GSTM1 NULL GENOTYPE ASSOCIATED WITH TYPE 2 DIABETIC NEPHROPATHY PATIENTS AMONG INDIAN POPULATION

Pulakes Purkait1,4; Kalpataru Halder2; Abhishikta Ghosh Roy3; B.N. Sarkar*3; J.M. Naidu4

1Anthropological Survey of India, Western Regional Centre, Pratapnagar, Udaipur -313001, Rajasthan, India
2Brahmananda Keshab Chandra College, 111/2 B.T Road, Bon Hooghly, Kolkata - 700108
3 Anthropological Survey of India, 27 Jawaharlal Nehru Road, Kolkata-700016, India
4Department of Anthropology, Andhra University, Visakhapatnam, Andhra Pradesh, India

ABSTRACT
Oxidative stress is an imperative factor in the etiology and pathogenesis of diabetes. Glutathione S-transferases are multifunctional enzymes, which act as a kind of antioxidant defense. The GSTM1null allele represents a deletion of the GSTM1 and result in a loss of enzymatic activity. Previous studies reported that GSTM1 null genotype is associated with type 2 diabetes and diabetic nephropathy in different populations. The aim of this study is to investigate the association of GSTM1 gene polymorphism in the type 2 diabetes mellitus patients with and without nephropathy. For the present study 356 age matched participants (123 T2DM, 84 T2DN patients and 152 healthy controls) belonging to Bengali population from Kolkata city were enrolled and 10ml of peripheral blood sample was collected from each individual. Genomic DNA was prepared from fresh whole blood by using the conventional method and polymerase chain reaction was carried out to identify the null genotype of GSTM1 gene. Data were analyzed using statistical software SPSS 16 and MINITAB 11. The present study reveals that anthropometric measurements and bio-chemical parameters were significantly varying in three groups, i.e. T2DM, T2DN and control. Distributions of GSTM1 null genotypes was 24 (19.51%) in the T2DM patients, 31 (36.90 %) in the T2DN patients and 20 (13.16 %) in the controls. The frequency of GSTM1 null genotype was significantly increased in the diabetic nephropathy patients. Our results suggest that GSTM1 gene polymorphisms
may play an important role in pathogenesis of type 2 diabetes mellitus with and without nephropathy patients among the Indian Bengali population.

**Key Words:** Type 2 diabetes mellitus, Type 2 diabetic nephropathy, *GSTM1*, Null genotype, Indian population.

**INTRODUCTION**

Diabetes Mellitus Type 2 (T2DM) is a multifactorial disease that develops through an exposure to environmental risk factors, daily life practice and genetic vulnerability. This heterogeneous syndrome is characterized by chronic hyperglycemia and other metabolic alterations. These mainly include dyslipidemia and hypertension that leads to a development of macro and microvascular complications. The disease pathogenesis involves a combination of β-cell insufficiency and insulin resistance. Much like other multifactorial diseases little is known about the genetics of T2DM (Robertson *et al*, 2004; Andreassi, 2009).

T2DM associated with an over-production of reactive oxygen species (ROS) and inefficiency of antioxidant defenses (Griesmacher *et al*, 1995; Giron *et al*, 1999; Opara, 2002; Fridlyand and Philipson, 2005). Oxidative stress is an imperative factor in the etiology and pathogenesis of diabetes (Raza and John, 2004) and is the consequence of accumulation of free radicals (oxygen free radicals and lipid peroxides) in tissues which particularly affects β-cells of pancreas (Moasser *et al*, 2012). Glutathione S-transferases are a multigene family of ubiquitous and multifunctional enzymes that include several classes of GSTs. This enzymes have important roles in decreasing of ROS and act as a kind of antioxidant defense (Richard *et al*, 2000; Sharma R *et al*, 2004; Hayes *et al*, 2005).

*GSTM1* encodes a cytoplasmic Glutathione S-transferases that belongs to the µ-class. The µ-class of enzymes functions in the detoxification of electrophillic compounds including carcinogens therapeutic drugs environmental toxins and products of oxidative stress by conjugation with glutathione (Prohaska, 1980). The genes encoding the µ-class of enzymes are organized in a gene cluster on chromosome 1p13.3 and are known to be highly polymorphic (Pearson *et al*, 1993; Xu S *et al*, 1998). Along with all the genetic polymorphisms described in this class of enzymes, the *GSTM1* is most significant because this gene is reported to be deleted resulting in absence of the respective isoform of the enzyme. The *GSTM1 null* (*GSTM1* deficiency) allele represent a deletion of the *GSTM1* and result in a loss of enzymatic activity (Ye Z. *et al*, 2004). Genetic variations of *GST* may influence individual susceptibility to some diseases associated with the deleterious effects of
oxidative metabolism (De Alvarenga et al, 2007). GSTM1 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 (Nowier et al, 2009; Sharma A et al, 2012).

