Association of Type 2 Diabetes Mellitus and Glutathione S Transferase (GSTM1 and GSTT1) Genetic Polymorphism

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Abstract: Oxidative stress, arising as a result of an imbalance between free radicals and anti-oxidant defenses, is associated with damage to lipids, proteins and nucleic acids, which could contribute to cellular dysfunctions leading to the pathophysiology of various diseases including atherosclerosis, cancer, and diabetes mellitus. Glutathione S-transferases (GSTs) belongs to a group of multigene and multifunctional detoxification enzymes, which defend cells against a wide variety of toxic insults. An important condition to assess whether the glutathione S-Transferase T1 (GSTT1) and M1 (GSTM1) genotypes are associated with type 2 diabetes mellitus and to ascertain whether the levels of blood lipids given exposure to diabetes are modified by the specific genetic polymorphisms of GSTT1 and GSTM1. Using a multiplex polymerase chain reaction, GSTT1 and GSTM1 gene polymorphisms were analy²³zed in 29 patients with type 2 diabetes mellitus compared to 16 healthy age and sex matched control group. The association between genotypes and blood lipids were assessed separately for all the study subjects (type 2 diabetes mellitus group and the control group) with GSTT1 null and also for GSTM1 null compared to GSTT1 present and GSTM1 present genotypes respectively The proportion of GSTT1 null genotypes was higher in diabetic patients as compared to controls (17.24% versus 6.25%). No significant difference of the frequency of GSTM1 null was observed between cases and controls (58.6% versus 62.5.%). The GSTT1 present genotype conferred a statistically significant 0.39 fold reduction in risk of type 2 diabetes mellitus relative to the null genotype of the GSTT1 genotype but the GSTM1 genotype did not differ with respect to their association with risk of type 2 diabetes mellitus. Among individuals with GSTT1 null and GSTM1 null, the serum cholesterol, triglycerides and high density lipoprotein were not significantly different from GSTT1 present or GSTM1 present genotypes. GSTT1 gene polymorphisms may play an important role in type 2 diabetes mellitus pathogenesis. The potential role of GSTM1 polymorphism as a marker of susceptibility to type 2 diabetes mellitus needs further studies in a larger number of patients. GSTT1 and GSTM1 null genotype do not have an effect on blood lipids.

Key words: Type 2 diabetes mellitus, Glutathione S transferase, Genetic polymorphism.

INTRODUCTION

Oxidative stress, arising as a result of an imbalance between free radicals and anti-oxidant defenses, is associated with damage to lipids, proteins and nucleic acids, which could contribute to cellular dysfunctions leading to the pathophysiology of various diseases including atherosclerosis, cancer, and diabetes mellitus[1]. Beta cells are very sensitive to cytotoxic stress because they express very little of the antioxidant enzymes. Hence, beta-cell is at greater risk of oxidative damage than other tissues with higher levels of antioxidant protection[2]. During pathogenesis of diabetes mellitus, oxidative and nitrosative stresses contribute to the destruction of insulin-producing beta-cells. Moreover, it is believed that increased oxidative stress is one of the main factors in the etiology and complications of diabetes mellitus[3].

Glutathione (GSH) is the major cellular antioxidant that protects against environmental toxicants as well as reactive oxygen species (ROS) mediated cell injury. GSH detoxifies ROS, reduces peroxides and detoxifies multiple compounds through glutathione-S-transferase (GST) conjunction[4]. Glutathione S-transferases (GSTs) belong to a group of multigene and multifunctional detoxification enzymes, which defend cells against a wide variety of toxic insults from chemical, metabolites, and oxidative stress[3]. An important condition affecting GST expression is oxidative stress, usually observed in diabetes[5]. Saito-Yamanaka *et al.*[6] found decreased GST activity in the liver of streptozotocin – induced diabetic rats as compared with

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normal rat livers. These data point to the fact that GSTs may offer protection against diabetes mellitus. The gene expressing GST enzymes is polymorphic and therefore, it is possible that individual variations in metabolic activities of each enzyme may regulate the clearance of toxic DNA intermediates and may be partially responsible for individual host susceptibility to oxidative stress damage of beta-cells[7].

