The rs10757278 Polymorphism of the 9p21.3 Locus Is Associated with Premature Coronary Artery Disease in Polish Patients

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Recently, genome-wide association studies have revealed a locus associated with coronary artery disease (CAD) and myocardial infarction, namely, 9p21.3. Its participation in the conditioning of the disease has been proven in many populations of European descent, but not yet in Slavs. Allelic variants of the rs10757278 polymorphism functionally affect the activity of the 9p21.3 locus; therefore, we conducted a study to determine whether the rs10757278 is associated with premature CAD in Polish patients. We studied 320 subjects aged 25–55 years, divided into two groups matched by sex and age: (1) patients with angiographically proven premature CAD ($n=160$), and (2) blood donors as a control group ($n=160$). The rs10757278 was genotyped using the method of fluorescently labeled allele-specific oligonucleotides. The frequency of the G allele was significantly higher in patients than in controls (58.2% vs. 42.8%, respectively, $p=0.011$) and was similar to the frequency of the GG homozygotes (30.6% vs. 17.5%, respectively, $p=0.006$). Both the GG homozygosity (odds ratio [OR]=2.08, 95% confidence interval [CI]: 1.19–3.66) as well as the G allele (OR=1.49, 95% CI: 1.08–2.07) have been associated with CAD in the analyzed population. These variants may be considered as risk factors, also in the Polish population.

Introduction

CORONARY ARTERY DISEASE (CAD) remains a serious clinical and economical problem especially in Poland where cardiovascular diseases are one of the most important causes of sickness absence, hospitalization, disability, and premature mortality (Mendis *et al.*, 2011).

CAD is a multifactorial condition and its phenotype results from the interactions between multiple genetic and environmental factors. Until now, many single-nucleotide polymorphisms (SNPs) of candidate genes as well as of regulatory loci have been discovered (Ogawa *et al.*, 2010; The IBC 50K CAD Consortium, 2011). In 2007, independent genome-wide association studies revealed nine highly correlated SNPs ($r^2>0.8$) in a new locus in the region of chromosome 9 (9p21.3), showing the greatest association with heterogeneous CAD of all of the loci analyzed so far (Helgadottir *et al.*, 2007; McPherson *et al.*, 2007; Samani *et al.*, 2007). Since then, numerous studies have confirmed the association of this locus with CAD or myocardial infarction (MI) in populations of European origin (Abdullah *et al.*, 2008; Anderson *et al.*, 2008; Assimes *et al.*, 2008; Lemmens *et al.*, 2009; Koch *et al.*, 2011) as well as Eastern (Shen *et al.*, 2008a; Ding *et al.*, 2009) and Southern Asia origin (AshokKumar *et al.*,

2011). However, genetic heterogeneity was shown in Black subjects (Assimes *et al.*, 2008). It was also documented that the 9p21.3 locus variants are associated with the risk of ischemic stroke (Anderson *et al.*, 2010) and diabetes type II (Cugino *et al.*, 2011) in various populations.

The susceptibility locus spans ~53 kb in section 21 on the short arm of chromosome 9. The SNPs associated with CAD lie in a gene desert flanked by cell-cycle-regulating genes: *CDKN2A* (cyclin-dependent kinase inhibitor 2A), encoding p16 (INK4, which is CDK4 inhibitor) and p14 (ARF, which binds MDM2) peptides, and *CDKN2B*, encoding p15 (an inhibitor of CDK4 and CDK6), and a large nonprotein-coding gene *ANRIL* (antisense RNA in the INK4 locus).

Harismendy *et al.* (2011) found that the 9p21 interval is the second densest gene desert for predicted enhancers in the human genome and they identified 33 enhancers within the locus. The authors also documented that the SNP rs10757278 (c.22114477A>G) is located in one of these enhancers and its risk allele (G allele) disrupts a binding site for STAT1, an interferon γ -activated transcription factor. They also showed that in the human vascular cells, interferon- γ activation strongly affects chromatin structure and transcriptional regulation of the 9p21 locus, including STAT1 binding, long-

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range enhancer interactions, and the expression of flanking genes, and that the rs10757278 polymorphism alters the cellular response to inflammation, angiogenesis, and atherogenesis (Harismendy *et al.*, 2011). Other studies have also shown that the rs10757278 strongly influenced the expression of the 9p21.3 genes, such as *CDKN2A*, *CDKN2B*, and *ANRIL*, while there was no such relationship with other polymorphisms of the 9p21.3 locus (Liu *et al.*, 2009; Burd *et al.*, 2010). Results of these studies point to the crucial role of this polymorphism in determining the activity of the 9p21.3 locus.

