• Clinical Research •

GSTM1-null and *GSTT1-active* genotypes as risk determinants of primary open angle glaucoma among smokers

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Abstract

• AIM: To evaluate glutathione transferase theta 1 and mu 1 (*GSTT1* and *GSTM1*) polymorphisms as determinants of primary open angle glaucoma (POAG) risk, independently or in combination with cigarette smoking, hypertension and diabetes mellitus.

• METHODS: A case-control study with 102 POAG patients and 202 age and gender-matched controls was carried out. Multiplex-polymerase chain reaction method was used for the analysis of *GSTM1* and *GSTT1* polymorphisms. The differences between two groups were tested by the *t*-test or χ^2 test. Logistic regression analysis was used for assessing the risk for disease development.

• RESULTS: The presence of *GSTM1-null* genotype did not contribute independently towards the risk of POAG. However, individuals with *GSTT1-active* genotype were at almost two-fold increased risk to develop glaucoma (*P*=0.044) which increased up to 4.36 when combined with *GSTM1-null* carriers (*P*=0.024). When glutathione transferase (*GST*) genotypes were analyzed in association with cigarette smoking, hypertension and diabetes, only carriers of *GSTT1-active* genotype had significantly increased risk of POAG development in comparison with *GSTT1-null* genotype individuals with no history of smoking, hypertension and diabetes, respectively (OR=3.52, *P*=0.003; OR=10.02, *P*<0.001; OR=4.53, *P*=0.002).

• CONCLUSION: The results obtained indicate that both *GSTM1-null* and *GSTT1-active* genotypes are associated with increased POAG risk among smokers, suggesting potential gene-environment interaction in glaucoma development.

• KEYWORDS: *GSTM1*; *GSTT1*; primary open angle glaucoma risk; smoking; hypertension; diabetes mellitus

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INTRODUCTION

Glaucoma, the second leading cause of blindness in the world, represents a group of diseases characterized by progressive degeneration of the retinal ganglion cells (RGC) and their axons[1]. Primary open angle glaucoma (POAG) is the most common form with multifactorial etiology, including genetic and environmental factors[2]. As for the genetic factors, until now, only three genes have been associated to POAG pathogenesis, optineurin, myocilin, and tryptophan-aspartic acid repeat containing protein 36[2]. Beside positive family history, the recent study indicated elevated intraocular pressure (IOP), older age, sub-Saharan African ethnic origin and high myopia as main risk factors for POAG[3-4]. Furthermore, other important contributing factors influencing primarily the increase of IOP are related to atherosclerosis development, specifically cigarette smoking, hypercholesterolemia, diabetes mellitus and obesity[5]. What is more, it has been proposed that oxidative stress, as a consequence of elevated IOP, might contribute to disease progression by inducing outflow resistance through trabecular meshwork (TM) and the RGC damage[5-8].

Glutathione transferases (GST) include a family of enzymes with distinct catalytic and non-catalytic functions[9]. Namely, GST catalyze the breakdown and the detoxification reactions of different xenobiotics and reactive oxygen species (ROS)[10].

Furthermore, literature date are indicating GST as modulators of cell proliferation and apoptosis, in terms of regulators of different protein kinases, one of them being apoptosis signal-regulating kinase 1 (ASK1)[11-12]. Almost all cytosolic GST classes exhibit genetic polymorphism[13]. Deletions polymorphisms occurring in *glutathione transferase mu 1* (*GSTM1*) and *glutathione transferase theta 1* (*GSTT1*) genes, resulting in a complete absence of enzyme activity[13] influence individual predisposition to environmental and oxidative stress[11,14]. Namely, due to a homozygous deletion of a 15-kb allele of the *GSTM1* gene, about half of the Caucasians are carriers of *GSTM1-null* genotype and do not have an active GSTM1 enzyme[15-17]. As for the *GSTT1* genotype, the lack of the enzyme activity is present in 20% of Caucasians carriers of the *GSTT1-null* genotype[18].

