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Glutathione S-transferase M1, T1 and P1 gene polymorphisms and the risk of developing type 2 diabetes mellitus in Egyptian diabetic patients with and without diabetic vascular complications

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Abstract *Background and aim of work:* Persistent oxidative stress is one of several factors that participate in the pathogenesis of type 2 diabetes mellitus (T2DM). Glutathione S-transferases (GSTs) are a family of antioxidant enzymes that exert important antioxidant roles in the elimination of reactive oxygen species. We aimed to assess the association of genetic polymorphisms in the GST isoenzymes M1, T1 and P1 with the risk of developing T2DM and its vascular related complications in Egyptian diabetic patients.

Subjects and methods: Fifty-four T2DM patients of whom twenty-seven were suffering from vascular complications were compared to fifty-one healthy volunteers. Null genotypes in the GST M1 and T1 genes were screened using polymerase chain reaction (PCR). The A313G single nucleotide polymorphism in the GSTP1 gene was detected using PCR–restriction fragment length polymorphism.

Results: No significant differences were noted between diabetic cases and control group regarding frequencies of null genotypes of GSTM1 and GSTT1 genes ($\chi^2 p = 0.631$ and $\chi^2 p = 0.832$, respectively). Furthermore, both null genotypes were not associated with the risk of developing T2DM or its related vascular complications whether alone or in combination. The frequency of the

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heterozygous mutation (AG) in the A313G GSTP1 polymorphism among diabetic cases with and diabetic cases without vascular complications was significantly higher compared to the control group ($p = 0.023$). The risk of developing T2DM was significantly higher in cases presenting with combined heterozygous GSTP1 and null GSTM1 genotypes (Odds ratio = 6.285, 95% confidence interval = 1.184–33.347, $p = 0.021$).

Conclusion: Our results could point out to potential roles of GSTP1 polymorphism alone or combined with GSTM1 gene polymorphism in the pathogenesis of T2DM related oxidative stress. Screening for other functional GST gene polymorphisms is important to understand the impact of interaction of multiple genetic factors in the pathogenesis of T2DM.

1. Introduction

Diabetes mellitus (DM) represents a group of metabolic diseases characterized by hyperglycemia resulting from defects in pancreatic insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels.[1] Diabetes remains a major public health issue. In 2010, it was estimated that 4.787 million Egyptians suffer from diabetes, particularly type 2 (T2DM), and that diabetes will increase to 8.615 million Egyptians by the year 2030.[2,3]

Oxidative stress is one of several mechanisms that contribute in the pathogenesis of T2DM and its related vascular complications. It represents a state of imbalance between pro-oxidants and antioxidant defense system. The hyperglycemia induced overproduction of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical, along with reactive nitrogen species (RNS) such as nitric oxide causes oxidation of DNA, proteins and other cellular components leading to their damage.[4,5] The metabolic abnormalities of diabetes cause increased mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, as well as the myocardium. This causes the activation of major pathways which increase intracellular ROS.[6,7]

Studies have shown that individuals with lowered antioxidant capacity are at increased risk of T2DM.[8,9] Alterations in the endogenous ROS scavenging defense mechanisms may lead to ineffective scavenging of ROS, resulting in oxidative damage and tissue injury.[3] Pancreatic β-cells have emerged as a putative target of oxidative stress-induced tissue damage being sensitive to cytotoxic stress because of their little expression of antioxidant enzymes. This seems to explain in part the progressive deterioration of β-cell function in T2DM.[10]

Different families have been identified in detoxification or reduction of ROS production. Glutathione S-transferases (GSTs) are the most important family of phase II isoenzymes known to detoxify a variety of electrophilic compounds, including carcinogens, chemotherapeutic drugs, environmental toxins, and DNA products generated by ROS damage to intracellular molecules. Detoxification via GSTs is achieved by conjugating them with glutathione. GSTs thus play a major role as cellular antimutagen and in antioxidant defense mechanisms.[11] Two distinct superfamilies of GST isoenzymes exist; one family comprises cytosolic, soluble dimeric enzymes,[12] and the other superfamily is composed of membrane bound trimeric proteins named the membrane associated proteins in eicosanoid and glutathione (MAPEG) metabolism.[13]

Human soluble GSTs collectively account for 4% of total soluble proteins in the liver. They exist as 50 KDa dimeric proteins with both subunits being from the same class of GSTs.[14] Based on sequence similarity, at least eight members of the cytosolic family have been identified in humans named Mu (M), Kappa (K), Alpha (A), Pi (P), Omega (O), Theta (T), Zeta (Z), and Sigma (S).[15]

