DOI : 10.35124/bca.2019.19.2.4485

Biochem. Cell. Arch. Vol. 19, No. 2, pp. 4485-4490, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

A STUDY TO INVESTIGATE THE POSSIBLE ASSOCIATION BETWEEN GSTM1 AND GSTT1 POLYMORPHISM AND THE OCCURRENCE OF COMPLICATIONS IN TYPE 2 DM

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(Received 11 August 2019, Revised 10 October 2019, Accepted 17 October 2019)

ABSTRACT : Oxidative stress has been identified to play a significant role in onset of diabetes and its complications .GST gene family is involved in detoxification and reduction of ROS. polymorphisms in these genes that result in null genotypes that may lead to reduced or loss of enzyme activity and thus adversely affects their functioning in protecting cells from ROS damage. This study aim to investigate the possible association between GSTM1 and GSTT1 polymorphism and the occurrence of complications in type 2 DM. A total of 100 established cases of type 2 DM patients without micro vascular complication and 100 cases of type 2 DM with micro vascular complication were included in the study. 160 healthy controls were taken. In this study we found that frequency of GSTT1 null genotype was 39% in T2DM patient without complication and 53% in patients with complications (OR = 1.76, 95%CI = 0.98-3.28, p = 0.04). This shows that higher frequency of GSTT1 null genotype is related to an increased predisposition for T2DM related complication, with a 1.76 fold increased risk of developing the complications relative to the present genotype. There was significant association of GSTT1 genotype with occurrence of diabetic complications. It was also observed that there was no significant association of the *GSTM1* null genotype with susceptibility to different diabetic complications (p = 0.57) in the population studied.

Key words : Type-2 diabetes, complications, GSTM1, GSTT1.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a common metabolic syndrome characterized by impaired metabolism of glucose and lipids due to defects in insulin secretion (beta cell dysfunction) or action (insulin resistance). Distribution of diabetes has been increased in the world in late years. This is because of urbanization, growth in population and high in obesity especially in developing countries. It was estimated by International Diabetes Federation (IDF) that a dramatic increase in the incidence of diabetes in India by 2030 (Ramachandran *et al*, 2010). Chronic hyperglycemia is associated with several micro vascular and macrovascular complications. Oxidative stress has been identified to play a major role in onset of diabetes and its complications. Enzymatic antioxidant like manganese superoxide dismutase (MnSOD), catalase (CAT) and two classes of multi-functional GST enzymes such as GSTM1 (GST-mu) and GSTT1 (GST-theta) have been reported to be important in detoxification of various toxic and carcinogenic compounds (Sorbin *et al*, 2011; Yalin *et al*, 2007; Bid *et al*, 2010).

GSTs involved in the defense mechanisms against raising free radicals, formed during oxidative stress in various tissues of diabetic (Kowluruand Chan, 2007). GSTs groups are those genes whose polymorphisms is well known to be associated with in the development of chronic diseases like T2DM and malignancies. Null genotypes of GSTM1 and GSTT1 polymorphism had been reported to be associated with lacking enzyme activity. It has been found that Null GSTM1 or GSTT1 have no functional enzyme activity in homozygous subjects(Xu *et al*, 1998; Landi, 2000).

Antioxidant compounds counter the effects of free radicals which include reactive oxygen species (ROS)

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among healthy subjects, antioxidants which are originated endogenously or are derived from dietary sources. These are categorized into 2 groups, enzymatic and non-enzymatic (Esteghamati *et al*, 2008). One of the most important antioxidant is glutathione (GSH). It provides the cell with multiple defenses against ROS as well as against their toxic products (Pompella *et al*, 2003). GSH is well known as a substrate in both the conjugation and the reduction reactions which is catalyzed by glutathione S-transferase (GST) enzymes in the cell.It is also capable of participating in non-enzymatic conjugation with some chemicals (Tesfamariam, 1994). So, it is hypothesized that GST polymorphisms may have a role in the pathogenesis of T2DM and its complications. In our study, the associated risk between GST and T2DM and its microvascular complications was investigated.