Many studies have investigated the association of *GSTM1* and *GSTT1* polymorphism with the risk of POAG, however with inconsistent results. The lack of association and controversial results for both *GSTM1* and *GSTT1* genotypes in relation to POAG might be due to the specific genetic background present in different world populations, the environmental factors and their interactions[19-20]. Both polymorphisms were extensively studied in oxidative stress-related conditions, such as obesity, type 2 diabetes, coronary heart disease, neurodegenerative diseases or smoking habits[21-27]. Based on the important role of GSTM1 enzyme in detoxifying benzodiolepoxide, present in tobacco smoke and environmental pollution, *GSTM1-null*-genotype-smoking interplay in POAG could be hypothesized[13,27]. To our knowledge, the effect of those polymorphisms in conjunction with hypertension and diabetes in POAG was not investigated as yet.

Owing to the fact that oxidative damage caused by increased production of ROS might play an important role in the pathophysiology of POAG, the aim of this study was to determine the relative risk associated with *GSTM1* and *GSTT1* polymorphisms, as well as possible association of those polymorphisms with cigarette smoking, hypertension and diabetes mellitus, as possible modifying risk factors for POAG.

SUBJECTS AND METHODS

Patients One hundred and two patients with POAG (45 men and 57 women, mean age 72.47±8.30y) were recruited from Eye Clinic, Zvezdara University Medical Centre, Belgrade and a group of 202 controls (91 men and 111 women, mean age 71.73±6.89y). Before the enrollment in the study, all patients were clinically examined and the diagnosed of POAG by an ophthalmologists. The following criteria were used for the diagnosis of POAG: IOP greater than 21 mm Hg before treatment with IOP lowering drugs, characteristic cupping of the optic disc, clinically present open angle of the anterior chamber on the gonioscopy and the typical glaucoma visual field changes detected by Humphrey's visual field analyzer. Only glaucoma patients who fulfilled the necessary criteria were included in our study. The exclusion criteria were: IOP bellow 21 mm Hg, the presence of the clinically open angle without any visual field and optic disc abnormalities, history of uveitis, trauma, primary angle closure glaucoma or secondary glaucoma. The demographic data together with the information about diabetes mellitus, hypertension and smoking status were gathered from each study subject using standard questionnaire at the moment of blood collection. In our study, smokers were delineated as individuals who smoked every day for a minimum of 60d period before their enrolment in the study. Furthermore, they gave the information how many cigarettes they smoked daily and the duration of smoking, as well. All the study participant have signed the informed consent before they were enrolled in the study. The study was approved by the Ethical Committee of the University Medical Centre Zvezdara, Belgrade and the research was carried out in a compliance with the Declaration of Helsinki.

Sample Collection and DNA Analysis Commercially available DNA kit (Qiagen, USA) was used to isolate DNA from blood leucocytes. The analysis of *GSTM1* and *GSTT1* polymorphisms was carried out by polymerase chain reaction (PCR) using the multiplex PCR method[28]. CYP1A1 was used as a housekeeping gene (Table 1). PCR protocol was as followed: denaturation at 94℃ for 4min followed by 94℃ for 30s; annealing: 59℃ for 30s; extension: 72℃ for 45s; number of cycles: 30; final extension: 72℃ for 5min. The blinded samples for quality control were included to ensure the validation of procedures and steps in the genotype identification and the investigators who performed the multiplex PCR analysis were uninformed of the case-control status. The similarity of the results for the blinded samples was 100%.

Statistical Analysis The data were analyzed using the Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS, Chicago, IL, USA). Categorical variables were presented using frequency [*n* (%)] counts and parametric variables were presented as mean±standard deviation. The *t*-test for continuous variables with normal distribution and χ^2 test for categorical variables were used to assess the differences between two groups. The logistic regression analysis was used for assessing the risk for disease development and the results were adjusted by age and gender. $P<0.05$ was considered statistically significant.

