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Original Article

Arachidonate 5-lipoxygenase (ALOX5) gene polymorphism (rs12762303) and arachidonate 5-lipoxygenase activating protein (ALOX5AP) gene polymorphism (rs3802278) and markers of carotid atherosclerosis in patients with type 2 diabetes mellitus

Sebastjan Merlo[1], Marija Šantl Letonja[2], Andreja Cokan Vujkovac[3], Delian Delev[4], Ioana Mozos[5], Peter Kruzliak[6,7], Danijel Petrovič[8]

[1]Institute of Oncology Ljubljana, Ljubljana, Slovenia; [2]General Hospital Rakičan, Murska Sobota, Slovenia; [3]General Hospital Slovenj Gradec, Slovenj Gradec, Slovenia; [4]Department Pharmacology and Clinical Pharmacology, Medical Faculty, Medical University of Plovdiv, Plovdiv, Bulgaria; [5]Department of Functional Sciences, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania; [6]2nd Department of Internal Medicine, Faculty of Medicine, Masaryk University, Brno, Czech Republic; [7]Laboratory of Structural Biology and Proteomics, Central Laboratories, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic; [8]Institute of Histology and Embryology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

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Abstract: Background: The study was designed to investigate the association between polymorphisms of the arachidonate 5-lipoxygenase (ALOX5) gene (rs12762303) and the arachidonate 5-lipoxygenase-activating proteingene (rs3802278), and markers of carotid atherosclerosis, such as carotid intima media thickness, the number of affected segments of carotid arteries and the sum of plaque thickness in patients with T2DM. Patients and methods: 595 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. Genotyping of the ALOX5 gene (rs12762303) and the ALOX5AP gene (rs3802278) was performed using KASPar assays. Results: In our study, we demonstrated an association between the rs3802278 and CIMT, and between the rs12762303 and coronary calcium score in subjects with T2DM. In our study, we did not demonstrate any association between tested polymorphisms (rs12762303 and rs3802278) and the sum of plaque thickness, the number of involved segments, hsCRP, the presence of carotid plaques or the presence of unstable carotid plaques. Conclusions: To conclude, in our study we demonstrated an association between the rs3802278 and CIMT, and between the rs12762303 and coronary calcium score in subjects with T2DM.

Keywords: Arachidonate 5-lipoxygenase, arachidonate 5-lipoxygenase activating protein, genetic polymorphism, association study, carotid atherosclerosis, type 2 diabetes mellitus

Introduction

Atherosclerosisis a chronic inflammatory disease [1]. Type 2 diabetes mellitus (T2DM) is considered a major epidemic of this century. T2DM is associated with an accelerated progression of atherosclerosis [2]. In patients with diabetes, cardiovascular complications are reported about 15 years earlier than in the population without T2DM [2-4].

Chronic, low-grade inflammation has been demonstrated to be involved in the pathogenesis of atherosclerosis in subjects at high risk to develop cardiovascular disease [5-8]. Moreover, genetic factors have long been known to modulate the risk of atherosclerosis and CVD, and they merit a search for the genes involved in the susceptibility to the atherosclerotic complications of T2DM [3, 9, 10]. Inflammation is involved in the pathogenesis of atherosclerosis,

Table 1. Baseline characteristics of subjects with T2DM and subjects without T2DM (control group)

	Subjects with T2DM N = 595	Control group N = 200	P
Age (years)	61.38 ± 9.65	60.07 ± 9.18	0.07
Male sex (%)	338 (56.8)	92 (46.0)	0.008
Diabetes duration (years)	11.25 ± 7.88	-	-
Cigarette smoking (%)	53 (8.91)	34 (17.0)	0.002
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	< 0.001
BMI (kg/m^2)	30.96 ± 4.74	27.90 ± 4.42	0.16
SBP (mmHg)	146.98 ± 19.98	143.3 ± 16.6	0.86
DBP (mmHg)	85.75 ± 11.62	84.7 ± 11.6	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	< 0.001
HbA1c (%)	7.89 ± 3.56	4.79 ± 0.29	< 0.001
Total cholesterol (mmol/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)	1.9 (1.2-2.7)	1.3 (0.9-1.9)	< 0.001
hs-CRP (mg/L)	2.2 (1.0-4.3)	1.3 (0.8-2.7)	< 0.001

Continuous variables were expressed as means ± standard deviations when normally distributed and as median (interquartile range) when asymmetrically distributed. Categorical variables were expressed as frequency (percentage). BMI-body mass index; SBP-systolic blood pressure; DBP-diastolic blood pressure; HbA1c-glycated haemoglobin; hs-CRP-high sensitivity C-reactive protein.

and the inflammatory process is triggered partially through the lipoxygenase pathway [1, 6, 11].

