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ORIGINAL ARTICLE

Deletion and Single Nucleotide Polymorphisms in Common Glutathione-S Transferases Contribute to Colorectal Cancer Development

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Abstract

Glutathione-S transferases (GSTs) are xenobiotic-conjugation enzymes involved in the detoxification process of heterocyclic aromatic amines and polycyclic aromatic hydrocarbons, widely recognized risk factors of colorectal cancer (CRC) development. Polymorphism in GSTs often leads to alteration or complete lack of enzyme activity, which might have an effect on CRC carcinogenesis. Aim of this study was to investigate GST gene variants as risk factors in patients with CRC. A total of 523 CRC patients administered for surgical resection and 400 matched controls were included. Deletion polymorphism of GSTs M1 and T1 was investigated by polymerase chain reaction. Single nucleotide polymorphism of GST A1 and P1 was investigated by restriction fragment length polymorphism method. The association between GST genotype and risk of CRC development was found in carriers of GSTT1-null and GSTP1-variant genotypes individually ($p = 0.050$ and $p = 0.016$, respectively). Furthermore, statistically significant association was found when combination of GSTP1-variant genotype with any of other three common GST genotypes was analyzed with respect to CRC susceptibility. Additionally, patients with combined GSTM1-null/GSTT1-null/GSTA1 low-activity/GSTP1-variant genotype showed 2.71-fold increased risk of developing CRC ($p = 0.037$). This study supports hypothesis that GST polymorphisms might have an important role in the process of the CRC development. Additionally, GSTM1-null/ GSTT1-null/ GSTA1 low-activity/ GSTP1-variant genotype could be combination of GST genotypes whose carriers are more prone to CRC development.

Keywords Colorectal cancer · Glutathione-S transferases · GST polymorphism · Carcinogenesis · Colorectal cancer development

Introduction

With 9.9% of worldwide-diagnosed cancers, colorectal cancer (CRC) accounts as the third most common, and one of the leading causes of cancer-related deaths [1]. More than 1 million people are diagnosed with CRC each year, which additionally emphasizes the statement that CRC represents one of the major global health issues [2]. Adenocarcinoma, deriving from colorectal mucosa epithelium, sums approximately 90% of all histological CRC subtypes [3].

While hereditary CRC is characterized by alterations of highly penetrant gene alleles, it accounts for only a small percentage of overall CRC. Majority of CRC cases develop due to sporadic alterations of low penetrant gene alleles, alone or in combination with environmental factors [4]. Apart from genetic, several modifiable risk factors have been recognized in CRC development, including nutritional habits, obesity, smoking and alcohol consumption [5].

Despite being quite common in general population, recognized CRC risk factors might not be single contributors of CRC development, suggesting that carcinogenesis is

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influenced by inter-individual genetic variations. Indeed, previous studies suggest that glutathione S-transferases could be linked with the development and progression of CRC [6, 7].

Glutathione-S transferases (GSTs) are large family of xenobiotic-conjugation enzymes [8]. GSTs are particularly important in the detoxification process of heterocyclic aromatic amines and polycyclic aromatic hydrocarbons, widely recognized risk factors of CRC development found in processed meat and tobacco [9]. Polymorphism in GSTs often leads to alteration or even complete lack of enzyme activity, which, due to their important role in detoxifying carcinogens, might have an effect on CRC carcinogenesis.

Bearing in mind that GST polymorphisms may play a significant role in the CRC development, a comprehensive study was conducted, aiming to determine the presence of established risk factors and specific GST gene variants in CRC patients, as well as, evaluation whether phenotype changes reflect genotype-associated risk.

Material & Methods

Study Population

Newly diagnosed CRC patients, treated and followed at the Digestive Surgery Clinic, Clinical Center of Serbia, Belgrade, were included in this study between the years of 2014–2016. All 523 patients (313 men, 210 women; average age 62.25 ± 11.38 years) had their diagnosis histologically confirmed in accordance with TMN and Dukes classification [10]. The control group included 400 individuals (203 men, 197 women, average age 60.40 ± 12.31 years) who had undergone surgery for benign conditions, unrelated to both non-malignant and malignant GI conditions at the same clinical center. The structured questionnaire composed at the Institute of Epidemiology, Faculty of Medicine University in Belgrade was used for acquiring patients' individual level-data. Regarding that, obese patients were classified as individuals with BMI above 25 and smokers as individuals who reported smoking during a minimum of 60-days period before they have entered the study. Additionally, for the purpose of calculating the pack-years data regarding number of smoked cigarettes, as well as, the duration of smoking were obtained. The study was approved by the Institutional Ethical board (approval number 56–6, Clinical center of Serbia) and was performed in accordance with principles of Helsinki declaration. Informed written consent was obtained from all recruited subjects.

