POSSIBLE INFLUENCE OF GSTM1 AND GSTT1 NULL GENOTYPE ON THE RISK FOR DEVELOPMENT OF SPORADIC COLORECTAL CANCER

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ABSTRACT
The glutathione-S-transferases (GSTs) constitute a family of xenobiotic-metabolizing phase II enzymes, which mediate exposure to cytotoxic and genotoxic agents and may be involved in the susceptibility to cancer in general, and to colorectal cancers, particularly. For two of the genes, coding the cytoplasmic isoenzymes GST-mu and GST-theta (GSTM1 and GSTT1), null variant alleles have been found, in which the entire gene is absent. Investigations on the association of GSTs’ null genotypes and colorectal cancer have reported quite controversial results.

The aim of the current pilot study was to examine the relation of GSTM1 and GSTT1 homozygous null genotypes with colorectal cancer risk in a case-control study of Bulgarian patients. The GSTM1 and GSTT1 genotyping was conducted in 46 patients with colorectal carcinoma and 42 controls. A modified multiplex (duplex) PCR-based method was applied to assess the GSTs’ genotypes.

We observed a statistically significant case-control difference in the presence of GSTT1 null genotype (0.30 vs. 0.07, p=0.006), and only a tendency for prevalence of GSTM1 null genotype in CRC patient (0.57 vs. 0.36, p=0.052). The combined null genotypes were determined only in patients (0.20), whereas none of the control individual was with such genotype (p<0.0001). We found a 5.69-fold (95% CI, 1.59-20.00) and 2.34-fold (95% CI, 0.99-5.49) increased risk associated with GSTT1 and GSTM1 null genotypes, respectively and 21.53-fold (95% CI, 3.56-128.71) increased risk associated with the combined null genotypes.

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In conclusion, based on our current results we suggest that the inherited absence of GST-theta alone or the simultaneous lack of GST-theta and GST-mu detoxifying enzymes due to the presence of homozygous null genotypes may be associated with development of sporadic colorectal cancer.

Keywords: colorectal cancer, GSTM1, GSTT1, null polymorphisms, genetic predisposition

Introduction
Colorectal cancer (CRC) is one of the most common cancers throughout European countries, including Bulgaria, with an incidence of more than 4000 new cases in 2004 (5). Colorectal carcinogenesis is a complex and multistep process in which interplay between the genetic and environmental factors occurs in the course of cancer development. Some of the most important environmental risk factors for CRC are considered to be the dietary factors, such as high fat intake, alcohol use; low consumption of fiber, fruits and vegetables, calcium, and selenium; chemical carcinogens (polycyclic aromatic hydrocarbons [PAH], heterocyclic amines, nitrosamines, and aromatic amines) in barbecue and burned read meat, in cigarette smoke, and in ambient air (3, 7, 15, 16, 24, 27).

In general, the environmental chemical carcinogens require metabolic changes, via which they can be first activated to reactive intermediates and further detoxified to water-soluble, more readily excretable compounds. Thus, it has been proposed that the polymorphisms in the genes encoding the host enzymes catalyzing the xenobiotic metabolism may modify the CRC risk associated with above mentioned carcinogen exposure and hence they may be involved in susceptibility to cancer (16, 22).

Glutathione S-transferases (GSTs, EC. 2.5.1.18) are a large family of a phase II xenobiotic-metabolizing enzymes that appear to form part of a protection mechanism against chemical carcinogenesis, including colorectal carcinogens, such as polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines (9, 18, 21). These enzymes catalyze conjugation of a wide variety of exogenic and endogenic electrophilic molecules with glutathione, giving rise in most
cases to less reactive, water-soluble metabolites that are more readily excreted in urine or bile (9, 13).

In humans, 8 classes of soluble GST isoenzymes, the cytosolic alpha (α), mu (μ), pi (π), sigma (σ), theta (θ), zeta (ζ), omega (ω), and the mitochondrial kappa (κ), have thus far been identified (1, 2, 9, 23). Most of GSTs are polymorphic enzymes and it is shown that the gene variations affect the enzyme activity and/or gene expression (4, 8, 9).

Two of the most relevant human GST isoenzymes are GST-mu and GST-theta. It has been reported that a substantial proportion of the Caucasian population has a homozygous deletion of the genes, encoding these enzymes: the GSTM1 gene (chromosome 1p13.3, encoding GST-mu) and the GSTT1 gene (chromosome 22q11.2, encoding GST-theta), resulting in a complete loss of these isoenzymes (2, 4, 6, 9, 19).

