ORIGINAL ARTICLE

PECAM-1 GENE POLYMORPHISM (rs668) AND SUBCLINICAL MARKERS OF CAROTID ATHEROSCLEROSIS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT

The platelet endothelial cell adhesion molecule 1 (PECAM-1) plays an important role in many inflammatory processes, including the development of atherosclerosis. Polymorphism rs668 of the *PECAM-1* gene (373C/G) is functional, and it was reported to be associated with increased serum levels of PECAM-1. We investigated the association between the rs668 polymorphism of PECAM-1 and subclinical markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus (T2DM). Five hundred and ninety-five T2DM subjects and 200 control subjects were enrolled. The carotid intima-media thickness (CIMT) and plaque characteristics (presence and structure) were assessed ultrasonographically. Biochemical analyses were performed using standard biochemical methods. Geno-typing of the *PECAM-1* gene polymorphism (rs668) was performed using KASPar assays. The control examinations were performed 3.8 ± 0.5 years after the initial examination. Higher CIMT was found in patients with T2DM in comparison with subjects without T2DM. Statistically sig-nificantly faster progression of the atherosclerotic markers was shown in subjects with T2DM in comparison with the control group. When adjusted to other risk factors, the rs668 GG genotype was associated with an increased risk of carotid plaques in subjects with T2DM. We concluded that our study demonstrated a minor effect of the rs668 PECAM-1 on markers of carotid atherosclerosis in subjects with T2DM.

Keywords: Association study; carotid atherosclerosis; Platelet endothelial cell adhesion molecule 1 (PECAM-1); rs668 polymorphism; type 2 diabetes mellitus (T2DM).

INTRODUCTION

The leukocyte adhesion and their transendothelial migration play an important role in the initial phase of atherogenesis [1]. Processes are regulated by various types of adhesion molecules, such as platelet endothelial cell adhesion molecule 1 (PECAM-1), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). The plasma level of adhesion molecules is elevated in individuals with atherosclerosis [2-4].

Platelet endothelial cell adhesion molecule 1, also called CD31, is a 130 kD member of the immunoglobulin superfamily, consisting of six extracellular immunoglo-bulin-like domains, one transmembrane domain, and one cytoplasmic domain. Their expression takes place on the surface of circulating platelets, monocytes, neutrophils and selected T cells [5,6]. The PECAM-1 is a signaling molecule that plays diverse roles in vascular biology, including

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modulation of platelet function [7,8], angiogenesis [9], vasculogenesis [10], integrin regulation [11], T-cell and B-cell activation [12] and mediation of leukocyte migration across the endothelium [13].

The *PECAM-1* gene is located at the end of the long arm of the chromosome 17 (17q23). Previous studies have reported the existence of 11 different single nucleotide polymorphisms (SNPs) of the *PECAM-1* gene. Three of them have been described that encode amino acid substitutions in the PECAM-1 molecule. A mutation in the *PECAM-1* gene in exon 3 at position +373 involves a C>G substitution, causing a leucine to valine substitution at position 125 (rs668) [14].

The interaction or activation of the PECAM-1 take place *via* homophilic binding with its first extracellular Ig-like domains [15,16]. This polymorphism might affect the homophilic binding capability and influence individual susceptibility to the development of atherosclerosis. The association between the rs668 PECAM-1 polymorphism and cardiovascular disease was studied in Caucasians [17-19], Japanese [20] and Chinese [21], but no clear answer on the association between the rs668 polymorphism of PECAM-1 and the development of cardiovascular diseases could be provided.

Platelet endothelial cell adhesion molecule 1 is important in the detection of mechanoreception (mechanical shear force) and mechanotransduction (conversion into chemical signals) by the endothelium [22,23]. Atherosclerotic lesion development occurs at sites of the vessel where flow and shear stress conditions are disturbed [24]. Pulsatile or oscillatory shear stresses induce pro inflammatory gene expression [25]. Using the mouse model, the effect of PECAM-1 deficiency (double knock-out mice model without the presence of the *PECAM-1* gene) on the development of atherosclerosis. They reported reduced atherosclerotic lesions in double knock-out mice models [21,25]. The purpose of this study was to investigate an association between the rs668 (+373C/G) polymorphism of the *PECAM-1* gene and subclinical markers of carotid atherosclerosis in patients with type 2 diabetes mellitus (T2DM).

PATIENTS AND METHODS

This study included 595 consecutive subjects with T2DM, admitted to the diabetes outpatient clinics of the general hospitals at Murska Sobota and Slovenj Gradec, Slovenia, and from the outpatient department at the Medical Center Medicor, Ljubljana, Slovenia. The inclusion criteria for the control group was the absence of T2DM, and consisted of employees of the General Hospital Murska Sobota, Slovenia. Another inclusion criteria for the subjects with T2DM and for the subjects in the control group was the age from 40 to 70. The exclusion criteria for subjects with T2DM and for the subjects in the control group was a history of either myocardial infarction (MI) or ischemic stroke. The study protocol was approved by the Slovene Medical Ethics Committee (98/08/10). The patients and control subjects were enrolled and followed in the period from 2008 to 2014.

