Biochemical and Biophysical Research Communications 407 (2011) 49–53

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Potential risk modifications of *GSTT1*, *GSTM1* and *GSTP1* (glutathione-S-transferases) variants and their association to CAD in patients with type-2 diabetes

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ARTICLE INFO

Article history:
Received 15 February 2011
Available online 23 February 2011

Keywords:
Coronary artery disease
Glutathione S-transferase
Polymorphism
Type-2 diabetes mellitus

ABSTRACT

Background: Type-2 diabetes mellitus (T2DM) is a major risk factor for coronary artery disease (CAD) resulting in high morbidity and mortality. Glutathione S-transferases (GSTM1, GSTT1 and GSTP1) are known for their broad range of detoxification and in the metabolism of xenobiotics. Recent studies revealed the relationship of *GSTs* variants with T2DM and CAD. In this case-control study we ascertained the association of GSTs variants in association with the development of CAD in patients with T2DM.
Methods: From the Southern part of India, we enrolled 222 T2DM patients, 290 T2DM patients with CAD and 270 healthy controls matched for age, sex and origin. Serum lipid profiles were measured and DNA was extracted from the blood samples. Multiplex PCR for *GSTM1/T1* (null polymorphism) and PCR-RFLP for *GSTP1* (105 A > G), were performed for genotyping of study participants. Gene frequency and lipid profiles were statistically analyzed for disease association.
Results: Regression analysis showed that, *GSTM1*-null genotype is associated with a 2-fold increase (OR = 2.925; 95% CI = 2.078–4.119; $P < 0.0001$) and *GSTT1*-null genotype is associated with a 3-fold increase (OR = 3.114; 95% CI = 2.176–4.456; $P < 0.0001$) to T2DM development. Ile/Val and Val/Val genotypes of *GSTP1* also showed a significant risk for T2DM (OR = 1.423, CI = 1.041–1.946; $P = 0.027$ and OR = 1.829, CI = 1.064–3.142; $P = 0.029$). Increased odds ratio showed that *GSTT1*-null genotype had a moderately higher occurrence in T2DM–CAD patients (OR = 1.918, 95% CI = 1.144–3.214; $P = 0.014$) than T2DM patients without CAD. The level of HDL has significantly decreased in *GSTT1*-present than in *GSTT1*-null genotype (43.50 ± 4.10 vs. 45.20 ± 3.90; $P = 0.004$) when compared with control and T2DM patients. However, LDL level showed a significant increase in *GSTT1*-null than *GSTT1*-present genotype (108.70 ± 16.90 vs. 102.20 ± 12.60; $P = 0.005$). Although the *GSTM1*-null polymorphism showed no correlation with lipid profiles among T2DM and T2DM with CAD patients, *GSTT1*-null polymorphism attained a statistical significance for the level of LDL (127 ± 28.20 vs. 134 ± 29.10; $P = 0.039$) and triglycerides in T2DM with CAD patients (182.10 ± 21.10 vs. 191.20 ± 24.10; $P = 0.018$).
Conclusion: Our work concludes that *GSTM1*, *GSTT1* and *GSTP1* variants might contribute to the development of T2DM and *GSTT1* variant alone is involved in the development of T2DM associated CAD complications in the South Indian population.

1. Introduction

Cardiovascular diseases are the leading cause of mortality among Type-2 diabetes mellitus (T2DM), which has a complex etiology that includes both atherogenic and myocardial components [1,2]. Reactive oxygen species (ROS) production induced by chronic hyperglycemia is implicated as a potential molecular mechanism behind diabetic vascular complications. ROS activates protein kinase C (PKC) and increases the production of advanced glycation end products (AGEs), leading to superoxide generation [3], which triggers artherosclerosis [4]. Pancreatic β-cells express low levels of anti-oxidant enzymes and become sensitive to cytotoxic stress that leads to higher risk of oxidative damage [5,6].

