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Association of the –262C/T polymorphism in the catalase gene promoter with carotid atherosclerosis in Slovenian patients with type 2 diabetes

Research Article

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Abstract: Genetic variations of the antioxidant enzymes may influence the susceptibility to oxidative stress and consequently the development and progression of diabetic complications. The aim of the current study was to test the association between the –262C/T polymorphism in the catalase gene promoter and carotid atherosclerosis in Slovenian patients with type 2 diabetes. Two-hundred and eighty six diabetics and 150 healthy controls were enrolled in the study. Carotid atherosclerosis was quantified ultrasonographiocally by carotid intima-media thickness (CITM), plaque score and plaque type. Genotypes were determined using the real-time PCR. Fibrinogen concentration showed a borderline statistically significant difference due to catalase genotypes (p=0,05). No difference in clinical characteristics, CIMT, plaque stability or plaque score was observed. Logistic regression model adjusted for age, gender, smoking, BMI, lipid parameters and duration of hypertension and diabetes showed significant association of T allele and lower risk for higher plaque score (OR=0,25; p=0,025). No association with CIMT>1mm and unstable plaques was observed. T allele of -262C/T is associated with lower risk for higher plaque score but it did not affect clinical parameters, CIMT and plaque stability. Whether this polymorphism can be used as a genetic marker for advanced carotid atherosclerosis in diabetic patients needs to be evaluated in the future.

Keywords: *Diabetes mellitus type 2 • Oxidative stress • Catalase polymorphism • Carotid atherosclerosis • Carotid intima-media thickness*

1. Introduction

Diabetes mellitus is one of the most common endocrine disorders affecting almost 6% of the world's population and its prevalence continues to increase. The majority of patients with diabetes suffer from increased morbidity and mortality from atherosclerotic vascular disease manifesting as coronary heart disease (CHD), cerebrovascular disease (CVD) and peripheral vascular disease (PVD).

It has been suggested that oxidative stress, defined as an imbalance between the production of free radicals and reactive metabolites and their elimination by protective mechanisms, plays a key role in the development and progression of both microvascular and macrovascular diabetic complications [1,2]. Antioxidant enzymes reduce oxidative stress through the inactivation of highly toxic free oxygen radicals and peroxides, and therefore play an important protective role in the pathogenesis of diabetic complications. Genetic variations within antioxidant enzymes which

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influence the gene expression could be responsible for quantitative changes in the enzymatic activity and therefore contribute to the susceptibility to oxidative stress and consecutively to the development of diabetic complications [3].

Catalase (EC 1.11.1.6) is a heme enzyme present in most eukaryotic organisms. It has a major role in controlling H_2O_2 concentrations in human cells, by converting H_2O_2 into H_2O and O_2 thereby directly reducing the production of HO and lipid hydroperoxides [4]. Catalase (CAT) enzymatic activity was induced by exposure to H_2O_2 in hamster tracheal epithelial cells [5] and in human retinal pigment epithelial cells [6], indicating a key role for catalase in antioxidant defence and its inducibility in response to oxidative stress.

A common polymorphism in the promoter region of the catalase gene consists of a C → T substitution at position -262 in the 5' region [7]. This polymorphism influences transcription factor binding, reporter gene transcription and was described as being functionally associated with blood catalase levels, but results are contradictory. Some studies proved a higher erythrocyte catalase level in carriers of the T allele compared to CC homozygotes [7], while others reported completely opposite results [8,9] or no association between catalase genotypes and enzymatic activity [10].

The reduction in catalase activity may play a role in the host response to oxidative stress and development of oxidative stress related diseases and also macrovascular diabetic complications. The variant CAT T allele has been associated with an increased risk of hypertension [11,12], vitiligo [13] and a higher risk of developing breast cancer [9]. Studies on diabetic population yielded very contrasting results. Some studies have shown a relationship between the -262C/T polymorphism and the development of type 1 diabetes [14] and its complications [3,15], but others failed to do so with type 2 diabetes [16] and diabetic retinopathy, nephropathy or ischemic cardiac disease [17]. Hungarian hypocatalasemic patients were found to have an increased prevalence of diabetes [18], higher plasma levels of homocysteine and lower levels of folate, suggesting that these patients are at greater risk for cardiovascular disease [19].

