Original Article

Association between a Marker on Chromosome 9 and Acute Coronary Syndrome
Confirmatory Study on Czech Population

(acute coronary syndrome / ANRIL / polymorphism / chromosome 9 / CDKN2A / CDKN2B)

J. A. HUBÁČEK[1], V. STANĚK[1], M. GEBAUEROVÁ[1], R. POLEDNE[1], M. ASCHERMANN[2], H. SKALICKÁ[2], J. MATOUŠKOVÁ[3], A. KRUGER[3], M. PĚNIČKA[4], H. HRABÁKOVÁ[4], J. VESELKA[5], P. HÁJEK[5], V. LÁNSKÁ[1], V. ADÁMKOVÁ[1,6], J. PIŤHA[1]

[1]Institute for Clinical and Experimental Medicine, Prague, Czech Republic
[2]2nd Department of Internal Medicine, General University Hospital, Prague, Czech Republic
[3]Department of Cardiology, Homolka Hospital, Prague, Czech Republic
[4]Cardiocenter, Department of Cardiology, University Hospital Královské Vinohrady, Prague, Czech Republic
[5]Department of Cardiology, Teaching Hospital Motol, Prague, Czech Republic
[6]University of South Bohemia, Faculty of Health and Social Studies, České Budějovice, Czech Republic

Abstract. Myocardial infarction (MI) is the leading cause of death in industrialized countries. All the classical risk factors for MI are responsible for approximately 50 % of MI cases. Attention has therefore recently been attracted to those genetic variants that are not associated with conventional risk factors. One of them is the marker rs10757274 in the "gene-free" zone on chromosome 9, which has been repeatedly recognized as a risk factor for development of MI in Western populations. We analysed the relationship between the rs10757274 variant on chromosome 9 and risk of the acute coronary syndrome (ACS) in Czech population. The rs10757274 (A > G) variant was successfully analysed (CR = 99.4 % for patients and 98.4 % for controls) by PCR-RFLP in consecutively examined 1,046 men and 281 women with ACS (age below 65 years) and in population-based controls – 1,162 men and 1,355 women (aged up to 65 years). ANOVA and χ^2 were used for statistical analysis. We confirmed that GG homozygotes are more frequent (codominant model of analysis) among patients with myocardial infarction than in the control group both in men (28.5 % vs. 22.0 %, P = 0.0001, OR 1.73, 95 % CI 1.36–2.19) and women (32.0 % vs. 24.6 %, P = 0.02, OR 1.62, 95 % CI 1.13–2.34). However, rs10757274 polymorphism was not associated with the classical risk factors either in control population or in ACS patients. We conclude that the rs10757274 variant at 9p23.1 is an important genetic risk factor for ACS development in the Czech population.

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Corresponding author: Jaroslav A. Hubáček, IKEM-DEM-LMG, Vídeňská 1958/9, 140 21 Prague 4, Czech Republic. Phone: (+420) 261 363 379; Fax: (+420) 241 721 666; e-mail: jahb@ikem.cz

Abbreviations: ACS – acute coronary syndrome, APO – apolipoprotein, bp – base pairs, BMI – body mass index, CAD – coronary artery disease, CVD – cardiovascular disease, *FTO* – fat mass and obesity-associated gene, GWAs – genome-wide association studies, MAF – minor allele frequency, MI – myocardial infarction, *MRAS* – muscle RAS oncogene, PCR – polymerase chain reaction, RF – risk factor, RFLP – restriction fragment length polymorphism, SNP – single-nucleotide polymorphism.

Introduction

Acute coronary syndromes (ACS) represent the most common cause of mortality and morbidity in industrially developed countries. Traditional five manageable risk factors (dyslipidaemia, hypertension, obesity, diabetes mellitus and smoking) explain only about the half of ACS cases. The rest seems to be independent of these risk factors.

Thus, the interest in the last years has focused on new, mainly genetic risk factors. Behind the variants within the well-known genes, such as for example *APOE* (Angelopoulos and Lowndres, 2008; Poledne et al., 2010) or *APOA5* (Hubáček et al., 2009), variants detected through the genome-wide association studies (GWAs) are new promising tools for detection of the individuals under increased risk of ACS.

Most interestingly, a couple of GWAs-detected variants associated with ACS/MI in different populations exhibit a risk which is partially (e.g. *FTO* – fat mass and obesity-associated gene) (Hubacek et al., 2010a) or completely (e.g. marker within *MRAS* – muscle RAS oncogene) (Erdmann et al., 2009) independent of the classical five risk factors.