Leukotrienes (LTs) have been implicated as mediators, and potential therapeutic targets in the development of atherosclerosis. LTs are arachidonic acid-derived lipid mediators of inflammation. The initial step in the formation of LTs is catalyzed by 5-lipoxygenase (5-LO) in collaboration with the lipoxygenase-activating protein, subsequently leading to the formation of the LT family [11].

The mouse 5-LO gene, ALOX5, has been shown to contribute to the development of atherosclerosis [12]. In 2004, an association between *ALOX5* promoter polymorphism and an increased carotid intima media thickness was reported [13]. Dwyer and co-workers reported an increase in CIMT in subjects with two copies of the non-wild-type alleles of a tandem SP1 binding motif polymorphism in the *ALOX5* promoter compared with subjects who had two copies of the wild allele at this site [13]. The 5-lipoxygenase-activating protein, encoded by the ALOX5AP gene, likely acts as an arachidonic acid-binding and transfer protein to facilitate 5LO activity [14]. In several studies polymorphisms and haplotypes of ALOX5AP were reported to be associated with either myocardial infarction or ischemic stroke [15-19].

The present study was thus designed to investigate the association between polymorphisms of the ALOX5 gene (rs12762303) and the ALOX5AP gene (rs3802278), and markers of carotid atherosclerosis, such as carotid intima media thickness (CIMT), the number of affected segments of carotid arteries and the sum of plaque thickness in patients with T2DM.

Material and methods

This cross-sectional study included 595 subjects with T2DM and 200 subjects without T2DM (control group), as described above [20]. The study protocol was approved by the Slovene Medical Ethics Committee.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The 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 described previously [20]. Plaques were defined as a focal intima-media thickening, and divided into 5 types according to their echogenic/echolucent characteristics, as described previously [20]. The inter-observer reliability for carotid plaque characterization was found to be substantial ($\kappa = 0.64$, $P < 0.001$).

In 215 out of 595 subjects with T2DM, coronary computed tomography angiography (CCTA) was performed for diagnostic purposes.

Blood samples for biochemical analyses were collected, as described previously [21]. The genomic DNA was extracted from 100 μL of whole blood using a Flexi Gene DNA isolation kit, in accordance with the recommended protocol (Qiagene GmbH, Hilden, Germany). Ge-

Table 2. Comparison of markers of carotid atherosclerosis in subjects with T2DM at the beginning of the study with regard to the ALOX5 gene (rs12762303) and the ALOX5AP gene (rs3802278) genotypes

	rs12762303			
	TT	TC	CC	P
Intima media thickness (μm)	1002 ± 190	1008 ± 196	917 ± 210	0.50
Number of involved segments	2.53 ± 1.59	2.48 ± 1.68	2.20 ± 1.80	0.89
Sum of plaque thickness (mm)	7.74 ± 4.40	8.29 ± 4.73	6.73 ± 4.60	0.556
hsCRP (mg/L)	3.26 ± 3.54	4.02 ± 3.59	3.00 ± 3.89	0.16
Presence of carotid plaques n (%)	339 (85.0)	152 (82.6)	9 (75)	0.89
Presence of unstable carotid plaques n (%)	183 (45.9)	95 (51.6)	7 (58.3)	0.63
Coronary calcium score	206 ± 282	429 ± 416	560 ± 215	0.005
	rs3802278			
	TT	TC	CC	p
Intima media thickness (μm)	982 ± 201	1023 ± 186	1037 ± 144	0.03
Number of involved segments	2.38 ± 1.51	2.63 ± 1.52	2.75 ± 1.54	0.24
Sum of plaque thickness (mm)	7.70 ± 4.40	8.23 ± 4.49	7.41 ± 4.68	0.53
hsCRP (mg/L)	3.38 ± 3.33	3.71 ± 3.84	3.18 ± 2.83	0.64
Presence of carotid plaques n (%)	261 (82.9)	189 (83.6)	50 (92.6)	0.28
Presence of unstable carotid plaques n (%)	145 (46.0)	120 (53.1)	20 (37.0)	0.06
Coronary calcium score*	269 ± 342	290 ± 347	162 ± 282	0.5

*Coronary computed tomography angiography (CCTA) was performed for diagnostic purposes in 215 out of 595 subjects with T2DM.

