Association of glutathione S-transferase polymorphisms with the severity of mustard lung

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Abstract

Introduction: Glutathione S-transferase (GST) is one of the major detoxifiers in alveoli. Polymorphism in GST genes can influence the ability of individuals to suppress oxidative stress and inflammation. The present study was aimed to explore the hypothesis that the genetic polymorphisms of GST T1, M1 and P1 are associated with the severity of the mustard lung in the sulfur mustard-exposed individuals.

Methods: Blood samples were taken from 185 sulfur mustard-exposed and 57 unexposed subjects. According to the stage of the mustard lung, sulfur mustard-exposed patients were categorized in the mild/moderate and severe/very severe groups. A multiplex PCR method was conducted to identify GSTM1 and GSTT1 null genotypes. To determine the polymorphisms of GSTP1 in exon 5 (Ile105Val) and exon 6 (Ala114Val), RFLP-PCR method was performed.

Results: The frequency of GSTM1 homozygous deletion was significantly higher in the severe/very severe patients compared with the mild/moderate subjects (66.3% vs. 48%, P = 0.013). The GSTM1 null genotype was associated with the severity of mustard lung (adjusted odds ratio [OR], 2.257; 95% CI, 1.219-4.180). There was no significant association between GSTT1 and GSTP1 polymorphisms with the severity of the mustard lung.

Conclusion: The different distribution of GSTM1 null genotype in severe/very severe and mild/moderate groups indicated that the severity of the mustard lung might be associated with the genetic polymorphism(s).

Introduction

Sulfur mustard or 1-chloro-2-(2-chloroethylsulfanyl) ethane as an alkylating agent can induce a special type of chronic obstructive pulmonary disease (COPD), which is called “mustard lung”.1,2 The mustard lung begins with the acute bronchitis and airway hyperreactivity, then continues to form some long-term respiratory complications, which persist throughout the lifetime of most cases. Understanding the molecular mechanism of the mustard lung and the triggers of its severity is thus crucial to restrict the respiratory complications. Individuals exposed to sulfur mustard, develop ongoing lung inflammation and an imbalance in oxidant/antioxidant systems.3,4 For example, evaluation of inflammatory chemokines has indicated the elevated level of some acute and chronic inflammatory mediators in these individuals.6,7 Inflammation and oxidative stress-related disabilities are associated with genetic variation.8-11 Thus, the evaluation of genetic variations in patients with different severity of mustard lung may provide helpful evidence to address its underlying molecular mechanism.

Glutathione S-transferases (GSTs) as a detoxifying family of enzymes modulate the addition of glutathione to oxidative stress products. These enzymes are involved in the incidence of oxidative stress and inflammatory-related diseases for various reasons. Firstly, several GST genes are polymorphic and their allelic variations can affect disease susceptibility.12,13 Secondly, they detoxify a wide range

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of structurally different substrates produced through oxidative stress.\textsuperscript{13} For example, peroxides produced from lipids, DNA, and catecholamines are neutralized by GST enzymes.\textsuperscript{16} GSTs are also involved in intracellular signal transduction to regulate inflammation and oxidative stress signaling pathways.\textsuperscript{17,18}

According to their genetic sequences and amino acid content, the mammalian GSTs have been classified into seven distinct classes. These classes and their OMIM accession number are as follows: alpha (e.g., GSTA1; 138359), mu (GSTM1; 138350), kappa (GSTK1; 602321), theta (e.g., GSTT1; 600436), pi (GSTP1; 134660), omega (GSTO2; 605482), and zeta (GSTZ1; 603758). The main contribution of this study is to evaluate the association between the severity of mustard lung and some of GST polymorphisms. The studied variations were GSTM1 null genotype (GenBank accession no. X68676), GSTT1 null genotype (GenBank accession no. AP000351) and GSTP1 single nucleotide polymorphisms (SNPs) including Ile105Val (rs1695) and Ala114Val (rs1138272).

