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The Interleukin-1 Receptor Antagonist Gene and the Inhibitor of Kappa B-Like Protein Gene Polymorphisms Are Not Associated with Myocardial Infarction in Slovene Population with Type 2 Diabetes

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A B S T R A C T

In this study we investigated the association of the interleukin-1 receptor antagonist gene variable number tandem repeat (IL1RN VNTR) polymorphism and of the inhibitor of kappa B-like protein (IKBL) gene polymorphism with myocardial infarction (MI) in a group of patients with type 2 diabetes. The IL1RN VNTR and the IKBL+738T>C gene polymorphisms were tested in 374 Caucasians: 151 cases with MI and 223 subjects with no history of coronary artery disease. The IL1RN VNTR polymorphism was not a risk factor for MI in Caucasians with type 2 diabetes (genotype 22 vs. the rest: odds ratio (OR) 1.6; 95% confidence interval (CI) = 0.8–3.5; p=0.2). We also failed to demonstrate that IKBL+738T>C gene polymorphism was associated with MI in patients with type 2 diabetes (OR =0.9; 95% CI = 0.3–2.6; p=0.9). We provide evidence that the IL1RN VNTR and the IKBL+738T>C gene polymorphisms are not risk factors for MI in Caucasians with type 2 diabetes.

Key words: *interleukin-1 receptor antagonist polymorphism, inhibitor of kappa B-like protein gene polymorphism, myocardial infarction, type 2 diabetes*

Introduction

Type 2 diabetes is a major risk factor for the development of coronary artery disease (CAD) and subsequent myocardial infarction (MI)[1]. Atherosclerosis is now generally accepted as a chronic inflammatory condition[2] and there is also growing evidence for an important role of chronic inflammation in type 2 diabetes[3]. Genetic markers involved in chronic inflammatory response may therefore be particularly important in the development of CAD and MI in patients with type 2 diabetes[4].

The proinflammatory cytokine interleukin-1 (IL-1) has been suggested to play a central role in the pathogenesis of both CAD and type 2 diabetes[2,5]. The extent of IL-1 signaling is determined by the local ratio of available IL-1 and its naturally occurring competitive inhibitor interleukin-1 receptor antagonist (IL-1ra)[6]. IL-1ra has vascular protective effects. It inhibits fatty streak formation in the apolipoprotein E deficient mouse[7], and its deletion using knockout technology results in lethal chronic inflammation of the arterial wall[8]. Allele 2 of the variable number tandem repeat (VNTR) polymorphism in intron 2 of the IL-1ra gene (IL1RN) has been associated with inflammatory diseases[9,10], including CAD[11,12].

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IL-1 induces the activation of transcription factor nuclear factor-κB (NF-κB), which leads to a coordinated increase in the expression of more than 160 genes whose products mediate inflammatory and immune responses, many of which have a documented role in atherogenesis[13,14]. Activated NF-κB has been detected in endothelial cells, smooth muscle cells, and macrophages in atherosclerotic plaques[15]. In unstimulated cells NF-κB is found in cytoplasm bound to inhibitory proteins (IκBs), which prevent it to enter the nucleus. When cells are stimulated by cytokines, IκB is phosphorylated by specific kinases causing its rapid degradation, thus enabling the transfer of NF-κB into the nucleus to induce gene expression necessary for the inflammatory reaction[13]. Inhibitor of kappa B-like protein (IKBL) is a member of IκBs. A thymine (T) to cytosine (C) substitution at position +738 in exon 4 of the IKBL gene (IKBL+738T>C) has been associated with multiple sclerosis[16] and with extensive and more severe course of ulcerative colitis[17], whereas polymorphisms in the promoter of the IKBL gene have recently been reported to be associated with type 1 diabetes[18].

Genetically determined variations in the IKBL and in IL-1/IL-1ra system may lead to a more pronounced inflammatory response and thus play an important role in the cascade of events that result in plaque rupture and subsequent MI, particularly in patients with type 2 diabetes. The prevalence of MI among diabetics in Slovenia is 2–4-times higher than in general population[19]. The objective of this study was to investigate the association of the IL-1ra gene polymorphism and the IKBL gene polymorphism with MI in Slovene patients of Caucasian origin with type 2 diabetes.