Among candidate genes related to oxidative stress, genes for cytosolic GSTs, particularly GSTM1, GSTT1 and GSTP1 were intensively studied in different disease states owing to their potential modulating roles in individual susceptibility to environmentally induced diseases. GSTM1 and GSTT1 genes are polymorphic in humans and the null genotypes are accompanied by lack of enzyme activity.[16,17] On the other hand, the GSTP1 single nucleotide polymorphism (SNP) present on exon 5 is characterized by guanine replacing adenine base at position 313 (A313G) of the gene nucleotides. This results in valine replacing isoleucine amino acid at position 105 in the GSTP1 isoenzyme protein. Such replacement results in the appearance of a new allele with alteration in specific activity for substrate compared to wild-type allele.[18]

Several investigators have determined the clinical or genetic factors associated with T2DM with interests to detoxification agents. As regards GSTM1, T1 and P1 isoenzymes, studies on Egyptian,[3] Chinese,[19] and Brazilian[20] populations reported a significant association of the null mutation of GSTT1 gene and T2DM, whereas in studies involving Turkish,[21,22] North Indian,[23] and Southern Iran[24] populations this association was observed between GSTM1 deletion and T2DM. Recently, studies conducted on Japanese[25] and South Indian population[26] as well as another meta-analysis study involving Asian, European and African diabetic populations[27] reported the association of both GSTM1 and GSTT1 null genotypes with the risk of developing T2DM. The North Indian[23] and another Egyptian study conducted in T2DM[28] were the only ones demonstrating a significant association of the GSTP1 SNP (A313G) with T2DM.

The different ethnic backgrounds creating such a controversy in results and scarcity of GST genetic studies conducted among Egyptian T2DM patients invited us to carry out this case-control study to assess the frequency of GSTM1, GSTT1 and GSTP1 genotypes in T2DM and explore any possible relation(s) between the genotypes and the risk of developing T2DM and its related vascular complications. To the best of our knowledge, the current study is one of few studies address-

ing the GSTP1 point mutation as well as the GSTM1 and GSTT1 gene polymorphisms among Egyptian T2DM patients.

2. Subjects

Informed consents were obtained from the one hundred and five individuals enrolled in this study. The Research Ethics Committee of the Medical Research Institute approved the study protocol. Fifty-four diabetic patients were selected from the Internal Medicine department of the Institute, with twenty-seven of them suffering from T2DM related vascular complications at the time of the study. Fifty-one apparently healthy volunteers obtained from the outpatient clinics of the Institute served as a control group. Cases suffering from any type of malignancy as well as bronchial asthma, hypertension preceding T2DM, cardiac, primary renal and liver diseases were excluded from this study.

3. Methods

3.1. Clinical examination and anthropometric measurements

To all the studied subjects, a thorough history was taken with stress on the duration of diabetes, as well as T2DM related vascular complications. Physical examination was done with stress on diabetes related vascular complications. Blood pressure was recorded. Ultrasonographic evaluation of the liver and kidneys was done. A slit lamp fundus examination to document retinopathy and a 12 lead standard electrocardiogram to document diabetic ischemic changes were done. Anthropometric measurements, namely body weight and height along with the calculation of body mass index (BMI) were done. Ultrasonographic determination of the right and left carotid arteries intima media thickness (CIMT) was done using a β-mode ultrasound to detect peripheral atherosclerotic changes.[29]

3.2. Laboratory investigations

3.2.1. Biochemical analysis

Following a twelve hour fasting period, concomitant venous blood samples and early morning midstream urine specimens were obtained from every participant. Fasting serum samples were used for the determination of concentrations of glucose, creatinine, total cholesterol and its high density fraction, triglycerides, and activity of alanine aminotransferase enzyme. Determination of urinary albumin and creatinine concentrations were done in the urine sample. Biochemical analysis was conducted on the Olympus AU400 clinical chemistry analyzer (Beckman Coulter Inc, Brea CA, USA). Calculations of serum low density lipoprotein fraction using Friedwald's formula and urinary albumin to creatinine ratio (ACR) were done.[30,31] Whole blood percent glycated hemoglobin (HbA_{1c}) value was determined using an ion exchange column chromatographic technique (Biosystems SA, Barcelona, Spain) according to the manufacturer's instructions.

3.2.2. Genomic analysis

Whole EDTA blood was used for genomic DNA extraction from peripheral mononuclear cells using a GeneJET™ column based genomic DNA purification kit (Fermentas, Thermo Fischer Scientific Inc., USA) according to the manufacturer's instructions. The integrity of the extracted DNA was assessed qualitatively by electrophoresis on a 1% agarose gel. Quantitative determination of concentration and purity of DNA was done using the NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, Delaware USA).

