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To cite this article: Hamid Nomani, Lida Hagh-Nazari, Ali Aidy, Asad Vaisi-Raygani, Amir Kiani, Zohreh Rahimi, Fariborz Bahrehmand, Ebrahim Shakiba, Hamid Reza Mozaffari, Heidar Tavilani & Tayebeh Pourmotabbed (2016) Association between GSTM1, GSTT1, and GSTP1 variants and the risk of end stage renal disease, Renal Failure, 38:9, 1455-1461, DOI: 10.1080/0886022X.2016.1214054

To link to this article: https://doi.org/10.1080/0886022X.2016.1214054

Published online: 08 Aug 2016.
LABORATORY STUDY

Association between GSTM1, GSTT1, and GSTP1 variants and the risk of end stage renal disease

Hamid Nomania,b, Lida Hagh-Nazaria,b, Ali Aidya,e, Asad Vaisi-Raygania,b, Amir Kiania,b, Zohreh Rahimi,c, Fariborz Bahrehmanda,b, Ebrahim Shakiba,b, Hamid Reza Mozaffarif, Heidar Tavilanig and Tayebeh Pourmotabbedd

aFertility and Infertility Center Research, Kermanshah University of Medical Sciences, Kermanshah, Iran; bDepartment of Clinical Biochemistry, Medical School, Kermanshah University of Medical Sciences, Kermanshah, Iran; cMedical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran; dDepartment of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA; eBiotechnology and Medicinal Plant Research Center, Ilam University of Medical Sciences, Ilam, Iran; fDepartment of Oral Medicine, School of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, Iran; gUrology and Nephrology Research Center, Hamadan University of Medical Sciences, Hamadan, Iran

ABSTRACT

Introduction: There are some evidences indicating DNA damage by oxidant and mutant agents has an essential role in the chronic renal failure and end stage renal disease (ESRD). To investigate the possible association of GSTs variants with ESRD, we investigated the frequency of GST-T1, M1, and P1 genotypes, and the level of malondialdehyde (MDA) in patients with ESRD.

Materials and methods: The present case-control study consisted of 136 ESRD patients treated with maintenance hemodialysis and 137 gender- and age-matched, unrelated healthy controls from the population of west of Iran. The GST- T1, M1, and P1 genotypes were determined in all individuals using multiplex-PCR and PCR-RFLP. The level of MDA was measured by high-performance liquid chromatography (HPLC).

Results: We found that GSTM1 and GSTT1 null genotypes (GSTT1/C0/GSTM1/C0) increased the risk of ESRD by 1.8 times (p < 0.001) and the increased risk of ESRD for GSTM-null (T1+/M1-/C0) genotype was 3.04 times (p = 0.002). ESRD patients carriers the GST (GSTM1-null + GSTT1-null + GST-null) genotypes compared to GST normal genotype increased the risk of ESRD by 3.3 (p < 0.001) times. ESRD patients carriers of GST-null, GSTM1-null, and GSTT1-null genotypes had greater MDA concentration compared with the same genotypes of control subjects. Our results indicated that the GST-null allele (GSTT1-null/GSTM1-null) is a risk factor for ESRD and carriers of this allele have high levels of MDA.

Conclusion: Our findings indicate that oxidative stress, impairment of the antioxidant system and abnormal lipid metabolism may play a role in the pathogenesis and progression of ESRD and its related complications. These data suggest that patients with ESRD are more susceptible to vascular diseases.

Introduction

End-stage renal disease (ESRD) is associated with several disorders including atherosclerosis, arthritis, dyslipidemia, metabolic syndrome, and cardiovascular disease (CVD). CVD has been reported as one of the main causes of death in patients with ESRD.1 A multicenter study demonstrated that the patients with ESRD have 10–20-fold higher chances to develop CVD compared with the general population and this evidence cannot be solely due to the “traditional” risk factors.2 Therefore, early detection of susceptibility of patients to ESRD and to CVD is of significant value. Evidences indicate that oxidative stress is one of the important factors involved in the development of CVD and atherosclerosis in patients undergoing maintenance hemodialysis.