CAD is common in Slavs and tends to occur at an earlier age in Slavic populations than in any other European ones. This perhaps partly results from genetic factors. Therefore, we conducted a replication study to determine whether the rs10757278 is associated with CAD in a population of Polish patients with premature disease. We also analyzed the potential relations of the rs10757278 and atherosclerotic phenotype as well as traditional risk factors.

Materials and Methods

Subjects

We studied 320 subjects. Group 1: 160 patients with angiographically proven premature CAD, 30 women and 130 men, aged 25–55 years (mean 41.21 ± 5.62). Group 2: 160 blood donors, including 25 women and 135 men, aged 25–55 years (mean 39.89 ± 8.21). CAD subjects were selected from (1) patients admitted to the 1st Department and Clinic of Cardiology at the Upper Silesian Centre of Cardiology in Katowice and (2) patients admitted to the 1st Department of Cardiac Surgery at the Upper Silesian Centre of Cardiology in Katowice. The patients were classified for the study by the same cardiologist. The controls were recruited from the Regional Centre of Blood Donor and Blood Treatment in Katowice. Following the nationwide recommendations of the Polish Centres of Blood Donor and Blood Treatment, blood samples were obtained only from subjects with systolic blood pressure (BP) <140 and diastolic BP >90 mmHg on the day of blood collection. All subjects were Polish Caucasians, inhabitants of Upper Silesia.

Inclusion and exclusion criteria, details of medical interview, diagnosis, and evaluation, as well as criteria for CAD, MI, and traditional risk factors have been described previously (Niemic *et al.*, 2007).

The study protocol was approved by the Ethics Committee of the Medical University of Silesia in Katowice (Poland) and all subjects gave written informed consent.

Biochemical analyses

Total serum cholesterol (TC), high density lipoprotein (HDL)-cholesterol, and triacylglycerols (TGs) were measured by enzymatic methods (commercial Analco kit, Warsaw, PL). low density lipoprotein (LDL)-cholesterol levels were calculated according to the Friedewald formula (Friedewald *et al.*, 1972) in subjects with TG levels below 4.4 mM.

Genetic analyses

Genomic DNA was extracted from peripheral lymphocytes using the MasterPure genomic DNA purification kit (Epicentre Technologies). The rs10757278 polymorphism of the 9p21 locus was genotyped using the TaqMan® Predesigned SNP Genotyping Assay (Applied Biosystems). The polymer-

ase chain reaction amplification was performed according to the manufacturer's specifications. Genotyping was performed using the 7300 Real-Time PCR System (Applied Biosystems).

Statistical analyses

Data were analyzed using the SAS 9.1 (SAS Institute, Inc.) and Statistica 6.0 (STATSOFT) software. Normality of distribution was computed by the W Shapiro–Wilk's test and then comparison of quantitative data was performed by U Mann–Whitney's test or the T-student's tests. Allele frequencies were deduced from the genotype distribution. Hardy–Weinberg equilibrium was tested in all groups by a χ^2 test. Comparisons of genotype and allele frequencies between cases and control subjects were performed by a χ^2 test. When the number of subjects in the sample was lower than 10, the Fisher's correction was used. Statistical significance was accepted at $p < 0.05$. ORs as well as their 95% CIs were computed using a univariate analysis and multiple logistic regression analysis after adjustment for age, sex, and traditional risk factors of CAD.

Pearson correlation coefficients between rs10757278 variants and clinical and biochemical parameters were calculated. Gene–traditional risk factor interactions were additionally analyzed using the measures of additive interactions including the synergy index and attributable proportion due to interaction (Assmann *et al.*, 1996).