The A313G SNP of the GSTP1 gene was determined using a PCR – restriction fragment length polymorphism (RFLP) according to the method described by Harries LW et al. (1997).[32] Briefly, 12 μL (50–150 ng) of genomic DNA were mixed in a 0.2 mL sterile eppendorf tube with 0.2 μL of each forward (5′-ACCCCAGGGCTCTATGGGAA-3′) and reverse (5′-TGAGGGCACAAGAAGCCCCT-3′) primers (Fermentas – Thermo Fischer Scientific Inc., USA) in concentrations of 5 pmol per reaction tube, 12.5 μL DreamTaq™ Green PCR Master Mix (2×) (Fermentas – Thermo Fischer Scientific Inc., USA), and completed to a final reaction volume of 25 μL using nuclease free sterile water. The PCR thermal cycler (Quanta Biotech, UK) conditions were as follows; a 5 min initial denaturation phase at 95 °C, followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 30 s), and a final elongation step of 5 min at 72 °C. The resulting 176-bp fragment, generated by PCR, was electrophoretically separated on a 2% agarose gel and visualized by ethidium bromide staining to confirm its presence. The PCR product was subjected to an RFLP using an Alw261 restriction endonuclease (Fermentas – Thermo Fischer Scientific Inc., USA). The digestion reaction was carried out in a 1.5 mL sterile eppendorf tube, where 5 μL of PCR product were mixed with 0.5 μL enzyme, 1 μL enzyme buffer, and completed to a final reaction volume of 15 μL with nuclease free sterile water. The mixture was incubated at 37 °C for one hour using a thermomixer (Eppendorf AG Hamburg, Germany). The digestion products electrophoretically separated on a 2% agarose gel revealed one of three possibilities; a single undigested band at 176 base pairs indicating the presence of a homozygote AA allele (wild type), the presence of a restriction site resulting in two fragments (91 and 85 base pairs) indicating the presence of a GG homozygote mutant allele, and lastly three bands (176, 91 and 85 base pairs) indicating the presence of an A/G heterozygote mutant allele.

Screening for deletions in the GSTM1 and GSTT1 genes was done using PCR according to the method described by Bid HK et al. (2010) for both genes.[23] Briefly, 12 μL of genomic DNA (50–150 ng) was mixed in a 0.2 mL sterile epprendorf tube with 0.2 μL of forward and reverse primers (Fermentas – Thermo Fischer Scientific Inc., USA) in concentrations of 5 pmol per reaction tube, 12.5 μL DreamTaq™ Green PCR Master Mix (2×) (Fermentas – Thermo Fischer Scientific Inc., USA), and completed to a final reaction volume of 25 μL using nuclease free sterile water. An internal control was used with every reaction in a multiplex manner to verify the successfulness of PCR composed of forward and reverse primers that amplify exon-7 of the CYP1A1 gene. Details of primer sequences and thermocycler (S96 Quanta Biotech, UK) conditions are available in Table 1. The PCR products were visualized using electrophoretic separation on a 2% agarose gel. PCR products representing GSTM1 and GSTT1 positive genotypes yielded bands of 215 and 480 bp, respectively, while the internal positive control (CYP1A1) PCR product band corresponded to 312 bp. Such genotyping approach did

Table 1 Primer sequences and cycler conditions for amplification of GSTM1 and GSTT1 genes.

Genes	Primers	Cycler conditions		
GSTM1	Forward 5′-GAACTCCCTGAAAAGCTAAAGC-3′ Reverse 5′-GTTGGGCTCAAATATACGGTGG-3′	GSTM1 35 Cycles	Initial Denaturation Denaturation Annealing Extension Final elongation	95 °C–5 min 94 °C–30 s 55 °C–30 s 72 °C–30 s 72 °C–10 min
GSTT1	Forward 5′-TTCCTTACTGGTCCTCACATCTC-3′ Reverse 5′-TCACGGGATCATGGCCAGCA-3′	GSTT1 35 Cycles	Initial Denaturation Denaturation Annealing Extension Final elongation	94 °C–5 min 94 °C–1 min 59 °C–1 min 72 °C–1 min 72 °C–10 min
CYP1A1 (Exon-7)	Forward 5′-GAACTGCCACTTCAGCTGTCT-3′ Reverse 5′-CAGCTGCATTTGGAAGTGCTC-3′			

not allow for detecting heterozygous carriers of GSTM1 or GSTT1 deletion; hence, the GSTM1-0 or GSTT1-0 genotype group included only patients homozygous for GSTM1 or GSTT1 deletion. The GSTM1-1 or GSTT1-1 genotype group included homozygous and heterozygous carriers of the functional allele.