Glutathione S-transferases (GSTM1, GSTT1 and GSTP1) belong to a super family of detoxification enzymes that provide critical defence against toxicants [7]. They are highly polymorphic, and individuals with these variants pose an increased susceptibility to many disorders. Whole gene deletions of *GSTM1* and *GSTT1* leads to a loss of function and numerous studies reported the association

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doi:10.1016/j.bbrc.2011.02.097

of these variants with risks of urinary bladder, breast, lung, colon, brain cancers, various non-malignant diseases [8,9] and salivary gland carcinoma [10]. The implications of interactions between genetic variants of *GSTs* in diabetes [11] and the risk of coronary artery disease (CAD) have been well demonstrated [12,13]. Interaction studies between *GSTM1*, *GSTT1* variants and the risk of coronary heart disease [14,15] showed that individuals with *GSTM1* or/and *GSTT1*-null genotypes were at a higher risk of incidence of CAD.

The *GSTP1* gene has also been frequently investigated. A functionally significant A to G transition in exon 5 leads to $Ile^{104}Val$ amino- acid substitution, which lowers the activity of this enzyme [16,17]. Recently, Zhong et al. found that a combined *GSTP1* and *GSTM1*-null mutations resulted in significantly reduced GST activity [18]. Although several molecular epidemiologic studies have examined the association of GST variants with cancer, diabetes and CAD [19,20], there was no report on the association of these variants and CAD development among the patients with T2DM. Thus, we conducted a case-control study in South Indian population with age- and sex-matched T2DM and CAD confirmed T2DM patients to evaluate whether the *GSTM1*, *GSTT1* and *GSTP1* variants modulate the risk of CAD in T2DM patients.

2. Methods

2.1. Study population and clinical evaluation

During the study period we included 512 patients (T2DM without CAD; $n = 222$ and T2DM with CAD; $n = 290$) from Government Rajaji hospital (GRH), Madurai, South India. Out of 270 controls, none had a history of cardiac or diabetic ailments. The basic demographic data, including body mass index (BMI), age, gender and duration of diabetes were obtained from the study subjects during the time of blood collection. In addition, blood glucose, HbA1c and lipid profiles were measured in the clinical laboratory of GRH. The American Diabetes Association Guidelines [21] were followed to identify the T2DM patients. The assumed risk factors for coronary artery disease such as hypertension with blood pressure, dyslipidemia, the level of total cholesterol, LDL, HDL, triglycerides, the usage of antihypertensive and lipid lowering drug therapy and the incidence of coronary angiography were considered to classify the patients with or without CAD. Exclusion criteria were smokers and alcoholics. Informed consent was obtained from the subjects according to the protocol of institutional ethical committee.

2.2. Clinical characteristics

Blood samples were obtained by vein puncture after an overnight fast from the study subjects. The samples were centrifuged and stored at −80 °C for the lipid profiling and biochemical analyses in the clinical laboratory of GRH. Total cholesterol (TC) was estimated by enzymatic method with kits procured from BioCon (BioCon valley e.V., Rostock, Germany) while low density lipoprotein (LDL) and triglycerides (TG) were estimated using the kits from Agappe diagnostics (India). High density lipoprotein (HDL) determination was done enzymatically by kits purchased from Lab-Care diagnostics (India) on an auto analyzer (Randox, Rx Imola auto analyzer). HbA1c was measured with the kit from Bayer healthcare (Bayer Diabetes Care, Tarrytown, USA). Plasma fasting blood glucose was measured by a glucose oxidase kit obtained from Dr. Reddy's lab (India).

2.3. DNA extraction and genotype determination

Genomic DNA was extracted by phenol–chloroform method and stored at −20 °C [22]. All primers used in this study were purchased from BioServe Biotechnologies, India. The polymorphism of *GSTM1/T1* was determined by multiplex PCR [23]. This method detects the presence (at least one allele present, homozygote or heterozygote) or absence (complete deletion of both alleles, homozygote) of genotype. The fragments of the *GSTM1/T1* were amplified with the primers *GSTM1:* forward: 5′-GAACTCCCTGAAA AGCTAAAGC-3′ and reverse: 5′-GTTGGGCTCAAATATACGGTGG-3′, *GSTT1:* forward: 5′-TTCCTTACTGGTCCTCACATCTC-3′ and reverse: 5′-TCACCGGACATGGCCAGCA-3′, and *β-globin:* forward: 5′-CAACT TCATCCACGTTCACC-3′ and reverse: 5′-GAAGAGCCAAGGACAGGT AC-3′. *β-globin* gene served as an internal positive control. The PCR protocol consisted of a 5 min pre-incubation step at 95 °C, 35 cycles of 1 min at 95 °C, 50 s at 60 °C, 1 min at 72 °C, and a final extension for 7 min at 72 °C. Amplified products (*GSTM1*: 215 bp, *GSTT1*: 480 bp, *β-globin*: 268 bp) were resolved on 2% agarose gel.