RESULTS

The clinical characteristics of POAG patients and controls are presented in Table 2. A total of 102 patients (45 males and 57 females) and 202 controls (91 males and 111 females) were included in the study. We found no statistical significance regarding age ($P=0.412$) and gender ($P=0.877$) between the glaucoma patients and controls. As expected, the smoking

Table 1 PCR: primer sequences and fragment lengths

Gene	Primer sequences	Gel electrophoresis results
GSTM1	F, 5' -GAACTCCCTGAAAAGCTAAAGC-3'; R, 5' -GTTGGGCTCAAATATACGGTGG-3'	*GSTM1-active*: 215 bp band; *GSTM1-null*: no band
GSTT1	F, 5' -TTCCTTACTGGTCCTCACATCTC-3'; R, 5' -TCACCGGATCATGGCCAGCA-3'	*GSTT1-active*: 480 bp band; *GSTT1-null*: no band
CYP1A1	F, 5'-GAACTGCCACTT CAGCTGTCT-3'; R, 5'- CAGCTGCATTTG GAAGTGCTC-3	312 bp band

bp: Base pair.

Table 2 Baseline characteristic of patients with POAG and respective controls mean±SD, *n* (%)

Variables	Controls	Patients	OR (95%CI)	*P*
Age (y)	71.73±6.89	72.47±8.30		0.412
Gender				
Male	91 (45.0)	45 (44.1)		
Female	111 (55.0)	57 (55.9)		0.877
Smoking				
No	136 (67.3)	55 (53.9)	1.00 (reference group)	
Yes	66 (32.7)	47 (46.1)	2.00 (1.20-3.35)	0.008
Arterial hypertension				
No	115 (65.0)	19 (19.0)	1.00 (reference group)	
Yes	62 (35.0)	81 (81.0)	7.95 (4.37-14.46)	<0.001
Diabetes mellitus				
No	138 (86.8)	73 (72.3)	1.00 (reference group)	
Yes	21 (13.2)	28 (27.7)	2.70 (1.41-5.16)	0.003

OR: Odds ratio adjusted for age and gender.

prevalence among POAG patients was higher (46.1%) than in the control group (32.7%). After adjustment for age and gender we found that the history of cigarette smoking was associated with significantly increased POAG risk of 2.0 (95%CI 1.20-3.35, *P*=0.008).

Glaucoma patients and controls were further stratified according to the presence of either arterial hypertension or diabetes mellitus and the risk of POAG development was assessed afterwards. Indeed, almost eight-fold increased risk to develop glaucoma was found in individuals with the history of arterial hypertension (OR=7.95, 95%CI=4.37-14.46, *P*<0.001) in comparison with the healthy ones. As for diabetes mellitus, diabetics were in 2.7-fold increased risk to develop POAG during life compared to the individuals with no history of diabetes (OR=2.70, 95%CI=1.41-5.16, *P*=0.003).

Glutathione Transferase Genotypes and Primary Open Angle Glaucoma Risk The frequency of *GSTM1-null* genotype was slightly higher in glaucoma patients (52.6%) than in controls (50.0%), hence without statistical significance (OR=1.15, 95%CI=0.71-1.89, *P*=0.569). The *GSTT1-active* genotype was significantly more common in patients with POAG (84.5%) than in healthy controls (74.3%). Furthermore, the individuals with *GSTT1-active* genotype were at almost two-fold increased risk to develop glaucoma than carriers of *GSTT1-null* genotype (OR=1.92, 95%CI=1.02-3.64, *P*=0.044) while the risk was even higher when combined with *GSTM1-null* genotype (OR=4.36, 95%CI=1.21-15.65, *P*=0.024) (Table 3).

Modifying Effect of Smoking, Hypertension and Diabetes Mellitus on Glutathione Transferases Genotypes in Relation to Primary Open Angle Glaucoma Risk The significant association was found for both *GST* genotypes and the risk of POAG in smokers. However, modifying effect of smoking was observed only among smokers with *GSTT1-active* genotype who were at 3.5-fold higher risk to develop POAG (OR=3.52, 95%CI=1.53-8.08, *P*=0.003) (Table 4).

