GSTT1 null genotype is a risk factor for diabetic retinopathy in Caucasians with type 2 diabetes, whereas GSTM1 null genotype might confer protection against retinopathy

Ines Cilenšek[a,1], Sara Mankoč[a,1], Mojca Globočnik Petrovič[b] and Daniel Petrovič[a,*]

[a]*Institute of Histology and Embryology, Medical Faculty Ljubljana, University of Ljubljana, Ljubljana, Slovenia*
[b]*Eye Clinic, University Medical Centre Ljubljana, Ljubljana, Slovenia*

Abstract. *Aim:* Substantial data indicate that oxidative stress is involved in the development of diabetic retinopathy (DR). The aim of the present study was to investigate whether the genetic polymorphisms: polymorphic deletions of glutathione S-transferases M1 (GSTM1) and T1 (GSTT1) and Ile105Val of the GSTP1 are associated with DR in Slovenian patients with type 2 diabetes.
Methods: In this cross sectional case-control study 604 unrelated Slovene subjects (Caucasians) with type 2 diabetes mellitus were enrolled: 284 patients with DR (cases) and the control group of 320 subjects with type 2 diabetes of more than 10 years' duration who had no clinical signs of DR. Genotypes were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).
Results: In our study, the deletion of the GSTM1 was found less frequent in cases with DR than in the controls (27.5% versus 44.4%; $P < 0.001$), whereas the deletion of GSTT1 was found significantly more often in cases than in the controls (49.3% versus 29.7%; $P < 0.001$). We did not find statistically significant differences in the genotype distribution in GSTP1 (Ile105Val) polymorphism between cases and controls (40.5% versus 46.0%).
Conclusions: We may conclude that individuals homozygous for the deletion of GSTT1 are at an $\approx$2-fold-greater risk of DR, whereas the GSTM1 deficiency is associated with lower frequency of DR in type 2 diabetics.

Keywords: Diabetes mellitus type 2, diabetic retinopathy, oxidative stress, glutathione S-transferase

1. Introduction

Type 2 diabetes mellitus is a common multifactorial genetic syndrome, which is determined by several different genes and environmental factors. It now affects 350 million people world wide but its incidence is increasing rapidly because of secondary factors, such as obesity, hypertension, and lack of physical activity [16, 52]. Hyperglycaemia resulting from uncontrolled glucose regulation is widely recognized as a causal link between diabetes and diabetic complications, as it causes free radicals hyperproduction in endothelial cells at the mitochondrial level [11].

Substantial data indicate that oxidative stress is involved in the development of diabetic retinopathy (DR) [3,22,30,33]. Retina, a tissue rich in polyunsaturated fatty acid, uses more oxygen than any other tissue in the body, and is very susceptible to damage [34].

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[1]The authors contributed equally to the manuscript.

*Corresponding author: Danijel Petrovič, MD, PhD, Institute of Histology and Embryology, Medical Faculty of Ljubljana, Korytkova 2, 1105 Ljubljana, Slovenia. Tel.: +386 1 543 7367; Fax: +386 1 543 7361; E-mail: daniel.petrovic.@mf.uni-lj.si.

Oxidative stress is the result of an imbalance between the amount of reactive oxygen species (ROS) and the capacity of antioxidant defence systems [3].

Since long-term exposure to oxidative stress is strongly implicated in the pathogenesis of diabetic complications, polymorphic genes of detoxifying enzymes are implied in the development of DR. We assessed the potential role of glutathione S-transferase (GST) on the development of DR in patients with type 2 diabetes.

Glutathione S-transferases (GSTs) belong to a family of ubiquitous and multifunctional enzymes that work as one of the endogenous antioxidants in our body [8, 18].

GST enzymes are coded by at least eight distinct loci: α (GSTA), μ (GSTM), θ (GSTT), π (GSTP), σ (GSTS), κ (GSTK), o (GSTO), and τ (GSTZ), each containing one or more homodimeric or heterodimeric isoforms. Three loci in particular, GSTM1, GSTT1, and GSTP1, have received most of the attention. The GSTM1 locus has been mapped on chromosome 1p13.3, while the GSTT1 and GSTP1 locus exist on chromosome 22q11.2 and 11q13. Persons with homozygous deletions of either the GSTM1 or GSTT1 locus have no enzymatic functional activity of the respective enzyme [41,43].