One of the first detected and probably most widely analysed DNA markers is a common haplotype on chromosome 9, which is characterized by three SNPs, rs10757274, rs2891168, or rs2383207 (Helgadottir et al., 2007; McPherson et al., 2007). These SNPs are in almost complete linkage disequilibrium (Palomaki et al., 2010). The marker is located (9p21.3) in an intergenic region between the genes coding for cyclin-dependent kinase inhibitors CDKN2A and CDKN2B. The classical risk factor-independent association between this marker and risk of ACS/MI development was widely reproduced in different populations, predominantly in Western-European Caucasians (for review see Palomaki et al., 2010). It has recently been discussed that the DNA region coding for ANRIL (antisense non-coding RNA in the INK4 locus) (Pasmant et al., 2011) is responsible for the association between the rs10757274 marker and atherosclerosis (Congrains et al., 2012), but a clear explanation of the real causality is still missing and remains speculative.

To extend the recent knowledge we have investigated whether the rs10757274 DNA variant on chromosome 9 is associated with the risk of acute coronary syndrome in Czech Slavonic population.

Material and Methods

Selection of individuals

We have analysed 1,336 patients with ACS (1,052 males aged under 65 years and 284 females aged under 75 years), consecutively enrolled as described in detail before (Pitha et al., 2007; Hubacek et al., 2010b).

As controls, we have genotyped 2,559 individuals (1,191 males and 1,368 females, aged 25–64 years [mean: 48.8 ± 10.7 years]) selected in nine Czech districts according to the WHO MONICA Project protocol (Tunstall-Pedoe et al., 2003).

DNA analysis

Genomic DNA was extracted from peripheral blood white cells (Miller et al., 1988). rs10757274 was genotyped using the polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (PCR-RFLP). All PCR chemicals and restriction enzymes were purchased from Fermentas International Inc., Burlington, Ontario, Canada. PCR reactions were performed using the MJ Research DYAD Disciple thermal cycler. Briefly, DNA was amplified in a total volume of 25 µl using the oligonucleotide primers 5´ttg ctt ggt aga tct tcc tcc atc cct t and 5´ttc cca gat gca ctg tat tgt ttg cct tac, in a total volume of 25 µl. The final PCR product (225 base pairs (bp)) was cut using 5 units of *Bsm*AI restriction enzyme, and restriction fragments were separated in 10% polyacrylamide gel by microtitre array diagonal gel electrophoresis (Day and Humphries, 1994). Allele A was represented by restriction fragments of 125 bp and 100 bp, while the presence of uncut product represented allele G.

Risk factor analysis and assessment

The basic risk factors were defined as follows i/ self-reported current smokers ii/ dyslipidaemia as plasma total cholesterol higher than 5.2 mmol/l (for ACS patients) or LDL cholesterol higher than 3.4 mmol/l (for controls) and/or triglycerides higher than 2 mmol/l or self-reported hypolipaemic treatment; iii/ body mass index (BMI) equal to or higher than 30 kg/m^2; iv/ hypertension (self-reported antihypertensive treatment or seated systolic/diastolic blood pressure higher than 139/89; in the case of ACS patients only-self reported history or previous medical record of hypertension), and finally v/ diabetes as self-reported diabetes and/or fasting glucose over 7 mmol/l and/or antidiabetic drugs.

Both in controls and patients, the self-reported data were obtained through personal questionnaires filled in under the supervision of a trained nurse. Body weight was measured with a horizontally placed electronic weight scale (scaled to the nearest 0.1 kg). Height was measured with a stadiometer to the nearest 0.5 cm.

The lipoprotein parameters (obtained from fasting plasma after overnight fast or fasting next day after the admission to the coronary unit in the case of patients with ACS) were assessed using conventional enzymatic methods (reagents from Boehringer Mannheim Diagnostics (Indianapolis, IN) and Hoffmann-La Roche (Basel, Switzerland)) in CDC accredited laboratory (Atlanta, GA).

Systolic and diastolic blood pressures were measured after a 10 min rest in a sitting position as an average of three readings on the right arm with an automated blood pressure unit (Automated sphygmomanometer BP-203 NA, Nippon Colin co., Ltd., Komaki, Japan).

Statistical analysis

The Hardy-Weinberg test (http://www.tufts.edu/~mcourt01/ Documents/Court%20lab%20-%20HW-%20calculator.xls) was applied to confirm the independent segregation of the alleles. χ^2 tests and odds ratio (95% CI) were calculated according to http://www.physics.csbsju.edu/cgi-bin/stats/contingency_form.sh?nrow=2&ncolumn=3, and http://www.hutchon.net/ConfidOR.htm. Individuals carrying the risk factors are expressed in % of the total sample. Differences in risk score values between genotypes were calculated by ANOVA. Mean ± SD are reported for risk score, and P values lower than 0.05 with Bonferroni correction (for analysis of the risk factors) for multiple testing were considered to be significant.