Materials and Methods

The study population of this cross-sectional analysis consisted of 374 subjects with type 2 diabetes lasting more than 10 years: 151 with MI (MI group) and 223 subjects in the control group with no history of CAD, no signs of ischemic changes on electrocardiogram and no ischemic changes during submaximal stress testing. The diagnosis of MI was made according to the criteria by World Health Organization. Patients with MI were included in the study 1–9 months after the acute event. The patients and control subjects came from independent families. The data and blood samples of age-matched controls were obtained from general practitioners. The controls did not have a history of angina pectoris or MI, and had a normal electrocardiogram. All the subjects enrolled in the study were Slovenes of Slavic origin. After informed consent was obtained from the patients and control subjects, a detailed interview was made. Arterial hypertension and cigarette smoking were defined as binary variables. Patients were classified as having type 2 diabetes according to the current American Diabetes Association criteria for the diagnosis and classification of diabetes[20] Expert Committee. Body mass index (BMI) was calculated as weight in kilograms divided by the height in square meters. Total cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL) and triglycerides were determined by standard biochemical methods.

The IL1RN VNTR polymorphism was evaluated by PCR, using primers: 5´-CTC AGC AAC ACT CCT AT-3´ and 5´-TCC TGG TCT GCA GGT AA-3´[21]. Sequence-specific primer -based typing for the IKBL+738T>C gene polymorphism was achieved with the following forward primers: IKB738TF 5'AGAAGCAGAGGGATCCTG 3' (sense) position 722 to 739, IKB738CF 5'AGAAGCAGAGGGATCCCG 3' (sense) position 722 to 739and the reverse primer MidR1 primer 5'AGGAAGAGCTGCGTGAGA 3' (antisense) position 829 to 846. The cycling conditions were as follows: 95 C for 3 min followed by 35 cycles of 95 C for 30 s, 61 C (for IKB738TF) or 63 C (for IKB738CF) for 30 s and 72 C for 30 s. The 144 bp *IKBL* product was separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining as described previously[17]. Two investigators (BP, DP) who were blinded for case or control status of the DNA sample performed the genotype classification.

Differences in mean values were analyzed by Student t-test. Chi-square test was used to compare discrete variables and to compare genotype distributions. Genotypic odds ratios for MI with 95% confidence intervals with two-tailed p-values were calculated by Chi-square test. Statistical analysis was performed using the SPSS program 13 for Windows (SPSS Inc. Illinois).

Results

The characteristics of the cases and control subjects are listed in Table 1. The cases were younger, predominantly of male sex and had a higher incidence of cigarette smoking compared to the control group. Additionally they had higher total cholesterol and LDL cholesterol levels, higher BMI, longer duration of type 2 diabetes, and lower HDL cholesterol levels than the controls (Table 1). There were no significant differences in the incidence of hypertension, and triglyceride levels between the cases and control subjects (Table 1).

The IKBL+738 genotype distributions in cases and controls were compatible with Hardy-Weinberg expectations (IKBL+738: cases $\chi^2=2.3$, $p=0.13$; IKBL+738: controls $\chi^2=0.015$, $p=0.9$). Univariate analysis (Table 2) failed to demonstrate an association between the IL1RN VNTR polymorphism and MI (OR=1.6; 95% CI=0.8–3.5; p=0.2). Less frequent alleles (alleles 3, 4 and 5) were grouped together with allele 1 into a single group (Table 2 and 3). Likewise, univariate analysis also failed to demonstrate an association between the TC genotype of the IKBL+738 gene polymorphism and MI (OR=0.9; 95% CI=0.3–2.6; p=0.9) (Table 1). There were no subjects with the CC genotype of the IKBL+738 gene polymorphism either in MI group or in the control group.