Patients were classified as having T2DM according to the current report of the American Diabetes Association [26]. After informed consent was obtained from the patients, a detailed interview was conducted concerning smoking habits, the duration and treatment of diabetes, arterial hypertension, and hyperlipidemia. Patients were asked whether they were smokers at the time of recruitment (current smoker). Subjects with T2DM with systolic blood pressure ≥140.0 mm Hg or diastolic blood pressure ≥85.0 mm Hg and/or subjects who were taking anti hypertensive drugs were considered to be hypertensive.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The carotid intima-media thickness (CIMT), defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured as previously described [27]. Plaques were defined as a focal intima-media thickening, and divided into five types according to their echogenic/ echolucent characteristics, as previously described [27]. The interobserver reliability for carotid plaque characterization was found to be substantial ($\kappa = 0.64$, $p < 0.001$).

After the patients with T2DM and subjects without T2DM (control group) were enrolled, they were prospectively followed-up for a few years. From the group with T2DM, 426 responded and participated in the control ultrasound examination of the neck artery, whereas, 132 re-sponded from the group of subjects without T2DM; 3.8 ± 0.5 years passed between the first and the control ultrasound examination.

Biochemical Analyses. Fasting blood samples for biochemical analysis were collected twice patients: upon enrollment and upon follow-up after a few years. Analyses were made at the hospital ac-

credited laboratory. The following parameters were determined: total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), blood sugar and high-sensitive C-reactive protein (hsCRP).

Genotyping. Genomic DNA was extracted from 100 µL of whole blood using a *FlexiGene* DNA isolation kit (Qiagen GmbH, Hilden, Germany), in accordance with the recommended protocol. The rs668 polymorphism of the *PECAM-1* gene was determined with the KASPar assay (LGC Genomics Ltd., Hoddesdon, Hertfordshire, UK) system.

Statistical Analyses. Continuous variables that were normally distributed, were reported in the form of mean ± standard deviation (SD). Variables that were not normally distributed, were presented in the form of median (inter-quartile range). The normality of distribution of continuous variables was examined using the Kolmogorov-Smirnov test. We used the Student's *t* test or the analysis of variance (ANOVA) to compare the numerical values of the continuous variables, to identify the distribution of variables. If the variables were asymmetrically distributed, we used the Mann-Whitney U test or the Kruskal-Wallis H test. The χ^2 test was used to compare the frequency's categorical variables, statistical evaluation of differences in the frequencies of different alleles and genotypes between the two groups, as well as in the case of determining the Hardy-Weinberg equilibrium.

The Pearson analyses was performed to examine the correlation between the independent variables. The results showed a high degree of correlation between the serum levels of total and LDL cholesterol ($r = 0.86$; $p <0.001$), as well as systolic and diastolic blood pressure ($r = 0.65$; $p <0.001$). In the case of a high degree correlation between two variables, only one variable from each pair was included in the multivariate statistical models.

The change in the value of ultrasound markers of carotid artery atherosclerosis was calculated by deducting the values measured at two ultrasound examinations. The criteria for a statistically significant difference was a *p* value of less than 0.05. To reduce the possibility of error due to the small number of subjects, we used the Bonferroni correction. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) computer program for Windows, version 20 (SPSS Inc., Chicago, IL, USA).

RESULTS

The clinical characteristics of subjects with T2DM and control subjects are shown in Table 1. Patients with T2DM had a greater waist circumference and there

Table 1. Initial clinical and biochemical characteristics of patients with type 2 diabetes mellitus and the control group.

Parameters	Patients with T2DM ($n = 529$)	Control Group ($n = 200$)	*p* Value
Age (mean ± SD)	61.38 ± 9.65	60.07 ± 9.18	0.07
Sex: M (%); F (%)	M: 338 (56.8); F: 191 (43.2)	M: 92 (46.0); F: 108 (54.0)	**0.008**
Duration of T2DM	11.5 ± 7.88	–	–
Smokers: M (%); F (%)	M: 30 (8.9); F: 23 (12.0)	M: 19 (20.6); F: 15 (13.9)	**0.002**
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	**<0.001**
BMI (kg/m²)	30.96 ± 4.74	27.90 ± 4.42	0.16
Systolic pressure (mm Hg)	146.98 ± 19.98	143.30 ± 16.60	0.86
Diastolic pressure (mm Hg)	85.75 ± 11.62	84.70 ± 11.60	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	**<0.001**
Hb A$_{1c}$ (%)	7.89 ± 3.56	4.79 ± 0.29	**<0.001**
Total cholesterol (nmol/L)	4.70 ± 1.19	5.36 ± 1.08	**<0.001**
HDL cholesterol (mmol/L)	1.19 ± 0.35	1.43 ± 0.37	**<0.001**
LDL cholesterol (mmol/L)	2.63 ± 0.94	3.24 ± 0.98	**<0.001**
Triglycerides (mmol/L)	1.90 (1.20-2.70)	1.30 (0.90-1.90)	**<0.001**
High sensitivity CRP (mg/L)	2.20 (1.00-4.30)	1.30 (0.80-2.70)	**<0.001**
CIMT (µm)	1013.00 ± 208.00	979.00 ± 141.00	**0.030**