The objective of this study was to investigate the possible association between GSTM1 and GSTT1 polymorphism and the occurrence of complications in type 2 DM.

MATERIALS AND METHODS

This was a case-control study conducted in Department of Biochemistry, Central Research lab and Central clinical Lab of Integral Institute of Medical Sciences and Research and Molecular and cell biology laboratory, Department of Biochemistry, KGMU, Lucknow. A total of 100 established cases of type 2 DM patients without microvascular complication and 100 cases of type 2 DM with microvascular complication were included in the study. 160 apparently healthy controls were taken. Patient of age > 35years <70 years and diabetic patients with at least 8 years disease duration were included in the study. Patients with acute illness, acute myocardial infarction, liver disorder, known inherited disorder of lipids, hypothyroidism, pregnancy and gestational diabetes, history of type 1 DM, primary renal disease and any, primary fundal affection and uveitis and retinal dystrophy were excluded from the study.

The different micro vascular complications included were: (a) Diabetic nephropathy proved by microalbuminuria and renal function tests; (b) Diabetic retinopathy by fundus examination and (c) Diabetic neuropathy proved by CNS examination.

Collection of samples

Under aseptic condition 5 ml of 10-12 hrs fasting venous blood was drawn from the antecubital vein. Fasting blood samples were used for the determination of concentration of glucose, creatinine,urea and analysis was done on Olympus AU400 clinical chemistry autoanalyser (Beckman Coulter Inc, Brea). Glycated hemoglobin HbA1c value was determined using HPLC technique (Bio rad D10). Plasma malondialdehyde level were determined by method as described by (plaser ZA, Cushman LL,JBC 1966). All the kits and reagents were used as per manufacturer's instructions.

Molecular assessment

The genomic DNA was extracted from whole blood cells using a QIAamp DNA blood Mini Kit (Qiagen, Chatsworth, CA, USA). The DNA samples were stored at -40^{o}C. All kits and reagents were used according to their manufacturer's instructions. The quality of the extracted DNA was checked by electrophoresis on 1% agarose gel. Quantity and purity of DNA was checked by NanoDropTM 1000 Spectrophotometer. Amplification was performed in a 11-µl reaction mixture containing genomic DNA 1 ìl (4-12ì g), 0.4 ìl of each forward and reverse primer set, 5.4 ìl of green PCR master mix and 3.8 ìl of nuclease free sterile water. The PCR products were visualized PCR products was visualized against a 50 bp DNA ladder using 2% agarose gel. PCR products representing GSTM1 and GSTT1 positive genotypes yielded bands of 215 and 480 bp, respectively, A part of exon-7 CYP1A1 gene was amplified and used as an internal control to avoid false-negative readings in this method. Primer for Exon7-CYP1A1: forward: 5'-GAACTGCCACTTCAGCTGTCT-3' reverse: 5'-CAGCTGCATTTGGAAGTGCTC-3'the internal positive control (CYP1A1) PCR product band

Table1 : Primer sequence and cycler conditions for amplification of GSTM1 and GSTT1.

Genes	Primers	Cycler condition		
GSTM1	Forward 5'GAACTCCCTGAAAAGCTAAAGC-3' Reverse 5'GTTGGGCTCAAATATACGGTGG-3'	GSTMI 35 cycle	Initial Denaturation Denaturation Annealing Extension Final elongation	94 ^{o}C- 5min 94 ^{o}C–30 s 60^{o}C-1min 72^{o}C-1min 72^{o}C-5min
GSTT1	Forward 5'TTCCTTACTGGTCCTCACATCTC-3' Reverse 5'TCACGGGATCATGGCCAGCA-3'	GSTT1 35 cycle	Initial Denaturation Denaturation Annealing Extension Final elongation	94 ^{o}C- 5min 94 ^{o}C–30 s 59^{o}C-1 min 72^{o}C-30 s 72^{o}C-5min

Table 2 : Demographic and biochemical profile of diabetic patients.