A GSTP1 variant with a substitution in the active site of valine for isoleucine at codon 105 (Ile105Val) has a reduced ability to conjugate reactive electrophiles with glutathione and may therefore sensitize cells to free radical–mediated damage. The Val105 variant has been associated with susceptibility to smoking-related cancer and cardiovascular disease [21].

In contrast to the extensive investigation of the GSTM1 and GSTT1 gene variants in relation to malignancy [9,31,35,41,46,59] there are few studies investigating the associations between GSTM1 and GSTT1 gene variants and macrovascular disease in diabetics [21,28,40]. Moreover, the association between the GSTM1 and GSTT1 gene variants and microvascular complications, namely DR and diabetic nephropathy, in type 2 diabetes has been evaluated only in two studies so far [17,21]. Moreover, recent studies have revealed significant interethnic differences in allelic frequencies of polymorphic GSTM1 and GSTT1 genes and susceptibility to certain diseases [25,44].

The aim of the present study was to investigate whether the genetic polymorphisms: polymorphic deletions of glutathione S-transferases M1 (GSTM1) and T1 (GSTT1) and Ile105Val of the GSTP1 are associated with DR in a large sample of Caucasians with type 2 diabetes.

2. Patients and methods

In this cross-sectional case-control study 604 unrelated Caucasians with type 2 diabetes mellitus with a defined ophthalmologic status were enrolled (they have not been controlled for the glycemic history). Patients were classified as having type 2 diabetes according to the current American Diabetes Association criteria [26].

Fundus examination was performed by a senior ophthalmologist (M.P.) after pupil dilatation (tropicamide and phenylephrine 2.5%) using slit lamp biomicroscopy with non-contact lens, and was electronically documented with a 50°-angle fundus camera (Topcon-TRC 40-IX; Tokyo, Japan). Staging of diabetic retinopathy was determined according to the ETDRS retinopathy severity scale [60].

The study group consisted of 604 subjects: 284 subjects with DR (cases) and the control group of 320 subjects with type 2 diabetes of more than 10 years' duration who had no clinical signs of diabetic retinopathy. The DR group consisted of 198 subjects with proliferative diabetic retinopathy (new vessel formation and/or fibrous proliferation with or without vitreous hemorrhage) and 86 subjects with non-proliferative diabetic retinopathy (microaneurysms, retinal hemorrhages, hard exudates) [60].

To avoid the confounding effect of impaired kidney function, the patients with overt nephropathy were not enrolled. The study was approved by the national medical ethics committee. After an informed consent for the participation in the study was obtained, a detailed interview was made.

Genomic DNA was extracted from 200 μl of whole blood using a FlexiGene DNA isolation kit according to the recommended protocol (Qiagene, Hilden; Germany).

Genotypes of GSTM1 and GSTT1 were determined by multiplex PCR amplification, as described previously [6]. For GSTT1-null/GSTM1-null genotype no bands were obtained, necessitating the use of albumin as internal positive control, in order to distinguish the null genotype from aborted PCR reactions. Amplification results in a 480 bp GSTT1 fragment, 215 bp GSTM1 fragment and 350 bp albumin fragment [6]. By using this protocol it was not possible to distinguish homozygous and heterozygous carriers of GSTM and GSTT alleles [7].