3.3. Statistical analysis

Statistical analysis of data was performed using statistical packages of Social Science (SPSS) version 20 (SPSS, Inc., Chicago, IL, USA).[33] Data were coded and fed to the SPSS software package. D'Agostino–Pearson *K*-squared test for normality was used to test for the degree of deviation from normal distribution across all quantitative variables in all groups and subgroups. For normally distributed variables, descriptive measures namely mean and standard deviation were applied and independent samples *t*-test for comparison between groups. For abnormally distributed quantitative variables, descriptive measures namely median, and range were applied, and Mann–Whitney test for comparison between groups. For comparing nominal clinical data variables between groups, Chi-square test with a Monte Carlo estimate of exact *p*-values as well as Fisher's exact test were used depending on the expected frequencies. Being a case control study, Odds ratio was used to measure the impact of GST genotypes or alleles on the risk of developing type 2 diabetes mellitus. Chi-square test for goodness of fit was used to compare the observed frequencies of different GSTP1 genotypes among all subjects to expected frequencies according to Hardy–Weinberg equilibrium equation.[34] A *p*-value less than 0.05 was considered statistically significant.

4. Results

A total number of 105 individuals (54 T2DM patients and 51 healthy volunteers) were genotyped for the three members of the GST family. Screening for the GSTT1, M1 and P1 gene polymorphisms was done using multiplex PCR for the first 2 isoenzymes and PCR-RFLP for the third one. The demographical characteristics, namely age and sex, are summarized in Table 2. The duration of disease in all diabetic cases varied from five to fifteen years. A significantly higher BMI mean value was demonstrated in all diabetic cases compared to the control group (Table 2). Atherosclerosis in diabetics was evidenced by a significantly higher median value of CIMT compared to the control group (Table 2). Also median values of both systolic and diastolic blood pressure were significantly higher in diabetic cases with vascular complications compared to those without vascular complications and control group (Table 2).

Poor glycemic control noted in diabetic cases was evidenced by the significantly higher mean values of whole blood glycated hemoglobin, and serum fasting glucose compared to the control group. Renal affection in cases suffering from diabetic nephropathy was noted by the significantly higher median value of urinary albumin to creatinine ratio in diabetics with vascular complications compared to those without vascular complications and control group (Table 2). The hypertensive state present in diabetics with vascular complications could be an aggravating factor. Furthermore the disturbed lipid pattern secondary to poor glycemic control was also noted in diabetic cases (Table 2).

As regards the GSTs gene polymorphisms, the GSTP1 genotypes in controls and diabetic cases as well as the total subjects were in agreement with Hardy Weinberg equilibrium (Table 3). No significant differences were noted between diabetic patients with and without vascular complications and control group regarding GSTM1 and GSTT1 both inserted and deleted ($p = 0.631$ and $p = 0.832$, respectively) (Table 4). The only difference noted was in the GSTP1 SNP where diabetic cases had a lower wild genotype (AA) and a higher heterozygous (AG) genotype compared to control group with *p*-value approaching statistical significance ($p = 0.053$) (Table 4). Furthermore, in cases with vascular complications the GSTP1 wild (AA) genotype was significantly lower and the heterozygous (AG) genotype was significantly higher compared to cases without vascular complications and control group ($p = 0.023$) (Table 4). When diabetic cases with vascular complications were categorized according to the type of vascular complications, we came across a significantly higher frequency of GSTT1 gene insertion in cases presenting with retinopathy and neuropathy ($p = 0.034$ and $p = 0.019$, respectively) (Table 5). No statistically significant differences were noted in the frequencies of GSTM1, GSTT1 and GSTP1 gene polymorphisms when diabetic cases were categorized according to the presence of one or more of vascular complications of diabetes (Table 6).

Table 2 Some demographical and clinical characteristics, anthropometric and radiological data, as well as biochemical parameters among the studied groups.

Items		Controls (n = 51) mean/median ± SD/Min–Max	Diabetic patients(n = 54) mean/median ± SD/Min–Max	p-value
Gender				
Male		n = 16 (31.4%)	n = 20 (37.0%)	$\chi^2 p = 0.541$
Female		n = 35 (68.6%)	n = 34 (63.0%)	
Age	(years)	43.37 ± 11.07	49.96 ± 8.99	$^{t}p1 = 0.001^{*}$
BMI	(kg/m^2)	27.39 ± 2.8	30.56 ± 3.97	$^{t}p1 < 0.001^{*}$
CIMT	(cm)	0.44 (0.3–0.62)	0.58 (0.40–2.30)	$^{MW}p1 < 0.001^{*}$
Duration of disease	(years)	–	10.5 ± 5.49	–
Whole blood HbA_{1c}	(%)	4.84 ± 0.38	8.52 ± 2.25	$^{t}p1 < 0.001^{*}$
Serum (Fasting)				
Glucose	(mg/dL)	90 (74–99)	168 (78–448)	$^{MW}p1 < 0.001^{*}$
Creatinine	(mg/dL)	0.9 (0.6–1.2)	1.0 (0.7–5.1)	$^{MW}p1 < 0.001^{*}$
ALT	(U/L)	15 ± 7	26 ± 12	$^{t}p1 < 0.001^{*}$
Total cholesterol	(mg/dL)	174 ± 22	207 ± 12	$^{t}p1 < 0.001^{*}$
HDL-cholesterol	(mg/dL)	55 ± 12	44 ± 13	$^{t}p1 < 0.001^{*}$
LDL-cholesterol	(mg/dL)	100 ± 19	130 ± 45	$^{t}p1 < 0.001^{*}$
Triglycerides	(mg/dL)	94 ± 31	172 ± 77	$^{t}p1 < 0.001^{*}$
Items	Control group(n = 51)	T2DM without vascular complications(n = 27)	T2DM with vascular complications(n = 27)	$p2/p3/p4$ values
Blood pressure				
Systolic (mmHg)	115 (90–135)	130 (90–135)	140 (110–180)	$^{t}p2 < 0.001^{*}$ $^{t}p3 < 0.001^{*}$
Diastolic (mmHg)	70 (60–85)	80 (60–85)	90 (60–110)	$^{t}p4 < 0.001^{*}$
Urine ACR				
(mg/gm)	11.0(2.0–28.5)	15.7 (2.3–29.6)	60.0 (1.5–1749.0)	$^{t}p2 = 0.153$ $^{t}p3 < 0.001^{*}$ $^{t}p4 < 0.001^{*}$

