INVESTIGATION OF GSTP1, GSTM1 AND GSTT1 GENE POLYMORPHISMS AND SUSCEPTIBILITY TO COPD.

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is characterized by decreased expiratory flow rate, with cigarette smoking being most important risk factor. The glutathione-S-transferases (GSTs) constitute a family of xenobiotic-metabolizing phase II enzymes mediating exposure to cytotoxic and genotoxic agents, including cigarette smoking. We studied the relationship of GSTP1, GSTM1 and GSTT1 gene polymorphisms with COPD risk in case-control studies of Bulgarian patients and controls. A PCR-RFLP and modified multiplex (duplex) PCR-based method were applied for detection of GSTs’ genotypes. A significant case-control difference was observed for the presence of null GSTM1 (0.67 vs. 0.36, p=0.003), but not for GSTT1 (0.13 vs. 0.07, p=0.364) homozygous genotype. There was a prevalence of at least one null genotype among patients compared to control (0.72 vs. 0.50, p=0.060). We found a 3.60-fold (95% CI, 1.52-8.54) increased risk associated with GSTM1 null genotype and 2.54-fold (95% CI, 0.96-6.73) increased risk associated with carrying of at least one GST null genotype. No associations were detected for the frequencies of A313G (Ile105Val) GSTP1 alleles and genotypes with the risk of developing COPD (p=0.807 and p=0.958). We suggest that the inherited absence of GST-mu, but not GST-theta detoxifying enzymes, due to the presence of homozygous null genotypes may be associated with COPD.

Key words: COPD, GSTP1, GSTM1, GSTT1, null polymorphism, genetic predisposition

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by decreased expiratory flow rate. It is generally accepted that cigarette smoking is the most important risk factor for COPD (1-3): it is estimated that over 90% of patients diagnosed with COPD have been chronic heavy smokers. Aetiology of COPD remains unknown, but the fact that only 10-20% of chronic smokers develop the severe impairment of pulmonary function associated with COPD, indicates the presence of genetic predisposing factors in its pathogenesis (4-7). Based on the pathophysiological characteristics of COPD, a number of genetic variants have been proposed as risk factors. Of the candidate genes investigated in relation to COPD development, those coding for xenobiotic-metabolizing enzymes have attracted attention because of their function in detoxifying of cigarette smoke products (6-10). These enzymes include glutathione-S-transferases (GSTs), some cytochrome isoenzymes (CYP), microsomal epoxide hydrolase (mEPHX) etc, (6-11). Although the association between genetic polymorphisms in these enzymes and development of COPD has been reported in some studies, quite contradictory findings were also published (5, 6, 9).

GSTs are a superfamily of enzymes involved in phase II of xenobiotic-metabolism catalyzing the conjugation of a wide range of electrophilic substances with reduced glutathione, thereby facilitating detoxification and further metabolism and excretion. Most of GSTs are polymorphic enzymes and it is shown that the gene variations affect the enzyme activity and/or gene expression (12-15).
The acidic cytoplasmic GST isoenzyme GST-pi is coded by a single gene GSTP1, in which two single nucleotide polymorphisms (SNPs) have been identified. They consist of transitions at A\textsuperscript{1578}G (exon 5, A\textsuperscript{13}G) and C\textsuperscript{2293}T (exon 6, C\textsuperscript{341}T), resulting in amino acid substitutions Ile\textsuperscript{105}Val and Ala\textsuperscript{114}Val, respectively, which appear to be within the active site of the GST-pi protein (13, 15, 16).

Two of the most relevant human GST isoenzymes, GST-mu and GST-theta, are genetically deleted in a high proportion of human population because of the homozygosity of non-functional null alleles of GSTM1 and GSTT1 genes, respectively (11, 13, 14).

The aim of the current study was to identify A\textsuperscript{313}G (Ile\textsuperscript{105}Val) GSTP1, GSTM1 and GSTT1 genotype frequencies and to evaluate their impact on the susceptibility to COPD in a Bulgarian population from Stara Zagora region.

**MATERIAL AND METHODS**

**Patient and control populations**

The patient group consisted of 48 patients with COPD, stage III, 37 (77%) males and 11 (23%) females, aged from 45-84 years (median of 68 years).

The inclusion criteria for COPD were as follows: age higher than 40 years; forced expiratory volume in one second (FEV\textsubscript{1}) of <50%; forced expiratory volume in one second (FEV\textsubscript{1})/ forced vital capacity (FVC) ratio of \leq 70%; FEV\textsubscript{1} reversibility after inhalation of 400 \textmu g Salbutamol of <12%.

The control group consisted of 120 healthy voluntaries or individuals with an ordinary flue infection; 65 (54%) males and 55 (46%) females, aged between 30 and 85 years (median of 57 years). For each of the genetic polymorphisms different control group was constituted.