Results

Basic description of the patients and controls is summarized in Table 1. Surprisingly, total plasma cholesterol was significantly higher in controls than in patients with ACS and no significant difference was found in plasma triglycerides in males; this finding was unchanged even after excluding patients with hypolipaemic drugs. The mean risk score was almost three times higher in patients than in controls (P < 0.001) and this ratio was consistent both in males and females.

The genotyping call rate was 99.4 % for ACS patients and 98.4 % for healthy controls. The frequencies of individual genotypes were within the Hardy Weinberg equilibrium (P = 0.57 for control males; P = 0.81 for control females and P = 0.87 for male patients; P = 0.49 for female patients) and did not differ significantly from the frequencies described in Western populations (MAF is 0.48 in Czechs, MAF in Western Europeans is ~ 0.47). Finally, no significant gender differences were observed (P = 0.12 for controls and P = 0.46 for patients).

We have confirmed the association between the G allele of the rs10757274 marker and ACS both in males and females.

GG homozygotes were more common among the ACS patients in males (28.5 % vs. 22.0 %, P = 0.0001 for

Table 1. Basic characteristics of the analysed individuals

	Males			Females		
	Controls	ACS patients	P	Controls	ACS patients	P
N	1 191	1 046		1 368	281	
Age (years)	49.0 ± 10.8	55.1 ± 7.6	0.001	48.6 ± 10.6	62.1 ± 8.3	0.001
Cholesterol (mmol/l)	5.76 ± 1.06	5.20 ± 1.18	0.001	5.80 ± 1.15	5.44 ± 1.22	0.001
Triglycerides (mmol/l)	1.97 ± 1.28	2.03 ± 1.44	n.s.	1.47 ± 0.82	1.79 ± 1.13	0.001
BMI (kg/m^2)	28.2 ± 4.0	28.5 ± 4.4	n.s.	27.6 ± 5.5	28.7 ± 5.7	0.001
Never smokers (%)	67.3	33.0	0.001	78.5	65.3	0.001
Diabetes prevalence (%)	8.9	37.3	0.001	6.8	52.8	0.001
Hypertension prevalence (%)	40.7	86.5	0.001	33.1	87.0	0.001

n.s. – not significant
values are expressed as mean (SD)

Table 2. Frequencies of the rs10757274 genotypes between healthy controls and acute coronary syndrome (ACS) patients. P value for the codominant model of analysis is also given.*

Males	Controls		ACS patients		OR	P	P*
	N	%	N	%	Crude		
GG	256	22.2	298	28.5	1.7	0.0001	
AG	569	48.9	532	50.0	1.4	0.001	0.0001
AA	338	29.0	225	21.5	1.0		
Females							
GG	333	24.6	90	32.0	1.6	0.008	
AG	673	49.7	133	47.3	1.4	0.31	0.02
AA	349	25.7	58	20.6	1.0		

Table 3. rs10757274 genotypes and presence of the traditional ACS risk factors in general population. Uncorrected P values are given.

	Controls, Czech post-MONICA							
	Males				Females			
	AA	AG	GG	P	AA	AG	GG	P
N	**338**	**569**	**256**		**349**	**673**	**333**	
Risk factor								
Smoking	37.3 %	29.9 %	32.0 %	0.07	24.9 %	20.8 %	22.2 %	0.32
DM2	8.1 %	8.3 %	11.3 %	0.31	7.5 %	6.0 %	7.5 %	0.54
Dyslipidaemia	73.5 %	72.2 %	71.4 %	0.85	61.4 %	60.0 %	65.9 %	0.20
Hypertension	41.7 %	39.2 %	42.6 %	0.59	32.4 %	33.3 %	33.3 %	0.95
Obesity	27.1 %	29.5 %	31.1 %	0.57	30.6 %	27.7 %	30.5 %	0.51
No. of RF	1.89 ± 1.13	1.75 ± 1.12	1.80 ± 1.10		1.57 ± 1.18	1.47 ± 1.13	1.58 ± 1.14	

Table 4. rs10757274 genotypes and presence of the traditional ACS risk factors in ACS patients. Uncorrected P values are given.