Materials and Methods

Materials

All standard chemicals for DNA extraction, including EDTA, NAOH pellets, Tris base, HCl, MgCl\(_2\), Triton X100, sucrose, NaCl, sodium dodecyl sulfate, and sodium perchlorate were purchased from Sigma-Aldrich (Germany). Primers were obtained from Pishgam Biotech Company (Iran). PCR master mix (2X) was provided by Ampliqon (Denmark). GelRed\textsuperscript{TM} Nucleic Acid Gel Stain was purchased from Biotium (USA). Agarose, Alw26I and AciI restriction enzymes, RFLP reagents, and DNA ladder were obtained from Thermo Scientific (USA).

Study design and participants

The study was performed on 185 male Iranian subjects with long-term respiratory complications 25 years after exposure to sulfur mustard. About 57 unexposed male subjects with mean age of 41.9±9.6 were also used as the control. GOLD spirometric criteria based on FEV\textsubscript{1} were used as an amplification template together with 10pmol of each primer and 10 μL 2X PCR master mix with 1.5 mM MgCl\(_2\) Amplification was conducted as follows: initial denaturation step for 5 minutes at 95°C, followed by 30 cycles at 94°C for 30 seconds, 61.5°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The PCR products from co-amplification of GSTT1, GSTM1 and CYP1A1 genes were then analyzed by electrophoresis on 2% agarose gels stained with GelRed\textsuperscript{TM}.

In GSTP1 gene 2 polymorphic sites (Ile105 → Val105; Ala114 → Val114) were assessed. For GSTP1 105Val variant, RFLP-PCR was performed according to Harries et al.\textsuperscript{22} The forward and reverse primers used were 5’ ACC CCA GGG CTC TAT GGA AA 3’ and 5’ CAG GTG CAC GGG CAC AAG CCC CT 3’, respectively. In a total volume of 20 μL, isolated DNA (50 ng) was used as an amplification template together with 10pmol of each primer and 10 μL 2X PCR master mix with 1.5 mM MgCl\(_2\). Amplification was carried out with a primary denaturation step at 95°C for 5 minutes, continued with 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The digestion of PCR product accomplished using 5 units of Alw26I, in a total volume of 20 μL. The digested products were analyzed by electrophoresis on 3.5% agarose gels.

For determining GSTP1 114Ala variant, RFLP-PCR was performed according to a method described by Ishii et al.\textsuperscript{7} The forward and reverse primers used were 5’ GGGGAGCAGCAGGAGAAT 3’ and 5’ CAGGTTGTAAGCAGGAGAAG 3’, respectively. In a total volume of 20 μL, isolated DNA (50 ng) was used as an amplification template together with 10pmol of each primer and 10 μL 2X PCR master mix with 1.5 mM MgCl\(_2\). Amplification was carried out with a primary denaturation step at 95°C for 5 minutes, continued with 30 cycles at 95°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The PCR product was digested using 5 units of AcI in a total volume of 20 μL. The digested products were analyzed using electrophoresis on 3.5% agarose gels.

Statistical analysis

The chi-square test was used to explore the distribution GSTM1 and GSTT1 null genotypes were determined in a single assay by multiplex polymerase chain reaction (PCR) technique.\textsuperscript{21} The forward and reverse primers used were 5’ GAACTCCCTGAAAGCCTAAAGC3’ and 5’ GTGGGGCTCAAATACGGTGG 3’ for GSTM1 null genotype; 5’ TCACCGGATCATGGCGACAGCA 3’ and 5’TTCCTTACTGGCTCCTCACATCTC 3’ for GSTT1 null genotype; 5’ GAACTGGCAGTCTAGCTT 3’ and 5’ CAG CTG CAG TTTGGAAGTGCT 3’ for CYP1A1 gene. Co-amplification of CYP1A1 gene was carried out to document successful PCR amplification. PCR was accomplished in a final volume of 20 μL containing 50ng isolated DNA, 10 pmol of each primer, and 10 μL 2X PCR master mix with 1.5 mM MgCl\(_2\). Amplification was conducted as follows: initial denaturation step for 5 minutes at 95°C, followed by 30 cycles at 94°C for 30 seconds, 61.5°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The PCR products from co-amplification of GSTT1, GSTM1 and CYP1A1 genes were then analyzed by electrophoresis on 2% agarose gels stained with GelRed\textsuperscript{TM}.