Results

Clinical and biochemical characteristics

Clinical and biochemical parameters of the patients and controls are shown in Table 1. There were 78.1% cases who had suffered from MI ($n = 125$) and 61.3% patients with critical stenoses (>90%) in coronary vessels ($n = 98$). CAD patients showed an increased level of TC, LDL-cholesterol, and TG. The level of HDL-cholesterol did not differ significantly between CAD patients and controls (Table 1). The high value of the OR for hypertension resulted from the fact that, according to the nationwide recommendations of Blood Donor and Blood Treatment Polish Centres, the blood samples were obtained only from patients with systolic BP <140 and diastolic BP >90 mmHg on the day of blood collection. Only the TG levels, hypertension, and cigarette smoking significantly differentiated the groups in the multivariate model after adjustment for age, sex, and the rs10757278 polymorphism.

Analysis of rs10757278 polymorphism

Genotype frequencies were compatible with the Hardy–Weinberg equilibrium in both groups. Data from genotyping the rs10757278 polymorphism are shown in Table 2. Frequencies of the G allele and GG homozygotes were significantly higher in patients than in controls ($p = 0.011$ and $p = 0.006$, respectively). Results of logistic regression analysis confirmed that the GG homozygosity was a risk factor for CAD in the analyzed population (OR = 2.08) also after adjustment for traditional risk factors (Table 2).

rs10757278 and clinical phenotype

There was no correlation between genotypes and carrier state of both alleles and MI, the severity of atherosclerosis estimated on the basis of the number of coronary stenoses,

TABLE 1. BIOCHEMICAL AND CLINICAL CHARACTERISTICS IN THE GROUPS OF CORONARY ARTERY DISEASE PATIENTS AND BLOOD DONORS

Characteristics	CAD (n=160)	BD (n=160)	Crude OR (95% CI) (univariate analysis)	p-Value
	Mean $\pm$ SD	Mean $\pm$ SD		
Age (years)	41.21 $\pm$ 5.62	39.89 $\pm$ 8.21	—	0.110
TC (mM)	5.83 $\pm$ 1.44	5.28 $\pm$ 1.35	—	<0.000
LDL (mM)	3.93 $\pm$ 1.26	3.46 $\pm$ 1.22	—	<0.000
HDL (mM)	1.13 $\pm$ 0.45	1.14 $\pm$ 0.39	—	0.907
TG (mM)	1.87 $\pm$ 0.99	1.45 $\pm$ 0.69	—	<0.000
BMI	26.92 $\pm$ 4.17	25.15 $\pm$ 3.72	—	0.337
	% (No.)	% (No.)		
Sex (male)	81.3 (130)	84.4 (135)	0.80 (0.43–1.49)	0.459
TC $\geq$ 5 mM	68.8 (110)	51.3 (82)	2.09 (1.29–3.39)	0.001
LDL $\geq$ 3 mM	71.9 (115)	60.0 (96)	1.70 (1.04–2.80)	0.025
TG $\geq$ 1.7 mM	54.4 (87)	31.3 (50)	2.62 (1.62–4.25)	<0.000
Cigarette smoking	57.5 (92)	21.9 (35)	4.83 (2.88–8.13)	<0.000
Hypertension	56.3 (90)	3.8 (6)	33.00 (13.13–88.12)	<0.000
Diabetes mellitus	6.30 (10)	0 (0)	—	0.004
BMI $\geq$ 25	52.5 (84)	44.4 (71)	1.39 (0.87–2.20)	0.146
Familial history of CAD	33.8 (54)	0 (0)	—	<0.000

BD, blood donor; BMI, body mass index; CAD, coronary artery disease; CI, confidence interval; HDL, high density lipoprotein; LDL, low density lipoprotein; OR, odds ratio; TC, total serum cholesterol; TG, triacylglycerol.

and critical arterial occlusions observed during coronary angiography (data not shown).

Gene-traditional risk factor interactions

We did not find any statistically significant correlations and interactions between genotypes of the rs10757278 polymorphism and respective traditional risk factors of CAD (data not shown).