Table 3. Baseline characteristics of subjects with T2DM with unstable plaques and subjects with T2DM with stable plaques

	Subjects with T2DM with unstable plaques N = 190	Subjects with T2DM with stable plaques N = 140	P
Age (years)	63.42 ± 9.41	64.94 ± 8.83	0.44
Waist circumference (cm)	107.82 ± 13.24	110.86 ± 12.15	0.62
BMI (kg/m^2)	29.92 ± 4.85	28.90 ± 4.72	0.56
SBP (mmHg)	145.7 ± 17.7	144.3 ± 16.9	0.92
DBP (mmHg)	85.6 ± 11.5	84.9 ± 11.6	0.19
Fasting glucose (mmol/L)	7.98 ± 2.47	8.16 ± 2.65	0.58
HbA1c (%)	8.38 ± 2.56	7.63 ± 0.29	0.20
Total cholesterol (mmol/L)	4.82 ± 1.12	4.68 ± 1.20	0.27
HDL cholesterol (mmol/L)	1.15 ± 0.30	1.21 ± 0.36	0.10
LDL cholesterol (mmol/L)	2.66 ± 0.89	2.58 ± 0.99	0.46
Triglycerides (mmol/L)	2.43 (1.4-3.3)	2.34 (1.3-2.9)	0.65
hs-CRP (mg/L)	4.1 (0.8-6.3)	3.4 (1.1-5.7)	0.44
Coronary calcium score	294 ± 371	290 ± 305	0.90

Continuous variables were expressed as means ± standard deviations when normally distributed and as median (interquartile range) when asymmetrically distributed. Categorical variables were expressed as frequency (percentage). BMI-body mass index; SBP-systolic blood pressure; DBP-diastolic blood pressure; HbA1c-glycated haemoglobin; hs-CRP-high sensitivity C-reactive protein.

notyping of ALOX5 gene (rs12762303) and the ALOX5AP gene (rs3802278) was performed using KASPar assays. Details of the method used can be found on http://www.kbioscience.co.uk/.

Continuous variables were expressed as means ± standard deviations, when normally distributed, and as median (interquartile range) when asymmetrically distributed. Normality of the continuous variables was examined by the Kolmogorov-Smirnov test. Continuous clinical data were compared using an unpaired Student's *t* test or analysis of variance (ANOVA) when normally distributed and the Mann-Whitney U-test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson X^2 test was used to compare discrete variables and to test whether the genotypes distribution is in Hardy-Weinberg equilib-

rium. A two-tailed *p*-value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 21 (SPSS Inc., Chicago, IL).

Results

Patients with T2DM had a greater waist circumference, higher fasting glucose and HbA1c levels compared to controls, whereas there were no statistically significant differences in age, gender distribution, BMI, systolic and diastolic blood pressure between patients with T2DM and control subjects (**Table 1**). Patients with T2DM had lower total, HDL and LDL cholesterol levels, and a higher triglyceride level compared to controls (**Table 1**). Plasma levels of inflammatory markers (i.e. hs-CRP and fibrinogen) were statistically significantly higher in patients with T2DM compared to controls (**Table 1**).

The genotype distributions in both patients with T2DM and controls were in Hardy-Weinberg equilibrium for the ALOX5 gene (rs12762303)-T2DM (genotype frequencies: TT genotype 67.1%, TC genotype 30.9%, CC genotype 2.0%; $\chi^2 = 3.08$; P = 0.08) and controls (genotype frequencies: TT genotype 69.0%, TC genotype 30.0%, CC genotype 1.0%; $\chi^2 = 2.69$; P = 0.1). The genotype distributions in both patients with T2DM and controls were in Hardy-Weinberg equilibrium for the ALOX5AP gene (rs3802278) polymorphism-T2DM (genotype frequencies: TT genotype 52.9%, TC genotype 38.0%, CC genotype 9.1%; $\chi^2 = 2.09$; P = 0.15) and controls (genotype frequencies: TT genotype 53.0%, TC genotype 39.0%, CC genotype 8%; $\chi^2 = 0.09$; P = 0.75).

The comparison of atherosclerosis parameters was performed with regard to different genotypes of both polymorphisms (rs12762303 and rs3802278) upon enrolment. In our study, we demonstrated an association between the rs3802278 and CIMT, and between the rs-12762303 and coronary calcium score in subjects with T2DM (**Table 2**). In our study, we did not demonstrate any association between tested polymorphisms (rs12762303 and rs-3802278) and the sum of plaque thickness, the number of involved segments, hsCRP, the presence of carotid plaques or the presence of unstable carotid plaques (**Table 2**).

