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GENETIC POLYMORPHISM OF GLUTATHION S-TRANSFERASE P1 (GSTP1) *Ile105Val* AND SUSCEPTIBILITY TO ATHEROGENESIS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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One of the characteristics of type 2 diabetes mellitus (T2DM) is the state of persistent oxidative stress (OS) that has been implicated in the pathogenesis of diseases such is atherosclerosis mainly through chronic hyperglycemia that stimulates production of reactive oxygen species (ROS) and increases OS.

Glutathione S-transferase P1 (GSTP1) is a member of the cytosolic GST superfamily. It plays an important role in neutralizing OS as an enzyme. Also, it participates in regulation of stress signaling and protects cells against apoptosis via its noncatalytic ligand-binding activity. *GSTP1 Ile105Val* functional polymorphism influences protein catalytic activity and stability and the aim of this study was to determine whether this gene variation influences susceptibility to atherogenesis in T2DM patients.

A total of 240 individuals (140 patients with T2DM, accompanied with clinical manifestations of atherosclerosis, and 100 healthy controls) were

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included in this study. Genomic DNA was isolated from peripheral blood cells and genotyping was performed using polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) analysis.
We obtained no statistically significant differences in the distribution of alleles and genotypes between cases and controls (P>0.05) but association between *Ile/Val* (OR=0.6, 95%CI=0.35-1.05, P=0.08) and *Val/Val* (OR=0.45, 95%CI=0.18-1.11, P=0.08) genotypes and disease approached significance (P=0.08).
Our results indicated that a larger study group is needed to establish the true relationship between potentialiy protective allele *Val* and the disease, and to determine the influence of other *GSTP1* polymorphisms on atherogenesis in T2DM patients.

Key words: GlutathioneS-transferase P1, gene polymorphism, type 2 diabetes mellitus, atherosclerosis

INTRODUCTION

Diabetes is one of the most frequent chronic diseases and it is a major public health problem worldwide. Serbia belongs to the group of European countries with the highest diabetes mortality rates where diabetes is the fifth leading cause of death and the fifth cause of the burden of disease. The number of deaths is even higher because of the errors in coding the causes of death particulary in those who died from myocardial infarction, stroke or other microvascular and macrovascular long term comlications. At least a half of the persons, especially elderly, with type 2 diabetes mellitus (T2DM) have not been diagnosed and are not aware of their disease until mainly cardiovascular and renal complications are already present (INSTITUTE OF PUBLIC HEALTH OF SERBIA "DR MILAN JOVANOVIĆ BATUT, 2011). Diabetes prevalence grows with age and ageing is associated with decrease in insulin secretion, action and clearance even in non-obese healthy elderly subjects (STEVIC *et al.* 2007).

As the result of diabetes there is a state of increased oxidative stress (OS) that leads to the development of accelerated atherosclerosis. Chronic hyperglycemia stimulates reactive oxygen species (ROS) production and increases oxidative stress. Excessive generation of ROS, such as superoxide, hydrogen peroxide and hydroxyl radical, along with reactive nitrogen species (RNS) such as nitric oxide, oxidize DNA, proteins and other cellular components leading to their damage (PITOCCO *et al*. 2010; KANETO *et al.* 2010; PEREIRA *et al*. 2008). Studies have shown that individuals with lowered antioxidant capacity are at increased risk of T2DM (BAYNESS *and* THORPE, 1999; GALLOU *et al*. 1993).

Among candidate genes related to OS, genes for cytosolic glutathione S-transferases (GSTs), especially mu (GSTM1), theta (GSTT1) and pi (GSTP1) are intensively studied. Although these cytosolic GSTs detoxify cytotoxic agents and protect cellular macromolecules catalyzing the conjugation of electrophilic compounds with glutathione (GSH), the role of GSTs in catalyzing the conjugation with endogenous products of oxidative damage to lipids (acrolin, crotonaldehyde, 4-hydroxynonenal) and DNA (base propenals) is well established (BERHANE *et al.* 1994). At physiological concentrations, GSTs regulate ROS levels influencing cellular proliferation and preventing ROS accumulation (HAYES *and* MCLELLAN, 1999) and thereby every change in GSTs genes can lead to oxidative stress.

Glutathion S-transferase P1 (GSTP1) is a member of the cytosolic GST superfamily (COWELL *et al*. 1988; MANNERVIK *and* DANIELSON, 1988). Because of its enzymatic and nonenzymatic multiple roles (WANG *et al.* 2001; HOLLEY *et al.* 2007) variation in the expression and activity of GSTP1 has been associated with a variety of human diseases (HARRIES *et al.*1997; MATTHIAS *et al*. 1998; MCILWAIN *et al*. 2006; NIKOLIĆ *et al*. 2011; SUVAKOV *et al.* 2012; AMER *et al*. 2012; GÖNÜL *et al*. 2012; KARIŽ *et al*. 2012; SANTL LETONJA *et al.* 2012; GRUBISA *et al.* 2012).