DNA Isolation and Genotyping

Genomic DNA was isolated from 200 μl of the whole peripheral blood by *QIAamp DNA Blood Mini Kit* (*Qiagen*, *USA*) according to the manufacture's protocol. The multiplex PCR technique used to detect homozygous deletions of *GSTM1* and *GSTT1* included primers for *GSTM1, GSTT1* and *CYP1A1* housekeeping gene, according to the method by *Abdel-Rahman* et al. [11].

PCR-restriction fragment length polymorphism (RFLP) method with *Eam1104I* (*Thermo Fisher Scientific*, *USA*) restriction enzyme was used for the analysis of the *GSTA1 C69T* (rs3957357) SNP according to the method by *Ping* et al. [12].

The analysis of *GSTP1 Ile105Val* (rs1695) SNP was performed according to the manufacturer's instructions using the *Applied Biosystems TaqMan® Drug Metabolism Genotyping* assay (*Life Technologies*, *Applied Biosystems*, *USA*, *assay ID*: *C__3237198_20*).

Statistical Analysis

Differences in investigated parameters were assessed using *Student's T* test/ANOVA for continuous data with normal distribution and *Mann–Whitney* rank-sum test for continuous data with non-normal distribution. $\chi 2$ test was used for categorical variables. The genetic variants and their risk for disease were computed by odds ratios (OR) and 95% confidence intervals (CI) by logistic regression analysis. OR was either crude or adjusted by BMI, as well as by age and gender. Results were considered to be statistically significant if p value was ≤ 0.050.

Results

Baseline and, clinical characteristics of patients and respective controls are summarized in Tables 1 and 2. As presented, CRC group comprised 1.5 times more male than female patients. CRC patients and controls did not differ in terms of age and BMI ($p > 0.05$). However, more than a half of the patients (58%) suffered from hypertension compared to controls (30%) and the CRC patients' group had more active smokers (68% vs. 48%). As far as clinical characteristics of CRC, the most frequent location was rectum (55%), whereas the majority of patients were diagnosed with well-differentiated CRC (77) and with T3 stage (49%).

GST Genotypes and CRC Risk

The frequency of *GST* genotypes, as well as, the relation to the CRC risk in both CRC patients and controls is presented in Table 3. While individual *GSTM1-null* and *GSTA1 CT + TT (low activity)* genotypes did not significantly contribute to the risk of CRC development, *GSTT1-null* and *GSTP1 IleVal + ValVal* (*variant)* genotypes were significantly associated with higher risk of CRC. Namely, the carriers of the *GSTT1-null*

Table 1 Baseline characteristic of CRC patients and controls

	CRC (n %)	Controls (n %)	OR (95%CI)	*P* value
Age (mean ± SD)[a]	62.25 ± 11.38	60.80 ± 11.79	/	0.067
Gender				
Male	310 (60)	203 (51)	1.00[e]	
Female	207 (40)	197 (49)	0.69 (0.53–0.89)	<0.05
Hypertension, n (%)[b]				
No	207 (42)	262 (70)	1.00[e]	
Yes	286 (58)	111 (30)	3.26 (2.45–4.33)	<0.001
Obesity, n (%)[b]				
BMI < 25	200 (42)	155 (41)	1.00[e]	
BMI > 25	272 (58)	224 (59)	0.94 (0.72–1.24)	0.664
Smoking, n (%) [c]				
Never	156 (32)	201 (52)	1.00[e]	
Ever[c]	336 (68)	189 (48)	2.29 (1.74–3.02)	<0.001
Pack-years[d]	30 (1.25–150.0)	27 (1.0–120.0)	/	0.105

[a] Mean ± SD; [b] Based on the data available; [c] minimum of 60-days period any time prior to the study onset; [d] Median (Min-Max); [e] Reference group. CI, confidence interval;

were at 1.35-fold increased risk for CRC development (OR = 1.35, 95%CI: 0.99–1.83, $p = 0.050$) when compared to individuals with *GTST1-active* genotype, whereas carriers of *GSTP1-variant* genotype were more frequent among patients (63% of CRC patients compared to 55% of controls) contributing to 1.39-fold increased risk of CRC development (95%CI: 1.07–1.82, $p = 0.016$) (Table 3).