	T2 DM with complication (n=100)	T2 DM without complication (n=100)	p-value[1]
Age in years	57.86±5.13	55.18±7.45	0.003*
Gender No. (%)			
Male	53 (53.0)	51 (51.0)	0.77
Female	47 (47.0)	49 (49.0)	
Diabetic duration in years	11.47±2.54	11.06±2.08	0.21
Blood sugar fasting (mg/dl)	184.82±31.33	153.13±33.54	0.0001*
Blood sugar PP (mg/dl)	256.64±22.83	212.70±52.47	0.0001*
HbA1c (%)	7.75±1.45	6.45±1.07	0.0001*
Serum creatinine (mg/dl)	3.79±2.49	0.97±0.26	0.0001*
Serum urea(mg/dl)	90.11±38.16	37.31±6.23	0.0001*
MDA(nmol/ml)	5.12±1.34	3.77±1.24	0.0001*

[1]Unpaired t-test/Chi-square test P <0.05 considered statistically significant.

Table 3 : Comparison of GST genotypes among the three groups.

GST	T2DM with complication (n=100)		T2DM without complication (n=100)		Controls (n=160)		χ^2	p-value
	No.	%	No.	%	No.	%		
GSTM1								
Present	51	51.0	55	55.0	99	61.9	3.18	0.20
Null	49	49.0	45	45.0	61	38.1		
GSTT1								
Present	47	47.0	61	61.0	111	69.4	12.93	0.002*
Null	53	53.0	39	39.0	49	30.6		

* P <0.05 considered statistically significant.

corresponded to 312 bp. The PCR products were visualized against a 50 bp DNA ladder using 2% agarose gel.

Statistical analysis

Data is expressed as mean ± standard deviation, or proportions for categorical variables. Categorical variables were compared using the Chi-square test. Continuous variables were compared by using unpaired t-test between the groups. The odds ratio (OR) with its 95% confidence interval (CI) was calculated. All the analysis was carried out on SPSS 16.0 version (Chicago, Inc., USA). A p-value less than 0.05 were considered statistically significant.

RESULTS

The mean age of T2 DM with complication and without complication was 57.86±5.13 and 55.18±7.45 years respectively. About half of patients of T2 DM with complication (53%) and without complication (51%) were males. There was no significant (p>0.05) difference in duration of diabetes between the groups. Blood sugar fating, blood sugar PP, HbA1c, serum creatinine, serum urea and MDA were significantly (p=0.0001) higher among patients of T2 DM with complication than without complication (Table 2).

Table 3 shows that GSTT1 null genotype was significantly more frequent in T2DM patients with and without complications than in control (53%, 39%, 30.6%; χ^2=12.93, p = 0.002). It shows association of this gene with pathogenesis of Diabetes and its complications. There was no significant association of GSTM1 null among the groups (49%, 45%, 38.1%, χ^2 = 3.18, p = 0.20).

Table 4 shows the comparison of GST genotypes frequencies between T2DM patient and control group. GSTM1 null genotype was present in 47% and 38.1% in T2DM patients and control respectively. It was observed that there was no significant association of the GSTM1 deletion with susceptibility to onset of Diabetes (p = 0.09).