Because GST-mu and GST-theta are important in the detoxification of carcinogens implicated in colorectal cancer, the absence of these enzymes may increase the risk of this common malignancy. A number of epidemiological studies have investigated the association of GSTM1 and GSTT1 genetic polymorphisms with colorectal cancer risk (4, 28). However the results from these studies have been very inconsistent.

In this respect the aim of the current pilot study was to assess the frequency of the null GSTM1 and GSTT1 genotypes in Bulgarian control’s and CRC patient’s cohorts and to evaluate the impact of these null polymorphisms on the susceptibility to colorectal cancer.

Materials and Methods

Patient and control populations

The patient group consisted of 46 patients with CRC, who had undergone tumor resections between January 2004 and December 2006 at the University hospital of Trakia University in Stara Zagora. Twenty nine (63%) of the patients were males and the rest of 17 (37%) were females, all aged between 42 to 95 years (median of 67 years). Thirty three percent of the patients suffered from carcinoma of the colon and 37% from carcinoma of the rectum. Tumor grading and staging was performed according to the TNM classification. Three percent of the patients had tumors in stage I, 50% in stage II, 17% in stage III and the rest (30%) of the patients had tumors in stage IV. The tumors were with the following histological characteristics: Grade of differentiation: low – 17%; moderate – 76%; high – 7%; Histological type: type I - 8%; type II - 20%; type III- 56%; type IV- 16%; Tumor cell invasion in blood vessels – 8%; in lymph vessels – 28%; perineural invasion – 20%; combined invasion of tumor cells in blood or lymph vessels or perineurally (VELIPI) – 44%; Inflammatory cell infiltration in invasive front: absent – 28%; weak – 40%; dense – 32%.

The control group consisted of 42 healthy voluntary or in Stara Zagora. twenty nine (63%) of the patients were males and 50% in stage ii, 17% patients had tumors in stage i, 50% in stage ii, 17% 10 years (median of 67 years).

DNA extraction

Genomic DNA of the patients was obtained from 10 µm tick slides (4 to 6) from the formalin-fixed paraffin-embedded tumor biopsies using a modified proteinase K digestion followed by phenol-chloroform-isomyl alcohol extraction or applying a commercial kit for isolation of genomic DNA from tissue (Macheray-Nagel, Germany), based on the proteinase K digestion followed by silica column filtration and collection of DNA. In some cases DNA was also isolated from fresh frozen biopsies using the same DNA extraction kit.

Genomic DNA of controls was isolated from cell mass of 2 ml of blood applying the conventional proteolytic 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

The null polymorphisms in GSTM1 and GSTT1 were assessed by a modified by us multiplex (duplex) PCR (26). 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 referent gene were carried out in a 25 µl mixture containing 2 to 4 µl of the genomic DNA, 1x standard PCR buffer, 2 mM MgCl2 (for GSTM1) or 1.5 mM MgCl2 (for GSTT1), 250 µM dNTP, 1U Taq polymerase (STS DNA polymerase, STS Ltd., Bulgaria) and 0.5 µM of the following primers: T1F (5’-TTC CTT ACT GGT CCT CAC ATC TC-3’) and TIR (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 (5’- ACC CCA GGG CTC TAT GGG AA-3’) and P105R (5’- TGA GGG CAC AAG AAG CCC CT-3’) (8, 25) for GSTP1 in both mixtures. Amplification consisted of 10 min at 94°C for initial denaturation and 40 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds, and extension at 72°C for 1 minute, followed by final extension at 72°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).

Although this assay can not distinguish between heterozygote and homozygote positive genotypes, it conclusively identifies the homozygous null genotypes.

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 χ2 test and Fisher’s exact test. The differences in the non-normally distributed quantitative variables between unpaired groups
were compared with Mann-Whitney U test. Factors with p<0.05 were considered statistically significant. The Odds ratio was calculated by using an interactive Online Software Package at the web site http://statpages.org/#Package (http://statpages.org/cstab2x2.html). If there were cells with a value of 0 in 2x2 contingency table, we applied the modification of Haldane for calculating the OR (20):

\[ \text{OR} = \frac{(2A+1)(2D+1)}{(2B+1)(2C+1)} \]

**Results and Discussion**

The *GSTM1* and *GSTT1* null polymorphisms were analyzed in parallel duplex PCR reactions, using *GSTP1* as a referent gene in each of the reactions. Thus in the electrophoresis of the PCR reaction products of patients homozygous for *GSTM1* null polymorphism (*GSTM1 null*) there was visible only one band of 176 bp corresponding to the PCR product of the referent *GSTP1* gene, whereas the electrophoresis of the PCR products of DNA template from patients with one or two wide type alleles of *GSTM1* (*GSTM1 non-null*) presented two bands: 219 bp band of *GSTM1* PCR product and 176 bp band of *GSTP1* referent gene (Fig. 1).