When modifying effect of hypertension was analyzed in relation to *GST* genotypes, it was observed that *GSTT1-active* hypertensive carriers were at 10-fold increased risk to develop glaucoma (OR=10.02, 95%CI=3.26-30.73, *P*<0.001). Namely, the overall risk was higher in combination, than when the risk of *GSTT1* or hypertension was assessed alone. Although hypertensive *GSTM1-null* carriers were at increased risk to develop glaucoma (OR=7.12, 95%CI=3.06-16.56, *P*<0.001), this can be attributed to independent effect of hypertension (Table 5).

Further, the aim of our attention was POAG patients with diabetes mellitus (Table 6). We found that diabetic carriers of *GSTT1-active* genotype were at 4.5-times increased risk to develop glaucoma (OR=4.53, 95%CI=1.76-11.66, *P*=0.002). The risk was higher than the risk of *GSTT1* or diabetes when assessed alone. Similarly to hypertension, increased risk in *GSTM1-null* carriers with diabetes was probably the consequence of independent effect of diabetes (OR=2.85, 95%CI=1.09-7.41, *P*=0.032).

Table 3 *GSTT1* and *GSTM1* genotypes in relation to the risk of POAG *n* (%)

Variables	Controls	Patients	OR (95%CI)	*P*
GSTT1				
Null	52 (25.7)	15 (15.5)	1.00 (reference group)	
Active	150 (74.3)	82 (84.5)	1.92 (1.02-3.64)	0.044
GSTM1				
Active	101 (50.0)	46 (47.4)	1.00 (reference group)	
Null	101 (50.0)	51 (52.6)	1.15 (0.71-1.89)	0.569
GSTT1/GSTM1				
GSTT1-null/GSTM1-active	22 (10.9)	3 (3.1)	1.00 (reference group)	
GSTT1-null/GSTM1-null	30 (14.9)	12 (12.4)	3.11 (0.77-12.45)	0.110
GSTT1-active/GSTM1-active	79 (39.1)	43 (44.3)	4.14 (1.16-14.74)	0.028
GSTT1-active/GSTM1-null	71 (35.1)	39 (40.2)	4.36 (1.21-15.65)	0.024

OR: Odds ratio adjusted for age and gender.

Table 4 Modifying effect of smoking on *GST* genotypes in relation to the risk of POAG *n* (%)

Variables	Controls	Patients	OR (95%CI)	*P*
GSTT1/smoking				
GSTT1-null/non-smoker	37 (18.3)	10 (10.3)	1.00 (reference group)	
GSTT1 null/smoker	15 (7.4)	5 (5.2)	1.41 (0.41-4.91)	0.586
GSTT1-active/non-smoker	99 (49.0)	40 (41.2)	1.51 (0.68-3.36)	0.311
GSTT1-active/smoker	51 (25.2)	42 (43.3)	3.52 (1.53-8.08)	0.003
GSTM1/smoking				
GSTM1-active/non-smoker	64 (31.7)	19 (19.6)	1.00 (reference group)	
GSTM1-active/smoker	37 (18.3)	27 (27.8)	3.13 (1.48-6.63)	0.003
GSTM1-null/non-smoker	72 (35.6)	31 (32.0)	1.69 (0.86-3.36)	0.129
GSTM1-null/smoker	29 (14.4)	20 (20.6)	2.96 (1.32-6.62)	0.008

OR: Odds ratio adjusted for age and gender.