GSTP1 is genetically polymorphic and two common nonsynonymous single nucleotide polymorphisms (SNPs) (*Ile105Val* and *Ale114Val*) and the methylation state of a CpG islands in its promoter have been studied extensively. The *Ile105Val* genotype constitutes an A313G change causing an Ile to Val substitution at amino acid 105 within the active site of the enzyme which results in substantial reduction of enzyme activity by altering its catalytic activity (TOWNSEND *and* TEW, 2003).

Since the susceptibility to OS is partially determined by genetic background there has been a deep interest in discovering additional genetic variants as markers of OS, which may have a role in predicting risk of oxidative stress-based diseases. So, in the present study we have evaluated possible interactions between *GSTP1 Ile105Val* gene polymorphism and clinical manifestations of atherosclerosis in type 2 diabetes mellitus patients from Serbia.

MATERIALS AND METHODS

The study comprised of 140 patients with T2DM and clinical manifestations of atherosclerosis (86 males and 54 females, age 40-70 years) treated at Dedinje Cardiovascular Institute and Zvezdara University Medical Center, Belgrade, Serbia from 2010-2011. The control group was composed of 100 healthy individuals, age and sex matched with cases. Participants were not related and originated from different parts of Serbia and only those who have signed the informed consent have been included in the study. The study was approved by the ethical Committee of Zvezdara University Medical Center.

All patients had diagnostic criteria for diabetes based on subsequent values of glycemia (2 values of glycemia in 2 subsequent days): fasting plasma glucose concentration ≥7,0 mmol/L (126 mg/dL) or glycemia in any random blood sample (regardless of meals) ≥ 11,1 mmol/L (200 mg/dL) with the presence of typical diabetes symptoms (polyuria, polydipsia, weight loss) or based on the value of glycemia during an Oral Glucose Tolerance Test (OGTT): plasma glucose concentration during an OGTT in the 120th minute ≥ 11,1mmol/L (200 mg/dL) ((INSTITUTE OF PUBLIC HEALTH OF SERBIA "DR MILAN JOVANOVIĆ BATUT, 2011). Diagnosis of atherosclerosis was based on medical and familiy history, physical exam, blood test and some of noninvasive and invasive test-methods (echocardiogram, ankle/brachial index, stress testing, computed tomography scan, angiography) or according to anamnesis data about: lower extremity arterial surgery, carotid artery surgery, previous myocardial infarction, previous cerebro-vascular diseases, vascular dementia, angina.

Genomic DNA was extracted from peripheral blood cells by DNeasy Blood & Tissue Kit (Qiagen, GmbH, Germany), and isolated DNA was stored at +4 °C until further analysis.

GSTP1 Ile105Val genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as previously described (HARRIES *et al.* 1997). A 176bp PCR products were digested by *Alw26* restriction enzyme (MBI

Fermentas, Lithuania) according to manufacturers recommendations. Digestion products were separated on 3 % agarose gel, stained with ethidium bromide (0.5 μg/mL) and visualized under UV light for genotype determination. The absence of restriction site indicated wild type (WT) genotype *Ile/Ile* (176bp), and presence of restriction site resulting in two fragments 91bp and 85 bp indicated mutant genotype *Val/Val*.

Statistical analysis was carried out using SPSS software (version 17). Allele and genotype frequencies in cases and controls were compared using chi-square test (X^2 test) and the risk of disease was assessed using logistic regression analysis. A P value <0.05 was considered significant.

RESULTS

The allele and genotype frequency distribution for the *GSTP1 Ile105Val* polymorphism in T2DM patients with clinical manifestations of atherosclerosis and controls are given in Table 1.

Low-activity (*Val)* allele was more frequent in the control group (38.5%) compared to cases (32%) and heterozygotes *Ile/Val* and homozygotes *Val/Val* genotypes frequencies were higher in controls than in patients (55% versus 45% for *Ile/Val* and 11% versus 9.3% for *Val/Val*), the difference however did not reach statistical significance between groups (P>0.05).

Similarly, none of the values obtained by logistic regression analysis showed a statistical significance though *Val* allele (OR=0.7, 95%CI=0.5-1.8, P=0.13), *Ile/Val* (OR=0.6, 95%CI=0.35-1.05, P=0.08) and *Val/Val* (OR=0.45, 95%CI=0.18-1.11, P=0.08) genotypes showed 1.43, 1.67 and 2.22 fold decrease in risk of disease, respectively. Also, grouped genotypes *Ile/Val+Val/Val* showed a nonsignificant 1.37 fold decrease in risk (OR=0.73, 95%CI=0.43-1.25, P=0.26) in compare with reference *Ile/Ile* (Table 1).