![Fig. 1. Genotyping of *GSTM1* and *GSTT1* by duplex PCR, which distinguishes between two genotypes for each gene: homozygous null genotype and non-null genotypes. The PCR product of the referent *GSTP1* gene is detected in the 2.5% agarose gel as a band of 176 bp, the PCR product of the wide-type *GSTM1* allele is with a length of 219 bp, and the PCR product of the wide-type *GSTT1* allele is with a length of 459 bp. Patients G43 and G44 are with *GSTM1* non-null/*GSTT1* non-null genotype, G45, G47 and G48 are *GSTM1* null/*GSTT1* non-null genotypes, and G46 is *GSTM1* null/*GSTT1* null genotype (M – 100 bp ladder marker).](image)

Homzygous genotype frequencies of the *GSTM1* and *GSTT1* null polymorphism in controls and patients with CRC.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Patients</th>
<th>Controls</th>
<th>OR (95% CI), p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 46</td>
<td>n = 42</td>
<td></td>
</tr>
<tr>
<td><em>GSTM1</em> genotype frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-null</td>
<td>20</td>
<td>27</td>
<td>1.0 (referent)</td>
</tr>
<tr>
<td>Null</td>
<td>26</td>
<td>15</td>
<td>2.34 (0.99 - 85.49), p=0.052</td>
</tr>
<tr>
<td><em>GSTT1</em> genotype frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-null</td>
<td>32</td>
<td>39</td>
<td>1.0 (referent)</td>
</tr>
<tr>
<td>Null</td>
<td>14</td>
<td>3</td>
<td>5.69 (1.59-20.00), p=0.006</td>
</tr>
<tr>
<td>Combined <em>GSTM1</em> and <em>GSTT1</em> genotype frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-null/non-null or null/null</td>
<td>37</td>
<td>42</td>
<td>1.0 (referent)</td>
</tr>
<tr>
<td>null/null (both null)</td>
<td>9</td>
<td>0</td>
<td>21.53 (3.56- 128.71), p&lt;0.0001</td>
</tr>
</tbody>
</table>

Analogously, the electrophoretic profile of the PCR products from patients with at least one wide type *GSTT1* allele (*GSTT1 non-null*) demonstrated two bands: one with a 459 bp size, corresponding to the *GSTT1* PCR product, and a band of 176 bp, corresponding to the *GSTP1* referent gene. The PCR products of genomic DNA from patients homozygous for the deleted *GSTT1* allele (*GSTT1 null*) were visible as a single 176 bp band of *GSTP1* referent gene only (Fig. 1).

Altogether our preliminary study included a small cohort of 46 patients with CRC and a group of 42 controls consisted of voluntaries and non-cancer hospital patients. There was no significant difference in the gender distribution between the case and control groups (p=0.148), however the control group was slightly younger than the patient group (p=0.05), which in addition to the small number of the enrolled groups did not admit the age adjustment of this case-control study.

The genotype frequencies of *GSTM1* and *GSTT1* null polymorphisms in patients with colorectal cancer cases and controls are presented in the Table. We observed a statistically significant case-control difference in *GSTT1* homozygous null genotype frequencies (p=0.006, Fisher’s exact test) and only a tendency for prevalence of *GSTM1* null genotype in patients compared to the controls (p=0.052, χ²-test). In the small population studied of 42 non-cancer ethnic Bulgarian controls only 3 (0.07) were homozygous for the null *GSTT1* allele and 15 (0.36) for the null *GSTM1* allele. The distribution and the frequencies of the null genotypes among the CRC patients were as follows: 14 (0.30) for *GSTT1* null genotype and 26 (0.57) for *GSTM1* null genotype (Table). Compared with the referent non-null *GSTT1* genotypes, supposed to be the putative lowest risk group, the Odds ratio (OR) for the *GSTT1* null genotype was very high: 5.69 (95% CI, 1.59-20.00, p=0.006). Analogously, however without reaching a statistical significance, the OR for the *GSTM1* null genotype compared to the referent non-null *GSTM1* genotypes was high with a value of 2.34 (95% CI, 0.99 -85.49, p=0.052). When both null
polymorphisms were analyzed in combination, we found that none of the controls had double null genotype (\textit{GSTM1 null and GSTT1 null}), whereas 9 out of 46 (20\%) CRC patients were with such double null genotype. This could account for the very high value of the OR of the double null genotype obtained in the analysis (OR=21.53, 95\% CI, 3.56- 128.71, \(p<0.0001\)) (Table 1).