Table 5 Modifying effect of hypertension on *GST* genotypes in relation to the risk of POAG *n* (%)

Variables	Controls	Patients	OR (95%CI)	*P*
GSTT1/hypertension				
GSTT1-null/normotensive	28 (15.8)	4 (4.2)	1.00 (reference group)	
GSTT1 null/hypertensive	15 (8.5)	10 (10.5)	4.38 (1.16-16.58)	0.029
GSTT1-active/normotensive	87 (49.2)	15 (15.8)	1.18 (0.36-3.89)	0.775
GSTT1-active/hypertensive	47 (26.6)	66 (69.5)	10.02 (3.26-30.73)	<0.001
GSTM1/hypertension				
GSTM1-active/normotensive	57 (32.2)	10 (10.5)	1.00 (reference group)	
GSTM1-active/hypertensive	33 (18.6)	41 (43.2)	7.26 (3.19-16.49)	<0.001
GSTM1-null/normotensive	58 (32.8)	9 (9.5)	0.90 (0.34-2.41)	0.842
GSTM1-null/hypertensive	29 (16.4)	35 (36.8)	7.12 (3.06-16.56)	<0.001

OR: Odds ratio adjusted for age and gender.

Based on relatively high prevalence of hypertensive smokers among POAG patients in our study, we further stratified them according to both *GST* genotypes, as well as smoking and hypertension status. We found that hypertensive smokers, carriers of the *GSTT1-active* or *GSTM1-null* genotype, were at increased risk of developing glaucoma compared to normotensive non-smokers, carriers of *GSTT1-null* or *GSTM1-active* genotype (P<0.001, data not shown). However, due to the limited number of study participants in each subgroup, the observed joint effect of hypertension and smoking on *GST* genotypes in relation to POAG risk requires further statistical analysis on a larger population.

DISCUSSION

In the present study, independent *GSTT1-active* genotype was associated with almost 2-fold increased risk to develop POAG. The risk was even higher when this genotype was combined with *GSTM1-null* genotype. Furthermore, we have shown that these genotypes in conjunction with cigarette smoking, hypertension and diabetes increase the risk of POAG.

The research data underline the important role of ROS and

Table 6 Modifying effect of diabetes mellitus on *GST* genotypes in relation to the risk of POAG *n* (%)

Variables	Controls	Patients	OR (95%CI)	*P*
GSTT1/diabetes				
GSTT1-null/non-diabetic	31 (19.5)	12 (12.5)	1.00 (reference group)	
GSTT1 null/diabetic	6 (3.8)	3 (3.1)	1.40 (0.27-7.22)	0.683
GSTT1-active/non-diabetic	107 (67.3)	57 (59.4)	1.49 (0.70-3.18)	0.295
GSTT1-active/diabetic	15 (9.4)	24 (25.0)	4.53 (1.76-11.66)	0.002
GSTM1/diabetes				
GSTM1-active/non-diabetic	67 (42.1)	30 (31.2)	1.00 (reference group)	
GSTM1-active/diabetic	11 (6.9)	15 (15.6)	3.38 (1.37-8.36)	0.008
GSTM1-null/non-diabetic	71 (44.7)	39 (40.6)	1.29 (0.71-2.33)	0.398
GSTM1-null/diabetic	10 (6.3)	12 (12.5)	2.85 (1.09-7.41)	0.032

OR: Odds ratio adjusted for age and gender.

oxidative stress in pathophysiology and progression of POAG[5-8]. Oxidative stress, due to an imbalance between the generation of ROS and the antioxidant defense, can result in cell membrane lipid peroxidation and damage to DNA and proteins affecting different eye structures. Specifically, it has been proposed that oxidative stress provides a setting for RGC damage and along these lines directly participates in optic nerve neuropathy[29]. Moreover, ROS may compromise TM integrity facilitating the increase of IOP which is considered to be the most important risk factor for POAG[6].

GST are polymorphic enzymes serving as cellular guardians against the oxidative stress. In our study *GSTM1-null* genotype was more frequent in the group of glaucoma patients but without statistical significance in comparison with healthy controls which is in agreement with several other investigations[30-33]. On the other hand, the association of either *GSTM1-null* genotype, described in Brazilian[34-35], Italian[36], Turkish[37] and Greek[38] population or *GSTM1-active* genotype described in an Estonian[33] and Turkish population[39] supported the notion of *GSTM1* as a risk factor of POAG. Regarding *GSTT1* deletion polymorphism, we found that carriers of *GSTT1-active* genotype were at significantly increased risk to develop POAG, which is in concordance with the results of a research conducted in the Brazilian population[34], while, in contrast to this, several others studies found association for *GSTT1-null* genotype[39-40]. In the studies of Chinese[31], Mexican[41], Estonian[33] and Turkish population[37] no association was found of *GSTT1* genotype and the risk for POAG.