**Methods**

Genomic DNA was isolated from 2 ml of whole blood applying the conventional proteinase K digestion followed by protein precipitation with over-saturated solution of NaCl, and deposit of genomic DNA with absolute ethanol. In some cases a commercial kit for isolation of genomic DNA from blood (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma, USA) was also used.

**Genotyping**

A\textsuperscript{313}G (Ile\textsuperscript{105}Val) SNP in GSTP1 was analyzed by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique as described by Harries et al. and performed earlier by us (12, 17). Briefly, 2 to 4 \textmu l of the genomic DNA was used as a template in a final volume of 30 \textmu l PCR mix containing 1xPCR buffer and 1 pmol/\textmu l of each primer P105F (5'- ACC CCA GGG CTC TAT GGG AA-3') and P105R (5'- TGA GGG CAC AAG AAG CCC CT-3'), 100\textmu M dNTP, 1.5 mM Mg\textsubscript{Cl\textsubscript{2}}, and 1 U Taq polymerase (STS DNA polymerase, STS Ltd., Bulgaria). The annealing temperature was 55\(^\circ\)C, and 35 cycles were carried out. The PCR product (20 \textmu l) was digested with 5 U Alw26I in a total volume of 25 \textmu l of 1xTango buffer (Fermentas Ltd) at 37 \(^\circ\)C for 4 hours. The fragments were separated on a 2.5% agarose gel, visualized with ethidium bromide and documented with Gel documentation system (Syngene, Synoptics Ltd, UK).

The null polymorphisms in GSTM1 and GSTT1 were assessed using multiplex (duplex) PCR (18), which we modified. GSTP1 was used as a referent gene for successful amplification and presence of sufficient amount of DNA template. In brief: two parallel PCRs for GSTM1 and GSTT1 polymorphisms with GSTP1 as reference gene were carried out in a 25 \textmu l mixture containing 2 to 4 \textmu l of the genomic DNA, 1xPCR buffer, 2 mM Mg\textsubscript{Cl\textsubscript{2}} (for GSTM1) or 1.5 mM Mg\textsubscript{Cl\textsubscript{2}} (for GSTT1), 250 \textmu M dNTP, 1U Taq polymerase (STS DNA polymerase, STS Ltd., Bulgaria) and 0.5 \mu M of the following primers: T1F (5'-TTC CTT ACT GGT CCT CAC ATC TC-3') and T1R (5'-TCA CCG GAT CAT GGC CAG CA-3') in the mix for GSTT1, M1F (5'-GAA CTC CCT GAA AAG CTA AAG C-3'), and M1R (5'-GTT GGG CTC AAA TAT ACG GTG G-3') in the mix for GSTM1 and P105F and P105R for GSTP1 in both mixtures. Amplification consisted of 10 min at 94\(^\circ\)C predenaturation and 40 cycles of denaturation at 94\(^\circ\)C for 1 minute, annealing at 62\(^\circ\)C for 30 seconds, and extension at 72\(^\circ\)C for 1 minute, followed by final extension at 72\(^\circ\)C for 7 minute. This results in a fragment of 459 bp for GSTT1, 219 bp for GSTM1, and 176 bp for GSTP1. A positive and negative control, the latter containing water instead of DNA, was
included in all PCRs. PCR products were analyzed on a 2.5% agarose gel, stained with ethidium bromide and documented with Gel documentation system (Syngene, Synoptics Ltd, UK).

Statistical analyses

Statistical analyses were performed using StatView™ v.4.53 for Windows (Abacus Concepts, Inc.). The frequencies of distribution in contingency tables were analyzed using $\chi^2$ test and Fisher’s exact test.

RESULTS

The overall allele and genotype frequencies of GST genes in patients with COPD and controls are presented on Table 1.

**Table 1: Allele and genotype frequencies of the GSTP1, GSTM1 and GSTT1 genes in cases and controls**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Cases</th>
<th>Controls</th>
<th>OR (95% CI), p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Frequency</td>
<td>n</td>
</tr>
<tr>
<td><strong>GSTP1 genotype frequency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A (Ile/Ile)</td>
<td>21</td>
<td>0.54</td>
<td>54</td>
</tr>
<tr>
<td>A/G (Ile/Val)</td>
<td>16</td>
<td>0.41</td>
<td>39</td>
</tr>
<tr>
<td>G/G (Val/Val)</td>
<td>2</td>
<td>0.05</td>
<td>4</td>
</tr>
<tr>
<td><strong>GSTP1 allele frequency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>58</td>
<td>0.74</td>
<td>147</td>
</tr>
<tr>
<td>Val</td>
<td>20</td>
<td>0.26</td>
<td>47</td>
</tr>
<tr>
<td><strong>GSTM1 genotype frequency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-null</td>
<td>16</td>
<td>0.33</td>
<td>27</td>
</tr>
<tr>
<td>null</td>
<td>32</td>
<td>0.67</td>
<td>15</td>
</tr>
<tr>
<td><strong>GSTT1 genotype frequency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-null</td>
<td>40</td>
<td>0.87</td>
<td>39</td>
</tr>
<tr>
<td>null</td>
<td>6</td>
<td>0.13</td>
<td>3</td>
</tr>
<tr>
<td>Combined GSTM1 and GSTT1 genotype frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-null/ non-null</td>
<td>11</td>
<td>0.28</td>
<td>16</td>
</tr>
<tr>
<td>null or null</td>
<td>28</td>
<td>0.72</td>
<td>16</td>
</tr>
</tbody>
</table>

