Effect of \textit{GSTM1} and \textit{GSTT1} double deletions in the development of oxidative stress in diabetic nephropathy patients

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Association of diabetic nephropathy (DN) with the deletion of \textit{GSTT1} and \textit{GSTM1} genes is well reported. Oxidative stress (OS) has also been associated with the development of DN. The present study was conducted to find out, whether these deletions had any contributory role in the development of OS in patients with DN. Pre-dialysis venous blood samples were obtained from 60 patients with diabetic end-stage renal disease (stages 4 and 5). Reduced-glutathione (GSH), glutathione S-transferase (GST) activity and malondialdehyde (MDA) levels were measured for the assessment of OS. Genetic polymorphism analysis of DN patients revealed the following distribution pattern: \textit{GSTM1} null 46.7%; \textit{GSTT1} null 55%; both null 30% and both positive 28.3%. Patients with both null genotypes were found to have significantly increased levels of MDA and low GST activity as compared to other genotypic groups. Lower GSH levels were observed in all the genotypic groups as compared to both positives. Double deletions involving \textit{GSTT1} and \textit{GSTM1} may result in decreased GST levels, leading to increased OS as reflected by increased MDA levels. As GST is a multi-functional enzyme involved in xenobiotic metabolism, this double null genotype population has a greater risk of development of DN. Further studies using increased sample size to find out the allelic distribution and their role in the development of DN are in progress.

Keywords: Diabetic nephropathy, Polymorphism, Glutathione S-transferase, \textit{GSTM1}, \textit{GSTT1}, Oxidative stress

Diabetic nephropathy (DN) has emerged as the most common cause of chronic kidney disease accounting for almost 44% cases in the US\(^1\). The scenario is further grave in India as most of the diabetic patients are in the age range of 45-64 yrs, while in developed countries most of them are above 65 yrs. Therefore, diabetic patients in developing countries are more vulnerable to develop the micro-vascular complications of diabetes including DN\(^2\), but despite relentless research into the etiopathogenesis of DN, much still remains unclear.

Various mechanisms have been shown to play a role in the development and progression of diabetic kidney disease. These include activation of protein kinase C \(\beta\) (PKC \(\beta\)), a serine/threonine kinase\(^3\), increased in advanced glycation end products (AGEs)\(^4\), increased transforming growth factor \(\beta\) (TGF \(\beta\))\(^5\), leading to fibrosis in the kidney, angiotensin II-mediated hypertension and hyperfiltration in the glomerulus\(^6\), increased aldose reductase producing sorbitol\(^7\), etc. A very common finding in all tissues affected by diabetes, including the kidney is the presence of increased oxidative stress (OS)\(^8,9\).

In the last few years, there has been a surge of reports studying the association of DN with the genetic polymorphism of glutathione S-transferase (GST), a multi-functional antioxidant enzyme, primarily involved in xenobiotic metabolism. Although no association of \textit{GSTM1} deletion has been found with DN in Japanese type-2 DM patients\(^10\), \textit{GSTT1} null genotype has been shown to be a risk factor for development of DN in the Chinese\(^11\). In the Korean population, \textit{GSTM1} null genotype is found to be associated with type-2 DN\(^12\). However, no report is available regarding the association of \textit{GST} polymorphism with DN in Indian population. Hence, the present study has been designed to investigate whether the deletions of \textit{GSTM1} and \textit{GSTT1} genes are related with higher OS in DN patients and the possible role of these polymorphisms in the development of DN.
Materials and Methods

Patients
We recruited 60 patients with DN (stages 4 and 5) from the dialysis unit of UCMS and GTB Hospital, Delhi after obtaining Institutional Ethical Clearance and informed written consent from each patient. Patients having type-2 DM for more than 5 yrs with albuminur >300 mg/day and having evidence of diabetic retinopathy were recruited. Patients on systemic steroids or with evidence of systemic/urinary tract infection were excluded.

Routine biochemical tests like serum urea and creatinine levels, fasting and post-prandial blood glucose, 24 hr urinary protein estimation and routine and microscopic examination of urine were done from the Hospital Laboratory Services, GTB Hospital, Delhi.

Genotyping
Genomic DNA for genotyping was isolated from peripheral venous blood using a DNA isolation kit (Omniprep™, G-Biosciences, USA). A single assay using a multiplex polymerase chain reaction (PCR) was performed for simultaneous gene amplification using the Eppendorf Mastercycler Gradient-5331 thermocycler. Briefly, ~50 ng of DNA was amplified in a 50 µl multiplex reaction mixture containing 30 pM of each of the following: GSTM1 primers (G<sub>R</sub>-5’ GAA CTC CCT GAA AAG CTA AAG C 3’ and G<sub>R</sub>-5’ GTT GGG CTC AAA TAT ACG GTG G 3’) and GSTT1 primers (T<sub>R</sub>-5’ TTC CTT ACT GGT CCT CAC ATC TC 3’ and T<sub>R</sub>-5’ TCA CCG GAT CAT GGC CAG CA 3’). As an internal control, the exon 7 of the CYP1A1 gene was also co-amplified (C<sub>R</sub>-5’ GAA CTG CCA CTT CAG CTG TCT 3’ and C<sub>R</sub>-5’ CAG CTG CAT TTG GAA GTG CTC CTC 3’) in a medium consisting of 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs (Bangalore Genei, Bangalore, India), 5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0) and 2U Taq DNA polymerase (New England BioLabs, Beverley, MA).