Regarding recognized risk factors for POAG development in this study, an increase in POAG risk was observed among individuals with smoking habits, hypertension and diabetes, which is in agreement with previous findings[42-46]. Beside more than 60 carcinogens, cigarette smoke is also an abundant source of the free radicals. Both polycyclic aromatic hydrocarbon (PAH) metabolites and free radicals are detoxified by GST[13]. We found that *GSTT1-active* smokers were at 3.5-fold increased risk in comparison with non-smoker *GSTT1-null* carriers. These results are biologically plausible since *GSTT1* mediated detoxification of various xenobiotics sometimes produces even more toxic products[13]. Such a mechanism could give a possible explanation for the increased risk of POAG among carriers of *GSTT1-active* genotype especially if they are smokers. Moreover, hypertension and diabetes contributed to genotype-associated POAG risk in the analyzed polymorphisms, especially in *GSTT1-active* genotype carriers, thus emphasizing the important role of gene-environmental interactions in POAG development.

The results of our study showing more than 4-fold risk of POAG in carriers of *GSTM1-null/GSTT1-active* genotype are in agreement with the recent study by Rocha *et al*[34]. In their study, they have found a powerful association of *GSTM1-null* genotype with higher levels of IOP, more drastic optic nerve and visual field damage. On the grounds that prolonged oxidative stress may contribute to the increase of IOP[6], our results on increased risk in *GSTM1-null*/*GSTT1-active* genotype indicate that oxidative damage might significantly affects the pathophysiology of POAG.

Recent findings strongly suggested the apoptosis of the RGC as one of the main events in both pathogenesis and progression of POAG, with special emphasis on activation of ASK1-mediated apoptotic pathway[47]. On the other hand, deletion of *ASK1* gene prevents RGC death, including retinal ischemia and optic nerve injury as shown in various experimental glaucoma models[47]. The probable molecular mechanism underlying the role of GSTM1 in disease deterioration might be the GSTM1 non-catalytic regulatory role in apoptotic ASK1-MAPK (mitogen-activated protein kinase) signaling cascade. Several studies have shown that, independently of its transferase activity, GSTM1 protein can regulate ASK1 activity by protein-protein interaction. Thus, under non-stimulated conditions, GSTM1-1 suppresses ASK1-mediated activation of c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling cascade, and inhibits the apoptotic cell death dependent of ASK1. On the contrary, different stressors,

such as ROS, can induce dissociation of GSTM1:ASK1 protein complex, consequently initiating ASK1-mediated apoptotic signaling pathway[48]. In that way, it can be speculated that in *GSTM1-null* POAG patients, apoptosis is more intense in RGC, even after antioxidant treatment. Thus, determination of *GSTM1* and *GSTT1* genotypes might be the valuable indicator in order to assess the risk for POAG and also as potential therapeutic targets.

Certain limitations are recognized in our study. Relatively small number of the study participants might be the source of potential biases and decrease the power of the study which may influence the study findings. In our study, all the participants were Caucasians, therefore the possible effect of ethnicity could not be assessed. Future studies with detailed patient information and rigorous designs are needed to increase the power of the study and to confirm the findings of our research.

Even though, the present study justifies the assumption that *GSTM1* and *GSTT1* polymorphisms modulate the risk of POAG, with special emphasis on *GSTT1-active* genotypes. Since POAG is a condition where early diagnosis and treatment are of a great importance, diagnostic tests to determine those who are at risk to develop glaucoma can be very valuable. The final purpose of the research would be the identification of a full set of genes eligible to contribute to glaucoma and to develop not just diagnostic but prognostic tests. Such a set of genes would potentially provide a way to identify those more susceptible to develop POAG and introduce early treatment before permanent optic nerve degeneration and blindness develop.

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