Table 2 Clinical characteristic of patient's tumor

Location	N (%)
Rectum	277 (55)
Colon	225 (45)
Patological differentiation[a]	
Well	385 (77)
Moderate	96 (19)
Poor	21 (4)
T stage[b]	
1	78 (15)
2	85 (17)
3	245 (49)
4	94 (19)
N stage[b]	
0	256 (51)
1	129 (26)
2	116 (23)
Metastases[b]	
0	413 (82)
1	89 (18)
Stage	
I	82 (27.3%)
II	74 (24.7%)
III	92 (30.7%)
IV	52 (17.3%)

[a,b] Data available data on patients' tumor grade and T stage, depending on the type of surgery and histopathology diagnostics

Combined Effect of *GST* Genotypes on CRC Risk

Combined effect on CRC development for both *null* and *variant GST* genotypes was further examined (Table 4). No significant relationship in terms of CRC risk was observed when the combined effect of any two of *GSTM1*, *GSTA1* and *GSTT1* genotypes was assessed ($p > 0.05$, data not shown).

On the other hand statistically significant association was found in all combinations of *GSTP1-variant* genotype and any of other three *GST* genotypes (*GSTP1 variant* and *GSTM1-null*: OR = 1.53, 95%CI: 1.01–2.18, $p = 0.03$; *GSTP1-variant* and *GSTA1 low-activity*: OR = 1.83, 95%CI: 1.22–2.75, $p = 0.004$; *GSTP1-variant* and *GSTT1-active:* OR = 1.95, 95%CI: 1.27–2.99, $p = 0.002$) when compared to referent genotype combination. Moreover when combined effect of any three *GST* genotypes was analyzed statistically significant connection regarding *GST* polymorphism and susceptibility to CRC was noticed for all triple genotype combinations involving *GSTP1* genotype (*GSTM-null/GSTT1-active/GSTP1-variant* OR = 2.07, 95%CI: 1.06–4.02, $p = 0.03$; *GSTM-null/GSTA1 low activity/GSTP1-variant OR* = 2.24, 95%CI: 1.24–4.04, $p = 0.001$; *GSTT1-active/GSTA1 low activity/ GSTP1-variant* OR = 2.62, 95%CI: 1.41–4.87, p = 0.001) when compared to referent genotype combination (Table 5).

Last but not least, we have examined combined effect of all four variant *GST* genotypes and observed an increasing trend in OR with the highest risk of 2.71-fold (95%CI: 1.06–6.91, $p = 0.037$) in individuals carrying all four variant *GST* genotypes (*GSTM1-null*, *GSTT1-null*, *GSTA1 low-activity* and *GSTP1-variant)* in comparison with the reference genotype combination (Table 6).

Table 3 GST genotypes in relation to the risk of CRC

GST genotype	CRC patients n, %	Controls n, %	Crude OR (95%CI)[e]	*p*- value
GSTM1				
active[a]	249 (49)	204 (49)	1.00[f]	0.509
null[b]	260 (51)	195 (51)	1.09 (0.84–1.42)	
GSTA1 (rs 3,957,357)				
CC (active)	186 (36)	160 (40)	1.00[f]	0.210
CT + TT (low activity)[c]	326 (64)	236(60)	1.19 (0.91–1.56)	
GSTT1				
active[a]	145 (29)	91 (23)	1.00[f]	0.050
null[b]	364 (71)	308 (77)	1.35 (0.99–1.83)	
GSTP1 (rs1695)				
IleIle (wild-type)	185 (37)	181 (45)	1.00[f]	0.016
IleVal + ValVal (variant)[d]	309 (63)	217 (55)	1.39 (1.07–1.82)	

[a] *Active*, if at least one active allele present; [b] *Null* if no active alleles present; [c] *Low activity*, if at least one *T* allele present. [d] *Variant*, if at least one *Val* allele present; CI, confidence interval; [f] Reference group; Deletion *GSTM1* and *GSTT1* genotypes were investigated in 523 cases and all recruited controls. SNP polymorphism *GSTA1**C69T and *GSTP1**Ile105Val were analyzed in 523 CRC cases and all recruited controls

The Association between *GST* Genotypes and Pathological Differentiation and Stages of CRC

The possible effect of *GST* polymorphisms on CRC pathological differentiation, as well as, progression was further assesed and no association was observed in case of *GSTM1* and *GSTT1* genotypes (figures not shown). However, results concerning the *GSTA1 low activity* genotype showed borderline significant difference with respect to tumor stage ($p = 0.054$, Fig. 1). Moreover, significant association was found in the case of *GSTP1* genotype and the level of tumor pathological differentiation, where the vast majority of patients, diagnosed with poor tumor differentiation (89%) had *GSTP1-variant* genotype (Fig. 2a). Additionally, an increasing trend in the frequency of *GSTP1-variant* genotype was noticed when the association between this genotype and tumor stage was investigated in CRC patients. ($p = 0.163$, Fig. 2b).