The comparison of subjects with T2DM with unstable plaques and subjects with T2DM with stable plaques did not demonstrate any differences in lipid parameters, waist circumference, blood pressure, inflammatory parameters (hsCRP), or coronary calcium score (**Table 3**).

Discussion

In our study, we demonstrated an association between the rs3802278 of the ALOX5AP gene and CIMT, whereas the rs12762303 of the ALOX5 gene was not associated with CIMT in subjects with T2DM. Our findings are in accordance with some previous reports demonstrating that the variability in the *ALOX5* gene might be associated with CIMT [13, 22]. Burdon and co-workers, on the other hand, failed to demonstrate an association between another ALOX5 gene polymorphism (rs3780906) and CIMT in the Diabetes Heart Study [23]. Similarly, Assimes and co-workers demonstrated no association between the ALOX5 gene polymorphism (rs12762303) and CIMT either [24].

In our study, we did not demonstrate any association between tested polymorphisms (rs12-762303 and rs3802278) and the sum of plaque thickness, or the number of involved segments, or hsCRP, or the presence of carotid plaques, or the presence of unstable carotid plaques. Our findings indicate differential effects of the ALOX5/ALOX5AP genes on the markers of carotid atherosclerosis (i.e. CIMT) and on the markers of inflammation (i.e. hsCRP). Moreover, our findings are in accordance with a recently published report of van der Laan and co-workers who found no association between ALOX5/ALOX5AP polymorphisms and carotid plaque phenotypes [25]. Moreover, they found no association between ALOX5/ALOX5AP polymorphisms and either serum ALOX5 or ALOX5AP levels [24]. Additionally, Zhang and co-workers, who enrolled a total of 501 ischemic stroke patients and 497 healthy controls in their recent study, failed to demonstrate a statistically significant association between ALOX5AP rs4073259 and ischemic stroke in the Chinese Han population [19].

An interesting study demonstrating the importance of lipoxygenase was reported by Zhou and co-workers [26]. They performed immunohistological analysis of atherosclerotic plaques

with/without T2DM from 60 patients undergoing carotid endarterectomy [26]. They demonstrated increased 5-LO expression in diabetic plaques compared to non-diabetic plaques, and increased 5-LO expression was associated with increased MMP-2 and MMP-9 expression. They speculated that the over expression of 5-LO and LTB [6] in atherosclerotic plaques might promote an MMP-induced plaque rupture in diabetes [26]. Moreover, the over expression of 5-LO was reported in atherosclerotic symptomatic plaques in comparison with asymptomatic plaques [21].

In our study, we demonstrated an association between the rs12762303 of the ALOX5 gene and coronary calcium score in subjects with T2DM, whereas the rs3802278 of the ALOX5AP gene was not associated with coronary calcium score in subjects with T2DM. Similarly, Burdon and co-workers demonstrated an association between either the ALOX5 gene polymorphism (rs2115819) or the ALOXAP gene polymorphism (rs9506352) and coronary calcium score in the Diabetes Heart Study [23]. In a few other studies polymorphisms and haplotypes of ALOX5 and ALOX5AP were reported to be associated with myocardial infarction [15-18, 24, 27]. Several reports demonstrating no/minimal effect on subclinical carotid atherosclerosis and several reports on the association with MI might be additional evidence that markers of carotid atherosclerosis and atherothrombotic events (i.e. MI) are most probably not regulated via similar genetical/biological mechanisms [15-19, 22-25, 27].

Conclusion

To conclude, in our study we demonstrated an association between the rs3802278 and CIMT, and between the rs12762303 and coronary calcium score in subjects with T2DM. Our findings suggest that tested polymorphisms in the ALOX5/ALOX5AP genes play a minor role (if any) in the development of subclinical atherosclerosis in subjects with T2DM.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Peter Kruzliak, Department of Internal Medicine, Faculty of Medicine, Masaryk University, Pekarska 53, 656 91 Brno, Czech Republic, E-mail: kruzliakpeter@gmail.com; Dr. Daniel Petrovic, Institute of Histology and Embryology, Faculty of Medicine, University of Ljubljana, Korytkova 2, 1000 Ljubljana, Slovenia. E-mail: daniel.petrovic@mf.uni-lj.si

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