The GSTP1 genotype was evaluated by PCR using the following primers: forward 5'- ACC CCA GGG CTC TAT GGG AA -3' and reverse 5'- TGA GGG

Table 1
Characteristics of patients with diabetic retinopathy (cases) and patients without diabetic retinopathy (controls)

Characteristics	Cases n (%)	Controls n (%)	P
Number	284	320	
Age (years)	65.6 ± 9.6	64.9 ± 10.3	0.4
Male sex (%)	139 (49)	140 (43.8)	0.2
Duration of diabetes (years)	19.1 ± 8.9	11.9 ± 8.1	< 0.001
Patients on insulin therapy (%)	201 (70.8)	119 (37.3)	< 0.001
HbA_{1c} (%)[1]	8.0 ± 1.6	7.7 ± 1.6	0.05
Systolic blood pressure (mm Hg)	144 ± 22	143 ± 19	0.6
Diastolic blood pressure (mm Hg)	84 ± 10	84 ± 9	0.7
BMI (kg/m^2)	28.3 ± 4.7	31.7 ± 14.9	0.01
History of hypertension (%)	149 (52.6)	174 (54.4)	0.7
Smokers (%)	33 (11.6)	36 (11.2)	1.0
Total cholesterol (mmol/l)	4.9 ± 1.3	4.8 ± 1.1	0.8
HDL cholesterol (mmol/l)	1.2 ± 0.3	1.2 ± 0.3	0.96
LDL cholesterol (mmol/l)	2.7 ± 0.9	2.6 ± 0.9	0.4
Triglycerides (mmol/l)	2.9 ± 3.1	2.5 ± 1.5	0.4

Data are expressed as means $\pm$ standard deviations or frequencies (percentages).
[1]The average value of HbA_{1c}.

CAC AAG AAG CCC CT-3' as described previously [49]. The PCR product was then digested with 3U Alw261 (New England Biolabs, Hertfordshire, UK). In homozygotes for A allele a fragment of 176 bp is seen. Restriction site appears when the G allele is present and two fragments (91 bp+ 85 bp) are seen in homozygotes.

Genotyping was performed by two researchers (D.P., I.C.), blinded to the retinopathy status of the patients. Chi-square test was used to compare discrete variables. Continuous clinical data were compared by unpaired Students t test. In addition, all variables that showed significant differences by univariate methods (chi-square test, unpaired Students t test) were analysed together in a logistic regression analysis. A $p < 0.05$ was considered statistically significant. Assuming the significance level of 0.05, we calculated the power of our study sample (284 DR cases, 320 controls – diabetics without DR) to be 99% for GSTT1-null and GSTM1-null genotypes.

Statistical analysis was performed using the SPSS program for Windows version 19 (SPSS Inc. Illinois).

3. Results

The characteristics of the cases and control subjects are summarized in Table 1. Cases had a longer duration of type 2 diabetes compared to the diabetics without DR. Additionally, they had a higher prevalence of insulin therapy than the controls (diabetics without DR). Body mass index (BMI) was higher in controls, but there were no significant differences in hypertension,

smoking, LDL and HDL cholesterol levels and triglyceride levels between the cases and controls.

The GSTP1 codon 105 variant was present in Hardy-Weinberg equilibrium (HWE) (GSTP1 cases: $^2 = 0.856$, $p = 0.35$; GSTP1 controls: $^2 = 0.15$, $p = 0.7$); however, HWE was not assessed for the GSTM1 and GSTT1 variants because heterozygous individuals could not be distinguished from the homozygous wild type. The distribution of GSTT1, GSTM1 and GSTP1 genetic polymorphisms among patients with DR (cases) and patients without DR (controls) are shown in Table 2. A significantly higher frequency of the GSTT1-null genotype was found in cases in comparison with controls (diabetics without DR). Moreover, the GSTM1-null genotype was significantly less frequent in controls.

Univariate analysis showed that the following variables were associated with the DR: GSTM1-null genotype, GSTT1-null genotype, duration of diabetes, patients on insulin therapy and BMI.

The variables showing an association ($p < 0.05$) were then put into a stepwise multiple logistic regression in order to study the possible effect of the GSTT1-null and GSTM1-null with other risk factors on DR development.

In the study, we found out that the absence of the GSTT gene (GSTT1-null genotype) was associated with an increased risk of DR. Contrary, the GSTM1-null genotype conferred a reduced risk of DR (Table 2). There was no evidence that GSTP1 variants were associated with DR in this population (Table 2).