We divided the subjects into high- and low-risk groups, based on the number of traditional concomitant risk factors of CAD. The analysis included nine risk factors: family history of CAD/MI, elevated levels of total cholesterol (TC $\geq$ 5 mM), triglycerides (TG $\geq$ 1.7 mM) and LDL-cholesterol (LDL $\geq$ 3 mM), hypertension, type II diabetes, cigarette smoking, overweight/obesity (body mass index $\geq$ 25), and male gender. High-risk subjects (individuals exposed to 5–9 risk factors) were more frequent in CAD group (61.2% vs. 17.5%,

$p < 1 \times 10^{-8}$); thus, low-risk subjects (individuals exposed to 0–4 risk factors) were more frequent between the controls. We analyzed the frequencies of rs10757278 genotypes in low- and high-risk subgroups and we found that the frequency of GG genotype and G allele differed only in low-risk subgroups, while these frequencies were quite similar in high-risk patients (Table 3).

Discussion

In our present study we confirmed an association between the rs10757278 polymorphism of the 9p21.3 locus and CAD in Polish patients. The G allele independently increased the risk of CAD in the analyzed population. To compare the results of this report with previously published studies, we presented compiled data from the analysis of the G allele association in populations of European origin (Table 4). We did not include the study on the Italian population (Shen *et al.*, 2008b) due to

TABLE 2. FREQUENCY OF GENOTYPES AND ALLELES OF THE rs10757278 POLYMORPHISM IN THE GROUPS OF CORONARY ARTERY DISEASE PATIENTS AND BLOOD DONORS

Genotype allele	CAD (N=160) % (n)	BD (N=160) % (n)		OR (95% CI), p
AA	25.0 (40)	31.9 (51)	vs. AG + GG	0.71 (0.42–1.19), $p=0.170$
AG	44.4 (71)	50.6 (81)	—	—
GG	30.6 (49)	17.5 (28)	vs. AA + AG	2.08 (1.19–3.66), $p=0.006^a$
AA + AG	69.4 (111)	82.5 (132)	vs. GG	0.48 (0.27–0.84), $p=0.006^b$
GG + AG	75.0 (120)	68.1 (109)	vs. AA	1.40 (0.84–2.36), $p=0.170$
A	47.2 (151)	57.2 (183)	vs. G	0.67 (0.48–0.92), $p=0.011$
G	52.8 (169)	42.8 (137)	vs. A	1.49 (1.08–2.07), $p=0.011$

^aOR=2.46 (95% CI: 1.35–4.49), $p=0.003$.

^bOR=0.41 (0.22–0.74), $p=0.003$.

Adjusted for sex, age, TC, LDL-cholesterol, HDL-cholesterol, TG, BMI, cigarette smoking status, hypertension, and familial history of CAD in multivariate logistic regression analysis model.

TABLE 3. FREQUENCY OF GENOTYPES AND ALLELES OF THE rs10757278 POLYMORPHISM IN GROUPS OF CORONARY ARTERY DISEASE PATIENTS AND BLOOD DONORS

Genotype allele	CAD % (n)	BD % (n)
<i>Low-risk subgroups (subjects exposed to 0–4 risk factors)</i>		
AA	29.0 (18)	35.6 (47)
AG	35.5 (22)	48.5 (64)
GG	35.5 (22) ^a	15.9 (21)
AA + AG	64.5 (40) ^b	84.1 (111)
GG + AG	71.0 (44)	64.4 (85)
A	46.8 (58)	59.8 (158)
G	53.2 (66) ^c	40.2 (106)
<i>High-risk subgroups (subjects exposed to 5–9 risk factors)</i>		
AA	22.4 (22)	14.3 (4)
AG	50.0 (49)	60.7 (17)
GG	27.6 (27)	25.0 (7)
AA + AG	72.4 (71)	75.0 (21)
GG + AG	77.6 (76)	85.7 (24)
A	47.4 (93)	44.6 (25)
G	52.6 (103)	55.4 (31)

^aOR = 2.91 (95% CI: 1.37–6.20), *p* = 0.0022.^bOR = 0.34 (0.16–0.73), *p* = 0.0022.^cOR = 1.70 (1.08–2.67), *p* = 0.016.

mismatches in age and sex between study groups. The frequency of both alleles in the Polish population, as well as the strength of the observed effect, was similar to those observed in the German population (Koch *et al.*, 2011), which is ethnically different but adjacent to the Polish one. In both populations, the strength of the effect was greater than the overall effect, calculated for all studies combined (Table 4).