It has been suggested that deficiency and alterations in the antioxidant mechanism and increased production of reactive oxygen species, lipid oxidation index of malondialdehyde (MDA) and DNA damage are important participating agents in the initiation and progression of oxidative and atherogenic event.3–5 In addition to the well-established link of oxidative stress with renal failure, the role of genetic predisposition in enhanced oxidative damage6 is demonstrated. Soluble glutathione S-transferase (GST, N-acetyltransferase epoxide hydroxylase, and sulphotransferase) is also known as phase II detoxifying enzymes.7,8 play an essential role in

CONTACT Lida Hagh Nazari  Lida.Haghnazari@yahoo.com; Ebrahim Shakiba  m.shakiba.d91@yahoo.com Department of Clinical Biochemistry, School of Medicine, Kermanshah University of Medical Sciences, Daneshgah Avenue, PO Box 6714869914, Kermanshah, Iran

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protecting DNA from genotoxins damage by inhibiting the formation of DNA adducts.9 Human GST genes are divided into four major subfamilies designated as GST α or A, GST μ or M, GST θ or T, and GST π or P.8 GSTM1 and T1 are polymorphic in populations and the null genotype for each of these genes has a high prevalence in human populations (10–65%).10,11 Deletions of these genes lead to lack of enzyme activity.12 The GSTP1 gene shows polymorphism within its coding region, of which the most well-known is an A–G transition at nucleotide position 1578, causing an isoleucine to valine substitution at codon 105 (Ile105 Val) in exon 5. This polymorphism results in decreased enzyme activity.5 This mutation is associated with high level of hydrophobic DNA adducts.13 In addition, altered GST activity associated with polymorphisms is expected to affect coronary artery disease (CAD), kidney disease, and cancer risk through decreased protection against DNA damage from reactive electrophiles.14

In this study, we investigated the relationship of GST genotypes with the risk of ESRD, and detected the levels of MDA, and total antioxidant capacity (TAC) in ESRD patients from west population of Iran.

**Patients and methods**

**Subjects**

The study protocol was approved by the Ethics Committee of the Kermanshah University of Medical Sciences and was in accordance with the principles of the Declaration of Helsinki II. All subjects provided written informed consent. The subjects were 136 hemodialysis patients with ESRD (mean age 58.1 ± 13.3 years; 89 male and 47 female) that were going under the dialysis patients from west population of Iran.

Controls and patients were sex and age matched.

**Blood collection**

A total of 5 mL of venous blood sample were collected from patients (pre-dialysis) and control subjects. Three milliliter of the blood samples were collected in EDTA vials for genomic DNA extraction and 2 mL blood without EDTA was used for biochemical analysis.

**DNA extraction**

DNA was extracted from blood by phenol-chloroform extraction method.16 The purity and concentration of extracted DNA was measured by Nanodrop spectrophotometer (Thermo 2000C model). The forward and reverse primers used for multiplex PCR and PCR conditions for determination of GSTM1, GSTT1, and GSTP1 genotypes are listed in Table 1. β-globin gene was co-amplified and used as an internal control for multiplex PCR. Multiplex PCR was carried out using 20 pmol of each primer,17 200 µM dNTPs, 1.5 mM MgCl2, 1 U Taq polymerase enzyme in a 10x PCR buffer, and 300–500 ng genomic DNA in total volume of 25 µL. After the DNA was denatured at 95 °C for 8 min, the reaction mixture was subjected to 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min with a final extension time of 10 min at 72 °C. PCR products were electrophoresed on a 2% agarose gel. The presence of GSST1 (a 480 bp fragment) and GSTM1 (a 230 bp fragment) was confirmed in the presence of amplified fragment of β-globin gene (268 bp). In the presence of GSTM1-null or GSTT1-null genotype, no specific PCR product of GSTM1 and GSTT1 was observed (Figure 1).