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of genotypes in 3 groups. Furthermore, the odds ratio was calculated between mild/moderate and severe/very severe groups to determine the risk of mustard lung severity in different genotypes. PFT parameters were compared between study groups with the t test. Data are expressed as the mean ± standard deviation (SD) and the frequency (percentage). All the statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

Results
The demographic characteristics of patients with severe/very severe and mild/moderate symptoms are shown in Table 1.

GSTM1 and GSTT1 gene polymorphisms
GSTM1 and GSTT1 genotypes were detected by the presence or absence of a band at 480 bp (corresponding to GSTT1 gene) and a band at 215 bp (corresponding to GSTM1 gene). A band at 312 bp (corresponding to CYPlAl gene) was used as an internal control (Fig. 1).

The distributions of GST genotypes were explored to evaluate the probable association between genetic variation in GST genes and the severity of pulmonary complications. In the case of GSTM1, the frequency of homozygous null genotype in severe/very severe patients was 66.3% in comparison with mild/moderate individuals (48%) and unexposed subjects (57.9%), (P = 0.044), (Table 2).

Comparing the 2 sulfur mustard-exposed groups for GSTM1 polymorphism indicated that the carriers of homozygous null genotype were more likely to develop the severe and very severe form of pulmonary complications (P = 0.013; adjusted odds ratio [OR], 2.257; 95% CI, 1.219-4.180), (Table 2). The association between GSTM1 null genotype and the presence of symptoms like a chronic cough and sputum was also explored. However, there was no statistical association between GSTM1 null genotype with sputum (P = 0.793) and chronic cough (P = 0.717), (Table 3).

The frequencies of GSTT1-null genotype in severe/very severe, mild/moderate and unexposed groups were 18.1%, 24.5%, and 17.5%, respectively. Although there was a 6.5 percent statistical difference between 2 exposed groups, it was not significant (adjusted OR, 0.676; 95% CI, 0.322-1.419). A combinational study of GSTM1- and GSTT1-null genotypes was also conducted. Although the association among 3 groups in the combinational study was of borderline statistical significance (P = 0.069), the comparison of severe/very severe subjects with mild/moderate patients indicated the elevated risk of pulmonary complications (adjusted OR: 2.058, when 95% CI, 1.099-3.953, P = 0.033), (Table 2).

GSTP1 gene polymorphisms
The Ile105Val transition occurs within the exon 5 of GSTP1 gene. The digestion of PCR product determined 3 different genotypes in this region (Ile/Ile, Ile/Val and Val/Val). The presence of a single 176 bp band indicates the wild-type genotype (Ile/Ile), whereas the conversion of Ile to Val results in 3 bands of 176, 91 and 85 bp in heterozygotes and 2 bands of 91 and 85 bp in homozygotes (Fig. 2). Statistical differences among these genotypes were negligible. For example, the percentage of Ile/Ile

Table 1. Clinical characteristics and age distribution in sulfur mustard-exposed patients

<table>
<thead>
<tr>
<th>Pulmonary Assessment (GOLD)</th>
<th>Mild/Moderate</th>
<th>Severe/Very Severe</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Chronic cough</td>
<td>No</td>
<td>5</td>
<td>4.9%</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>97</td>
<td>95.1%</td>
</tr>
<tr>
<td>Sputum</td>
<td>No</td>
<td>14</td>
<td>13.7%</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>88</td>
<td>86.3%</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td>45.6</td>
<td>7.2</td>
</tr>
<tr>
<td>FVC (%)</td>
<td></td>
<td>89.26</td>
<td>6.08</td>
</tr>
<tr>
<td>FEV1 (%)</td>
<td></td>
<td>59.07</td>
<td>4.05</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td></td>
<td>66.5</td>
<td>6.6</td>
</tr>
</tbody>
</table>

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Table 2. Distribution of GSTM1, GSTT1 and GSTP1 genotypic frequencies in sulfur mustard-exposed patients and non-exposed individuals