$p1$-value describes the statistical difference between control group and whole diabetic cases.
$p2$-value describes the statistical difference in urinary ACR between control group and diabetic cases without vascular complications.
$p3$-value describes the statistical difference in urinary ACR between control group and diabetic cases with vascular complications.
$p4$-value describes the statistical difference in urinary ACR between diabetic cases without and with vascular complications.
Abbreviations: ACR, Albumin to creatinine ratio; ALT, Alanine aminotransferase; BMI, body mass index; CIMT, Carotid intima media thickness, HbA1c, Glycated hemoglobin, HDL-C, high density lipoprotein cholesterol, LDL-C, Low density lipoprotein cholesterol.
*A p-value less than 0.001 was considered highly significant.
* A p-value less than 0.05 was considered statistically significant.

Table 3 The agreement of GSTP1 genotypes with Hardy Weinberg (HW) equilibrium.

Genotype	Observed		HW expected		x^2	p-value
	Number	%	Number	%		
Control group						
Wild (AA)	38	74.5	37.1	72.7	1.002	0.316
Heterozygous mutant (AG)	11	21.6	12.8	25.1		
Homozygous mutant (GG)	2	3.9	1.1	2.2		
Diabetic group						
Wild (AA)	29	53.7	30.4	56.3	0.995	0.318
Heterozygous mutant (AG)	23	42.6	20.3	37.6		
Homozygous mutant (GG)	2	3.7	3.4	6.1		
Total subjects						
Wild (AA)	67	63.8	67.2	64.0	0.014	0.902
Heterozygous mutant (AG)	34	32.4	33.6	32.0		
Homozygous mutant (GG)	4	3.8	4.2	4.0		

A p-value less than 0.05 was considered statistically significant.

Table 4 Distribution of GSTM1, GSTT1, and GSTP1 gene polymorphisms among the studied groups.

GSTs genepolymorphisms	Control group (n = 51)		Total number of diabetic cases (n = 54)		Diabetic cases with vascular complications (n = 27)		Diabetic cases without vascular complications (n = 27)		p-value
	Number	%	Number	%	Number	%	Number	%	
GSTM1 gene									$\chi^2 p1 = 0.631$
Present	25	49.0	29	53.7	14	51.9	15	55.6	$\chi^2 p2 = 0.859$
Deleted	26	51.0	25	46.3	13	48.1	12	44.4	
GSTT1 gene									$\chi^2 p1 = 0.832$
Present	33	64.7	36	66.7	16	59.3	20	74.1	$\chi^2 p2 = 0.507$
Deleted	18	35.3	18	33.3	11	40.7	7	25.9	
GSTP1 gene									$^{MC}p1 = 0.053$
Wild (AA)	38	74.5	29	53.7	18	66.7	11	40.7	$^{MC}p2 = 0.023^*$
Heterozygous mutant (AG)	11	21.6	23	42.6	9	33.3	14	51.9	
Homozygous mutant (GG)	2	3.9	2	3.7	0	0	2	7.4	

$p1$: Statistical differences between control group and total number of diabetic cases.
$p2$: Statistical difference between control and each of diabetic cases with and without vascular complications of T2DM.
Abbreviations: MC, Monte Carlo test; χ^2, Chi square test.
* A p-value less than 0.05 was considered statistically significant.

Table 5 Frequency of diabetic patients with the three GST gene polymorphisms according to each vascular complication.