Table 1. The genotype and allele frequencies and logistic regression analysis of GSTP1 Ile105Val gene polymorphism

	Cases (140)	Controls (100)	OR	95%CI	P-value
Genotype frequency, N (%)					
Ile/Ile	64 (45.7)	34 (34)	1.00	Standard	
Ile/Val	63 (45)	55 (55)	0.6	0.35-1.05	0.08
Val/Val	13 (9.3)	11 (11)	0.45	0.18-1.11	0.08
Ile/Val+Val/Val	76 (54.3)	66 (66)	0.73	0.43-1.26	0.26
Allele frequency, n (%)					
Ile	191 (68)	123 (61,5)	1.00	Standard	
Val	89 (32)	77 (38.5)	0.7	0.5-1.8	0.13

DISCUSSION

The most of diabetes-related deaths and complications are due to cardiovascular diseases. As a result of diabetes, there is a state of increased oxidative stress and compromised antioxidant defense that leads to the development of accelerated atherosclerosis and both microvascular and macrovascular long-term complications.

Excess mortality from coronary heart disease (CHD) among diabetic people compared to the general population has been demonstrated (HUXLEY *et al*. 2006). Cross-sectional study of 657 consecutive patients with symtomatic carotid atherosclerotic disease from our population showed that half of these patients have features of the metabolic syndrome (55.6% of studied patients) (MAKSIMOVIC *et al.* 2009). Patients with premature atherosclerosis (≤ 50 years of age) more frequently had 4 and 5 risk factors for atheosclerosis and DM is among the most significant for atherosclerotic disease of the supra-aortic branches. In patients with coronary artery disease (CAD), DM is a less prevalent risk factor in patients with premature coronary disease compared with elderly (RADAK *et al.* 2012).

In the present case-control study we have investigated the association of *GSTP1 Ile105Val* with cardiovascular complications in state of increased oxidative stress. According to our results there are no statistically significant differences in the distribution of alleles and genotypes for the *GSTP1 Ile105Val* polymorphism between cases and controls, which is in accordance with the results of some other similar studies (GÖNÜL *et al.* 2012; SANTL LETONJA *et al.* 2012; KARIŽ *et al.* 2012; MOASSER *et al.* 2012).

Though this *GSTP1* gene polymorphism has been widely studied in malignant diseases and many previous studies reported the association between *Val* with a variety of human cancers (HARRIES *et al.*1997; MATTHIAS *et al.* 1998; MCILWAIN *et al*. 2006; NIKOLIĆ *et al.* 2011), controversial results have been reported so far about the importance of this allele in oxidative stress-based diseases (FRYER *et al.* 2000; MATTEY *et al*. 1999; ISHII e*t al.* 1999; MANN *et al.* 2000; SUVAKOV *et al.* 2012; AMER *et al.* 2012; GÖNÜL *et al.* 2012; KARIŽ *et al*. 2012; SANTL LETONJA *et al*. 2012; RAMPRASATH *et al*. 2011; BID *et al.* 2010; GRUBISA *et al.* 2012).

The controversial results obtained by studies of different populations might be atribbuted to the interaction of many factors, such as differences in ethnic background and differential susceptibility to diseases. Our study confirmed previously reported results from other studies from Serbia reporting effects of *GSTP1 Ile105Val* polymorphism on T2DM in patients with pancreatic disease (NIKOLIĆ *et al*. 2011), on oxidative stress in haemodialysis patients, including patients with diabetic nephropathy (SUVAKOV *et al.* 2012), and effects of this polymorphism on patients with clinical manifestations of atherosclerotic disease without T2DM (GRUBISA *et al.* 2012).

Our results showed statistically nonsignificant decrease in the risk of atherosclerosis for allele *Val* and genotypes *Ile/Val* and *Val/Val*, but the association between these two genotypes and disease approached significance (P=0.08). Similar results have been reported in patients with manifest atherosclerotic disease (GRUBISA *et al.* 2012) and in the study on rheumatoid arthritis (MATTEY *et al*. 1999). In a study performed by Fryer and coworkers (FRYER *et al*. 2000), authors found that the *Val1/Val* genotype conferred a 6-fold decrease in asthma risk and that this genotype correlated with decreased severity of airway hyperresponsiveness (AHR). Mann et al (MANN *et al.* 2000) found that *GSTM3 AA* and homozygosity for both *GSTM1*0* and the *GSTP1 Ile* were linked with severe disability in multiple sclerosis (MS) and association between *Ile* and chronic obstructive pulmonary disease (COPD) was also found (ISHII *et al.* 1999).