The PCR protocol included an initial denaturation at 94°C for 5 min, followed by 35 cycles of 2 min at 94°C, 1 min at 59°C and 1 min at 72°C, followed by a final extension of 10 min at 72°C. The final PCR product from co-amplification of GSTM1 (215 bp), GSTT1 (480 bp) and CYP1A1 (312 bp) was visualized after electrophoresis in ethidium bromide-stained 2% agarose gel on UVP DIGI- DOC IT gel documentation system.

Oxidative stress parameters
GST activity in serum was measured spectrophotometrically (Shimadzu UV-2450) using 1-chloro-2,4-dinitrobenzene as substrate and expressed as nmol/min/mg of protein. Malondialdehyde (MDA) was determined by measuring the thiobarbituric acid reactive substances in serum. Glutathione (GSH) content in blood was measured using dithio-nitrobenzene and expressed as µmol/dl.

Statistical analysis
One-way ANOVA with Tukey’s multiple comparison procedure as post-hoc test was used for comparison of data. P-value of <0.05 was considered as the level of significance for all statistical analysis.

Results and Discussion
Genotypic analysis of the samples using a multiplex PCR revealed that 28 (46.6%) of the cases had GSTM1 null, while 33 (55%) had GSTT1 null genotype (Table 1). Further, 18 (30%) cases were found to have deletions in both the genes (both-null) simultaneously, while 17 (28.3%) had no deletions (both positive). A sample representative gel picture showing all possible genotypes is shown in Fig. 1.

Studies on healthy population, including a previous study from this laboratory have reported significant presence of GSTM1 and GSTT1 null genotypes amongst Indians. Mishra et al. have reported the frequencies of GSTM1 and GSTT1 null alleles in normal healthy north-Indian individuals to be 33.0% and 18.4%, respectively, with concomitant lack of both genotypes in 7.0% of population, while Singh et al. have reported the prevalence of GSTM1 and GSTT1 null genotypes to be 21% and 27.4% respectively, with simultaneous double deletion in 0.7% population. From these data, it can be observed that the null genotypes of these two genes have a much higher representation amongst DN patients. A similar trend has been reported in some Asian populations. The GSTM1 null genotype has been observed in 48.6% of Japanese DN patients, whereas the prevalence of GSTM1 and GSTT1 deletions in Chinese DN cases has been found to be

<table>
<thead>
<tr>
<th>DN cases (n = 60)</th>
<th>% of Total cases</th>
</tr>
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<tbody>
<tr>
<td>GSTM1 positive</td>
<td>32</td>
</tr>
<tr>
<td>GSTM1 null</td>
<td>28</td>
</tr>
<tr>
<td>GSTT1 positive</td>
<td>27</td>
</tr>
<tr>
<td>GSTT1 null</td>
<td>33</td>
</tr>
<tr>
<td>Both null</td>
<td>18</td>
</tr>
<tr>
<td>Both positive</td>
<td>17</td>
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Table 1—Distribution of different genetic polymorphisms amongst diabetic nephropathy (DN) patients.
58.0% and 42.0%, respectively. Literature regarding association of concomitant deletion of these genes in DN patients is scarce.

Analysis of demographic profile of the patients with respect to genotypes did not reveal any significant differences with regard to age, sex or levels of urea, creatinine and blood glucose (fasting and post-prandial). Though the duration of chronic kidney disease (CKD) did not vary amongst the four groups, it was observed that patients with both positive genotype had a significantly higher duration of DM in comparison to other genotypes (Table 2).

The observation from our study that the concomitant deletion of GSTM1 and GSTT1 genes i.e. both null genotype is quite high amongst DN patients, as compared to the data reported in healthy populations led us to postulate that this genotype might be associated with higher OS in these patients.

Increased production of oxidants occurs for a variety of reasons due to hyperglycaemia. The high glucose is shown to activate superoxide production from mitochondria. Decreased activity of antioxidants also occurs in diabetic kidney disease. The increased oxidant stress caused by diabetes lead to increased activity of the antioxidant enzymes, such as glutathione peroxidise, catalase and superoxide dismutase (SOD), but this is not observed in diabetics with nephropathy.

To investigate whether the genotypic variation in GST affects its phenotypic expression, we measured GST levels in serum as shown in Table 3. GST levels were 20 to 25% lower in GSTM1 null and GSTT1 null groups compared to both positive group, whereas in the both null groups, it was 31% lower, indicating a somewhat combined effect of double deletion. In order to see the effect of this differential expression of GST on OS, we measured MDA in serum and GSH in whole blood (Table 3). MDA levels altered significantly in case of deletions in GST gene either single or both, indicating that GST gene polymorphism might have some contributory effect towards development of OS observed in DN patients. In case of either GSTM1 or GSTT1 deletions, MDA level was about 20% higher, whereas in both null cases, it was 35% higher again, indicating a possible additive effect of these gene deletions. The levels of intracellular antioxidant GSH were also found to be diminished correspondingly, signifying increased OS. It was inferred that deletion of GSTM1 and GSTT1 genes led to lower levels of GST in the cells causing OS, which possibly plays an important role in the development of DN.

OS is an integral feature of DN as has been shown by several reports. However, this was the first
study, indicating the role of concomitant deletions of \textit{GSTM1} and \textit{GSTT1} genes in the development of OS in patients of DN. As the sample size in this study was small, the statistical relevance of the results of genetic polymorphism was not attempted. Further studies using increased sample size to find out the allelic distribution and their role in the development of DN are in progress.

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