The GST isoenzymes expressed in human tissues comprise alpha, mu, pi, theta, Kappa, sigma, zeta and omega gene families. Because many GST genes are polymorphic, there has been considerable interest in determining whether particular allelic variants are associated with altered risk of a variety of pathologies including cancers and cardiovascular and respiratory diseases[8].

Three of the GST genes, GSTM1, GSTT1 and GSTP1 have been found to have functional polymorphisms that are frequently present in the general population. Expression of GST alpha, mu, pi and theta in the pancreas, varies by cell type. For example expression of GST mu and GST alpha have been reported in human islet tissue. The GST mu null gene polymorphism (GSTM1) has been associated with chronic pancreatitis, leukemia and other cancers, rheumatoid arthritis and asthma. The GST theta null gene polymorphism (GSTT1) has been reported to be associated with breast cancer and colorectal cancer[4]. Some studies indicated that genetic variations of GSTTT enzyme are associated with the development of end-stage renal disease in diabetes mellitus patients[9].

GSTM1 and GSTT1 polymorphisms are the most common polymorphisms of GST enzymes in the human population with major ethnic differences and have been studied most extensively in many studies. Five mu class genes are situated (GSTM1 – GSTM5) on chromosome 1. Polymorphism has been identified in the GSTM1 are GSTM1*0, GSTM1*A and GSTM1*B. GSTM1*0 is deleted and homozygote (GSTM1-null genotype) express no protein and leads to absence of phenotypic activity, GSTM1-positive genotype, namely GSTM1*A and GSTM1*B, differ by one base, and the catalytic effectiveness of the enzymes encoded by these alleles is similar. The homozygous GSTM1-null genotype has attracted interest because its frequency varies from 45% to 50% in different populations[10].

There are two theta class genes, GSTT1 and GSTT2, located on chromosome 22. GSTT1 is represented by two alleles: a functional or wild allele (GSTT1*1) and a nonfunctional or null allele (GSTT1*0). The homozygous genotype for the null allele has been defined as GSTT1*0, and the genotype with at least one functional allele has been denoted as GSTT1*1. The GSTT1*0 frequency ranges from 16% to 38% of the overall population[11].

To facilitate understanding the multifactorial causes of diabetes mellitus, it is reasonable to study whether genetic polymorphism of antioxidant enzymes contribute to the pathogenesis of diabetes mellitus. We hypothesized that if environmental toxicants and oxidative stress are associated with type 2 diabetes mellitus then GSTs, may be a modifying factor that contributes to type 2 diabetes progression and thus the GST M1 and GSTT1 null genotypes will be associated with type 2 diabetes mellitus.

The aim of this study is to investigate the distribution of GSTM1, GSTT1 polymorphisms in patients with type 2 diabetes mellitus and controls in order to explore the possible association between GST variants and the occurrence of type 2 diabetes mellitus and also to evaluate the role of these polymorphic genes as a genetic risk modifier in the etiology of type 2 diabetes mellitus and the levels of blood lipids.

Specimen Collection: Ten cc venous blood was withdrawn from the anticubital vein and was divided into two parts:
- Two ml was collected in a tube containing ethylenediamine tetraacetate (EDTA) as an anticoagulant for determination of glycosylated haemoglobin (Hb A1c) for assessment of glyceamic state.
- Two ml was collected in a tube containing ethylenediamine tetraacetate (EDTA) as an anticoagulant for DNA extraction.
- Six cc was collected in a clean dry centrifuge tube. Blood was allowed to clot at $37\ ^0$ C water bath. Clot was separated and centrifuged for 10 minutes at 3000 g. Serum was divided into aliquots and stored at $-20\ ^0$ C until analyzed. Samples were assayed for measurements of Cholesterol, Triglycerides and HDL.

MATERIALS AND METHODS

Methods: Hb A1C was measured with a cation exchange chromatography method assessed glycaemic control. The procedure is a microchromatographic methodology for the quantitation of glycosylated haemoglobin (non- diabetic reference 5.5 % - 7.7%) (GLYCO Hb Quick column procedure)[12].