In the last few years some investigations have been done on the association of DN with the genetic polymorphism of GSTs. Although no association of GSTM1 deletion has been found with DN in Japanese T2DM patients (Fujita et al, 2000; Hori et al, 2007), GSTT1 has been reported to be a risk factor for development of DN in Chinese population (Yang et al, 2004). In the Korean population GSTM1 null genotype is associated with T2DM patients (Kim et al, 2005), whereas GSTT1 polymorphism is a risk factor but not GSTM1, for development of T2DM in case of Brazilian population (Pinheiro et al, 2013). The effect of GSTM1 and GSTT1 double null genotype is associated with the development of oxidative stress in Indian DN patients (Datta et al, 2010), and also the combined effect of GSTM1, GSTT1 and GSTP1 polymorphism have been found to play a significant role in North Indian T2DM patients (Bid et al, 2010).

This led us to investigate whether any significant association between GSTM1 null genotype and type 2 diabetes with and without nephropathy patients in Eastern Indian population is present or not.

MATERIAL AND METHODS

Participants

The present study on GSTM1 gene null genotype was carried out among 356 age matched participants belonging to Bengali population from Kolkata. The subjects include 123 Type 2 Diabetes patients without nephropathy (T2DM), 84 Type 2 Diabetes patients with Nephropathy (T2DN) and 152 healthy controls from Kolkata city.

Registered patients were recruited from B.P. Poddar Hospital and Research Centre, Kolkata as well as Calcutta Medical College and Hospital, Kolkata and a detailed medical history of each patient was recorded accordingly. The detection of Type 2 diabetic as well as diabetic nephropathy patients was based on physician’s recommendation. The healthy unrelated age matched controls with no history of renal disease and diabetes mellitus were randomly selected and recruited from local community centers. Prior to the recruitment of subjects the
ethical committee clearance was obtained from the respective medical institutions and accordingly informed consent was obtained from all the participants.

**Anthropometric measurements**

Anthropometric measurement and Clinical data included information on duration of diabetes, presence of any complication, history of other disorders, systolic and diastolic blood pressure also collected. The weight (kg) and height (cm) were recorded and the body mass index (BMI) was calculated using the formula: weight (kg) divided by height (m) squared (kg/m²).

**Collection of Blood samples and biochemical analysis**

Approximately 10 ml of peripheral venous blood sample was collected from each individual participated in the study into two separate tubes, one in a 5ml BD K2 vacutainer® (BD, NJ, USA) containing EDTA as anticoagulant for genetic analysis and another in a 5ml BD Serum vacutainer® without EDTA for biochemical analysis. Blood samples were stored at 4°C to avoid haemolysis and cellular damage. Samples were transported to the laboratory within 3 hours of collection to ensure good results. Thereafter, blood samples were transferred to labeled sterile polypropylene centrifuge tubes. The blood samples were centrifuged, serum separated, collected and stored at 4°C as well as at -86°C until further analysis. DNA was extracted from the blood sample collected in EDTA contain tube.

Venous blood samples were drawn from the fasting subjects in the morning into 4 ml BD Serum vacutainer® without EDTA for biochemical analysis. Blood glucose was measured using the Breez 2 glucometer (blood glucose monitor) on the spot. All laboratory tests were conducted at the DNA laboratory in the Anthropological Survey of India. The levels of total Cholesterol (mg/dl), Triglycerides (mg/dl), high-density lipoprotein cholesterol HDL (mg/dl), low density lipoprotein cholesterol LDL (mg/dl), creatinine (mg/dl), Urea (mg/dl), BUN (mg/dl), Total Protein (g/dl), Albumin (g/dl) and Chloride ( mmol/L) were measured enzymatically on an auto analyzer EM360 (TRANSASIA) with kits supplied by Transasia Bio-Medical LTD.