The performed PCR-RFLP technique for analyzing the allele and genotype frequencies of $A^{313}G$ (Ile$^{105}$Val) SNP in GSTP1 showed a single PCR band of 176 bp for patients homozygous for the wild GSTP1 allele for (A/A, Ile/Ile), three PCR bands of 176 bp, 81 bp and 95 bp for patients heterozygous and two short DNA bands of 81 bp and 95 bp for patients homozygous for the variant GSTP1 allele (G/G, Val/Val) (Figure 1).

The study concerning the GSTP1 polymorphism comprised 39 COPD patients and 97 unaffected controls. The gender distribution was not different between the cases and controls (p=0.156, $\chi^2$ test), although there was a prevalence of males in the patient group (72% vs. 59% in control group). The median age of controls was 57 years (mean 58±SD of 10 years) and 67 years for patient group (mean 67±8 years, p<0.0001, Mann-Whitney U test). This significant difference did not admit the age adjustment of this case-control study.

We did not observe a significant case-control difference in genotype frequencies according to $A^{313}G$ (Ile$^{105}$Val) SNP in GSTP1 (p=0.958, $\chi^2$-test). In the population of 97 unaffected controls, 54 (0.56) were homozygous for the wild GSTP1 allele (A/A, Ile/Ile), 39 (0.40) controls were heterozygous, and the rest of 4 (0.04) were homozygous for the variant GSTP1 allele (G/G, Val/Val) (Figure 2a).

The allele frequencies did not also differ significantly between patients and controls (p=0.807) (Figure 2b).

The GSTM1 and GSTT1 null
polymorphisms were analyzed in parallel PCR reactions, using \textit{GSTP1} as a reference gene in each of the reactions. Thus in the electrophoresis gel of the PCR reaction products of patients homozygous for \textit{GSTM1} null polymorphism (\textit{GSTM1} null) there was only one visible band of 176 bp corresponding to the PCR product of the reference \textit{GSTP1} gene, whereas the electrophoresis of the PCR products of DNA template from patients with one or two wide type alleles of \textit{GSTM1} (\textit{GSTM1 non-null}) presented two bands: 219 bp band of \textit{GSTM1} PCR product and 176 bp band of \textit{GSTP1} reference gene (Figure 3).

Analogously, the electrophoretic profile of the PCR products from patients with at least one wide type \textit{GSTT1} allele (\textit{GSTT1} non-null) demonstrated two bands with a 459 bp band, corresponding to the \textit{GSTT1} PCR product, and a band of 176 bp, corresponding to the \textit{GSTP1} reference gene. The PCR products of genomic DNA from patients homozygous for the deleted \textit{GSTT1} allele (\textit{GSTT1} null) were visible as a single 176 bp band of \textit{GSTP1} reference gene only (Figure 3).

In the analysis of \textit{GSTM1} null polymorphism the patient population consisted of 48 individuals and the control group comprised 42 individuals. In the study for \textit{GSTT1} null polymorphism there were 46 patients and 42 controls enrolled. In both analyses there was a significantly different gender distribution between the cases and controls (\(p=0.002\) for \textit{GSTM1} and \(p=0.011\) for \textit{GSTT1} analysis, \(\chi^2\) test), as there was a prevalence of males in the patient group compared to the controls (77\% vs. 44\% in \textit{GSTM1}, and 76\% vs. 50\% in \textit{GSTT1} analysis). The age of the patients was not significantly different from that of controls in both analyses (\(p=0.471\) and \(p=0.207\), Mann-Whitney U test), which permitted only age-adjusted but not gender-adjusted case-control analyses.