T2DM: type 2 diabetes mellitus; BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; CRP: C-reactive protein; CIMT: carotid intima-media thickness.

Table 2. Changes in ultrasound markers of atherosclerosis at carotid artery in patients with type 2 diabetes mellitus and the control group between the initial and control ultrasound examinations.

Parameters	Patients with T2DM (n = 426)	Control Group (n = 137)	0.50
Annual CIMT increment (µm/year)	20.33 (11.74-29.86)	12.83 (8.82-20.66)	**0.00**
Δ number of plaque segments	2.00 (1.00-3.00)	1.50 (0.70-2.20)	**0.00**
Δ sum of the plaque thickness (mm)	5.40 (2.40-7.05)	3.64 (2.88-5.48)	**0.02**

CIMT: carotid intima-media thickness; Δ: variable value changes during the observation period, expressed as a percentage of baseline values.

Table 3. Comparison of ultrasound markers of the carotid artery atherosclerosis in patients with type 2 diabetes mellitus according to the *PECAM* gene polymorphism 373 C/G (rs668) genotypes at the beginning of the study.

Parameters	CC Genotype	CG Genotype	GG Genotype	p value
CIMT (µm)	1041.00 ± 198.00	990.00 ± 187.00	988.00 ± 211.00	0.19
Number of segments with plaques	2.56 ± 1.65	2.39 ± 1.72	2.51 ± 1.54	0.36
Sum of the plaque thickness (mm)	8.14 ± 4.77	7.99 ± 5.23	7.66 ± 4.02	0.16
Presence of plaques: [+] [−]	149.00 (85.60) 25.00 (14.40)	256.00 (84.50) 47.00 (15.50)	94.00 (79.70) 24.00 (20.30)	0.36
Presence of unstable plaques: [+] [−]	86.00 (57.70) 63.00 (42.30)	144.00 (56.30) 112.00 (43.70)	58.00 (61.70) 36.00 (38.30)	0.66

CIMT: carotid intima-media thickness.

Table 4. Changes in ultrasound markers of carotid artery atherosclerosis in patients with type 2 diabetes mellitus between the first and the second ultrasound examination of the carotid arteries according to the *PECAM* gene polymorphism 373 C/G (rs668) genotypes.

Parameters	CC Genotype	CG Genotype	GG Genotype	p Value
Annual CIMT increment (µm/year)	20.34 (11.64-28.04)	20.69 (7.14-32.28)	14.28 (10.71-35.97)	0.71
Δ number of plaque segments	2.00 (0.50-2.50)	2.00 (1.00-3.00)	2.00 (2.00-3.00)	0.74
Δ sum of the plaque thickness (mm)	7.30 (3.85-8.70)	5.45 (2.30-9.20)	4.40 (1.40-8.42)	0.38

CIMT: carotid intima-media thickness; Δ: variable value changes during the observation period, expressed as a percentage of baseline values.

were more smokers compared to the control group. There were no statistically significant differences between patients with T2DM and controls in other clinical characteristics [age, body mass index (BMI), systolic and diastolic pressure]. A biochemical examination of patients with T2DM showed statistically significant higher levels of fasting glucose, Hb A$_{1c}$, total cholesterol, HDL, LDL, triglyceride and hsCRP compared with the control group (Table 1). Moreover, higher CIMT was found in patients with T2DM in comparison with subjects without T2DM (Table 1).

The ultrasound examination of the carotid artery was performed at the time of enrollment in the study, and 3.8 ± 0.5 years after the initial examination. Changes in the progression of atherosclerotic markers (change in annual CIMT increase, change in the number of plaque segments and change in the sum of the plaque thickness) between subjects with T2DM and the control group are shown in Table 2. Statistically significantly faster progression of the atherosclerotic markers was shown in subjects with T2DM in comparison with the control group (Table 2).