	ACS patients							
	Males				Females			
	AA	AG	GG	P	AA	AG	GG	P
N	**225**	**532**	**298**		**58**	**133**	**90**	
Risk factor								
Smoking	60.3 %	65.0 %	64.1 %	0.50	45.5 %	43.5 %	43.5 %	0.97
DM2	39.9 %	34.5 %	40.4 %	0.19	69.6 %	49.4 %	52.9 %	0.01**
Dyslipidaemia	68.7 %	65.2 %	66.5 %	0.66	72.4 %	62.4 %	60.0 %	0.28
Hypertension	84.5 %	88.9 %	91.8 %	0.05*	82.1 %	89.0 %	89.5 %	0.36
Obesity	30.2 %	30.8 %	33.2 %	0.75	48.1 %	36.1 %	30.5 %	0.12
No. of RF	2.84 ± 1.03	2.81 ± 0.98	2.93 ± 1.00		3.08 ± 0.98	2.78 ± 1.17	2.71 ± 1.13	

* 0.47 after Bonferroni correction for multiple testing
** 0.09 after Bonferroni correction for multiple testing

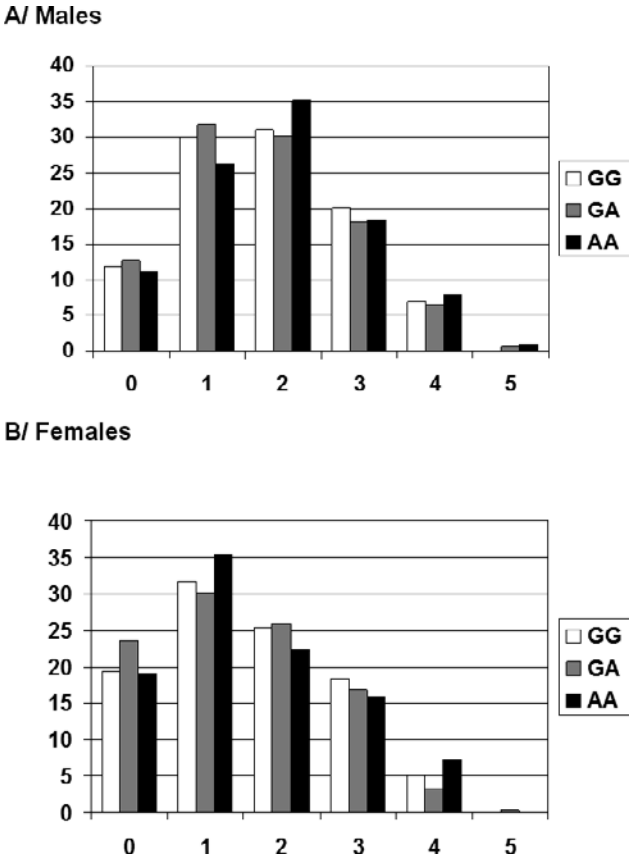

Fig. 1. Distribution of the risk score according to the rs10757274 genotype in control population
Individuals were divided into six subgroups according to the total number (0–5) of risk factors (dyslipidaemia, hypertension, BMI, smoking and diabetes mellitus type 2) present. Males and females were analysed separately.

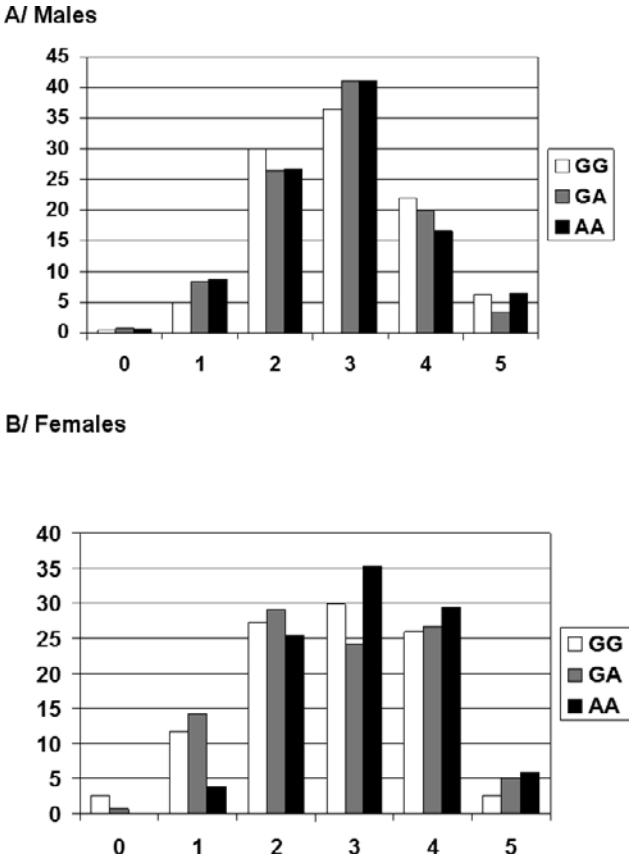

Fig. 2. Distribution of the risk score according to the rs10757274 genotype in the patients with acute coronary syndrome
Individuals were divided into six subgroups according to the total number (0–5) of risk factors (dyslipidaemia, hypertension, BMI, smoking and diabetes mellitus type 2) present. Males and females were analysed separately.