**Genotyping of GSTM1 gene null genotype**

Approximately 5mL peripheral blood samples were collected in BD Vacutainer K2 EDTA tube with written and informed consent from patients and from normal individuals as controls, without any history of diabetes. Genomic DNA was prepared from fresh whole blood by using the conventional phenol–chloroform method (Miller et al, 1998). Genomic
DNA was dissolved in TE (10mM Tris-HCl and 0.1mM EDTA, pH 8.0). Polymerase chain reaction (PCR) was carried out to identify the null genotype of \textit{GSTM1} gene in a DNA thermo cycler (Gene Amp PCR 9700 - Applied Biosystems, USA) using 20 pmoles of each of the following primer (flanking primer pair): oligonucleotide sense primer: 5’- TTTCTTACTGTCCTCCTC 3’ and anti-sense primer: 5’ – TCACCGGATCAGGCCAGCA – 3’ (Sayed \textit{et al}, 2010) in a final volume of 10µl containing 50-100 ng genomic DNA, 10X PCR buffer, 25 mM MgCl2, 100 mM of each dNTP and 1 U/ µl of Taq polymerase (Invitrogen, Carlsbad, CA). PCR was performed with a gradient standardized PCR condition with an initial denaturing time at 95°C for 5 min. Then the DNA was amplified for 35 cycles with denaturation at 94°C for 1 min, annealing at 68.9°C for 1:30 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. PCR products were directly visualized in UV light using ethidium bromide staining after electrophoresis in a 2.5% Agarose gel. The presence or absence of \textit{GSTM1} gene was detected by the presence or absence of a 206 bp band.

![Figure 1: Agarose gel stained with ethidium bromide showing the amplified PCR products for \textit{GSTM1}. Lane 1,2,4,5,6,11,12,13,14 & 15 shows the presence of \textit{GSTM1} gene, Lane 3,7,8 & 10 shows the absence of the gene and lane 9 represents the 50 bp ladders.](image)

**Statistical analysis**

Data were analyzed using statistical software SPSS Version 16. Data were expressed as mean and SD. Statistical differences between groups were assessed by analysis of variance (ANOVA). Genotype frequencies of \textit{GSTM1} gene polymorphism were compared between type 2 diabetic patients (T2DM), type 2 diabetic nephropathy on hemodialysis patients (T2DN) and healthy match controls using \chi^2-test (MINITAB 11). Statistical significance was assumed at the \(p<0.05\) level.

**RESULTS**

Table 1 shows the clinical characteristics of 84 T2DN patients (Null genotype =31, Present=53), 123 T2DM patients (Null genotype= 24, Present= 99) and 152 control
participants (Null genotype = 20, Present = 132). The glucose and lipid parameters (cholesterol, Triglyceride, HDL) also revealed highly significant differences between T2DN, T2DM patients and control ($P < 0.05$). The most important renal functional parameters i.e. serum creatinine, total protein and Albumin, Urea, BUN and chloride levels showed significant differences ($P < 0.05$) between three groups. Whereas anthropometric variables i.e. height, weight, BMI, SBP and DBP also showed significant difference between the three study groups ($P < 0.05$).