Diabetic complications	GSTM1				GSTT1				GSTP1				p-value
	Present		Deleted		Present		Deleted		Wild type		Heterozygous		
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
CAD													$\chi^2 p1 = 0.082$
Positive	5	35.7	9	69.2	9	56.3	5	45.5	9	50.0	5	55.6	$\chi^2 p2 = 0.581$
Negative	9	64.3	4	30.8	7	43.8	6	54.5	9	50.0	4	44.4	$\chi^2 p3 = 1.000$
PAD													$^{FE}p1 = 1.000$
Positive	3	21.4	2	15.4	5	31.3	0	0.0	3	16.7	2	22.2	$^{FE}p2 = 0.060$
Negative	11	78.6	11	84.6	11	68.6	11	100.0	15	83.3	7	77.8	$^{FE}p3 = 1.000$
CVD													–
Positive	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	
Negative	14	100.0	13	100.0	16	100.0	11	100.0	18	100.0	9	100.0	
Retinopathy													$\chi^2 p1 = 0.568$
Positive	8	57.1	6	46.2	5	68.8	3	27.3	10	55.6	4	44.4	$\chi^2 p2 = 0.034^*$
Negative	6	42.9	7	53.8	11	31.3	8	72.7	8	44.4	5	55.6	$\chi^2 p3 = 0.695$
Neuropathy													$^{FE}p1 = 0.326$
Positive	13	92.9	10	76.9	0	100.0	7	63.6	17	94.4	6	66.7	$^{FE}p2 = 0.019^*$
Negative	1	7.1	3	23.1	16	0.0	4	36.4	1	5.6	3	33.3	$^{FE}p3 = 0.093$
Nephropathy													$^{FE}p1 = 0.695$
Positive	10	71.4	8	61.5	10	62.5	8	72.7	10	55.6	8	88.9	$^{FE}p2 = 0.692$
Negative	4	28.6	5	38.5	6	37.5	3	27.3	8	44.4	1	11.1	$^{FE}p3 = 0.193$

$P1$: statistical differences of GSTM1according to each vascular complication.
$P2$: statistical differences of GSTT1 according to each vascular complication.
$P3$: statistical differences of GSTP1 according to each vascular complication.
Abbreviations: CAD, Coronary artery disease; χ^2, Chi square test; FE, Fisher Exact test; PAD, peripheral arterial disease; CVD, cerebrovascular disease.
* A p-value less than 0.05 was considered statistically significant.

Our results were further analyzed to determine whether different combinations of genotypes from the GST genes in both control and diabetic cases could be involved in the risk of T2DM development. Our study revealed a significantly higher frequency of combined heterozygous (AG) GSTP1 and null GSTM1 genotypes among diabetic cases (Odds ratio = 6.285, 95% confidence interval = 1.184–33.347, p = 0.021) (Table 7).

In diabetic cases with and without vascular complications as well as in the whole group of diabetic cases, no significant associations were noted between the frequencies of GST-M1, -T1 and -P1 gene polymorphisms and each of glycated hemoglobin, fasting serum levels of glucose, triglycerides, total cholesterol, and high and low density lipoprotein fractions of cholesterol (Our unpublished results).

Table 6 The frequency of diabetic patients with GSTM1, GSTT1, and GSTP1 gene polymorphisms according to the presence of one or more vascular complications of diabetes.

GSTs gene polymorphisms	Number of diabetic microvascular and macrovascular complications										*p*-value
	1		2		3		4		5		
	No.	%	No.	%	No.	%	No.	%	No.	%	
GSTM1											
Present	3	21.4	3	21.4	3	21.4	4	28.6	1	7.1	0.921
Deleted	4	30.8	1	7.7	4	30.8	3	23.1	1	7.1	
GSTT1											
Present	2	12.5	3	18.8	3	18.8	6	37.5	2	12.5	0.132
Deleted	5	45.5	1	9.1	4	36.4	1	9.1	0	0.0	
GSTP1											
A allele	11	78.6	7	87.5	12	85.7	13	92.9	2	50.0	0.393
G allele	3	21.4	1	12.5	2	14.3	1	7.1	2	50.0	

A *p*-value less than 0.05 was considered statistically significant.
Monte Carlo test was used for comparing between numbers of complications categories.

Table 7 Distribution of combinations of glutathione S-transferase genotypes among diabetic patients and control group and the risk of developing diabetes mellitus.