![Fig. 2. Association of the age at the diagnosis with \textit{GSTM1} and \textit{GSTT1} genotypes. The figures represent the mean values ± SE (standard error).](image)

![Fig. 3. Association of \textit{GSTM1} and \textit{GSTT1} genotypes with the tumor pTNM staging and presence of distant metastases of patients with CRC.](image)

![Fig. 4. Association of \textit{GSTM1} and \textit{GSTT1} genotypes with invasion of tumor cells in lymph vessels and with the combined criteria for invasion of tumor cells in blood vessels, lymph vessels or perineurally (VELIPI).](image)

The presence of \textit{GSTM1} and \textit{GSTT1} null genotypes among the CRC patients was analyzed in association with several clinical and histological tumor characteristics. There was no difference in the frequencies of \textit{GSTM1} or \textit{GSTT1} null genotypes between the genders (\(p=0.322\) for \textit{GSTM1} BIOTECHNOL. & BIOTECHNOL. EQ. 23/2009/1

and \(p=0.520\), \(\chi^2\)-test and Fisher’s exact test), locations of the primary tumor (colon vs. rectum, \(p>0.999\), Fisher’s exact test), degree of differentiation (\(p=0.967\), \(\chi^2\)-test) and presence of inflammatory infiltrate in the invasive front (absent vs. weak/moderate, \(p=0.407\), Fisher’s exact test). The age at the time of diagnosis did not distinguish between the patients with null and non-null \textit{GSTT1} genotypes (\(p=0.914\) for \textit{GSTT1}, Mann-Whitney U test) (Fig. 2), but there was a tendency for earlier diagnosis of the disease in patients homozygous of \textit{GSTM1} null allele (mean 62.6±3 (SE) years) than those possessing at least one wide type \textit{GSTM1} allele (68.9±2 (SE) years, \(p=0.107\), Mann-Whitney U test) (Fig. 2). Moreover, carriers of the \textit{GSTM1} null genotype, but not \textit{GSTT1} null genotype, appeared to be more frequently with tumors in a more advanced stage (stage III/IV, \(p=0.033\), Fisher’s exact test) (Fig. 3), with synchronous distant metastases at the time of diagnosis (\(p=0.042\), Fisher’s exact test) (Fig. 3), and to have tumors with lymph vessel invasion (\(p=0.008\), Fisher’s exact test) (Fig. 4) or with at least one type of tumor cell invasion: blood vessel, lymph vessel or perineural invasion (\(p=0.042\), Fisher’s exact test) (Fig. 4).

The development of colorectal cancer is a complex process and it is now widely accepted that the risk for this relatively common and lethal disease is determined by an intricate interaction of both genetic and environmental factors such as susceptibility genes, carcinogen exposure and dietary factors (14). Inherited susceptibility is associated with high-penetrance genes such as \textit{APC}, \textit{hMLH-1}, \textit{hMSH-2}, \textit{p53}, playing an important role in familial colorectal cancer, which represents only 5 to 10\% of all CRCs (17). In the great majority of colorectal cancers a consequence of sporadic somatic changes in tumor-associated genes are supposed to have more pronounced effect in tumor development and progression (11, 12). However, several other low-penetrance susceptibility genes may alter the predisposition to colorectal cancer.

Such low-penetrance susceptibility genes are considered to be the genes encoding the xenobiotic-metabolizing enzymes because of the important role of these metabolic enzyme in the activation and further detoxification of wide variety of procarcinogens and chemicals contained in most of the environmental factors such as cigarette smoke, diets and air pollutants (14). \textit{GSTM1} and \textit{GSTT1} have been suggested as some of these low-penetrance susceptibility genes since they code for cytoplasmic GST-mu and GST-theta enzymes. They are involved in conjugation and in most cases detoxification with reduced glutathione of numerous electrophilic compounds with exogenic and endogenic origin, turning out to be potent colorectal carcinogens (3, 4, 9).