Genotyping of the single-nucleotide polymorphism of the GSTP1 was performed by PCR-RFLP. The GSTP1 allele was detected by treating their corresponding PCR products with 1 U of ALW261 restriction enzyme for at

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**Table 1. Primers and PCR conditions for all the GSTs variants.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer sequence</th>
<th>PCR condition</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>GST T1 &amp; M1 &amp; globin multiplex PCR</td>
<td>M1: 5′-TTC TGG ATT GTA GCA GAT CA-3′&lt;br&gt;M2: 5′-CCG CAT CTT GTG CTA CAT TGCCG-3′&lt;br&gt;T1: 5′-TTC CTT ACT GGT CCT CAC ATC TC-3′&lt;br&gt;T2: 5′-TCA CCG GAT CAT GCC GAG CA-3′&lt;br&gt;B1 (β-globin): 5′-CAA CTT CAT CCA GGT TCA CC-3′&lt;br&gt;B2 (β-globin): 5′-GAA GAG CCA AGG ACA GGT AC-3′&lt;br&gt;P1: 5′-AGT GTA TGG TCC AGA AGT AG-3′&lt;br&gt;P2: 5′-AGC CAC CTG AGG GGT AAG-3′</td>
<td>Initial denaturation 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, annealing at 60 °C for 30 s, extension at 72 °C for 30 s with a final extension at 72 °C for 5 min</td>
<td>21</td>
</tr>
<tr>
<td>GST P1</td>
<td>M1: 5′-TTC TGG ATT GTA GCA GAT CA-3′&lt;br&gt;M2: 5′-CCG CAT CTT GTG CTA CAT TGCCG-3′&lt;br&gt;T2: 5′-TCA CCG GAT CAT GCC GAG CA-3′&lt;br&gt;B1 (β-globin): 5′-CAA CTT CAT CCA GGT TCA CC-3′&lt;br&gt;B2 (β-globin): 5′-GAA GAG CCA AGG ACA GGT AC-3′&lt;br&gt;P1: 5′-AGT GTA TGG TCC AGA AGT AG-3′&lt;br&gt;P2: 5′-AGC CAC CTG AGG GGT AAG-3′</td>
<td>Initial denaturation 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, annealing at 61 andn JM, Sanchez P, Ruiz-Reguena E. C for 30 s, extension at 72 °C for 1 min with a final extension at 72 °C for 5 min</td>
<td>21</td>
</tr>
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</table>
at least 16 h in 37 °C. The digested fragments were electrophoresed on a 12% polyacrylamide gel. The genotypes of GSTP1 were determined as follows: The PCR product (433 bp) corresponding to wild type of GSTP1 allele was cleared by ALW26I enzyme and generating 329 and 104 bp, while the PCR products containing of the homozygote genotype generating 222 and 104 bp DNA fragments and heterozygotes of GSTP1 were cleared by the enzyme producing 329, 222 and 104 bp fragments (Figure 2).17

Figure 1. Agarose gel electrophoresis (2%) pattern of GSTM1 and GSTT1 multiplex PCR. From left to right lane 1 shows 100 base pairs DNA molecular weight marker; GSTT1 yields a product of 480 bp, whereas the amplification product of GSTM1 is 230 bp and β-globin gene (268 bp) as an internal control. Lanes 2 GSTT1/GSTM1 genotypes, lanes 3 GSTT1/GSTM1null genotypes, lanes 4 and 7 GSTT1null/GSTM1 genotypes, lanes 5, and 6 GSTT1null/GSTM1null genotypes.

Measurement of serum levels of MDA and TAC

Plasma MDA was measured by an Agilent Technologies 1200 Series high-performance liquid chromatography (HPLC) system (Agilent Corp., Germany) using a fluorescence detector. The column was EC 250/4.6 Nucleodur 100–5 C18ec (Macherey-Nagel, Duren, Germany). Butylated hydroxytoluene (BHT), MDA, methanol, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), were analytical grade and purchased from Sigma Chemical Co. (St Louis, MO), all other reagents were products of Merck (Darmstadt, Germany).