codominat model; OR 1.73; 95 % CI 1.36 – 2.19) as well as in females (32.0 % vs. 24.6 %, P = 0.02 for codominant model; OR 1.62; 95 % CI 1.13 – 2.34) (Table 2).

Similarly to other studies we were not able to detect significant associations between the DNA marker rs10757274 and standard risk factors of ACS when i/ analysed individually (dyslipidaemia, hypertension, BMI, smoking and diabetes mellitus type 2) or ii/ mean score values were compared. For details, see Table 3 for controls and Table 4 for ACS patients.

Also, the distribution of individuals divided into six subgroups according to the total number of risk factors

(0–5 risk factors present) did not significantly differ between the three analysed CDKN2A-2B genotypes (see Fig. 1 for details in controls and Fig. 2 for details in ACS patient groups).

Discussion

As in all Caucasian populations studied so far, we have detected the rs10757274 DNA variant at CDKN2A-2B (9p21.3) locus as an independent risk factor for ACS also in Czech Slavonic population. According to our findings, carriers of the GG genotype exhibit about 1.6 higher odds for the occurrence of ACS than carriers of at least one A allele. Thus, we have extended the risky effect of this marker on ACS also to Western Slavonic population(s).

Further, the detected risk was similar in males and females and was completely independent of traditional cardiovascular risk factors (BMI, hypertension, dyslipidaemia, smoking and diabetes mellitus).

This observation is in agreement with already published reports focused on the same topic. As summarized previously (Palomaki et al., 2010), the 9p21.3 marker is associated with elevated risk of CAD/MI/CVD in almost all so far analysed populations of Western European descent and also in a few studies on Asian populations. Interestingly, the magnitude of the effect is similar between the studies and varies, mostly according to the age of the participants, between 1.3 and 1.6. The consistent results, together with the fact that employment of this marker has improved assessment of the coronary artery disease risk development (Talmud et al., 2008), suggest that it could have clinical implications.

However, the risk of CAD seems to be independent of this marker in other ethnic groups (black individuals, Hispanics and American Indians) (Franceschini et al., 2011; Gong et al., 2011). Nevertheless, in black individuals, the risky GG homozygotes occur with minimal frequency (< 1%) and heterozygotes are about four times less common than in Caucasians (http:www.ncbi.nlm.gov/projects/SNP/snp_ref.cgi?rs=10757274).

In previous studies, no link between the conventional risk factors and 9p21.3 marker was detected. To analyse this in further detail we have defined the risk score for each individual (for controls and patients separately), based on their personal risk profile/presence of clearly defined risk values. These cumulative risk values also show no association with this marker.

As there is no clear link to any of the classical risk factors, the major effort was spent to detect potentially functional gene located in the close proximity of the rs10757247 variant at 9p21.3. Indeed, a gene designated *ANRIL* was described in detail in the same region (Jarinova et al., 2009). Although the expression studies suggest association between the 9p21.3 polymorphisms and *ANRIL* expression (Pasmant et al., 2011), this finding has not substantially improved our knowledge; the human *ANRIL* gene is transcribed into non-coding RNA. The mouse model suggested (Sato et al., 2010), however, that ANRIL might have a regulatory/silencing effect on some genes and modify expression of other genes, thus indicating some regulatory function.

Analysis of another set of SNPs at the same locus suggests that variants within this region are possibly associated with the platelet reactivity and coronary artery calcification in adults without manifest CVD (Musunuru et al., 2010). If confirmed for the CVD-associated SNPs/haplotype, these results could be a plausible explanation for its effect on the CVD risk.

The detection of 9p21.3 and similar markers (although in other cases neither extensive confirmatory studies nor negative studies were published) stress the fact that our knowledge of cardiovascular risk factors still needs to be substantially improved.

In summary, we have detected a strong association between DNA marker rs10757247 on chromosome 9 (9p21.3) and acute coronary syndrome in Czech/Slavonic population of males and females, which was independent of traditional cardiovascular risk factors.

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