Discussion

The role of GST polymorphisms as potential contributing risk factors in the process of CRC carcinogenesis is disputable. In this study, we have assessed four most common *GST* polymorphisms in terms of CRC susceptibility and found that individuals carrying *GSTT1-null* or *GSTP1-variant* genotypes had significantly higher risk of CRC development, which was even more noticeable in their combination or when third *GST* genotype, either *GSTM1* or *GSTA1* was added. Additionally, CRC patients with combined *GSTM1-null*/*GSTT1-null*/*GSTA1 low-activity*/*GSTP1-variant* genotype had 2.71-fold increased susceptibility to CRC.

Given that modifiable environmental risk factors have an important role in the complex course of CRC development and progression, genetic polymorphisms in xenobiotic metabolyzing enzymes GSTs have been widely investigated

Table 4 Combined effect of GST genotypes and CRC risk

Combined genotype	CRC patients, n %	Controls, n%	Crude OR (95%CI)	*p* value
GSTM1/GSTP1 genotype				
GSTM1-active[a]/GSTP1-wild type	88 (36)	88 (46)	1.00 [e]	
GSTM-null[b]/GSTP1-variant[c]	156 (64)	102 (54)	1.53 (1.01–2.18)	0.031
GSTA1/GSTP1 genotype				
GSTA1-active[a]/GSTP1-wild type	59 (24)	77 (36)	1.00 [e]	
GSTA1-low activity[d]/GSTP1-variant[c]	185 (76)	132 (64)	1.83 (1.22–2.75)	0.004
GSTT1/GSTP1 genotype				
GSTT1- active[a]/GSTP1-wild type	129 (60)	139 (73)	1.00 [e]	
GSTT1- null[b]/GSTP1-variant[c]	87 (40)	51 (27)	1.95 (1.27–2.99)	0.002

[a] *Active*, if at least one active allele present; [b] *Null*, if no active alleles present; [c] *Variant*, if at least one *Val* allele present; [d] *Low activity*, if at least one *T* allele present; [e] Reference group;

Table 5 Combined effect of three GST genotypes and CRC risk

Combined genotype	CRC patients, n %	Controls, n%	Crude OR (95%CI)	p value	Adjusted OR (95%CI)	*p* value
GSTM1/GSTT1/GSTA1						
GSTM1-active[a]/GSTT1-active/GSTA1-active	57 (60)	59 (70)	1.00 [e]		1.00 [e]	
GSTM-null[b]/GSTT1-null/GSTA1-low activity	38 (40)	25 (30)	1.57 (0.84–2.93)	0.153	1.48 (0.77–2.85) [f]	0.243
GSTM1/GSTA1/GSTP1 genotype						
GSTM1-active[a]/GSTA1-active/GSTP1-wild type	26 (22)	39 (38)	1.00 [e]		1.00 [e]	
GSTM-null[b]/GSTA1-low activity[d]/GSTP1-variant[c]	94 (78)	63 (62)	2.24 (1.24–4.04)	<0.001	2.37 (1.27–4.40)	0.001
GSTM1/GSTT1/GSTP1 genotype						
GSTM1-active[a]/GSTT1-active/GSTP1-wild type	35 (36)	18 (19)	1.00 [e]		1.00 [e]	
GSTM-null[b]/GSTT1-null/GSTP1-variant[c]	63 (64)	67 (81)	2.07 (1.06–4.02)	0.032	2.06 (0.99–4.27)	0.05
GSTT1/GSTA1/GSTP1 genotype						
GSTT1-active[a]/GSTA1-active/GSTP1-wild type	38 (44)	58 (67)	1.00 [e]		1.00[f]	
GSTT1-null[b]/GSTA1-low activity[d]/ GSTP1-variant[c]	48 (56)	28 (33)	2.62 (1.41–4.87)	<0.001	2.23 (1.14–4.39)[f]	0.020

[a] *Active*, if at least one active allele present; [b] *Null*, if no active alleles present; [c] *Variant*, if at least one *Val* allele present; [d] *Low activity*, if at least one *T* allele present; [e] Reference group; [f] OR odds ratio adjusted to age, gender, pack-years, BMI, hypertension;

[9, 13]. Nevertheless, although vast, existing results on GST polymorphism and CRC are quite debatable.