Genotyping of *GSTP1* was determined by PCR-RFLP [24]. A mutation by A to G transition in exon 5 introduces *Alw261* digestion site. On *Alw*261 digestion of the PCR product (176 bp), the GG allele resulted in two fragments (91 and 85 bp) while the AG allele resulted in three fragments (176, 91 and 85 bp). Primers used to amplify the *GSTP1* fragment (176 bp) were forward 5′-ACCC CAGGGCTCTATGGGAA-3′ and reverse 5′-TGAGGGCACAAGAAGCCC CT-3′. Initial denaturation was carried out at 95 °C for 5 min. Cycling conditions (35 cycles) were: denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and polymerization at 72 °C for 30 s. A final polymerization step of 72 °C for 5 min was carried out to complete the elongation process. The amplified PCR product was then digested with *Alw261* restriction enzyme and separated on a 3% agarose gel.

2.4. Statistical analysis

Data of continuous variables were expressed as mean ± standard deviation (SD) and data of noncontinuous variables as frequency (*N*, %). Categorical variables were presented using frequency counts and compared by χ^2-test. The χ^2 goodness-of-fit test was used to identify significant departures from the Hardy–Weinberg equilibrium. The genetic variants and their risk for disease were computed by odds ratios (OR) and 95% confidence intervals (CI) by logistic regression analysis. Continuous variables without skewness were compared by unpaired Student's *t*-test. The data for three or more independent groups were analyzed by analysis of variance (ANOVA) and Turkey's honestly significant Post-hoc difference test was used to test differences based on continuous variables. Calculations were performed using the SPSS software version 17.0 (Chicago, IL, USA). *P* value of <0.05 was considered to be statistically significant.

3. Results

3.1. Baseline characteristics

A total of 512 patients (T2DM = 222 and T2DM–CAD = 290) and 270 gender- and age-matched controls were involved in this study. Anthropometric measurements and clinical characteristics of the study populations were summarized in Supplementary Table 1. No significant difference was found between three populations in age (40.10 ± 7.20 vs. 40.93 ± 6.10 vs. 41.00 ± 4.80) and gender (72.96% vs. 74.32% vs. 73.10%) respectively. In the patients (both T2DM and T2DM–CAD) the established risk factors (hypertension, BMI, HbA_{1c} and high level of fasting glucose) for diabetes and CAD were elevated than in control. The lipid profile also showed higher levels of total cholesterol, LDL and TG but lower level of HDL in patients.

3.2. GST genotypes and allele frequencies

The distributions of GST genotypes and alleles of the study populations were given in Table 1. The *GSTM1*-null genotype occurs at higher frequencies in T2DM and T2DM–CAD patients than in control (42.34%, 44.10% and 20.74%, $\chi^2 = 14.70$, $P < 0.0001$). *GSTT1*-null genotype is also occurring at higher frequencies in T2DM and T2DM–CAD patients, than in control (31.53%, 46.90% and 17.80%, $\chi^2 = 19.40$, $P < 0.0001$). Allelic distribution of $Ile^{105}Val$ genotype in both cases (diabetic, diabetic with CAD) and control was consistent with those predicted by the Hardy–Weinberg equilibrium ($\chi^2 = 0.532$, $P = 0.466$; $\chi^2 = 3.50$, $P = 0.062$, $\chi^2 = 1.05$, $P = 0.306$) and the frequency of homozygous variant was higher than that of control group (12.62% and 11.04% vs. 8.14%).