Combined GST polymorphisms	Control group ($n = 51$)		Diabetic patients ($n = 54$)		*p*-value	OR	95% Confidence interval
	No.	%	No.	%			
GSTM1 and GSTT1							
Both present ®	16	31.4	22	40.7			
Both deleted	26	51.0	21	38.9	$\chi^2 p = 0.226$	0.587	0.247–1.392
One present + one deleted	9	17.6	11	20.4	$\chi^2 p = 0.832$	0.889	0.298–2.647
GSTM1 and GSTP1							
Present M1 + wild (AA) P1 ®	16	31.4	14	25.9			
Present M1 + hetero mutant (AG) P1	9	17.6	12	22.2	$\chi^2 p = 0.461$	1.523	0.495–4.685
Present M1 + homo mutant (GG) P1	0	0	2	3.7	$^{FE}p = 0.484$	-	-
Deleted M1 + wild (AA) P1	22	43.1	15	27.8	$\chi^2 p = 0.615$	0.779	0.294–2.060
Deleted M1 + hetero mutant (AG) P1	2	3.9	11	20.4	$\chi^2 p = 0.021^*$	6.285	1.184–33.347
Deleted M1 + homo mutant (GG) P1	2	3.9	0	0.0	$^{FE}p = 0.492$	-	-
Present M1 + AG and GG-P1	9	17.6	14	25.9	$\chi^2 p = 0.305$	1.778	0.590–5.354
Deleted M1 + AG and GG- P1	4	7.8	11	20.4	$\chi^2 p = 0.090$	3.142	0.814–12.131
GSTT1 and GSTP1							
Present T1 + wild (AA) P1 ®	24	47.1	23	42.6			
Present T1 + hetero mutant (AG) P1	7	13.7	11	20.4	$\chi^2 p = 0.379$	1.639	0.542–4.960
Present T1 + homo mutant (GG) P1	1	2.0	2	3.7	$^{FE}p = 0.552$	2.087	0.176–24.616
Deleted T1 + Wild (AA) P1	14	27.5	6	11.1	$^{FE}p = 0.152$	0.447	0.146–1.362
Deleted T1 + hetero mutant (AG) P1	4	7.8	12	22.2	$\chi^2 p = 0.070$	3.130	0.881–11.123
Deleted T1 + homo mutant (GG) P1	1	2.0	0	0.0	$^{FE}p = 1.000$	-	
Present T1 + AG and GG-P1	5	15.7	13	24.1	$\chi^2 p = 0.091$	2.713	0.834–8.823
Deleted T1 + AG an GG- P1	5	9.8	12	22.2	$\chi^2 p = 0.124$	2.504	0.762–8.230

Abbreviations: FE, Fisher exact test; OR, Odds ratio; ®, reference group; χ^2: Chi square test; Hetero, heterozygous; Homo, homozygous.
* A *p*-value less than 0.05 was considered statistically significant.

5. Discussion

A number of epidemiological studies have tested possible associations between polymorphisms of the GST isoforms particularly deletions in the GSTM1 and GSTT1 genes (null genotypes) and the GSTP1 A313G SNP with disease risk or therapy outcome in different types of pathologies.[3,35–37]

Several population-based studies have reported a GSTM1 prevalence ranging from 16% to 60%.[38] Asians and Caucasians have the highest frequencies (50–53%) while black populations including Africans, African–American and black populations of Brazil have the lowest ones.[39–41] This was in agreement with our result as 51% of controls showed GSTM1 deletion which was also close to the respective frequencies reported for Middle East Arabs[41] and Egyptians[42] with frequencies in the range of Caucasian healthy populations. Such finding may be explained by the fact that Egyptians belong to the Mediterranean Caucasian race. No significant difference was noted in the frequency of GSTM1 null genotype polymorphism between diabetic patient group and control group (Table 4). Such finding is in agreement with results reported by some studies[20–22] whereas other studies showed a significant association between the frequency of GSTM1 genotype and T2DM.[3,22–24]

As regards the GSTT1 polymorphism among controls in our study, we demonstrated a frequency rate of GSTT1 null genotype (35.3%) that did not vary too much from European and Mediterranean that ranged from 10.4% to 42.5%[38], being the highest among Chinese (64%), followed by Koreans (60%), African–Americans (22%), Caucasians (29%) and Asian–Indians (16%), and the lowest among Mexican–Americans (10%).[43] When considering gender differences in the GSTT1 genotype among our control group, the female (68.6%) to male ratio (31.4%) was high which might explain the higher frequency of GSTT1 deletion among female controls. A meta-analysis study did report a significantly higher frequency of GSTT1 deletion among healthy Caucasian females, yet was not able to explain it on biological grounds, since GSTT1 gene is not located on the sex chromosome.[38]

The GSTT1 null genotype polymorphism did not show any significant association with T2DM in our study ($\chi^2 p = 0.832$) since it did not show any significant difference between control group and diabetic cases (Table 4). This was in agreement with some studies.[21–24] The results of our study are opposed to Egyptian,[3] Chinese[19] and Brazilian[20] researchers who not only observed lack of association between GSTM1 genotypes and T2DM, but also observed that the prevalence of GSTT1-null genotype is a more critical risk factor in T2DM development. In South India, Ramprasath T et al. (2011)[26] in their study on South Indian T2DM patients observed significant associations between T2DM and both null genotypes of GSTM1 and GSTT1. Amer et al.[3] also indicated that the GSTM1 and GSTT1 genotype distributions significantly differ between T2DM patients and controls in Egypt. In addition, they reported that the combined genotype of GSTM1-null/GSTT1-null may increase the risk of T2DM development.