Discussion

In the study, in which we enrolled 374 patients with type 2 diabetes (151 patients with MI and 223 controls),

TABLE 1
CHARACTERISTICS OF PATIENTS WITH MI AND CONTROLS

Characteristics	MI group (%)	Controls (%)	p value
Number	151	223	
Age (years)	59.2±11.2	66.5±10.2	<0.001
Male sex	99 (65.6)	102 (45.7)	<0.001
BMI (kg/m^2)	28.8±3.6	27.9±4.5	0.03
Arterial hypertension	100 (66.2)	157 (70.4)	0.9
Smoking habit	52 (34.4)	33 (14.7)	<0.001
Diabetes duration (years)	21.6±7.4	17.8±8.4	0.003
Total cholesterol (mmol/l)	5.9±1.4	5.5±1.3	0.007
HDL cholesterol (mmol/l)	1.1±0.3	1.2±0.4	0.027
LDL cholesterol (mmol/l)	3.7±1.3	3.2±1.0	<0.001
Triglycerides (mmol/l)	2.4±1.4	2.5±1.7	0.6
TC genotype*	6 (4.0)	10 (4.5)	0.9

*IKBL+738T>C gene polymorphism

TABLE 2
THE IL1RN VNTR POLYMORPHISM IN PATIENTS WITH MI AND IN PATIENTS WITHOUT CAD

	MI group	Control group	p value	OR (95% CI) *
Allele 2	79 (0.26)	103 (0.23)	0.38**	
Allele X§	223 (0.74)	343 (0.77)		
Genotype 22	15 (9.9)	14 (6.3)	0.2§§	1.6 (0.8–3.5)
Genotype X2	49 (32.5)	75 (33.6)		
Genotype XX	87 (57.6)	134 (60.1)		

*Odds ratio (95% confidence interval), **p value for allele frequency (χ^2=0.337), §combined frequency for alleles 1, 3, 4, and 5, §§p-value and OR for recessive model (22 versus X2 plus XX).

TABLE 3
ALLELE DISTRIBUTION OF THE IL1RN VNTR POLYMORPHISM IN CASES AND CONTROLS

Allele	MI group	Control group	Number of repeats
Allele 1	216 (71.5)	332 (74.4)	4
Allele 2	79 (26.2)	103 (23.1)	2
Allele 3	7 (2.3)	9 (2)	5
Allele 4	0	2 (0.4)	3
Allele 5	0	0	6

we did not find an association between the IL1RN VNTR gene polymorphism and MI (OR=1.6; 95% CI=0.8–3.5; p=0.2) or between the TC genotype of the IKBL+738 polymorphism and MI (OR=0.9; 95% CI=0.3–2.3; p=0.9).

IL-1 is thought to play a central role in atherogenesis and thrombosis[22]. Several mechanisms have been suggested, promotion of leukocyte adherence to endothelium, stimulation of cytokine and adhesion molecule expression, procoagulant activity and induction of metalloproteinases that may lead to destabilization and rupture of the atherosclerotic plaque[23]. IL-1ra is a competitive inhibitor of IL-1 encoded by a polymorphic gene in the IL-1 family. The VNTR polymorphism of the IL1RN gene contains 5 alleles of between 2 and 6 repeats of an 86 bp sequence[21]. The four repeat sequence (allele 2, IL1RN*2) has been associated with single vessel CAD in a UK population[11]. However, subsequent studies reported lack of association between the IL1RN*2 allele and the risk of CAD in patients undergoing coronary angiography[24], as well as between IL1RN*2 and the risk of acute MI[25,26]. Conflicting results have also been reported in studies investigating the association of IL1RN*2 with protection from restenosis after coronary angioplasty[12,27,28]. Interestingly, Marculescu et al. have found an increased prevalence of CAD in IL1RN*2 carriers compared to noncarriers, but only among patients with type 2 diabetes[29]. In our study, however, we failed to demonstrate such an association in subjects with type 2 diabetes.