3.3. Association of GST polymorphism with T2DM and CAD

Regression analysis showed that, *GSTM1*-null genotype was associated with a 2-fold increase (OR = 2.9254; 95% CI = 2.078–4.119; $P < 0.0001$) and *GSTT1*-null genotype was associated with a 3-fold increase (OR = 3.114; 95% CI = 2.176–4.456; $P < 0.0001$) to T2DM development. Heterovariants and homovariants of *GSTP1*, showed a significant risk between controls and T2DM subjects (Table 2) (OR = 1.423, CI = 1.041–1.946; $P = 0.027$ and OR = 1.829, CI = 1.064–3.142; $P = 0.029$). Table 3 shows the risk estimates for CAD among the diabetic patients. There was no risk found for *GSTM1*-null genotype. However, *GSTT1*-null genotype had a moderately higher occurrence of CAD in T2DM–CAD patients (OR = 1.918, 95% CI = 1.144–3.214; $P = 0.014$) than T2DM patients without CAD. T2DM–CAD patients with *GSTP1* Ile/Val or Val/Val genotypes also did not change the risk for CAD as compared with T2DM patients without CAD (OR = 1.277, 95% CI = 0.872–1.870; $P = 0.208$ and OR = 0.997, 95% CI = 0.554–1.794; $P = 0.992$).

3.4. Correlations between GST genotypes and the blood lipid profile

Table 4 represents the level of HDL is significantly decreased in *GSTT1*-present than in *GSTT1*-null genotype (43.50 ± 4.10 vs. 45.20 ± 3.90; $P = 0.004$) when comparing control and T2DM patients. Whereas, LDL level showed a significant increase in *GSTT1*-null than the *GSTT1*-present genotype (108.70 ± 16.90 vs. 102.20 ± 12.60; $P = 0.048$). Similar comparison was carried out among the T2DM and T2DM with CAD patients (Table 5). Although the *GSTM1*-null polymorphism did not show any association with lipid profiles, *GSTT1*-null polymorphism attained a statistical significance for the level of LDL (127 ± 28.20 vs. 134 ± 29.10; $P = 0.039$) and triglycerides in T2DM with CAD patients (182.10 ± 21.10 vs. 191.20 ± 24.10; $P = 0.018$).

4. Discussion

Oxidative stress, resulting from an imbalance between free radicals and anti-oxidant defenses is associated with cellular dysfunctions leading to the pathophysiology of various diseases including diabetes mellitus, atherosclerosis, and cancer [25]. Excessive and highly reactive free radicals produced by hyperglycemia, further exacerbates the progression of diabetic associated cardiac ailments. GSTs are known for their broad range of detoxification processes [26] and in the metabolism of xenobiotics. They are expressed in human vessels and cultures of arteries. Hence, they might play a protective role against the development of atherosclerotic plaques [27]. Recent studies indicate that the risk of CAD is

Table 1
Comparison of GST genotypes among three populations.

	Controls ($n = 270$) n (%)	T2DM without CAD ($n = 222$) n (%)	T2DM with CAD ($n = 290$) n (%)	Association ($df = 2$)	P value
GSTM1					
Null	56 (20.74)	94 (42.34)	128 (44.10)	$\chi^2 = 14.70$	$P < 0.0001$
Present	214 (79.26)	128 (57.76)	162 (55.86)		
GSTT1					
Null	48 (17.80)	70 (31.53)	136 (46.90)	$\chi^2 = 19.40$	$P < 0.0001$
Present	222 (82.20)	152 (68.47)	154 (53.10)		
GSTP1					
Ile/Ile	118 (43.70)	82 (36.90)	94 (32.41)		
Ile/Val	130 (48.14)	112 (50.45)	164 (56.55)		
Val/Val	22 (8.14)	28 (12.62)	32 (11.04)		
Allele A	67.80	62.16	60.70	$\chi^2 = 1.21$	$P = 0.55$
Allele G	32.20	37.84	39.30		

Distributions of the $Ile^{105}Val$ genotypes in control, T2DM and T2DM–CAD were in Hardy–Weinberg equilibrium ($\chi^2 = 1.05$, $P = 0.306$, $\chi^2 = 0.532$, $P = 0.466$ and $\chi^2 = 3.5$, $P = 0.0615$, respectively, calculated by χ^2 goodness-of-fit test).