GSP1 performs as a phase II detoxification enzyme. Also, it participates in regulation of stress signaling and protects cell against apoptosis via its noncatalytic ligand-binding activity (HOLLEY *et al.* 2007). In low levels of OS or unstressed conditions GSTP1 is found catalytically active and it is an inhibitor of c-Jun-N-terminal kinase (JNK) (ADLER *et al.* 1999). Exposure to

higher levels of OS causes GSTP1 dissociation from GSTP1-JNK complexes and JNK activation. This activates signalling pathways for stress response and apoptosis, and GSTP1 forms covalently linked and enzymatically inactive dimers and oligomers (TEW and RONAI, 1999). Some studies suggested that suppression of GSTP1 leads to elevated JNK activity, increased proliferation and reduced apoptosis (RUSCOE *et al.* 2001). Also, oxidative stress causes activation of transcription factor nuclear factor-kappa B (NF-kB) that induces expression of NF-kB responsive genes including *GSTP1* (XIA *et al.* 1996), while GSTP1 expression rises in response to OS or proinflammatory stimuli.

When the expression of GSTP1 decreases or protein has low activity, it has been speculated that cell becomes more susceptible to mutation and damage as a result of exposure to electrophiles and oxidative stress (KINZLER and VOGELSTEIN, 1997). It has been assumed that genetic variation is responsible for alteration in the level of enzyme activity as a result of at least two mechanisms-changes in the active site (Ile to Val substitution in the hydrophobic substrate binding site), and differences in levels of enzyme protein (MOYER *et al.* 2008). Probably, alteration of only one or two amino acids as a result of genetic polymorphism can be associated with drastic changes in the level of protein often due to rapid protein degradation through a ubiquitin-proteasome-mediated process (WANG *et al.* 2003; WEINSHILBOUM and WANG, 2004). In studies on *GSTP1* gene sequence variation and functional genomic, about 35 SNPs have been identified and some of them are uncaracterized yet. Authors showed that the variant nucleotide at (-18) resulted in protein binding that was not observed in WT sequence. Because this functional SNP is tighly linked with *Ile105Val* it is possible that a portion of the association of the codon 105 polymorphism with various clinical phenotypes may result from the effect of transcription of G(-18)A SNP (MOYER *et al.* 2008). We evaluated only one polymorphism in polymorphic *GSTP1* that probably limited this association study.

Although our data provide evidence that *Ile105Val* polymorphism is not associated with increased oxidative stress in T2DM and atherogenesis, a larger study group is needed to establish true relationship between potentialy protective allele *Val* and individual susceptibility to disease in our population. Also, study on other *GSTP1* gene polymorphisms is recommended.

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GENETIČKI POLIMORFIZAM GLUTATION S-TRANSFERAZE P1 (GSTP1) *Ile105Val* I OSETLJIVOST NA ATEROGENEZU KOD PACIJENATA SA DIJABETESOM TIPA 2

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Izvod

Jedna od osobina tipa 2 dijabetes mellitus (T2DM) je stanje perzistentnog oksidativnog stresa (OS) koji je sastavni deo patogeneze bolesti kao što je ateroskleroza i to najčešće putem hronične hiperglikemije koja stimuliše nastanak reaktivnih vrsta kiseonika (tzv ROS) i povećava nivo OS.

Glutation S-transferaza P1 (GSTP1) pripada superfamiliji citosolnih GST. Kao enzim, ima važnu ulogu u neutralizaciji OS. Takođe, učestvuje u regulaciji signalnog puta stresa ali i štiti ćelije od apoptoze putem nekatalitičke ligand-vezujuće aktivnosti. Funkcionalni polimorfizam *Ile105Val GSTP1* gena utiče na katalitičku aktivnost proteina i na njegovu stabilnost i cilj ove studije je bio da se utvrdi da li ova genska varijanta utiče na osetljivost na aterogenezu kod pacijenata sa T2DM.

Ukupno 240 osobe (140 pacijenata sa T2DM i nekom od kliničkih manifestacija ateroskleroze i 100 zdravih kontrola) je bilo uključeno u ovu studiju. Genomska DNK je izolovana iz ćelija periferne krvi a genotipizacija je urađena primenom reakcije lančane polimeraze posle koje je urađena analiza dužine restrikcionih fragmenata (PCR-RFLP analiza).

Nismo dobili statistički značajne razlike u raspodeli alela i genotipova između obolelih i kontrola (P>0.05) ali asocijacija između *Ile/Val* (OR=0.6, 95%CI=0.35-1.05, P=0.08) i *Val/Val* (OR=0.45, 95%CI=0.18-1.11, P=0.08) genotipova i bolesti se približila značajnosti (P=0.08).

Naši rezultaati pokazuju da je potrebna veća grupa ispitanika da bi se utvrdila prava veza između potencijalno protektivnog alela *Val* i bolesti kao i da bi se utvrdio uticaj drugih polimorfizama gena *GSTP1* na aterogenezu kod T2DM pacijenata.

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