Genomic DNA was extracted from peripheral venous blood using a salting out protocol, as described by[13]. GSTM1 and GSTT1 genetic polymorphisms were evaluated using multiplex polymerase chain reaction (PCR) technique. The PCR primers were synthesized according to[14]. Primers for GSTM1 were 5' – GAA CTC CCT GAA AAG CTAA AGC and 5' GTT GGG CTC AAA TAT ACG GTG G and for GSTT1 were 5' – TTC CTT ACT GGT CCT CAC

ATC TC and 5' – TCA CCG GACAT GGC CAG CA. The ß – globin locus was used as an internal control to avoid false-negative readings. Primers for ß – globin were 5' – CAA CTT CAT CCA CGT TCA CC and 5' – GAA GAG CCA AGG ACA GGT AC. PCR reaction was carried out in a total volume of 25 ul containing 10 pmol of each primer, 2.5 mmol / L of MgCl2, 0.2 mmol/L of each deoxynucleotide triphosphate, 1 unit of Taq polymerase. And 100 ng of genomic DNA. Amplification was performed by initial denaturation at 94 ^{0} C for 5 minutes, followed by 30 cycles at 94 ^{0} C for 1 minute, 64 ^{0} C for 1 minute and 72 ^{0} C for 1 minute and a final extension of 72 ^{0} C for 7 minutes. The amplified products were identified by electrophoresis in a 1.5% agarose gel and stained with 0.5 ug/ml ethidium bromide. The product lengths were 215 bp, 480 bp, and 268 bp for GSTM1, GSTT1 and ß – globin, respectively. Absence of PCR product for GSTM1 or GSTT1 in the presence of the ß – globin band was indicative of a null genotype for GSTM1 or GSTT1. Individuals with one or two copies of the relevant gene were classified as a positive genotype and individuals with homozygous deletions as a null genotype.

Serum total cholesterol[15] and triglycerides[16] were measured using enzymatic methods; HDL-cholesterol[17] was measured with the phosphotungestic acid-magnesium precipitation method. LDL by the[18,17].

Statistical Analysis: Age of the patient and the control group were compared with student's t test. The chi-square test was applied to compare differences in gender between patients and controls. GSTT1 and GSTM1 genotypes were classified as either null (homozygous deletion) or non-deleted. Odds ratio (OR) with 95% confidence limits calculated by logistic regression was used to analyze the occurrence of frequencies of the GSTM1 and GSTT1 genotypes. P-values were two-tailed and a value of < 0.05 was considered statistically significant. All analyses were performed using SPSS v. 11.5 statistical analysis software.

RESULTS AND DISCUSSION

Results: Table 1 shows the demographic data of the patients and the control group.

The mean age of the control group was 53.19 ± 9.89 years, 4 of them (25 %) were males and 12 of them (75 %) were females. The mean age of the diabetes mellitus group was 58.48 ± 7.22 years, 19 of them (65.52 %) were females and 10 of them (34.46 %) were males. The groups were not statistically different with respect to age and gender (p > 0.05). Diabetic cases had higher levels of glycosylated hemoglobin compared to the control group (p<0.001).

The frequencies of GSTM1 and GSTT1 – null genotypes were 17.24 % and 58.62 % respectively in diabetic patients. The proportion of GSTT1 null genotypes was higher in diabetic patients as compared to controls (17.24% versus 6.25%). No significant difference of the frequency of GSTM1 null was observed between cases and controls.

The GSTT1 present genotype conferred a statistically significant 0.39 fold reduction in risk of type 2 diabetes mellitus relative to the null genotype of the GSTT1 genotype (OR 0.39, 95% CI 0.16 – 0.80) (p < 0.05). But the GSTM1 genotype did not differ with respect to their association with risk of type 2 diabetes mellitus., after controlling for major confounders including age and sex (OR: 1.18, 95% CI: 0.34 – 4.16) (p>0.05) (Table 2).