After the adjustment for BMI, the duration of diabetes, insulin therapy and age, the carriers of GSTT1-

Table 2
Distribution of different genetic polymorphisms amongst subjects with DR (cases) and amongst diabetics without DR (controls)

	Cases (284)		Controls (320)		OR (95% CI)[1]	P
	No.	%	No.	%		
GSTP1						
Genotype AA	115	40.5	147	46.0	0.863 (0.628–1.187)	0.373
Genotype AG	137	44.4	142	44.4	1.252 (0.908–1.725)	0.192
Genotype GG	32	11.4	31	9.6	Reference	
GSTM1						
GSTM1-null	78	27.5	142	44.4	0.475 (0.337–0.668)	< 0.001
GSTM1-present	206	72.5	178	55.6	Reference	
GSTT1						
GSTT1-null	140	49.3	95	29.7	2.303 (1.649–3.216)	< 0.001
GSTT1-present	144	50.7	225	70.3	Reference	

[1]OR, odds ratio; CI, confidence interval. The data were analyzed by the χ^2 test.

Table 3
Adjusted odds ratios for risk factors for diabetic retinopathy according to multiple logistic regression analysis

Risk factors	OR (95% CI)	P
GSTT1-null	2.167 (1.437–3.267)	< 0.001
GSTM1-null	0.589 (0.387–0.896)	0.013
Duration of diabetes	0.899 (0.874–0.924)	< 0.001
BMI	1.068 (1.021–1.068)	0.004
Insulin therapy	2.422 (1.583–3.705)	< 0.001
Age	1.044 (1.021–1.068)	< 0.001

null genotype showed a 2.167-fold higher risk for DR (OR = 2.167; 95% CI = 1.437–3.267; $p < 0.001$; Table 3). Carriers of GSTM1-null genotype showed as much as a 61.3% reduction in relative risk or as little as a 10.4% reduction in risk (OR = 0.589; 95% CI = 0.387–0.896; $p = 0.013$; Table 3).

4. Discussion

As GSTM1 and GSTT1 are involved in the processing of reactive oxygen, lipid peroxidation products and some key metabolites of toxicants, there are potential links between genetic polymorphisms of these enzymes and the pathogenesis of a number of chronic diseases. In particular, important insights into the effects of the GSTM1 and GSTT1 gene deletions on the pathogenesis of human diseases have been derived from molecular epidemiological studies [10]. To better understand the role of GST genotypes on the development of DR, the aforementioned loci were selected for the evaluation in a large sample of Caucasians with type 2 diabetes.

In our study, the GSTT1-null genotype was found to be statistically significantly more frequent in the cases with DR compared to the controls (49.3% vs. 29.7%; $p < 0.001$) suggesting the GSTT1-null genotype to the genetic risk factor for DR in subjects with type 2 di-

abetes. Our results are not in accordance with the results of the study in Slovenian Caucasians with type 1 diabetes [29]. In the study in children with type 1 diabetes, the prevalence of GSTT1-null genotype was 18.8% among patients with DR and 25% among diabetics without DR [29]. Our findings, on the other hand, are in agreement with reports by Datta et al. [17] and Doney et al. [21], who have recently reported the GSTT1-null genotype to be associated with diabetic nephropathy. Moreover, it was also reported that individuals homozygous for the GSTT1-null allele had a more generalized vasculopathy, with an increased risk of progression of both diabetic nephropathy and sight-threatening retinopathy, and this association was not influenced by smoking status [21].

In the study we demonstrate a lower frequency of the GSTM1 gene deletion polymorphism in DR in comparison to diabetics without DR (27.5% vs. 44.4%). We report for the first time that the GSTM1 deficiency might confer protection against the development of DR in people with type 2 diabetes. Moreover, the association between the GSTM1-null genotype and DR remained significant after adjusting for age, duration of diabetes, insulin therapy and BMI. To our knowledge, only one study [29] revealed similar evidence of association between the GSTM1-null genotype and the reduced risk of DR in a smaller prospective study in type 1 diabetes.

