not significantly different in either genotype distribution or in allele frequencies (data not shown).

3.4. Association of rs10757278 and rs518394 variants with relative CDKN2B mRNA expression in MI patients

We analyzed relative *CDKN2B* mRNA expression in PBMC of 72 MI patients with regard to the rs10757278 and rs518394 genotypes.

Compared with the AA (n = 14) genotype, *CDKN2B* mRNA expression was lower in the AG (n = 38) genotype (mean factor of 0.576, SE 0.216–1.447, p = 0.011) and in the GG (n = 20) genotype (mean factor of 0.584, SE 0.225–1.466, p = 0.035) (Fig. 2a). The rs10757278 G allele containing genotypes, assessed using a dominant model of inheritance, exhibited a significant decrease in *CDKN2B* mRNA expression compared with the common AA homozygote (mean factor of 0.579, SE = 0.217–1.448, p = 0.012).

In the control group, mRNA expression was lower in G allele carriers but not in a statistically significant way (data not shown).

The rs518394 genotypes were not significantly associated with *CDKN2B* mRNA expression (GC, n = 37 vs. GG, n = 28, mean factor of 1.44, SE = 0.216–1.447, p = 0.054; GG, n = 28 vs. CC, n = 7, mean factor of 1.24, SE = 0.482–2.983, p = 0.46; Fig. 2b).

We haven't found significant association of *CDKN2B* mRNA expression with clinical and angiographic parameters that could influence mRNA expression: heart insufficiency, infarct location, post PCI TIMI flow (thrombolysis in myocardial infarction flow), disease severity (number of diseased arteries) and the medication use after MI (data not shown).

4. Discussion

We did not observe an association between 9p21.3 rs10757278 and the first non-fatal MI in this case-control study performed with a southeastern European population from Serbia. Contrary to many studies that have replicated the association of 9p21.3 rs10757278 or rs1333049 with CAD in western and northern European populations [20], few studies failed to replicate this association with MI (in coronary heart disease patients) [21] or CAD/MI [6].

Previous studies that addressed the association of rs10757278 and rs1333049 with disease reported a frequency of the risk alleles in Caucasian control subjects ranging from 0.43 in northwestern Europe to

Table 2
Genotype and allele frequencies of genetic variants rs10757278 and rs518394 from the 9p21.3 region in the controls and the patients with first non-fatal MI.

Genetic variant		Controls n = 583 (%)	Patients n = 523 (%)	OR	95% CI	p	Adjusted OR[a]	95% CI	p
rs10757278	AA	144 (24.7)	112 (21.4)	1			1		
	AG	284 (48.7)	254 (48.6)	1.15	0.84–1.56	0.36	1.08	0.73–1.61	0.67
	GG	155 (26.6)	157 (30.0)	1.29	0.92–1.81	0.12	1.19	0.77–1.82	0.41
	Alele A/G	0.49/0.51	0.46/0.54	1.06	0.87–1.29	0.54			
rs518394	GG	200 (34.3)	212 (40.5)	1			1		
	GC	290 (49.7)	240 (46.0)	0.78	0.60–1.01	0.07	0.81	0.61–1.08	0.16
	CC	93 (16.0)	71 (13.5)	0.70	0.48–1.02	0.06	0.69	0.45–1.03	0.07
	Alele G/C	0.59/0.41	0.63/0.37	0.83	0.70–0.99	0.04			

OR odds ratio; 95% CI confidence interval.
[a] OR adjusted for gender, age, BMI, LDLC, HDLC, Tg, hypertensive and smoking status; p values < 0.05 were considered nominaly significant.

over 0.5 in the southeastern Mediterranean (Italians and in our study) [22] to 0.59 in the Middle East (Turkey, Iran) [23,24]. Given that the frequency of the rs10757278 risk G allele in the current study fell in the range of that of other European CAD patients, we suggest that the lack of an association between rs10757278 and MI may be due to the high frequency of rs10757278 G allele in Serbian control subjects. A recent study noted that markers in the 9p21.3 region showed signal of the positive and balancing selection in Europe and noteworthy differences in LD block structure among 19 populations [25]. However, we cannot exclude that our study was slightly underpowered. Therefore, additional studies in neighboring populations should be performed in order to more accurately elucidate the risk for MI.