Considering the possible additive effect of different GST genotypes, we also examined the association between the genotype profile and type 2 diabetes mellitus risk. The risk of type 2 diabetes mellitus was not significantly different in subjects with combinations of GSTM1 and GSTT1 (null and positive genotypes) (Table 3).

The association between genotypes and blood lipids were assessed separately for participantants with GSTT1 null or GSTT1 null in both diabetic patients and control population. Among individuals with GSTM1 null and GSTT1-null, the serum cholesterol, triglycerides and high density lipoprotein were not significantly different from GSTM1 present or GSTT1 present genotype (Table 4 and Table 5).

Discussion: Type 2 diabetes mellitus is characterized by being a polygenic disorder and generally thought of as a syndrome, rather than a single specific entity. This suggests that a common adverse force is exerted on beta-cells in all patients. One such force is the oxidative stress. Oxidative phosphorylation during anaerobic glycolysis generates reactive oxygen species (ROS). The islet is unusually at risk for damage by pro-oxidant forces, because it expresses very low levels of antioxidant mRNA, protein, and activity. GSTs can work as endogenous antioxidants to protect cells from oxidative stress. The GSTs catalyze the conjugation of glutathione to a wide range of electrophiles and represent a protective mechanism against oxidative stress. The GST family of genes is critical in the protection of cells from ROS because they utilize as substrates a wide variety of products of oxidative stress[3].

Most genetic polymorphisms do not cause an identifiable change in the organism in which they occur. However, some either cause disease outright or alter disease susceptibility. A large number of studies

Table 1: Characteristics and Genotype Distribution of the Study Population

	Control Group	Type 2 DM Group	P valueNumber
Number	16	29	
Gender			
Male N (%)	4(25)	10 (34.48 %)	> 0.05
Female N (%)	12 (75)	19 (65.52%)	NS
Age (years)	53.19 ± 9.89	58.48 ± 7.22	> 0.05 NS
Hypertension N (%)	___	13 (44.32 %)	
Duration of Diabetes	____	7.24 ± 5.6	
Cardiac N (%)	____	2 (6.70 %)	
Family History N (%)	____	14 (48.28 %)	
Consanguinity N (%)	_____	6 (20%)	
HbA1c (%)	5.1 ± 0.22	9.92 ± 2.4	< 0.001
GSTT1 null (%)	1 (6.25%)	5 (17.24 %)	
GSTM1 null (%)	10 (62.5 %)	17 (58.62 %)	

Table 2: Association between Glutathione S Transferase (GST) genotypes And risk of type 2 Diabetes mellitus Development among the studied population

Genotype	Control Group (N = 16)	Type 2 DM(N= 29)	OR	95% CI	P value
GSTM1 [a]					
Positive N (%)	6 (37.5 %)	12 (41.38 %)	1.0	Reference	NS
Null N (%)	10 (62.5 %)	17 (58.62 %)	1.18	0.34 – 4.16	
GSTT1 [a]					
Positive N (%)	15 (93.75 %)	24 (82.76 %)	1.0	Reference	P<0.05
Null N (%)	1 (6.25 %)	5 (17.24 %)	0.39	0.16 – 0.80	

OR: odds ratio   CI: Confidence interval from binary logistic regression

[a] : Carriers of at least one intact allele are used as reference

Table 3: Association between Type 2 Diabetes Mellitus Development And Combinations of GSTM1 and GSTT1 Genotypes among the studied population

Genotype Combination						
GSTM1	GSTT1	Control GroupN =16	Type 2 DM group N 29	OR	95 %CI	P value
Positive	Positive	5 (31.25%)	7 (24.14 %)	0.71	0.18 – 2.77	NS
Positive	Null	1 (6.25 %)	5 (17.24 %)	0.86	0.45 – 1.90	NS
Null	Positive	10 (62.5 %)	17 (58.62 %)	0.73	0.21 – 2.55	NS

OR: odds ratio   CI: Confidence interval from binary logistic regression

Type 2 DM: Type 2 diabetes mellitus

Table 4: The relationship between GSTT1 genotype and blood lipids In diabetic patients