Table 2
Frequency distributions of GST genotypes and their relationship with the risk of T2DM.

	Controls ($n = 270$) n (%)	T2DM ($n = 512$) n (%)	OR	CI = 95%	P-value
GSTM1					
Null	56 (20.74)	222 (43.36)	1 (Reference)	–	
Present	214 (79.26)	290 (56.64)	2.925	2.078–4.119	$P < 0.0001$
GSTT1					
Null	48 (17.80)	206 (40.24)	1 (Reference)	–	
Present	222 (82.20)	306 (59.76)	3.114	2.176–4.456	$P < 0.0001$
GSTP1					
Ile/Ile	118 (43.70)	176 (34.38)	1 (Reference)	–	
Ile/Val	130 (48.14)	276 (53.90)	1.423	1.041–1.946	$P = 0.027$
Val/Val	22 (8.14)	60 (11.71)	1.829	1.064–3.142	$P = 0.029$

OR – odds ratio and CI – confidence interval from conditional logistic regression analysis.

Table 3
Frequency distributions of GST genotypes and their relationship with the risk of T2DM associated CAD risk.

	T2DM without CAD (n = 222) n (%)	T2DM with CAD (n = 290) n (%)	OR	95% CI	P-value
GSTM1					
Present	128 (57.76)	162 (55.86)	1 Reference		
Null	94 (42.34)	128 (44.10)	1.076	0.756–1.531	P = 0.685
GSTT1					
Present	152 (68.47)	154 (53.10)	1 Reference		
Null	70 (31.53)	136 (46.90)	1.918	1.144–3.214	P = 0.014
GSTP1					
Ile/Ile	82 (36.90)	94 (32.41)	1 Reference		
Ile/Val	112 (50.45)	164 (56.55)	1.277	0.872–1.870	P = 0.208
Val/Val	28 (12.62)	32 (11.04)	0.997	0.554–1.798	P = 0.992

ORs – odds ratio and CI – confidence interval from conditional logistic regression analysis.

Table 4
The relationship between GST genotypes and blood lipids in T2DM patients.

	GSTM1-present (128)	GSTM1-null (94)	P-value*	GSTT1-present (152)	GSTT1-null (70)	P-value*	GSTP1-Ile/Ile (82)	GSTP1-Ile/Val (112)	GSTP1-Val/Val (28)	P-value#
Cholesterol (mg/dl)	131 ± 22.70	132.80 ± 23.50	0.569	133.70 ± 25.70	137 ± 24.90	0.366	134 ± 23.10	133 ± 24.30	135.20 ± 26.50	0.992[a] 0.941[b] 0.904[c]
LDL (mg/dl)	103.2 ± 12.70	102.20 ± 13.60	0.579	102.20 ± 12.60	108.70 ± 16.90	0.005	104.2 ± 15.80	103.20 ± 18.10	106.50 ± 17.30	0.846[a] 0.869[b] 0.639[c]
HDL	44.60 ± 3.80	44.30 ± 3.60	0.550	43.50 ± 4.10	45.20 ± 3.90	0.004	45.10 ± 3.80	44.70 ± 4.30	44.20 ± 4.10	0.783[a] 0.580[b] 0.834[c]
TG	143 ± 16.70	143.50 ± 16.20	0.823	143.50 ± 18.10	144 ± 17.10	0.843	145.20 ± 15.10	143 ± 19.30	146.70 ± 17.10	0.579[a] 0.954[b] 0.584[c]

Values are expressed as ± SD.
* P value – compared by Student's t-test.
# P value determined by Turkey's honestly significant Post-hoc difference test.
[a] P-value Ile/Ile vs. Ile/Val.
[b] P-value Ile/Ile vs. Val/Val.
[c] P-value Ile/Val vs. Val/Val.

Table 5
The relationship between GST genotypes and blood lipids in T2DM with CAD patients.