The distribution of genotypes in the population of patients with T2DM was in Hardy-Weinberg equilibrium (T2DM: χ^2 = 0.45; p = 0.50; control group: χ^2 = 1.46; p = 0.23). Table 3 outlines differences in the ultrasound markers of carotid artery atherosclerosis (initial ultrasound examination) in patients with T2DM with regard to the rs668 genotypes. The differences were not statistically significant with regard to rs668 genotypes (Table 3). We did not demonstrate statistically significant differences in the markers of subclinical carotid atherosclerosis between different genotypes in subjects with T2DM (Table 4).

Table 5 shows the relation between the rs668 and the incidence of either plaques or unstable plaques

Table 5. Relationship of the *PECAM* gene polymorphism 373 C/G (rs668) genotypes to the presence of plaques/ unstable plaques of the carotid arteries in patients with type 2 diabetes mellitus at the beginning of the study.

Parameters	Presence of Plaques		Presence of Unstable Plaques	
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value
Hypertension (0 = no; 1 = yes)	1.88	0.35	1.38	0.52
Systolic pressure (mm Hg)	0.24	0.68	0.26	0.32
Serum LDL (mmol/L)	1.41	0.41	1.34	0.25
Serum HDL (mmol/L)	0.11	**0.02**	0.31	0.34
Hb A$_{1c}$ (%)	0.89	**0.03**	1.23	0.43
CG allele	1.03	0.49	0.72	0.17
GG allele	1.18	**0.03**	0.68	0.51

OR (95% CI): odds ratio (95% confidence interval); LDL: low-density lipoprotein; HDL: high-density lipoprotien; all models were adjusted by age, sex, smoking habits and treatment with statins. The reference group are homozygotes for the C allele (the CC genotype).

in subjects with T2DM. When adjusted to other risk factors, the rs668 GG genotype was associated with an increased risk of carotid plaques in subjects with T2DM (Table 5).

DISCUSSION

In the present study, we demonstrated an association between the rs668 GG genotype and carotid artery plaque incidence in patients with T2DM when adjusted to other risk factors. On the other hand, we did not find any effect of the rs688 genotype on the progression of atherosclerosis in patients with T2DM.

Our study is the first demonstrating an association between the rs668 PECAM-1 and the presence of carotid plaques in patients with T2DM. In our study, we observed a greater number of plaques in subjects with the GG rs668 genotype. The importance of the *PECAM-1* gene in the pathogenesis of atherosclerosis was demonstrated [21,25]. The decrease in areas with atherosclerotic lesion in PECAM-1 double knock-out mice was reported [21,25].

An association between the rs668 PECAM-1 and car-diovascular disorders was reported several times, but not in all studies [29-33]. Similarly, an association between the rs668 *PECAM-1* gene and either the increased levels of soluble PECAM-1 or ischemic stroke was found in the Chinese population [21]. An association between the rs668 *PECAM-1* gene and increased levels of soluble PECAM-1 was confirmed in the setting of patients with acute MI [28].

Similarly, Fang *et al.* [29] demonstrated an association between the rs668 *PECAM-1* gene and either

increased levels of soluble PECAM-1 or the severity of coronary artery disease in the Asian Indian population in Singapore. Reschner *et al.* [17] reported an association between the PECAM-1 rs668 (the CC genotype) and MI in Caucasians with T2DM. Finally, the effect of several polymorphisms of the *PECAM-1* gene on cardiovascular disease was confirmed by Listi *et al.* [30] in the Northern Italian population. These findings were not confirmed in the general population from Germany, as rs688 was not found to be an independent risk factor for coronary artery disease (CAD) [31].

In a recently published meta-analysis of 15 studies, including 7636 subjects, no association between the rs668 PECAM-1 and cardiovascular diseases was demonstrated [35]. Moreover, the progression of subclinical markers of carotid atherosclerosis was statistically significantly faster in subjects with T2DM in comparison with subjects without T2DM. This finding is in accordance with expectations of other researchers as well [32,33].

Our study has some limitations due to its actual design (cross-sectional design at the enrollment of subjects with T2DM and control subjects) and relatively small sample size (*i.e.,* less than 1000 subjects with T2DM), however the study was appropriately powered to detect the differences in subclinical markers of carotid atherosclerosis upon enrollment as well as during follow-up. The lack of the effect of rs688 on the progression of atherosclerosis might be due to a rather short interval between the first and control examinations (3.8 ± 0.5 years). Moreover, it is impossible to exclude the impact of interactions with other

potentially relevant variables on the development of atherosclerosis. The second limitation of the study is the lack of an intermediate phenotype, *i.e.,* serum PECAM levels. We presume that the effect of rs668 is *via* increased serum PECAM levels.

In conclusion, our study demonstrated a minor effect of the rs668 PECAM-1 on the markers of carotid atherosclerosis in subjects with T2DM, as the GG genotype of the rs668 PECAM-1 was associated with a higher incidence of carotid plaques in subjects with T2DM. With that kind of associations established in genetic studies, we presumed that we might predict the genetic risk of carotid atherosclerosis in subjects with T2DM.

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