Due to our knowledge, based on the PubMed database, there was no study of association between the catalase polymorphism and carotid atherosclerosis in patients with type 2 diabetes mellitus. Therefore, the aim of the current study was to investigate whether the -262C/T polymorphism in the catalase gene promoter is associated with carotid atherosclerosis (quantified with carotid intima-media thickness, plaque score and plaque type assessed with carotid ultrasound) in Slovenian patients with type 2 diabetes.

2. Material and Methods

2.1. Patients

In this cross sectional study 286 subjects with type 2 diabetes from the diabetic ambulance of the General hospital Murska Sobota, Slovenia were enrolled. We also enrolled 150 healthy subjects to compare their CIMT with diabetics. All the subjects enrolled in the study were Slovenian and were not related. Inclusion criteria were age >40 years and diagnosis of diabetes mellitus type 2. Exclusion criterion was evident cardiovascular disease (CAD) such as myocardial infarction and cerebral stroke. The research protocol was approved by the National Medical Ethics Committee. Patients were classified as having type 2 diabetes according to the current report of the WHO Classification of Diabetes Mellitus [20]. After the informed consent was obtained from the patients, a detailed interview was made concerning smoking habits, the duration and treatment of diabetes, arterial hypertension, hyperlipidemia and consuming other drugs. Patients were asked whether they were smokers at the time of recruitment ("current smoker"). The body mass index (BMI) was calculated as weight in kilograms, divided by the height in square meters. We measured systolic blood pressure (SBP) and diastolic blood pressure (DBP) in the right upper arm of the patients while they were sitting. Subjects with systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mm Hg and/or subjects who were using antihypertensive drugs were considered to be hypertensive. In the diabetic patients group 119 patients were on insulin therapy, 163 used oral antidiabetic drugs and 4 patients were on diet. Due to indications diabetic patients used antihypertensive drugs (diuretics, calcium channel blockers, ACE inhibitors, angiotensin receptor antagonists, beta blockers), lipid-lowering drugs (statins and fibrates) but also aspirin (11 patients), clopidogrel (2 patients), proton pump inhibitors (2 patients), antiarrhythmic drugs (1 patient) and antihistaminic drugs (1 patient).

2.2. Ultrasound image analysis

Atherosclerotic changes on the carotid arteries were assessed by ultrasonography. High resolution B mode, colour Doppler, and pulse Doppler ultrasonography of both carotid arteries were performed with the commercially available ultrasound system (Toshiba Aplio) with a multi-frequency linear array transducer. All examinations were performed by a single expert radiologist blinded to the participant's diabetes status. Patients were examined in the supine position with the head tilted backwards. The protocol involved the

Table 1. Characteristics of patients with type 2 diabetes according to catalase genotypes.

Characteristics	CC n (%)	CT n (%)	TT n (%)	P (ANOVA)
Number	23	102	161	286
Age (years)	64.2 ± 9.4	59.6 ± 15.0	61.2 ± 14.2	0.34
Male sex (%)	8 (34,4)	50 (49)	75 (46.5)	0.53
Duration of diabetes (years)	11.5 ± 8.0	8.9 ± 8.5	9.9 ± 8.1	0.34
Patients on insulin therapy (%)	9 (39,1)	37 (36.1)	73 (45.6)	0.31
Systolic blood pressure (mm Hg)	137.7 ± 10.9	143.0 ± 18.9	143.4 ± 20.4	0.52
Diastolic blood pressure (mm Hg)	82.5 ± 8.4	84.5 ± 11.2	85.7 ± 16.0	0.67
BMI (kg/m2)	31.3 ± 4.3	30.9 ± 5.2	32.2 ± 10.2	0.44
History of hypertension (%)	21 (90,5)	83 (81,0)	129 (80,3)	0.75
Smokers (%)	3 (13)	2 (2.2)	8 (4,8)	0.08
Waist circumference (cm)	105.5 ± 12.8	108.0 ± 11.8	109.4 ± 13.7	0.33
Troponin I (mg/L)	0.0074 ± 0.014	0.0042 ± 0.012	0.064 ± 0.76	0.68
hsCRP (mg/L)	4.52 ± 4.9	3.78 ± 4.1	4.98 ± 9.9	0.50
Glucose (mmol/l)	8.65 ± 3.2	7.91 ± 2.5	8.01 ± 2.6	0.47
Fibrinogen (g/L)	4.55 ± 1.0	4.16 ± 1.1	4.51 ± 1.3	0.05
D-dimer (mg/L)	0.70 ± 0.8	0.60 ± 0.93	0.77 ± 1.1	0.42
Total cholesterol (mmol/l)	4.61 ± 1.2	4.73 ± 1.0	4.93 ± 1.2	0.21
HDL cholesterol (mmol/l)	1.24 ± 0.4	1.14 ± 0.3	1.20 ± 0.3	0.27
LDL cholesterol (mmol/l)	2.56 ± 0.7	2.60 ± 0.9	2.67 ± 0.9	0.77
Triglycerides (mmol/l)	2.54 ± 1.7	2.34 ± 1.4	2.63 ± 2.2	0.49
CIMT – left (mm)	1.08 ± 0.16	1.08 ± 0.14	1.09 ± 0.14	0.61
CIMT – right (mm)	1.1 ± 0.11	1.08 ± 0.14	1.10 ± 0.13	0.33