<table>
<thead>
<tr>
<th>Gene polymorphisms</th>
<th>Unexposed</th>
<th>Mild-moderate</th>
<th>Severe-very severe</th>
<th>P*</th>
<th>P#</th>
<th>P$</th>
<th>P%</th>
<th>OR$</th>
<th>AOR$</th>
<th>95% CI</th>
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</thead>
<tbody>
<tr>
<td>GSTM1 genotype</td>
<td>-/-</td>
<td>33</td>
<td>57.9</td>
<td>49</td>
<td>48.0</td>
<td>55</td>
<td>66.3</td>
<td>0.444</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/+ or +/+</td>
<td>24</td>
<td>42.1</td>
<td>53</td>
<td>52.0</td>
<td>28</td>
<td>33.7</td>
<td>0.233</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTT1 genotype</td>
<td>-/-</td>
<td>10</td>
<td>17.5</td>
<td>25</td>
<td>24.5</td>
<td>15</td>
<td>18.1</td>
<td>0.450</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/+ or +/+</td>
<td>47</td>
<td>82.5</td>
<td>77</td>
<td>75.5</td>
<td>68</td>
<td>81.9</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTP1 Ile105Val genotype</td>
<td>II</td>
<td>24</td>
<td>43.9</td>
<td>42</td>
<td>41.2</td>
<td>39</td>
<td>47</td>
<td>0.896</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>28</td>
<td>49.1</td>
<td>51</td>
<td>50</td>
<td>36</td>
<td>43.4</td>
<td>0.899</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VV</td>
<td>4</td>
<td>7.0</td>
<td>9</td>
<td>8.8</td>
<td>8</td>
<td>9.6</td>
<td>0.648</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AV</td>
<td>7</td>
<td>12.3</td>
<td>17</td>
<td>16.5</td>
<td>15</td>
<td>18.1</td>
<td>0.648</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTM1 and GSTT1 combinational genotype</td>
<td>M1/- and T1/- or +/+</td>
<td>34</td>
<td>59.6</td>
<td>61</td>
<td>59.8</td>
<td>62</td>
<td>74.7</td>
<td>0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1/- and T1/-</td>
<td>23</td>
<td>40.4</td>
<td>41</td>
<td>40.2</td>
<td>22</td>
<td>25.3</td>
<td>0.044</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: AOR: Adjusted odds ratio adjusted by age.

* comparison of 3 groups; † comparison of mild-moderate individuals with unexposed individuals; ‡ comparison of severe-very severe individuals with unexposed individuals; § comparison of exposed and unexposed individuals; ‡ comparison of severe-very severe individuals with mild-moderate subjects. * comparison of severe-very severe individuals with mild-moderate subjects. * data show significant differences with P < 0.05.

Table 3. Association of GSTM1 genotype with sputum and chronic cough in exposed population

<table>
<thead>
<tr>
<th>GSTM1 genotype</th>
<th>Sputum</th>
<th>Chronic Cough</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>-/-</td>
<td>14</td>
<td>53.8</td>
</tr>
<tr>
<td>+/+ or +/+</td>
<td>12</td>
<td>46.2</td>
</tr>
</tbody>
</table>

genotype was determined as 47%, 41.2%, and 43.9% for severe/very severe, mild/moderate and unexposed groups, respectively. Consequently, the association between severity of pulmonary complications and the Ile105Val polymorphism was not significant (adjusted OR: 0.695; 95% CI, 0.237- 2.042), (Table 2).

The Ala114Val variation occurs within the exon 6 of GSTP1 gene. The digestion of PCR product revealed 2 genotypes in this region (Ala/Ala and Ala/Val). The wild-type genotype (Ala/Ala) presents 2 recognition sites and creates 3 separated fragments (55, 118 and 247 bp). The conversion of Ala to Val removes one of the recognition sites and consequently, creates 3 separated fragments (55, 118 and 247 bp). The GSTM1 modulates signaling through interaction of lipid and DNA hydroperoxides. This is specifically important because sulfur mustard derivatives trigger the formation of lipid peroxidation biomarkers such as malondialdehyde.26, 27 In addition, GSTM1 modulates signaling through interaction and inhibition of ASK1 (apoptosis signaling kinase 1).17 This protective mechanism can preserve cells and is necessary to restrict oxidative stress. ASK1 is a MAP3K and acts through JNK and p38 signaling pathways, which respond to different stresses such as ROS and also regulate inflammation, morphogenesis and apoptosis.24
Other studied polymorphisms were Ile105Val and Ala114Val within exon 5 and 6 of GSTP1 gene. Isoleucine to Valine substitution at codon 105 does not change the enzymatic affinity for glutathione. However, electrophile substrate and its bulkiness can affect the efficiency of catalytic reaction. Thermal stability of the Val 105 variant is also 2.5 fold higher than the Ile 105 variant. The residue 114 (Ala) is located in a position far from the active site, and its variation effects are unclear exactly.