For MDA analysis, a 50-µL sample [plasma or TEP standard (stock standard solution containing 5 µM TEP in 40% ethanol solution) was spiked with 50 µL BHT (0.05%V/V BHT in ethanol), 400 µL H3PO4 solution, and 100 µL TBA (TBA 42 mM in 0.44 M phosphoric acid) solution in a 2-mL Eppendorf tube. Sample tubes were capped tightly, vortex mixed, then incubated for 1 h in a 100 °C water bath. Following heat derivatization, samples were cooled on an ice for 10 min, with 250 µL n-butanol subsequently added to each vial for extraction of the MDA–TBA complex. Tubes were vortex mixed 5 min then centrifuged 3 min at 14,000 g to separate the two phases. Aliquots of 100 µL were removed from the n-butanol layer of each sample and placed in HPLC vials for analysis without evaporation. Serum MDA was determined by injection of 20 µL of above solution onto HPLC reverse phase with a mobile phase comprised methanol-buffer (40/60, V/V) [phosphate buffer (KH2PO4 50 mM adjust pH = 6.7 with KOH) pumped at 1 mL/min. MDA was detected by its native fluorescence at 553 nm, excitation 515 nm. The concentration and identity of the eluted MDA was confirmed by comparison to a commercial standard and quantified by peak area using Agilent Technologies 1200 Series software.18

All analysis was conducted in duplicate and data was displayed as the mean ± the standard error of the mean of duplicate treatments.

The serum levels of TAC were measured using commercially available kits (Randox Laboratories Ltd., Crumlin, Antrim, N. Ireland, Cat. no. NX2332).

Statistical analysis

Allelic frequencies were calculated by the gene counting method. The χ² test was used to verify the agreement of the observed genotype frequencies with those expected according to the Hardy–Weinberg equilibrium. The genotypes and allele frequencies of GST in patients with ESRD were compared with the control group using the χ² test. Odds ratios (OR) were calculated as estimates of relative risk for disease and 95% confidence intervals obtained by SPSS logistic regression (SPSS Inc., Chicago, IL). A two-tailed Student’s t-test, analysis of variance and nonparametric independent sample Mann–Whitney analysis were used to compare quantitative data. Statistical significance was assumed at p < 0.05.
Results

Characteristics of ESRD patients and control subjects are shown in Table 2. There was no significant difference between the mean of age and sex of the two groups. The patients with ESRD had significantly higher plasma MDA concentration ($2.04 \pm 0.4 \text{ vs. } 1.1 \pm 0.3 \text{ mM, } p < 0.001$) and serum level of TAC ($8.9 \pm 2.4 \text{ vs. } 6.4 \pm 2.3 \text{ mM, } p < 0.001$) compared to control group.

**GST M1 and GST T1 genotype**

Frequencies of GST M1 & T1 normal and null genotypes are described in detail in Table 3. The observed GST M1, T1, and P genotypes distribution in patients with ESRD and healthy individuals was the same as the predicted genotype from the Hardy–Weinberg equilibrium. The frequencies of GST normal (T1+/−M1+) and GSTT1-null (T1−/−M1+) genotypes were higher in control subjects than patients, while the frequencies of GSTM1-null (T1+/−M−) and GST-null (T1−/−M−) genotypes were significantly higher in patients with ESRD compared to those in controls ($p < 0.001, \chi^2 = 22.6$). Odds ratio (OR) of GST T1 and GST M1 genotypes in individuals with either GSTM1-null or GSTT1-null genotype indicated that these genotypes increased the risk of ESRD by 2.61 times, and those genotypes with one of GSTM1-null or GSTT1-null or GST null (T1−/−M−) genotypes, increased 3.3 times risk of ESRD (Table 4). The presence of GSTM1-null genotype increased the risk of ESRD by 3.04 fold.