scanning of the common carotid artery (CCA), carotid bifurcations and origins of internal carotid arteries (ICA). The CIMT was measured at 3 sites along the 10 mm-long segment of the far wall of the CCA free of plaques in agreement with the carotid intima-media thickness consensus [21]. CIMT on the left and on the right were calculated as the mean of three readings, and the mean of the left and right CCA-CIMT measurement was used in the analysis. Plaque was defined as focal intima-media thickening greater than or equal to 1,2 mm. The plaque score (PS) was calculated by summing the total number of sites with plaques (each of the CCAs, bifurcations, and ICAs, bilaterally) [22]. Moreover, we determined the type of plaques from type 1 to type 5 [23,24]. Type 1 plaque is defined as dominantly echolucent with a thin echogenic cap, type 2 plaque as predominantly echolucent with small areas of echogenicity, type 3 plaque as dominantly echogenic with small areas of echolucency (less than 25 %), type 4 plaque as uniformly echogenic (equivalent to homogenous), and type 5 as predominantly calcified plaque. Plaque types 1,2 and 3 were considered unstable, while types 4 and 5 were considered stable [25].

2.3. Genotyping

Genomic DNA was extracted from 100 µl of whole blood using a Qiagen isolation kit. Genotyping for the CAT -262C/T polymorphism (rs 1001179) was performed using TaqMan (Applied Biosystems, Foster City, CA).

2.4. Biochemical analysis

Blood samples for biochemical analysis: total cholesterol, triglyceride levels, high-density lipoprotein (HDL), cholesterol level, fasting blood glucose, hsCRP, troponin, D-dimer and fibrinogen were collected after an overnight fasting. All blood biochemical analyses were determined by standard biochemical methods in the hospital's accredited lab.

2.5. Statistical methods

Data are expressed as means ± standard deviations or frequencies (percentages). The chi-square test was used to compare discrete variables. Continuous clinical data were compared by an unpaired Student's *t* test. Multiple logistic regression analysis was performed for the evaluation of the independent effect of catalase genotypes on CIMT>1mm, unstable plaques and higher plaque score, adjusted for the presence of established risk factors (age, gender, smoking, BMI, lipid parameters

Table 2. An average CIMT in diabetics and healthy controls.

	Left (mm)	Right (mm)	Mean (mm)	p
Diabetics	1.09 ± 0.14	1.1 ± 0.13	1.09 ± 0.12	<0.001
Controls	0.98 ± 0.15	0.97 ± 0.15	0.98 ± 0.14	

and duration of hypertension and diabetes). A p<0,05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 17 (SPSS Inc., Chicago, IL).

3. Results

The characteristics of diabetic patients according to catalase genotypes are listed in Table 1. According to catalase genotypes in patients with type 2 diabetes we did not observe significant differences in the following parameters: age, prevalence of male sex, duration of diabetes, treatment of diabetes with insulin, prevalence of arterial hypertension, systolic and diastolic blood pressure, BMI, hs CRP, blood glucose, troponin I and D-dimer (Table 1). There were no statistically significant differences in lipid parameters and CIMT on the left and on the right side among the three genotypes (Table 1). A borderline statistically significant difference was found in the fibrinogen concentration according to CAT genotypes (p value = 0,05; Table 1). We also observed a low smoking prevalence in the group of diabetic patients (2.2 to 13 %).