	Null	Present	P value
N	(5)	(24)	
Cholesterol (mg/dl))	201.6 ± 9.5	194.3 ± 4.6	NS
Triglycerides (mg/dl)	188.6 ± 9.2	176.5 ± 17.8	NS
HDL (mg/dl)	46.8 ± 47.3	45.7 ± 6.0	NS
LDL (mg/dl)	116.9 ± 9.9	112.4 ± 6.5	NS

HDL: high density lipoprotein cholesterol   LDL: low density lipoprotein cholesterol

Table 5: The relationship between GSTM1 genotype and blood lipids In diabetic patients

	Null	Present	P value
N	(17)	(12)	
Cholesterol (mg/dl))	200.8 ± 8.2	194.8 ± 9.6	NS
Triglycerides (mg/dl)	201.6 ± 3.1	181.2 ± 11.7	NS
HDL (mg/dl)	47.3 ± 3.5	46.2 ± 5.8	NS
LDL (mg/dl)	115.6 ± 7.4	112.6 ± 10.5	NS

HDL: high density lipoprotein cholesterol   LDL: low density lipoprotein cholesterol
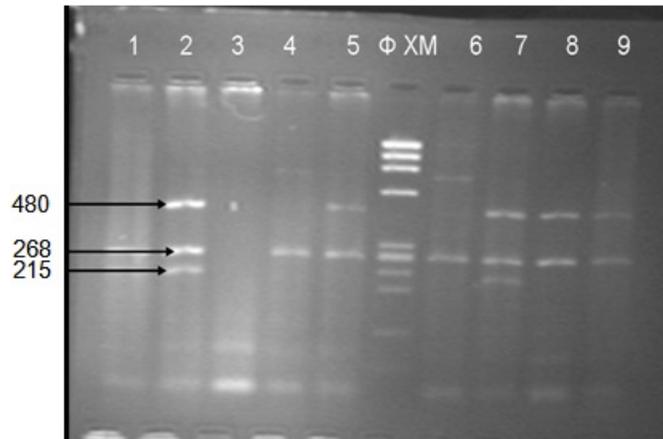
Fig. 1: 1.5% Agarose gel stained with ethidium bromide illustrating different band sizes in the control group Lane 1 homozygous deletion for GSTT1, Lanes (2 & 7) heterozygous for GSTT1 and GSTM1. Lanes (8&9) homozygous deletion for GSTM1.In Lane 3 there was no band due to failure in DNA extraction or in the PCR process.
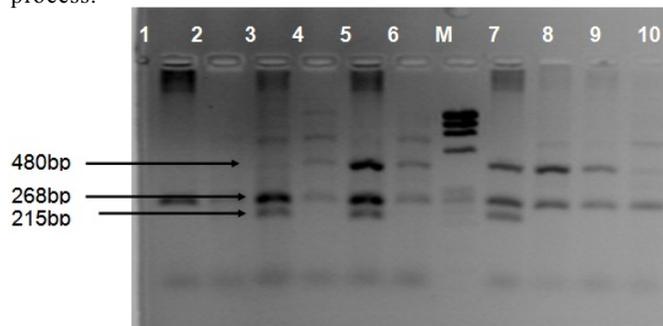

Fig. 2: This figure shows PCR products of Type 2 diabetes mellitus. The product lengths were 215bp for GSTM1, 480bp for GSTT1 and 268bp for beta globin. Lanes (3, 5 & 7) heterozygous for GSTT1 and GSTM1. Lanes (8&9) homozygous deletion for GSTM1. Lanes (1, 2 &10) absence of PCR product for GSTM1 or GSTT1 in the presence of beta globin band was indicative of a null genotype for GSTM1 or GSTT1. lane M is ΦXM.

have attempted to show links between the susceptibility to diseases and GST polymorphic variants[19].

The aim of this study is to investigate the GSTT1 and GSTM1 gene polymorphism in the patients with type 2 diabetes mellitus compared to healthy individuals. Also to evaluate the role of these polymorphic genes as a genetic risk modifier in the etiology of type 2 diabetes mellitus and the levels of blood lipids given exposure to diabetes.