There are various reports on the association between GSTM1-null genotype and reduced risk for some cancers [36,38,57]. Lin et al. [37] found that people who are deficient in GSTM1 and who consume broccoli are protected against colon cancer, due to the potentially slower excretion of isothiocyanates. The protecting effect of isothiocyanates against lung cancer was shown also in individuals consuming cruciferous vegetables

and bearing the GSTM1-null genotype [38]. Furthermore, studies by Xiao and Singh [56] provide support for isothiocyanate-mediated inhibition of angiogenesis. They showed that isothiocyanate supress expression of nuclear factor-κB-regulated genes, including vascular endothelial growth factor (VEGF), a primary suspect in induction of retinal neovascularization in diabetes [1, 4,58].

Independent of the genetic factors, insulin therapy was statistically significantly associated with DR. This finding suggests the existence of other factors, such as differences in the ability of insulin secretion, or differences in the frequency of episodes of hypoglycemia, or adverse events associated with insulin therapy (hypoglycemia, worsening diabetic retinopathy if HbA$_{1c}$ decreases rapidly) [20,45]. Logistic regression analysis demonstrated that beside genetic factors (GSTT1-null and GSTM1-null genotypes) and insulin therapy, the duration of diabetes was an independent risk factor for DR. The duration of diabetes [23,53], high BMI [32,47] and glycaemic control [12,51] are the most important factors associated with the development of retinopathy. Today, HbA$_{1c}$ measurement is regarded as the "gold standard" indicator for glycaemic control in diabetic patients, reflecting glucose levels over 2–3 month period [5,42].

Large ethnic differences in the prevalence of the homozygously deleted GSTT1 genotype have been observed. The prevalence of the GSTT1-null genotype is highest among Chinese (64%), followed by Koreans (60%), African-Americans (22%), Caucasians (29%) and Asian-Indians (16%), whereas it is lowest among Mexian-Americans (10%) [61,62]. In the present study, the GSTT1-null genotype frequency found in the control group (29.7%) did not vary very much from already known Slovenian frequency (25.5%) and from European and Mediterranean ones (from 10.4 to 42.5%) [25]. Similarly, the GSTM1 is polymorphic, and is absent in 10–65% of the human population [63]. In our study, the prevalence of the GSTM1-null genotype was 44.4% among the controls, and fits within the range of variability observed in the Mediterranean basin and in Europe [25,44].

It is now generally accepted that retinopathy is a low-grade chronic inflammatory condition associated with increased leukocyte entrapment in retinal capillaries, and areas of capillary non-perfusion and endothelial cell damage [2]. Apart from realising enhanced amounts of ROS when compared with normal cells [14,19,48,**?**], leukocytes from diabetic patients also synthesise leukotriene B4 [27,50], which in turn may

act along with other inflammatory mediators including prostaglandins [13] to promote changes in retinal vasculature. Since some members of GSTs are involved in the biosynthesis of prostaglandins and leukotriens [15] the absence of enzyme activity of GSTM1 might be protective against DR in type 2 diabetes.

Type 2 diabetes is strongly associated with atherosclerosis and it represents a major risk factor for cardiovascular disease (CAD). Despite the prominent role of oxidative stress in the development of CAD, few studies have indicated that GSTM1-null genotype is protective against CAD [54,55]. The up-regulation of another enzyme more effective at detoxification of atherogenic compounds and higher activity of CYP1A2 in individuals null for GSTM1 [39] might offer another reasonable explanation for the protective effect of GSTM1-null genotype.

However, it should be stressed that to date we do not have any firm evidence that such mechanisms promote efficient defence against the development and progression of DR in type 2 diabetics. Hence, additional research is mandatory for answering this question.

5. Conclusions

We may conclude that individuals homozygous for the deletion of GSTT1 are at an $\approx$2-fold-greater risk of DR, whereas the GSTM1 deficiency is associated with lower frequency of DR in type 2 diabetics. Finally, further research is required to clarify potential protective role of GSTM1 null genotype against DR in type 2 diabetes.

Acknowledgement

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