Table 1: Comparison of variables in different groups and genotype

<table>
<thead>
<tr>
<th>Variables</th>
<th>CONTROL (-) null genotype (n = 20)</th>
<th>CONTROL (+) Present (n = 132)</th>
<th>T2DM (-) null genotype (n = 24)</th>
<th>T2DM (+) Present (n = 99)</th>
<th>T2DN (-) null genotype (n = 31)</th>
<th>T2DN (+) Present (n = 53)</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Sig.</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>53.55 ± 5.31</td>
<td>54.55 ± 5.31</td>
<td>57.92 ± 11.73</td>
<td>54.55 ± 10.42</td>
<td>54.55 ± 9.56</td>
<td>53.83 ± 8.04</td>
<td>0.59</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.36 ± 9.35</td>
<td>162.83 ± 9.88</td>
<td>156.50 ± 6.64</td>
<td>158.97 ± 9.78</td>
<td>158.22 ± 9.82</td>
<td>158.56 ± 7.83</td>
<td>0.00*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60.70 ± 12.60</td>
<td>64.53 ± 11.99</td>
<td>63.68 ± 14.29</td>
<td>61.91 ± 12.80</td>
<td>54.81 ± 10.64</td>
<td>56.79 ± 8.42</td>
<td>0.00*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.34 ± 3.37</td>
<td>24.31 ± 3.85</td>
<td>26.03 ± 5.89</td>
<td>24.36 ± 3.80</td>
<td>21.86 ± 3.65</td>
<td>22.64 ± 3.40</td>
<td>0.00*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>121.30 ± 16.87</td>
<td>124.16 ± 21.97</td>
<td>140.25 ± 23.52</td>
<td>136.57 ± 19.20</td>
<td>148.13 ± 28.70</td>
<td>153.64 ± 24.13</td>
<td>0.00*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>78.10 ± 7.21</td>
<td>82.86 ± 10.56</td>
<td>88.21 ± 11.18</td>
<td>84.56 ± 10.17</td>
<td>87.32 ± 16.48</td>
<td>88.53 ± 12.04</td>
<td>0.00*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>109.11 ± 10.08</td>
<td>114.32 ± 21.29</td>
<td>158.75 ± 40.16</td>
<td>166.67 ± 77.06</td>
<td>108.48 ± 22.35</td>
<td>105.94 ± 20.82</td>
<td>0.00*</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>159.44 ± 30.11</td>
<td>172.66 ± 37.18</td>
<td>179.32 ± 20.40</td>
<td>185.90 ± 44.13</td>
<td>174.42 ± 28.47</td>
<td>177.58 ± 45.06</td>
<td>0.02*</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>140.85 ± 82.23</td>
<td>155.40 ± 68.70</td>
<td>192.39 ± 62.57</td>
<td>180.93 ± 93.79</td>
<td>151.14 ± 69.04</td>
<td>158.54 ± 68.33</td>
<td>0.00*</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>41.42 ± 12.12</td>
<td>44.73 ± 16.57</td>
<td>47.79 ± 16.03</td>
<td>51.44 ± 17.69</td>
<td>51.28 ± 14.02</td>
<td>49.96 ± 17.46</td>
<td>0.00*</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>89.85 ± 21.44</td>
<td>96.86 ± 24.72</td>
<td>93.73 ± 18.92</td>
<td>98.27 ± 32.37</td>
<td>92.91 ± 19.96</td>
<td>95.91 ± 36.27</td>
<td>0.80</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.91 ± 0.17</td>
<td>0.93 ± 0.17</td>
<td>1.52 ± 0.79</td>
<td>1.19 ± 0.41</td>
<td>6.41 ± 0.93</td>
<td>6.19 ± 0.82</td>
<td>0.00*</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>15.68 ± 5.65</td>
<td>18.55 ± 7.91</td>
<td>27.77 ± 18.09</td>
<td>28.64 ± 13.28</td>
<td>69.97 ± 22.49</td>
<td>73.53 ± 34.67</td>
<td>0.00*</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>7.32 ± 2.64</td>
<td>8.66 ± 3.69</td>
<td>12.97 ± 8.45</td>
<td>13.38 ± 6.20</td>
<td>32.68 ± 10.50</td>
<td>34.34 ± 16.19</td>
<td>0.00*</td>
</tr>
<tr>
<td>Total</td>
<td>7.16 ± 0.75</td>
<td>7.16 ± 0.75</td>
<td>7.99 ± 0.82</td>
<td>8.10 ± 0.71</td>
<td>7.71 ± 0.79</td>
<td>7.55 ± 0.75</td>
<td>0.00*</td>
</tr>
</tbody>
</table>
Table 2 shows the distribution of GSTM1 genotypes and their frequencies in the patients T2DM (n = 123), T2DN (n = 84) and in the control subjects (n = 152). The frequency of GSTM1 (null or deletion) genotype was 24 (19.51%) in the T2DM patients, 31 (36.90%) in the T2DN patients and 20 (13.16%) in the controls. The frequency of GSTM1 (deletion) genotype was significantly increased in the T2DN patients, 36.90% as compared with the control subjects (13.16%). The frequencies of GSTM1 (Null) and GSTM1 (present) genotypes were significantly different between patients and control subjects ($X^2 = 18.675$, $P = 0.000$).

Significant different was further verified and present in the table 3. It was observed from the table that T2DN was significantly differing from controls and T2DM ($P < 0.005$). No significant difference of the frequency of GSTM1 null was observed between T2DM and controls. The frequency of GSTM1 (null or deletion) genotype was (26.57%) in the total diabetes patients (T2DM + T2DN) and significantly increased than control subjects ($P = 0.002$).