In the present work, we report our preliminary results from a relatively small case-control study on the impact of the null genotypes of \textit{GSTM1} and \textit{GSTT1} null polymorphisms on CRC risk among Bulgarians. The frequency of the homozygous \textit{GSTT1} (0.07) null genotype in our control group was commensurable to than reported by Andonova et al., in a case-control study of Bulgarian patients with Balkan endemic nephropathy (BEN) (0.09) (1), but our results are relatively
lower than that in most of the Caucasian control populations (4, 6). More pronounced was the difference in the frequency of the GSTM1 null genotype between our control group (0.36) and that of BEN analysis (0.47) (1) and other case-control studies of Caucasian populations (1, 4, 6). The differences could be attributed to the relatively small number of our control group in this preliminary study.

Concerning the risk of CRC, our study shows some effect of the null polymorphisms, particularly of GSTT1 and to a less extent of GSTM1 on the susceptibility to this neoplastic disease. Our results are in line with other studies describing similar higher risk for developing CRC of individuals carrying null GSTT1 genotype compared to those having at least one wide-type GSTT1 allele, however the OR in our study is markedly higher (5.69) than the other reported (1.9 and 3.4) (4). We attribute this difference again to the small cohorts enrolled in our preliminary analysis, which could be confirmed also by the very wide range of the 95% confidence interval (1.59-20.00). In addition there are several other studies and surveys which do not find an impact of GSTT1 null polymorphism on the susceptibility to CRC (4). In this respect we consider that a notable enlargement of our study populations is necessary to guarantee the observed statistical association of the risk for CRC with the GSTT1 null genotype.

In our study we also obtained a quite high OR for CRC (2.34) in individuals with GSTM1 deficiency, although this association was only with marginal statistical significance (p=0.052). A lower, but statistically significant summary odds ratio (1.15) was found for Caucasians only in a meta-analysis based on 20 case-control studies with populations from different race and ethnic groups (28). However, the final results of this meta-analysis did not support the hypothesis that GSTM1 alone is an important risk factor for colon cancer and suggested that GSTM1 status alone had no effect on the risk of developing colon cancer. Analogously, results for several case-control studies were pooled earlier in another report and the conclusion was that no consistent association between GSTM1 genotype alone and colorectal cancer could be confirmed (4).

Based on those latter results and having in mind the complex nature of the process of xenobiotic-metabolism involved in colorectal carcinogen detoxification, we analyzed the combined effect of both null genotypes on the susceptibility of colorectal cancer. We found a very high odds ratio for CRC of carrying simultaneously homozygous deletions of GSTM1 and GSTT1, suggesting that the absence of both isoenzymes, GSTM-mu and GST-theta, might be an important risk factor for developing colorectal cancer.

Moreover, grounded on our observation that the GSTM1 null genotype was found in younger CRC patients with tumors in more advanced tumor stage and with presence of tumor cell invasion in lymph vessels, we proposed the notion that the lack of GST-mu due to the GSTM1 null genotype might determine an early onset of colorectal cancer with more aggressive phenotype. We can explain these observations with the presence in some extent of GST-mu in normal colorectal mucosa (10) and with its main substrate specificity to epoxides of polycyclic aromatic hydrocarbons, which are the metabolic intermediates after phase I biotransformation of these xenobiotics. Thus, the lack of GST-mu isoenzyme in individuals with GSTM1 null genotype, although there is an overlapping substrate specificity of most of the cytoplasmic GSTs, might result in a pronounced imbalance of ingested carcinogen detoxification. This in turn probably can cause more DNA mutations triggering earlier and more severe epithelial cell transformation that could possibly contribute to earlier appearance of the tumor with a more aggressive phenotype.

Conclusions
In conclusion, our current preliminary results suggest that the inherited absence of GST-theta alone or the simultaneous lack of GST-theta and GST-mu detoxifying enzymes due to the presence of homozygous null genotypes may be considered as risk factors for development of colorectal cancer. However, further larger and more extensive case-control studies are warranted to elucidate the role of GSTM1 and GSTT1 alone or in interaction with other genes and environmental exposures as predisposing factors in colorectal carcinogenesis.

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