In the current study, obtained results revealed that the GSTM1 null genotype frequency was 18% higher in severe/very severe patients in comparison with mild/moderate individuals. The frequency of GSTM1 null genotype was determined 58% in unexposed group, which is consistent with other studies in Iranian population. The frequencies of homozygous null genotype in severe/very severe patients (66.3%) and mild/moderate population (48%) indicate the different distribution of genotypes in 2 groups compared with standard population and therefore, imply that GSTM1 genotype affects the severity of mustard lung. Altogether, present study findings were consistent with the results reported by Cheng et al. According to their study GSTM1 deletion is a risk factor for developing the severe form of COPD while evaluated independently or in combination with other genes such as mEPHX.

In the case of GSTT1, there was not a remarkable association between gene deletion and the severity of mustard lung (adjusted OR, 0.676; 95% CI, 0.322-1.419). GSTT1 null genotype frequency in our studied population was in accordance with other studies in Iranian population. The combinatorial communication of GSTT1 and GSTM1 with the severity of mustard lung was also examined. This analysis showed that a significant association was established between the genotypes and phenotypes. Although the P value was on the border of reliability, the simultaneous association of the disease with GSTM1 and GSTT1 was controversial. Accordingly, more studies may be required to determine this association with severity of mustard lung.

Genetic variations of GSTP1 are associated with COPD. However, Associations between GSTP1 polymorphisms and the severity of pulmonary complications were doubtful in this study. Determined frequencies for GSTP1 polymorphisms in normal people were consistent with other studies in Iranian population.

One of the important functions of GSTM1 is to modulate inflammation-related signaling pathways. When the expression of GSTM1 increases through ASK1 and c-Jun activation, this factor acts as a negative feedback. This function can protect cells from developing oxidative stress and inflammation. As shown, GSTM1 homozygous null genotype induces the more severe form of the mustard lung that indicates the importance of ASK1 pathway in the patients. In the other words, high values of GSTM1 deletion may lead to more activation of ASK1 and consequently, the generation of an inflammatory response. Further investigations are needed to determine the impact of the ASK1 pathway on the severity and molecular mechanism of the mustard lung.

Conclusion
In the present study, we have reported the potential association between genetic polymorphism of GSTM1 with the severity of the mustard lung because the GSTM1 null genotype was found frequently in sulfur mustard-exposed patients with severe and very severe stage of lung complications. Although mustard gas is the main cause of the disease initiation, the exploration of genetic polymorphism indicated a complex interplay of genes and environment in the mustard lung patients. The examination of ASK1 over-activation is required to provide more information about the molecular mechanisms of long-term complications of sulfur mustard.

Ethical approval
The study was carried out according to the Declaration of Helsinki and on the basis of good clinical practice. All participants provided written informed consent in their native language.

Fig. 2. PCR-RFLP assay for identification of polymorphism at GSTP1 Ile 105 residue. Lanes 3-6 and 10-15: Ile/Ile; lanes 1 and 7: Ile/Val; lanes 2 and 8: Val/Val; lane 9: molecular weight ladder.

Fig. 3. PCR-RFLP assay for identification of polymorphism at GSTP1 Ala114 position. Lanes 1, 5 and 12: Ala/Val genotype; lanes 2-4, 6-9, 11, 13 and 14: Ala/Ala genotype; lane 10: molecular weight ladder.
Research Highlights

What is current knowledge?
✓ Sulfur mustard derivatives induce long-term complications in respiratory system. Inflammatory response, oxidative stress and airway obstruction are recognized as the main symptoms of mustard lung.

What is new here?
✓ The severity of mustard lung is affected by GSTM1 gene deletion, which proposes a complicated interplay among genes after exposure to mustard gas.

Competing interests
Authors declare no conflict of interests.

Acknowledgements
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