Table 2: Genotypic frequencies of GSTM1 gene null polymorphism in patients and control groups

<table>
<thead>
<tr>
<th>GSTM1 gene</th>
<th>T2DM (n = 123)</th>
<th>T2DN (n = 84)</th>
<th>CON (n = 152)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘-’ null genotype</td>
<td>24 (19.51%)</td>
<td>31 (36.90%)</td>
<td>20 (13.16%)</td>
</tr>
<tr>
<td>‘+’ present</td>
<td>99 (80.49%)</td>
<td>53 (63.10%)</td>
<td>132 (86.84%)</td>
</tr>
<tr>
<td>$X^2 = 18.675$, $p = 0.000$, DF = 2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Statistics of genotype of GSTM1 gene null polymorphism in study groups

<table>
<thead>
<tr>
<th>Between Study group</th>
<th>$X^2$</th>
<th>$p$</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2DM Vs. CON</td>
<td>2.042</td>
<td>0.153</td>
<td>1</td>
</tr>
<tr>
<td>T2DN Vs. CON</td>
<td>18.010</td>
<td>0.000</td>
<td>1</td>
</tr>
<tr>
<td>T2DM Vs. T2DN</td>
<td>7.739</td>
<td>0.005</td>
<td>1</td>
</tr>
<tr>
<td>T2DM + T2DN Vs. CON</td>
<td>9.540</td>
<td>0.002</td>
<td>1</td>
</tr>
</tbody>
</table>
DISCUSSION

Type 2 diabetes mellitus (T2DM) is characterized by being a polygenic disorder and Diabetic nephropathy (T2DN) is a complication of diabetes and multifactorial disease, where renal function has declined. Glutathione S-transferases (GSTs) a member of multigene and multifunctional detoxification enzyme family defend cells against a wide variety of toxic insults from chemical, metabolites, and oxidative stress (Nowier et al, 2009b).

The aim of this study is to investigate the GSTM1 gene polymorphism in the type 2 diabetes mellitus patients with and without nephropathy compared to healthy controls. Also to evaluate the role of these polymorphic genes as a genetic risk modifier in the etiology of type 2 diabetes mellitus with and without nephropathy and the levels of blood lipids given exposure to diabetes.

In this present study, we have observed that there were highly significant differences in the biochemical parameters between T2DN, T2DM patients and control subjects. The study showed that the frequency of GSTM1 (null) genotypes was significantly higher in the T2DN patients as compared with the healthy subjects in the present population. These results were consistent with previous studies (Agrawal et al, 2007; Said et al, 2013). A study from India (Datta et al, 2010; Agrawal et al, 2007) and Egyptian (Said et al, 2013) reported that the GSTM1 null genotype was associated with the higher risk for CRF and ESRD as well as chronic lymphocytic leukemia (Yuille et al, 2002), Renal Cell Carcinoma (Yang X et al, 2013) and colorectal cancer (Chen et al, 2005). Although, some case control studies reported that GSTM1 null genotypes have no associations with type 2 diabetes (Wang et al, 2006, Nowier et al, 2009b). Whereas Akgul et al, (2012) also reported that the GSTM1 null genotypes were not a risk factor for CRF development.

Previous studies reported a variety of associations between Type 2 diabetes and diabetic nephropathy, cancer, and GST gene polymorphisms. But little is known about the effect of GST gene polymorphisms on blood lipids and renal function. In the present study, we also compared blood glucose, serum cholesterol, triglycerides, high density lipoproteins cholesterol in both diabetic subjects (T2DM, T2DN) and the control group for GSTM1 genotypes (null compared to present genotypes). Among individuals with GSTM1 null the total cholesterol, triglycerides and high density lipoprotein as well as renal function parameters i.e. serum creatinine, total protein and albumin, urea, BUN, chloride levels and anthropometric variables were significantly different from GSTM1 present.
CONCLUSION
Our results suggest that GSTM1 gene polymorphisms may play an important role in type 2 diabetes mellitus pathogenesis and complication. GSTM1- null genotypes have an effect on blood lipids and renal function given exposure to diabetes mellitus and diabetic nephropathy. 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.

CONFLICT OF INTEREST
The authors declare no conflict of interest for the present research outcome.

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REFERENCE