There was a significant difference in the frequency of \textit{GSTM1} null genotype between the patients and controls (0.67 vs. 0.36, \(p=0.003\), \(\chi^2\) test) as the OR was 3.6 (95\% CI, 1.52 – 8.54) (Figure 4a). In contrast to \textit{GSTM1} in the analysis for \textit{GSTT1} null polymorphism we did not see significant prevalence of the homozygous null genotype in the patient population compared to the controls (0.13 vs. 0.07, \(p=0.489\), Fisher’s exact test) (Figure 4b). When both null polymorphisms were analyzed in combination, we found a tendency for higher frequency of at least one null homozygous genotype in patient group than in the controls (0.72 vs. 0.50, \(p=0.060\), \(\chi^2\) test) with an OR of 2.54 (95\% CI, 0.96 – 6.73) (Figure 4c).

\section*{DISCUSSION}

COPD is a complex multifactorial disease and it has been suggested that a complicated interplay between environmental and genetic factors is likely to be involved in its development (2-4, 7, 19). Cigarette smoking has been generally accepted as one of the most important environmental factors (1-3). However, only a relatively small proportion of heavy smokers develop COPD, which indicates that there is genetic basis for COPD.
Detoxification genes are potential candidates in the susceptibility of patients with COPD. Polymorphisms in these genes alter the metabolism of xenobiotics, such as polycyclic aromatic hydrocarbons (PAH) present in cigarette smoke (20).

In the present work, we report our preliminary results from a case-control study on the impact of the $A^{313}G$ (Ile105Val) SNP of GSTP1 and of the null genotypes of GSTM1 and GSTT1 null polymorphisms on COPD risk in Bulgarian population. The frequency of $A^{313}G$ (Ile105Val) GSTP1 genotypes in the group of Bulgarian individuals from the region of Stara Zagora (0.56; 0.40; 0.04) is in close consistence with those published for other Caucasian type control cohorts such as Finish control group (0.55, 0.38, 0.07) (21), European-Americans (0.42, 0.51, 0.07) (22), and for random control individuals of Caucasian type from the Edinburgh area (0.51, 0.425, 0.065) (12). Results from Bulgarian patients show slightly different genotype frequency distributions with respect to the controls in the case-control study amongst Bulgarians with Balkan endemic nephropathy (BEN) (0.47, 0.38, 0.15) (23). Analogously, the frequency of the homozygous GSTT1 (0.07) null genotype in our control group was comparable to that reported by Andonova et al. in the BEN analysis (0.09) (23). However, the difference in the frequency of the homozygous GSTM1 null genotype between our control group (0.36) and that of BEN analysis (0.47) (23) was more pronounced, which could be attributed to the relatively small number in our control group in this preliminary study.

Concerning the risk of COPD, our preliminary study does not draw positive conclusive results for the impact of $A^{313}G$ (Ile105Val) GSTP1 and GSTT1 null polymorphisms on the susceptibility to this chronic pulmonary disease. Based on the genotype and allele distribution amongst patient and control groups, we suggest, on the other hand, that the $A^{313}G$ (Ile105Val) GSTP1 polymorphism and GSTT1 null polymorphism are most likely not risk factors for developing COPD. These data are in accordance with other similar analyses.
in COPD patients from Korea (5) and Taiwan (9), but opposed to the results published for patients from India (10) and Japan (6, 7).

Figure 3: Genotyping of GSTM1 and GSTT1 by duplex PCRs, which distinguished between the null genotypes (homozygous for null GSTM1 or GSTT1 alleles, respectively) and non-null genotypes (containing at least one wild type GSTM1 or GSTT1 allele, respectively): non-null GSTM1 genotype – lines 4 and 8; null GSTM1 genotypes – lines 6 and 10; non-null GSTT1 – lines 5, 9 and 11; null GSTT1 genotype – line 7; lines 1 and 2 - negative controls for GSTM1 and GSTT1 duplex PCRs; line 3 – 100 bp DNA ladder (Fermentas Ltd.)

(a)

(b)

(c)

Figure 4: Distribution of GSTM1 (a) and GSTT1 (b) null genotypes independently and in combination (c) among controls and patients with COPD

Due to the observed higher frequency of homozygous GSTM1 null genotype in our patients compared to controls we proposed a role of this null polymorphism in genetic
predisposition for COPD. This result is in line with the reports demonstrating increased risk of COPD in Caucasian and Taiwanese population with homozygosity for GSTM1 null allele (7, 9). Having in mind the complex nature of the process of xenobiotic-metabolism involved in cigarette smoke compound detoxification and based on our results we suggest that the absence of at least one of the isoenzymes, GSTM-mu or GST-theta, due to homozygous null genotype of the genes encoding them, might be a risk factor for developing COPD.

In conclusion, we suggest that the null GSTM1 genotype, but not the null GSTT1 genotype and genotypes containing the variant $^{313}G$ (Val$^{105}$Val) GSTP1 allele, might be considered as a risk factor for developing COPD in Bulgarian population from the region of Stara Zagora. However, larger and more extensive studies are indispensable to confirming these findings.

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REFERENCES