The CAT genotype distribution was compatible with Hardy-Weinberg expectations (χ^2=1,4; p=0.23).

We measured the average CIMT in 286 diabetic patients and 150 healthy controls. The diabetics' average CIMT was 1,09±0,14 on the left and 1,1±0,13 on the right, while healthy controls had an average CIMT of 0,98±0,15 on the left and 0,97±0,15 on the right. The mean of the left and right CCA-CIMT was 1,09±0,12 in diabetics and 0,98±0,14 for healthy controls. There was a statistically significant difference between the mean CIMT between diabetics and healthy controls (p=0,000) (Table 2). Due to our results, we decided to divide all the diabetics into two groups due to the CIMT: those with CIMT≤1 mm and those with CIMT >1 mm.

The distribution of CAT genotypes with respect to CIMT (CIMT > 1mm vs. CIMT ≤ 1 mm), plaque stability (unstable plaques – types 1, 2, 3 vs. stable plaques – types 4, 5) and plaque score (score 0, 1, 2 vs. score 3, 4, 5, 6) is listed in Table 3. We did not observe significant differences in CAT genotypes distribution according to CIMT, plaque stability or plaque score.

Results of multiple logistic regression analysis adjusted for the presence of established risk factors (age, gender, smoking, BMI, lipid parameters and duration of hypertension and diabetes) are listed in Table 4. A significantly lower risk for higher plaque score was found in patients carrying the T allele (OR=0,25; 95%CI=0,07-0,84; p=0,025). Heterozygosity for T allele was associated with greater decrease in higher plaque score risk (OR=0,18; 95%CI=0,05-0,70; p=0,014) compared to homozygotes (OR=0,28; 95%CI=0,08-0,98; p=0,047).

4. Discussion

Our data indicate no statistically significant differences in clinical characteristics, lipid parameters and CIMT on the left and on the right side among the three genotypes. Fibrinogen concentration showed borderline statistically significant difference due to catalase genotypes. Carriers of T allele had significantly lower risk for higher plaque score, whereas no association with CIMT>1mm and unstable plaques was observed.

Our results did not show any statistically significant association between catalase genotypes and established risk factors for carotid atherosclerosis, such as systolic and diastolic blood pressure, BMI, triglyceride, HDL, LDL and total cholesterol levels, hs CRP, and glucose concentration. This may be due to different approaches for blood glucose lowering (diet, insulin or oral anti-

Table 3. Distribution of genotypes with respect to CIMT, plaque type and plaque score.

	CC genotype	CT genotype	TT genotype	Total	p
Total	23	102	161	286	0.48
≤ 1 mm	7 (30.4)	32 (31.4)	40 (24.8)	79	
> 1 mm	16 (69.6)	70 (68.6)	121 (75.2)	207	
No plaque	2 (8.7)	13 (12.8)	18 (11.2)	33	0.38
Types 1,2,3	16 (69.6)	59 (57.8)	82 (50.9)	157	
Types 4,5	5 (21.7)	30 (29.4)	61 (37.9)	96	
Score 0,1,2	13 (56.5)	37 (36.3)	67 (41.6)	117	0.19
Score 3,4,5,6	10 (43.5)	65 (63.7)	94 (58.4)	169	

Table 4. Multiple logistic regression analysis for the association between catalase genotypes and CIMT>1 mm, unstable plaques and higher plaque score, compared to CC genotype as a reference.

CIMT>1 mm			
	p	OR	95% CI
CTTTCT+TT	0.92	1.08	0.26-4.52
TT	0.53	0.64	0.16-2.52
CT+TT	0.69	0.78	0.20-2.91
Plaque type 1,2,3			
	p	OR	95% CI
CT	0.99	0.99	0.27-3.71
TT	0.43	1.64	0.48-5.54
CT+TT	0.58	1.40	0.43-4.58
Plaque score 3,4,5,6			
	p	OR	95% CI
CT	0.014	0.18	0.05-0.70
TT	0.047	0.28	0.08-0.98
CT+TT	0.025	0.25	0.07-0.84

diabetic agents), but also due to physical activity, eating habits, antihypertensive and lipid lowering drugs. It is also possible, that people with different catalase genotypes have different response to drugs such as statins, known to reduce oxidative stress in patients with type 2 diabetes mellitus beside lipid lowering [26].