	GSTM1-present (162)	GSTM1-null (128)	P-value*	GSTT1-present (154)	GSTT1-null (136)	P-value*	GSTP1-Ile/Ile (94)	GSTP1-Ile/Val (164)	GSTP1-Val/Val (32)	P-value#
Cholesterol (mg/dl)	199.20 ± 21	198.80 ± 19.84	0.868	202 ± 21.20	201 ± 19.10	0.673	203.80 ± 19	203.10 ± 21.30	204.80 ± 21.10	0.910[a] 0.989[b] 0.905[c]
LDL (mg/dl)	127 ± 27.10	125.5 ± 26.20	0.634	127 ± 28.20	134 ± 29.10	0.039	125.50 ± 29.40	125 ± 28.60	132 ± 28.80	0.952[a] 0.585[b] 0.426[c]
HDL	41 ± 4.20	40.30 ± 3.60	0.128	39.80 ± 4.20	39.70 ± 3.80	0.832	40.10 ± 4.10	40.30 ± 3.80	39.90 ± 3.60	0.973[a] 0.931[b] 0.856[c]
TG	183.10 ± 23.20	182 ± 23.40	0.690	182.10 ± 21.10	191.20 ± 24.10	0.018	185.30 ± 21.40	184.40 ± 23.60	186.30 ± 21.70	0.890[a] 0.993[b] 0.903[c]

Values are expressed as ± SD.
* P value-compared by Student's t-test.
# P value determined by Turkey's honestly significant Post-hoc difference test.
[a] P-value Ile/Ile vs. Ile/Val.
[b] P-value Ile/Ile vs. Val/Val.
[c] P-value Ile/Val vs. Val/Val.

modulated by variants of *GSTM1/T1* genes in smokers [20] and patients with diabetes [12]. In our study we cautiously eliminated the smokers and alcoholics, owing to the fact that their different physiologic status could affect the output of the study.

In this study the *GSTM1*, *GSTT1*, and *GSTP1* gene polymorphisms in T2DM patients with or without CAD and healthy controls were investigated. A statistically significant association was found for *GSTM1*, and *GSTT1*-null variants and the development of diabetes by regression analysis. A risk for diabetes could also be found for *GSTP1* $Ile^{105}Val$ and $Val^{105}Val$ variants when compared to control.

Dyslipidemia is the major factor for the development of diabetes as well as CAD. Thus, we examined the association between the genotype and lipid profile with the T2DM risk. In this analysis, among T2DM patients the LDL level showed significant increase in the *GSTT1*-null population which is consistent with the earlier study [11]. However, there was also a significant increase in the HDL level compared to the control group. Though the reason for increased level of HDL remains uncertain, the higher level of LDL might be one of the factors, why the *GSTT1*-null population is prone to diabetes. This result has supplemented the support for the earlier study which showed the irrelevancy of HDL and LDL cholesterol levels [28].

In quest of CAD risk in T2DM subjects, we found that the frequency of *GSTT1*-null showed higher indices of CAD risk compared with T2DM subjects in regression analysis. Although statistical analysis on the lipid profiles showed no correlation, the triglyceride and HDL levels in *GSTT1*-null polymorphic samples showed an increased value in T2DM–CAD patients. Hypertriglyceridemia is a theoretical risk factor for CAD and increased production of atherogenic factors lead to the development of coronary heart disease [29]. We therefore hypothesize that the *GSTT1*-null genotypes may enhance the risk of susceptibility to atherogenesis. Considering previous studies [15,30] the *GSTT1*-null polymorphic carriers of our study may have low GSTT1 activity which leads to decreased ROS removal, increased pro-inflammatory factor production and impaired atheroprotective properties, thus making them prone to atherosclerosis. We conclude that *GSTT1* allele play a pivotal role in the development of CAD in T2DM patients. *GSTM1/T1* and *GSTP1* gene polymorphisms play a vital role in the development of only T2DM. Although, this study represents a small sample size of a South Indian ethnic group; the results could nevertheless be used as a baseline for further studies with different ethnicity and environmental conditions that give competitive results.

Acknowledgments

We thank University Grants Commission (UGC), New Delhi, India, for the funding through CEGS, CAS and UPE schemes. T. Ramprasath expresses his gratitude to the UGC for the award of Genomics-Meritorious Research Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.097.

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