Transcription factor NF-κB plays a critical role in mediating immune and inflammatory responses and is considered as potentially one of the most important proinflammatory pathways in both atherosclerosis[30] and type 2 diabetes[31]. It regulates the expression of a large number of genes, including proinflammatory cytokines (*e.g.*, TNF-α and IL-1), growth factors, adhesion molecules,

and others[31]. In diabetes hyperglycemia causes the formation of advanced glycation end products due to increased nonenzymatic glycation of proteins[32]. Binding of advanced glycation end products to the receptors for advanced glycation end products (RAGE) results in intracellular oxidant stress and activation of NF-κB in endothelial cells and vascular smooth muscle cells[32]. Bierhaus et al. have provided evidence that ligands of RAGE can induce sustained activation of NF-κB in diabetic patients and therefore might contribute to the persistent NF-κB activation observed in hyperglycemia[33]. A thymine (T) to cytosine (C) substitution at position +738 in exon 4 of the IKBL gene causes an amino acid substitution of cysteine with arginine, which lies in a predicted protein kinase C phosphorylation site and possibly alters the function of the IKBL protein[34]. Despite the IKBL+738T>C polymorphism was associated with more severe form of some inflammatory diseases[16,17], we failed to demonstrate an association between this polymorphism and MI in subjects with type 2 diabetes.

We presume that the lack of an association between the IL1RN VNTR polymorphism and the IKBL+738T>C polymorphism and CAD in our study may be due to a multifactorial nature of the CAD. Besides, controversial results of association studies may be explained by differences in study design or by genetic heterogeneity within and between the populations from which the samples were derived. However, the distribution of IL1RN*2*2 genotype (6.0%) and of the TC genotype of the IKBL+738 polymorphism (4.5%) in our controls was similar to that reported in other studies[16,24,35].

Moreover, retrospective association studies are apt to survival bias. Both IL-1ra and IKBL polymorphisms may be related to increased early mortality after acute MI that could lead to underestimation of their true role in patients who survived MI as in our study. Namely, increased levels of IL-1ra are associated with greater mortality in patients with unstable angina pectoris[36] and acute MI[37]. Second, the exclusion of CAD on the basis of a negative history of MI or angina pectoris, and absence of ischemic changes on electrocardiogram and during exercise stress testing, has some disadvantages. It is not possible to rule out the possibility that a proportion of patients in the control group had asymptomatic CAD. In a recent study 18% of asymptomatic diabetic patients with a negative result of exercise stress testing presented silent CAD with significant (≥70%) angiographically documented coronary stenosis[38].

In conclusion, the IL1RN VNTR gene polymorphism and the IKBL+738T>C polymorphism are not risk factors for MI in Caucasians with type 2 diabetes.

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POLIMORFIZMI GENA ZA ANTAGONIST INTERLEUKIN-1 RECEPTORA TE GENA ZA INHIBITOR KAPPI B-SLIČNOG PROTEINA NISU POVEZANI SA INFARKTOM MIOKARDA U POPULACIJI SLOVENACA SA DIJABETESOM TIPA 2

S A Ž E T A K

U ovoj studiji istraživali smo povezanost polimorfizma promjenljivog broja uzastopnih ponavljanja gena antagonista interleukin-1 receptora *(engl. interleukin-1 receptor antagonist gene variable number tandem repeat – IL1RN VNTR*) te polimorfizma gena inhibitora kappa B-sličnog proteina *(engl. inhibitor of kappa B-like protein – IKBL)* sa miokardijlnim infarktom (MI) u grupi pacijenata sa dijabetesom tipa 2. Polimorfizimi gena IL1RN VNTR i IKBL+738T>C testirani su na 374 ispitanika: 151 slučaj sa MI i 223 ispitanika bez ikakve povijesti srčanih bolesti. Polimorfizam IL1RN VNTR nije bio factor rizika za MI kod ispitanika sa dijabetesom tipa 2 (genotip 22 vs. ostatak: (OR) 1.6; 95%; (CI) = 0,8-3,5; p=0,2).. Također nismo uspjeli pokazati da je polimorfizam gena IKBL+738T>C povezan sa MI u pacijenata sa dijabetesom tipa 2 (OR =0,9; 95% CI = 0,3-2,6; p=0,9). Iznjeli smo dokaze kako polimorfizmi gena IL1RN VNTR i IKBL+738T>C nisu faktori rizika za MI kod bolesnika sa dijabetesom tipa 2.