Many studies had confirmed an association between elevated fibrinogen level and carotid atherosclerosis (CIMT and plaque thickness) [27-29], but not all [30]. Fibrinogen has an affinity for binding to hydrophobic, ateromatous lipid surfaces and accumulation in plaques [31]. As atheromatous plaque forms, it incorporates fibrinogen and fibrin [32], which provide a scaffold for smooth muscle cell migration and proliferation but also for calcium deposition [33]. A recent study has showed that the association between fibrinogen level and atherosclerotic disease is attenuated over time. By the fourth decade, age and smoking cessation could modify fibrinogen synthesis and the association with cardiovascular disease [27]. We found the highest fibrinogen concentration in patients with CC catalase genotype and at the same time these group was the oldest. Our data did not show any association between catalase genotypes distribution and CIMT, plaque stability or plaque score, maybe due to the low smoking prevalence and/or older age in our study group. However, our data showed borderline statistically significant difference in fibrinogen concentration due to catalase genotypes, so we are unable to conclude that catalase genotypes really affect fibrinogen concentration. Further studies are needed to elucidate the association of the –262C/T polymorphism in the catalase gene and fibrinogen concentration.

Logistic regression model adjusted for age, gender, smoking, BMI, lipid parameters and duration of hypertension and diabetes showed that carriers of T allele had significantly lower risk for higher plaque score, whereas no association with CIMT>1mm and unstable plaques was observed. It seems that T allele has a protective role against advanced atherosclerotic process, defined as more than 3 sites with atherosclerotic plaques on carotid arteries bilaterally.

Diabetes is usually accompanied by an increased production of free radicals or impaired antioxidant defence, and increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications [34]. As the polymorphism -262C/T is associated with different catalytic activity of enzyme it could be expected that those with the lowest catalytic activity would have the highest risk for development of diabetic complications. Higher catalase activity in diabetic patients carrying T allele has already been reported as a result of an increased transcriptional activity of the -262T CAT promoter variant [3]. Higher catalytic activity of antioxidant enzymes may increase the tolerance to oxidative stress and prevent the development and progression of diabetic complications.

In our study we did not measure the catalase activity, so we could not say whether the catalase -262C/T polymorphism is associated with the catalase activity in patients with type 2 diabetes, but as there was no statistically significant difference in CIMT, plaque stability and plaque score due to catalase genotypes it can be presumed that there was no difference in catalytic activity due to different genotypes. Such a result might be a consequence of an oxidative modification of catalase caused by increased oxidative stress in diabetic population, which could be one of the reasons for lower enzymatic activity [35]. A decrease in catalase activity could favour the accumulation of deleterious hydrogen peroxide and affect the oxidant/antioxidant balance. Further studies, focusing on catalase activity due to different genotypes and different levels of oxidative stress are needed to elucidate the association between catalase gene polymorphism and development of vascular complications in patients with type 2 diabetes mellitus.

The limitations of our study, such as the lack of direct biochemical evidence indicating the correlation of gene polymorphisms with altered catalytic activities of enzymes, single centre study, small sample size and cross-sectional design, suggest that further studies,

preferably prospective in nature, are needed to elucidate the role of polymorphisms of antioxidant enzymes and other risk factors in atherogenesis.

5. Conclusions

The -262C/T polymorphism of the catalase gene was found to modify the fibrinogen level and increase the risk for higher plaque score but it was not associated with CIMT or plaque stability in patients with type 2 diabetes.

Carotid plaque and CIMT can be easily detected by B-mode ultrasonography, which enables not only the assessment of the full spectrum of the atherosclerotic process, from normal artery walls to arterial occlusion, but also a following of its progression over time.

The CIMT has been shown to be associated with cardiovascular risk factors and the incidence of cardiovascular disease. As several studies reported an association of CIMT and coronary atherosclerosis, and of carotid plaque type and diabetic retinopathy, alone or in combination with nephropathy, CIMT measurements and plaque detection can be used as a good primary tool for the detection and follow up of micro- and macrovascular diabetic complications.

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