Our results revealed that association of rs518394 with MI and disease burden was not independent of other investigated risk factors. Despite being a risk for prevalent MI in preexisting CAD patients, C allele containing haplotype (GCCG) has been shown to protect against disease severity [6]. However, these results have not been replicated. Researchers have been discussing whether the lead 9p21.3 variants are associated with stable and/or acute clinical presentations of CAD due to the heterogeneity of previously investigated phenotypes in CAD patients [5,26]. This study was designed to include a homogeneous group of patients with first non-fatal large MI, all of whom undervent PCI and had underlying CAD. Although depicting a homogeneous phenotype should increase the accuracy of a risk assessment, it could also lead to selection bias and consequently affect the allele frequency distribution. It has also been suggested that the 9p21.3 haplotypic effect on CAD is not homogeneous across different haplotypic backgrounds, which might explain the conflicting results [26]. However, none of other 9p21.3 variants contained predictive information beyond that of rs1333049 [26], which is in high LD with investigated variant rs10757278. We found that 6 months after the first non-fatal MI, CAD patients exhibited decreased expression of *CDKN2B* mRNA compared with control subjects. This finding is consistent with that of a previous study [27]. Decreased *CDKN2A* expression was noted in patients with ischemic stroke compared with controls in a Chinese Han population [28]. It was previously shown that *CDKN2A* and *CDKN2B* expression were positively correlated [9]. Therefore, these results are consistent with one another. Our study adds novel data pertaining to the down-regulation of *CDKN2B* in CAD patients six months after an acute event and revascularization/stenting of the diseased artery compared with control subjects. *CDKN2B* and *CDKN2A* are tumor suppressors that regulate cell cycle [29,30], an important process in atherosclerosis pathology [31]. Decreased expression of such anti-proliferative molecules may promote pathologic macrophage or vascular proliferation and further stimulate proatherogenic mechanisms [32]. For example, it has been shown that elevated PBMC counts 6 months after MI are an independent predictor of left ventricular remodeling in patients [33].

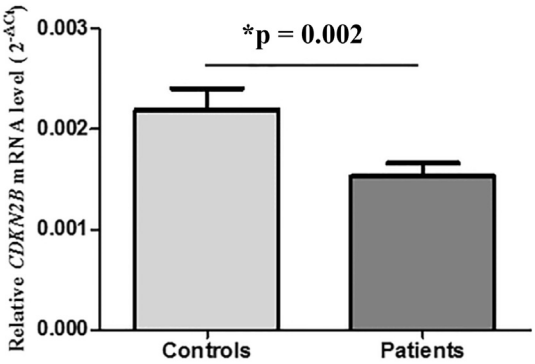

Fig. 1. Relative *CDKN2B* mRNA expression in PBMC of healthy controls and MI patients, 6 months after the first MI.
The cDNAs from PBMC specimens were used in real-time qPCR as a template for relative quantification of *CDKN2B* mRNA and *PPIA* (endogenous control) mRNA. Threshold cycle (Ct) values were used for quantification. For each specimen, the expression level of *CDKN2B* mRNA was normalized to endogenous control and the relative mRNA level is presented as a mean of $2^{-\Delta Ct}$ ± standard error. REST 2009 software was used for relative expression calculation between groups (QIAGEN, Hilden, Germany). Relative *CDKN2B* mRNA expression in PBMC of MI patients (n = 72) was significantly down–regulated compared with controls (n = 43) by mean factor of 0.65, SE = 0.278–1.556, *p = 0.002.