It was also found that GSTT1 null genotype was present in 46% and 30.6% in T2DM patients and control respectively (OR=1.93, 95% CI=1.15-3.12, p=0.003) This shows that GSTT1 null genotype is related to an increased predisposition for T2DM, conferring a 1.93 fold increased risk of developing the Diabetes relative to the

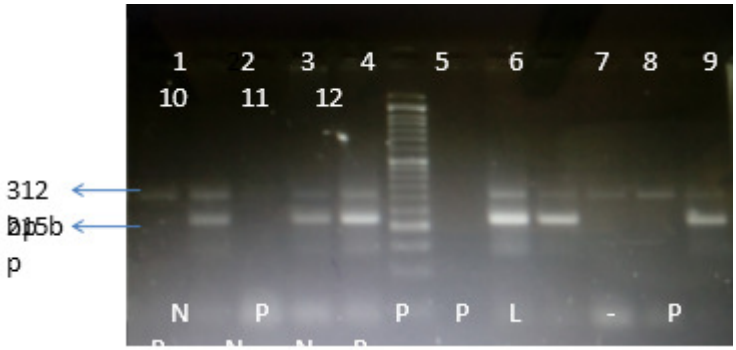

Fig. 1 : PCR amplification of GSTM1 gene in control group (215bp).
Lane 6, 50 bp DNA ladder; **lanes 2,4 5,8,9 and 12** GSTM1 Present **lane 1, 10 and 11**GSTM1 null.
The 312 bp fragment is the product of CYP1A1 gene internal control, seen in lanes **1, 2, 4, 5, 8, 9, 10, 11 and 12**.

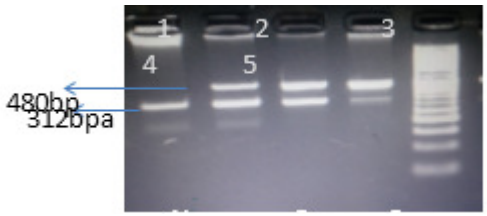

Fig. 2 : PCR amplification of GSTT1 gene in control group (480 bp).
Lane 5, 50 bp DNA ladder; **lanes 2, 3, 4** GSTT1 Present **lane1**, GSTT1 null
The 312 bp fragment is the product of CYP1A1 gene internal control, seen in lanes **1–4**.

Table 4 : Comparison of GST genotypes frequencies between T2DM and control groups.

GST	T2DM (n=200)		Controls (n=160)		OR	95%CI	p-value
	No.	%	No.	%			
GSTM1							
Present	106	53.0	99	61.9	1.00 (Ref.)	0.65-2.78	0.09
Null	94	47.0	61	38.1	1.44		
GSTT1							
Present	108	54.0	111	69.4	1.00 (Ref.)	1.15-3.12	0.003*
Null	92	46.0	49	30.6	1.93		

* P <0.05 considered statistically significant.

present genotype.

Table 5 shows the comparison of GST genotypes frequencies between T2DM patient without complication and with complications. GSTM1 null genotype was present in 45% and 49% patients of T2DM patient without complication and with complications, respectively. It was observed that there was no significant association of the *GSTM1* deletion with susceptibility to complications (p = 0.57) in the population studied.

For an analysis of the risk associated with the deletion polymorphism for *GSTT1*, it was found that GSTT1 null genotype was present in 39% and 53% of T2DM patient without complication and with complications respectively

Table 5 : Frequency distributions of GST genotypes and their relationship with the risk of T2DM complications.

GST	T2DM with compli-cation (n=100)		T2DM without compli-cation (n=100)		OR	95%CI	p-value
	No.	%	No.	%			
GSTM1							
Present	51	51.0	55	55.0	1.00 (Ref.)	0.51-2.48	0.57
Null	49	49.0	45	45.0	1.17		
GSTT1							
Present	47	47.0	61	61.0	1.00 (Ref.)	0.98-3.28	0.04*
Null	53	53.0	39	39.0	1.76		

* P <0.05 considered statistically significant

(OR=1.76, 95% CI=0.98-3.28, p=0.04) This shows that it is related to an increased predisposition for T2DM related complication, conferring a 1.76 fold increased risk of developing the complications relative to the present genotype.