Three meta-analyses of the associations of the 9p21.3 locus with CAD and/or MI have been published to date (Lemmens *et al.*, 2009; Palomaki *et al.*, 2010; Saleheen *et al.*, 2010). In two cases, the authors included different SNPs of the 9p21.3 locus tested in the context of CAD/MI, justifying their decision by a high linkage disequilibrium between the individual polymorphisms. In both meta-analyses, the overall effect of risk allele was calculated for populations of European and Asian origin together. In the meta-analysis of Palomaki *et al.* (2010) the gene-dose effect was analyzed. Data concerned the effect of high-risk homozygotes (within the risk genotypes of any of the 9p21.3 SNPs) in relation to heterozygotes and the effect of heterozygotes in relation to low-risk homozygotes. Neither the effect of the carrier state of the risk allele nor the effect of homozygosity in relation to the other two genotypes was analyzed (Palomaki *et al.*, 2010). The total effect for risk alleles obtained in the second meta-analysis (Lemmens *et al.*, 2009) was 1.29 (95% CI: 1.25–1.33), and it was lower than that obtained in our present comparison (Table 4). This may result from two reasons. The first is the inclusion of Asians in the analysis (with the effect usually weaker than that observed in populations of European descent); the second is an analysis of all studied polymorphisms of the 9p21.3 locus. As mentioned in the Introduction, only the rs10757278 polymorphism affects the activity of genes within the 9p21.3 locus and shows a functional relationship. This fact may slightly underestimate the observed effect. Third, meta-analysis by Saleheen *et al.* (2010), in terms of ethnicity (separate effect for the populations

TABLE 4. COMPARISON OF THE RESULTS OF STUDIES ON THE rs10757278 IN THE CONTEXT OF CORONARY ARTERY DISEASE AND/OR MYOCARDIAL INFARCTION IN THE POPULATIONS OF EUROPEAN ANCESTRY

Reference	Population	Number of alleles (G/A)		G allele frequency (%)		OR (95% CI)	p-Value	Phenotype	MI (%)
		Cases	Controls	Cases	Controls				
Abdullah <i>et al.</i> (2008)	U.S.	368/252	508/612	59.3	45.4	1.76 (1.43–2.16)	4×10^{-8}	CAD + MI	53.9
Anderson <i>et al.</i> (2008)	U.S. ^a	1023/957	498/576	51.7	46.4	1.24 (1.06–1.44)	0.005	CAD, MI?	?
	U.S. ^b		489/589	51.7	45.4	1.29 (1.11–1.50)	9×10^{-4}		
Assimes <i>et al.</i> (2008)	U.S. ^c	271/237	346/376	53.3	47.9	1.24 (0.98–1.57)	0.06	CAD + MI	?
	U.S. ^d	1028/864	634/716	54.3	47.0	1.34 (1.17–1.55)	3×10^{-5}		
Lemmens <i>et al.</i> (2009)	Belgian	1015/813	778/840	55.5	48.1	1.35 (1.18–1.55)	1×10^{-5}	CAD + MI	43.2
Helgadottir <i>et al.</i> (2007)	Icelandic	2322/2222	9399/11123	51.1	45.8	1.24 (1.16–1.32)	$< 1 \times 10^{-8}$	MI	100.0
Koch <i>et al.</i> (2011)	U.S.	2709/1921	2636/2376	58.5	52.6	1.27 (1.17–1.38)	$< 1 \times 10^{-8}$	MI	100.0
Present study	German	3855/3459	1053/1369	52.7	43.5	1.45 (1.32–1.59)	$< 1 \times 10^{-8}$	MI	100.0
	Polish	169/151	137/183	52.8	42.8	1.49 (1.08–2.07)	0.011	CAD + MI	78.1
Overall	—	12760/10876	16478/18760	54.0	46.8	1.34 (1.29–1.38)	$< 1 \times 10^{-8}$	CAD + MI	—

^aCompared to controls with angiographically excluded CAD.^bCompared to population of healthy subjects as controls (without angiography).^cYounger individuals (≤ 45 years for men and ≤ 55 years for women).^dOlder individuals.