We additionally found significantly decreased relative *CDKN2B* mRNA expression in MI patients with the rs10757278 risk G allele versus the AA genotype in peripheral blood mononuclear cells 6 months after the acute event. Several previous studies have attempted to elucidate the regulation of gene expression as a mechanism by which 9p21.3 region might increase CAD risk [8–13]. Among six 9p21.3 risk variants, only rs10757278 was significantly associated with diminished expression of proximate transcripts *CDKN2B*, *CDKN2A*, *ARF* and *ANRIL* in peripheral blood T-cells of healthy individuals [10]. Furthemore, decreased *CDKN2B* mRNA and protein levels were reported in rs1333049 risk (CC) genotype in vascular smooth muscle cells compared with the common homozygote [12].

Table 3
Association of genetic variant rs518394 with the risk for first non–fatal MI occurrence assesed by dominant model of inheritance.

Variant	Dominant genetic model	OR	95% CI	p	Adjusted OR[a]	95% CI	p
rs518394	GG	ref.			ref.		
	GC + CC	0.76	0.59–0.97	0.03	0.78	0.60–1.03	0.08

[a] OR adjusted for gender, age, BMI, LDLC, HDLC, Tg, hypertensive and smoking status; p < 0.05 was considered nominaly signifficant.

Table 4
Main characteristics of the controls and the patients included in the analysis of *CDKN2B* mRNA relative expression in PBMC.

Characteristic (unit)	Controls	Patients	p
	n = 43	n = 72	
Overall			
Age (years)	45.25 ± 10.14	55.21 ± 7.99	< 0.001[a]
Gender, males (%)	48.93	73.62	0.008[c]
BMI (kg/m2)	26.09 ± 3.56	27.14 ± 4.21	ns[a]
TC (mmol/L)	5.68 ± 1.07	5.64 ± 1.13	ns[b]
LDLC (mmol/L)	3.73 ± 0.92	3.66 ± 1.08	ns[b]
HDLC (mmol/L)	1.58 ± 0.27	1.13 ± 0.27	< 0.001[b]
Tg (mmol/L)	1.27 ± 0.73	1.89 ± 1.30	< 0.001[a]
Hypertension (%)	23.25	45.83	0.01[c]
Smokers (%)	39.53	20.08	ns[c]
DMT II (%)	0.00	33.33	N/A
Clinical and angiographic characteristics (%)			
Advanced Killip class[d]		18.05	N/A
NYHA > 2		27.58	N/A
Final TIMI–3 flow		90.19	N/A
Multiple vessel disease[e]		47.47	N/A
Collaterals		15.62	N/A
STEMI		80.55	N/A
Anterior wall MI		42.85	N/A
Proximal lesions		50.81	N/A
Post–MI discharge medications (%)			
ACE inhibitors		97.18	N/A
Beta–blockers		80.28	N/A
Diuretics		16.90	N/A
Statins		97.18	N/A
Nitrates		94.36	N/A
Heparin		60.56	N/A
Aspirin		100.0	N/A
Clopidogrel		98.59	N/A

Values are presented as mean ± SD for age, BMI, TC, LDLC, HDLC, and Tg.
DMT II - diabetes mellitus type 2; STEMI - ST elevation myocardial infarction; TIMI - thrombolysis in myocardial infarction; p values < 0.05 were considered statistically significant; ns - non significant; N/A - not applicable.

[a] p-Mann-Whitney *U* test.
[b] p-Student's *t*-Test.
[c] p-Pearson's Chi–square test.
[d] Defined as Killip class ≥2.
[e] Defined as obstruction of ≥50% of the epicardial vessel.

In order to prevent the acute phase response of MI from affecting gene expression, we deliberately analyzed the *CDKN2B* expression in patients 6 months after the acute event. To exclude the potential influence of downstream negative consequences of MI on gene expression (e.g., heart insufficiency, infarct location, post-PCI TIMI flow, severe atherosclerosis (number of diseased arteries) and medication use) 6 months after the event, we looked for an association between these parameters and *CDKN2B* expression. We did not observe any, so we suggest that a significant association between rs10757278 genotypes and *CDKN2B* expression is independent of the investigated clinical post-MI parameters. Because we did not find a significant association between genotype and *CDKN2B* expression in the control subjects, we speculate that the control sample should be larger or that other CAD- or MI-linked factors may modulate expression in disease. There is also a possibility that cell-specific and phenotype-driven effects of risk variants on gene expression in the 9p21.3 region may persist. A complex regulatory map has been demonstrated at the 9p21.3 locus; it was previously discussed that disease-associated alleles may have condition-specific effects [7].