As known, beside detoxifying exogenous electrophilic xenobiotics, GST enzymes including GSTT1 inactivate endogenous end-products formed as secondary metabolites during oxidative stress. It is protective against many toxicants, and it may be important to reach a proper balance between the detrimental and beneficial effects of the enzyme. Altered GST activity due to genetic mutations have been shown to modulate individual's susceptibility to environmental factor induced diseases; including cancers[20].

GSTT1-null genotype (homozygous for non-functional allele) has a decreased capability in detoxifying some carcinogens and oxygen metabolites. It is possible, therefore, that the effect of the GSTT1 genotype is the same in the development of cancer and diabetes mellitus. Therefore, it might be indicated that the GSTT1- present genotype is protective against diabetes mellitus type 2. The genetic contribution of GSTs to diabetes has been previously investigated, GST polymorphisms have been reported to be associated with trogitazone response in type 2 diabetes[21]. Another study reports lack of a direct association between GSTT1 and type 2 diabetes mellitus[22].

This study revealed that type 2 diabetes mellitus as a whole had a higher prevalence of the GSTT1-null genotype than controls. GSTT1-present genotype was associated with a 0.39-fold decreased risk of having type 2 diabetes mellitus relative to the GSTT1-null. Our results agree with those of Wang *et al*,[3] and Hori *et al*.,[23].

In the present study we have found that the frequency of the GSTM1 genotype was not statistically significantly higher in type 2 diabetes mellitus group as compared to the control group. Our study showed that the frequency of GSTM1 positive individuals was 41.38 % in type 2 diabetes mellitus and 37.5 % in the controls. There was no evidence of association between GSTM1 and type 2 diabetes mellitus in the studied population. This study supports the findings by Wang *et al*,[3], where 60 % of type 2 diabetes mellitus cases were GSTM1 positive and 56 % of the control group. Although, the majority of case control studies of GSTM1 and GSTT1 null genotypes have reported risk associations with disease[24,25]. A few studies have reported associations with disease protection[26,27]. For example, alcohol induced chronic pancreatitis protection is associated with the GSTM1 null genotype[26]. The results of this study regarding GSTM1 genotype prevalence in type 2 diabetic subjects is disagrees with the results of Hori *et al*.,[24] who stated that the incidence of type 2 diabetes mellitus was 1.5 fold higher in the GSTM1 null genotype.

Many factors may account for the different results in similar studies. First, it may reflect the differences of ethnic, genetic and environmental background of the populations studied. For instances, GSTT1 deficiency is less frequent than GSTM1 deficiency, but in both cases the frequency in the population varies among different ethnic groups. There may be differences even in the same population because of genetic and environmental factors. Second, the differences in number of the subjects studied in genetic researchers may also lead to different outcome. Third, methodological issues should also be considered[28].

Several members of the GST family exhibit selenium-independent glutathione peroxidase activity, which plays an important role in protecting cells against lipid and nucleotide hydroperoxides[3]. Some investigators have observed a variety of associations between asthma, cancer and diabetic nephropathy, and GST gene polymorphisms. But little is known about the effect of GST gene polymorphisms on blood lipids.

In the present study, we also compared serum cholesterol, triglycerides, high density lipoproteins in both diabetic subjects and the control group for GSTT1 and GSTM1 genotypes (null compared to present genotypes). Among individuals with GSTM1 null and GSTT1-null, the serum cholesterol, triglycerides and high density lipoprotein were not significantly different from GSTM1 present or GSTT1 present. Our results agree with Wang *et al*,[3].

Conclusion: Our results suggest that GSTT1 gene polymorphisms may play an important role in type 2 diabetes mellitus pathogenesis. GSTT1 and GSTM1-null genotypes do not have an effect on blood lipids given exposure to diabetes mellitus.

Recommendation: Although, some of our data were statistically significant, we acknowledge that the findings presented here are preliminary because of the small number of subjects and that the study requires confirmation in a separate larger cohort.

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