The present study did not relate the risk of developing diabetic vascular complications to the presence of the null genotype polymorphisms in the GSTM1 or GSTT1 genes (Table 5). On the contrary, the GSTT1 null genotype polymorphism showed a significantly decreased frequency in those suffering from neuropathy and retinopathy (Table 5). Cilenšek I et al. (2012)[44] proposed a protective effect for GSTM1 null genotype against retinopathy explained by an up-regulation of other antioxidant enzymes such as manganese superoxide dismutase which become more effective in detoxification of atherogenic compounds. Our explanation for a protective effect of GSTT1 null genotype could rely on the same reason. The high activity of cytochrome P450 system in such patients might offer another explanation.[45] However, it should be stressed that to date no firm evidence exists that such mechanisms promote efficient defense against the development and progression of T2DM vascular complications. Hence, additional research is mandatory in this area.[44,45]

As regards the GSTP1 gene polymorphism in the present study, the genotype distributions among controls, diabetics and total subjects were in agreement with the Hardy–Weinberg equilibrium (Table 3). Such genotype distribution was in agreement with other studies concerning GSTP1 genotype distribution among Egyptians.[28,46] Contrary to studies that did not report an association of GSTP1 (A313G) SNP with T2DM,[21,22,24] our study was able to demonstrate a statistical difference that approached the level of significance in the frequencies of GSTP1 genotypes between whole diabetic patient group and control group, with a lower frequency of wild (AA) genotype and a higher frequency of heterozygous (AG) mutant genotype demonstrated in all diabetic cases ($^{MC}p = 0.053$). Such a statistical difference became significant when diabetic cases divided according to the presence or absence of vascular complications were compared to the control group ($p = 0.023$) (Table 4). This finding is in agreement with results of Bid HK et al. (2010)[23] in North Indian T2DM cases and Amer MA et al. (2012)[28] in Egyptian T2DM cases, implicating the GSTP1 gene polymorphism in the susceptibility to and risk of T2DM development among Egyptians. The domination of the G allele in the GSTP1 polymorphism (A313G) results in reduction of GSTP1 enzyme activity. Consequently, the cell becomes more susceptible to mutation and damage from exposure to electrophiles and ROS.[47]

Despite the significance noted in the G allele in GSTP1 gene polymorphism among diabetic cases, our study failed to demonstrate any significant association between GSTP1 polymorphism and any of diabetic vascular complications (Table 5) which is not far from the result reported by Townsend D et al. (2003).[48] Furthermore, no significant associations were found between GSTM1, GSTT1 and GSTP1 gene polymorphisms and the number of vascular complications in T2DM cases presenting with them (Table 6).

When comparing the combined effect of the null genotypes of GSTM1 and GSTT1 as well as the GSTP1 SNP and the risk of developing T2DM, a significantly higher frequency of combined GSTP1 heterozygous (A/G) and GSTM1 deleted genotype in the whole diabetic group was demonstrated compared to the control group (Odds ratio = 6.285, 95% confidence interval = 1.184–33.347, $p = 0.021$) (Table 7). This result points out the value of investigating the combined effects of genotypes in population studies that might be synergistically associated with the risk of diseases.

The association studies that evaluate the impact of genotype on disease progression are usually limited by the fact that the duration of diabetes, together with the uncontrolled hyperglycemia and high BMI are the most important factors associated with the development of diabetic vascular complications.[45] Other factors involved in the hyperglycemia-induced cell damage could influence our results. An important contributor in that area is advanced glycation end products that modify ROS formation through their corresponding receptors and therefore influence the production of growth factors and cytokines by affected cells.[49] Our results thus represent only a part of the complex pathological network of diabetes and its related vascular complications.

6. Conclusion

In conclusion, our study was only able to demonstrate an increased risk of developing T2DM but not its vascular related complications among cases having heterozygous (AG) GSTP1 A313G polymorphism alone as well as when combined with GSTM1 null genotype. One of the limitations of this study was the evaluation of selected polymorphisms that may not be representative of the whole polymorphisms in the GST gene cluster. Thus, functional studies are needed to clarify the exact molecular mechanisms by which GST gene variants may exert influence on pancreatic beta cells destruction. Furthermore conducting a large-scale cohort study in Egyptian population may confirm the role of GSTM1, T1 and P1 gene polymor-

phisms in the pathogenesis of T2DM and its related complications. Moreover, performing genetic studies concerning genes involved in ROS elimination such as manganese activated superoxide dismutase (MnSOD), catalase, and glutathione reductase would be of value in exploring the genetic related antioxidant defense system in T2DM.

Conflict of interest

None to declare by the authors of this manuscript.

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