A major limitation of this study is that we did not have enough power to reject the null hypothesis. Boosting the sample size, particularly the number of controls, would afford a more precise estimation of the investigated variants' frequencies in our population. However, the difference of estimated risk among populations should not be underestimated. While we confirmed the association of rs10757278 with *CDKN2B* expression in MI patients, we did not find any association of this variant with MI. Future studies should address these inconsistencies in the context of plausible mechanistic hypotheses with which to examine how rs10757278 exerts effects. Validation and replication of our data in another sample group from a similar population and with the same CAD subphenotype would be beneficial as well.

In conclusion, our results point to a dysregulation of gene expression in the 9p21.3 region 6 months after acute MI, which is affected by a genetic variant in patients as well. Therefore, an rs10757278 rare allele is one of the factors that may lead to prolonged risk for proatherogenic complications. Additional research in specifically defined CAD subphenotypes and cell subtypes would be advantageous for resolving the complex role of 9p21.3 in atherosclerotic diseases.

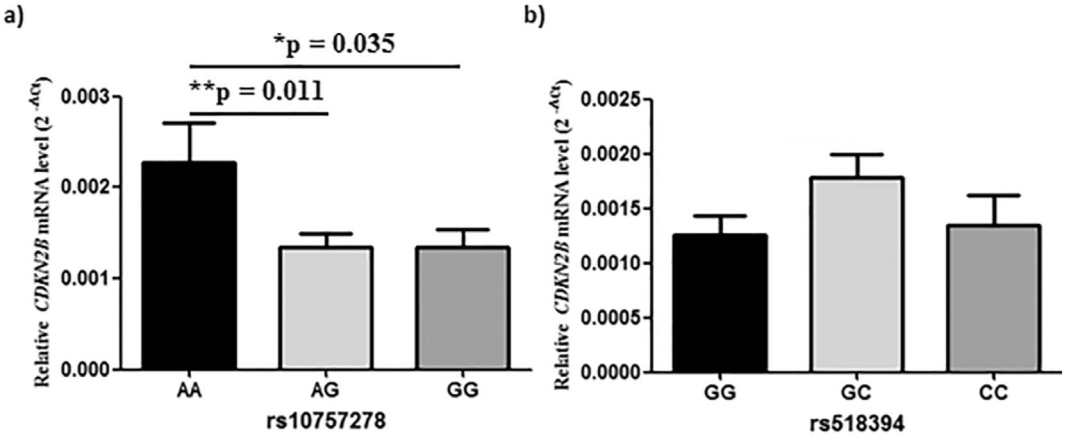

Fig. 2. Relative *CDKN2B* mRNA expression in PBMC of MI patients 6 months after the first MI, with regard to rs10757278 and rs518394 genotypes.
The cDNAs from PBMC specimens were used in real-time qPCR as a template for relative quantification of *CDKN2B* mRNA and *PPIA* (endogenous control) mRNA. Threshold cycle (Ct) values were used for quantification. For each specimen, the expression level of *CDKN2B* mRNA was normalized to endogenous control and the relative mRNA level is presented as a mean of $2^{-\Delta Ct}$ ± standard error. REST 2009 software was used for relative expression calculation betwen groups (QIAGEN, Hilden, Germany).
a) Compared with the AA (n = 14) genotype, relative expression was significantly down–regulated in the GG (n = 20) genotype by a mean factor of 0.584, SE 0.225–1.466, *p = .035; and in the AG (n = 38) genotype by a mean factor of 0.576, 0.216–1.447, **p = 0.011.
b) Relative *CDKN2B* mRNA expression in relation to rs518394 genotypes was not significantly different, GG (n = 28), CG (n = 37), CC (n = 7).

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Appendix A. Supplementary data

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