DISCUSSION

Oxidative stress has been identified to play a significant role in progression of diabetes. Since the GST genes are involved in breakdown of byproducts of oxidative stress, polymorphisms in these genes that result in null alleles lead to loss of enzyme activity and thus adversely affects their functioning in protecting cells from ROS damage (Wang *et al*, 2006; London *et al*, 2000). A number of studies have worked on possible association between polymorphism of GSTM1and GSTT1 gene and its relation with diabetes and its microvascular complications. In this study, we found that frequency of null GSTM1 and GSTT1 was comparatively higher in patients with diabetic complication than control supporting the associative role of polymorphism in pathogenesis of diabetic complications.

In this study, blood sugar fasting, blood sugar PP, HbA1c, serum creatinine, serum urea and MDA were significantly (p=0.0001) higher among patients of T2 DM with complication than without complication.

In this study, we found that GSTT1 null genotype was significantly more frequent in T2DM patients with and without complications than in control(53%, 39%, 30.6% ÷2=12.93 p=0.002) (Table 3). It was found that GSTT1 null genotype was present in 46% and 30.6% in T2DM patients and control respectively (OR=1.93, 95%CI=1.15-3.12, p=0.003) (Table 4). This shows that GSTT1 null genotype is related to an increased predisposition for T2DM, conferring a 1.93 fold increased risk of developing the Diabetes relative to the present genotype.

Such finding is in agreement with results reported by a study conducted by D.S Pin heiro *et al* (2013) in Brazilian population in which GSTT1 null genotype conferred a 3.2 fold increased risk to T2DM relative to present genotype.

In the present study for an analysis of the risk associated with the deletion polymorphism for *GSTT1*, it was found that GSTT1 null genotype was present in 39% and 53% of T2DM patient without complication and with complications respectively (OR=1.76, 95% CI=0.98-3.28, p=0.04). This shows that it is related to an increased predisposition for T2DM related complication, conferring a 1.76 fold increased risk of developing the complications relative to the present genotype. A meta-analysis study by Sun *et al* (2015) reported an increased risk of development of diabetes retinopathy associated with presence of null polymorphisms in both GSTM1 and GSTT1 genes.

In a study by Yang *et al* (2004), it has been found that GSTT1 homozygous deletion as a risk factor for developing ESRD, which develops due to nephropathy, in diabetic patients which is in agreement with our result.

There was no significant association of GSTM1 null among the groups (49%, 45%, 38.1% $\chi^2 = 3.18$ p=0.20) (Table 3).

Such finding were in agreement with results reported by a study conducted by D.S Pin heiro *et al* (2013) in Brazilian population and a study by Gonil *et al* (2012), whereas in contrast a study by Bid *et al* (2010) and E.Moasser showed a significant association between the frequency of GSTM1 genotype and T2DM.

GSTM1 null genotype was present in 45% and 49% patients of T2DM patient without complication and with complications, respectively. It was observed that there was no significant association of the *GSTM1* deletion with susceptibility to complications (p = 0.57) in the population studied (Table 5).

The results of our study are similar to Egyptian, study conducted by Amer *et al* (2011), 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.

A study carried out by Doney *et al* (2005) demonstrated that GSTT1-null individuals have a more generalized vasculopathy with an increased risk of progression of both retinopathy and nephropathy which was in agreement with our study.

Fujita *et al* (2000) suggested that GSTM1 null genotype was not contributive to the development of diabetic nephropathy in Japanese type 2 diabetic patients.

This allows us to conclude that the GSTT1 null genotype genes may contribute to T2DM and its related complications.

CONCLUSION

GSTT1 and GSTM1 gene polymorphism involved in T2DM pathogenesis and can be considered as a marker to determine the possible susceptibility to diabetes and its complications.

ACKNOWLEDGEMENT

The author wishes to thanks King George Medical University, Lucknow for supporting this research. The authors are also thankful to Integral University, Lucknow for providing the necessary facilities for research. The manuscript has been approved by the competent authority (Assigned Manuscript Communication Number – IU/R&D/2019-MCN000701).

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