As shown in Table 5, patients with GSTM1-null, GSTT1-null, or GST-null genotypes had significantly higher plasma concentrations of MDA and TAC compared to controls with the corresponding genotypes.

**GST P1 genotype and alleles**

The overall distribution of GST P1 genotype and alleles (as calculated using the actual allele number) in ESRD patients were similar to those in control group. GST P1 genotype and alleles did not significantly increase the risk of ESRD (Table 6).

Discussion

Chronic kidney disease (CKD) is increasingly recognized as a major public health problem in the world. ESRD is a progressive and irreversible deterioration in renal function associated with high levels of free radicals.

Alterations and modifications in plasma lipid concentration, the antioxidative defense mechanism, a significant increase in the levels of serum biomarkers for lipid peroxidation, such as MDA, and lower levels of non-enzymatic antioxidant systems have been observed in patients with ESRD. These results suggest that there is a relationship between ESRD and oxidative damage.

GSTs isoforms are responsible for cell defense against electrophile agents and unsaturated aldehyde atherogens. These enzymes catalyze the conjugation of glutathione to a wide range of electrophiles and play a protective role against oxidative stress.

As shown in Table 5, patients with GSTM1-null, GSTT1-null, or GST-null genotypes had significantly higher plasma concentrations of MDA and TAC compared to controls with the corresponding genotypes.
A/G 50 (37.6%) A/A 74 (55.6%) 70 (51.1%) GST P1 genotypes

G 68 (25.6%) A 198 (74.4%) 197 (71.9%) GST P1 alleles

The distribution of GST P1 genotypes and alleles in patients with ESRD was not found in Taiwanese or Asian Indians ESRD patients.

Human cytosolic GSTs have been well characterized and are known to be polymorphic, with variable frequency in different ethnic groups. The percentage of individuals who do not express the GSTM1 and GSTT1 enzymes due to homozygous gene deletion is higher in Caucasians and in Asians than in Africans. About 60% of Asians, 40% of Africans, and 20% of Caucasians do not express the GSTM1 and GSTT1 enzymes. These data suggest the existence of ethnic-specific GST genetic susceptibility to ESRD development.

The role of low-activity GST-P1 polymorphism has been addressed in only two studies that demonstrated an association between this polymorphism with increased risk of ESRD. Our result, however, did not show significant association between GST P1 polymorphism and ESRD.

Among GST genotypes, individuals with GST null (T1—M1—) genotype had maximum MDA levels when compared to the control group, indicating a high level of lipid peroxidation in these subjects. Recently, we have reported that GSTT1-null and GSTM1-null genotypes are involved in the pathogenesis of CAD in west of Iran and patients with SLE, psoriasis, or CVD have significantly high concentration of MDA compared to control individuals. These data together suggest a link between MDA level and high risk of CVD development in patients with ESRD.

In this study, the frequency of GST normal in control group was 32.8% and the frequency of GST mutant (GSTM1-null + GSTT1-null + GST-null) was 67.2%, whereas in ESRD group, they were 12.8 and 87.2%, respectively. Our data suggests that inactive forms of enzymes do not protect the individuals from oxidative damage that can lead to the development of ESRD.

Conclusion

Our results indicated that the GST-null allele (GSTT1-null/GSTM1-null) is a risk factor for ESRD. Carriers of this
allele have high levels of MDA. These results indicate that oxidative stress, impairment of the antioxidant system and abnormal lipid metabolism may play a role in the pathogenesis and progression of ESRD and its related complications. Our data also suggest that patients with ESRD are more susceptible to vascular diseases. However, due to the heterogeneous picture of ESRD and the influence of a subset of risk factors in the development of the disease further studies are needed to shed light on contribution of GST-null allele of GST polymorphism and their enzymatic activity in the development of ESRD in different ethnicities.

**Disclosure statement**

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This work was supported by the Kermanshah University of Medical Sciences, Kermanshah, Iran [grant No. 90064].

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