MI, myocardial infarction; ?, not specified.

of European and Asian origin), was carried out properly and showed results for each of the SNPs individually. The total effect of the rs10757278 G allele on CAD risk for populations of European origin is OR = 1.30 and 95% CI: 1.22–1.39. This result is similar to that obtained in our updated comparison.

Similarly to most of previous studies, there was no correlation between the rs10757278 and angiographically documented severity of atherosclerosis (the number of coronary stenoses and critical arterial occlusions) in our study. However, Patel *et al.* (2010) found that the G allele was responsible for both the severity of the disease and its progression and the effect was gene–dose dependent. The greatest risk of disease progression was shown by GG homozygotes (OR = 3.42, 95% CI: 1.54–7.60) and intermediate risk by AG heterozygotes (OR = 2.51, 95% CI: 1.26–4.99) compared with AA homozygotes.

Our current research also showed no correlation between the rs10757278 polymorphism and common traditional risk factors of CAD. However, an analysis of high-risk (individuals exposed to 5–9 risk factors) and low-risk (individuals exposed to 0–4 risk factors) subgroups showed that the effect of the GG genotype on CAD risk was statistically significant only in low-risk subgroups. These results indicate that the GG genotype may be a significant risk factor for CAD in patients exposed to few traditional risk factors. In high-risk groups, CAD results from the coexistence of traditional risk factors, rather than from the genotype. These results lead to the conclusion that the rs10757278 polymorphism influences the CAD susceptibility independently of the traditional risk factors, which is consistent with previous observations (Helgadottir *et al.*, 2007; Abdullah *et al.*, 2008; Anderson *et al.*, 2008; Assimes *et al.*, 2008; Lemmens *et al.*, 2009; Patel *et al.*, 2010; Koch *et al.*, 2011). However, Scheffold *et al.* (2011) showed that the rs10757278 may have a greater impact on the risk of CAD in patients with positive family history. The results of another study indicate that the risk of MI and CAD associated with the 9p21.3 locus appears to be modified by a prudent diet high in raw vegetables and fruits (Do *et al.*, 2011). The authors showed that the carriers of the risk allele whose diet is rich in raw fruits and vegetables had a lower risk of MI (OR = 1.02) in relation to those with an average (OR = 1.17) and low fresh vegetables' diet (OR = 1.32). Thus, there are fragmentary data on interactions of the 9p21.3 locus and common risk factors of CAD/MI. It is very probable that the 9p21.3 locus acts independently of all known risk factors, which may indicate that the rs10757278 contributes to the pathogenesis of CAD through mechanisms that are yet unknown. At the moment, there are only presumptions that the 9p21.3 locus is a link between the genetic susceptibility to CAD and vascular growth, remodeling, as well as inflammation signaling (Liu *et al.*, 2009; Burd *et al.*, 2010; Harismendy *et al.*, 2011). The effect of interferon- γ on chromatin structure and transcriptional regulation of the 9p21 locus may also indicate the participation of epigenetic processes, whose progress and intensity can influence the rs10757278 polymorphism (Liu *et al.*, 2009; Burd *et al.*, 2010; Harismendy *et al.*, 2011).

Over a period of the last 5 years, genome-wide association studies have identified about 30 genetic variants increasing CAD risk, of which only 6 act through the known risk factors (Roberts and Stewart, 2012). Although the exact function of the 9p21.3 locus is not yet known, recently conducted studies have linked its SNPs with CAD and/or MI in populations of

Europe and Asia. The G allele of the rs10757278 polymorphism occurs with a high frequency in European populations, so its relationship with CAD is also noticeable in relatively small groups, as evidenced by the results of the present study. It should be emphasized that, to our knowledge, this is the first Slavic population analyzed in the context of this polymorphism. There is a need to confirm the relationship in other Slavic populations as well as to determine the molecular background that shapes the susceptibility to CAD in risk allele carriers within the 9p21.3 locus. Understanding the action mechanism of this locus would enable a conscious use of this common marker in assessing the overall risk of CAD.

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Author Disclosure Statement

No competing financial interests exist.

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