GSTM1 null genotype is mostly examined GST polymorphism in relation to increased risk to various cancers. However, due to diverse ethnicity and geographical origin of patients included in various studies. Association of *GSTM1* polymorphism and CRC risk varies, since GST genotype is known to be influenced by both of these factors [14]. In a large meta-analysis by *Economopoulos* et al. [15] individuals with *GSTM1-null* or *GSTT1-null* genotype were at higher risk for CRC development, while presence of either one or both *GSTA1* or *GSTP1-variant* alleles did not further contribute to risk of CRC development. However, all four GST genotypes were investigated in Caucasian population in only three of the studies included in this meta-analysis [16–18]. Still, the results obtained in two of them [15, 17] indicated no significant association between *GSTM1-null* genotype and CRC risk, which is in accordance with the results obtained in our study. Regarding the distribution of *GSTM1* genotype in our study cohort, it was similar with the distribution in other studies conducted on Caucasians [19–21], while, as expected, in contrast to numerous studies on Asian populations [22]. Combined effect of *GSTM1-null* and *GSTT1-null* analyzed in the study of *Martinez* et al. showed an increased risk of CRC in carriers og this genotype combination [16], which is partially in agreement with our results, since the observed association in our case was not statistically significant (OR = 1.50, 95%CI: 0.95–2.35, $p = 0.080$).

Given that that deletion of *GST1* gene leads to lack of its expression in intestinal tract and detoxifying ability, *GSTT1-null* was also GST polymorphism of interest in numerous studies [23–25]. It seems that *GSTT1-null* genotype is

Table 6 Cumulative effect of *GST* risk-associated genotypes on the risk of CRC development

Number of *GST* genotypes associated to CRC risk	CRC patients n, %	Controls n, %	OR (95%CI)[a]	*p*-value
0	27 (3.9)	19 (6.8)	1.00[b]	
1	107 (21.8)	115 (29.1)	1.32 (0.69–2.51)	0.395
2	215 (43.8)	152 (38.5)	2.01 (1.08–3.74)	0.028
3	129 (26.3)	90 (22.8)	2.04 (1.07–3.85)	0.031

0: Reference genotype combination (*GSTM1-active/GSTT1-active/GSTA1 active/GSTP1-wild type)*; 1, 2, 3, 4: 1, 2, 3, 4: The number of the present risk-carrying genotypes: either one of each risk-carrying, or two of each risk-carrying, or three of each risk-carrying or all four risk-carrying GST genotypes (*GSTM1-null* or *GSTT1-active* or *GSTA1 low-activity* or *GSTP1-variant*); [a] OR odds ratio adjusted to age, gender; CI, confidence interval; [b] Reference group;

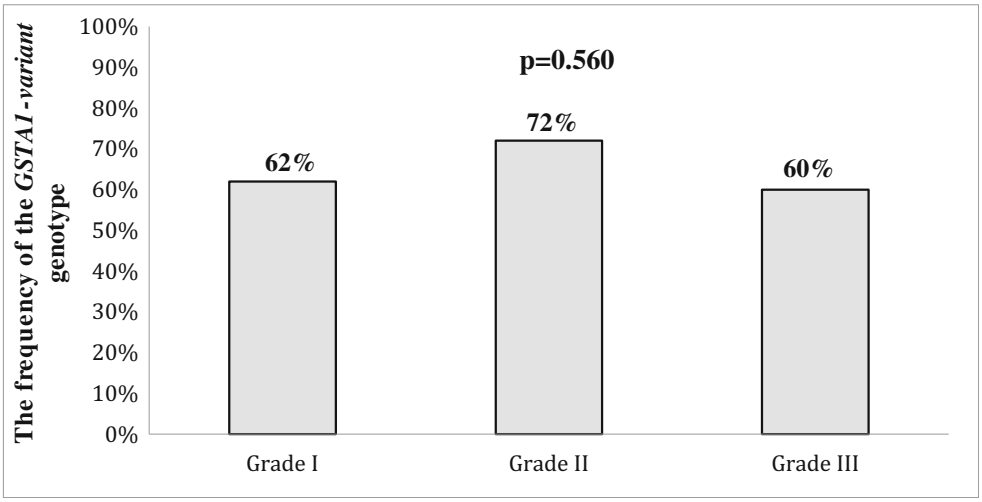

Fig. 1 The frequency of *GSTA*1 variant risk-associated genotypes in CRC patients stratified according to tumor grade

recognized as a CRC risk factor, especially in Caucasians [24]. Previous meta- analysis of *Qin* et al. and *Wan* et al. [25, 26] suggested that *GSTT1-null* genotype conferred a 1.21- fold and 1.32-fold increased risk of CRC, respectively. What is more, data from the recent case-control study of *Masood* et al. [27] also showed that *GSTT1* deletion is associated with

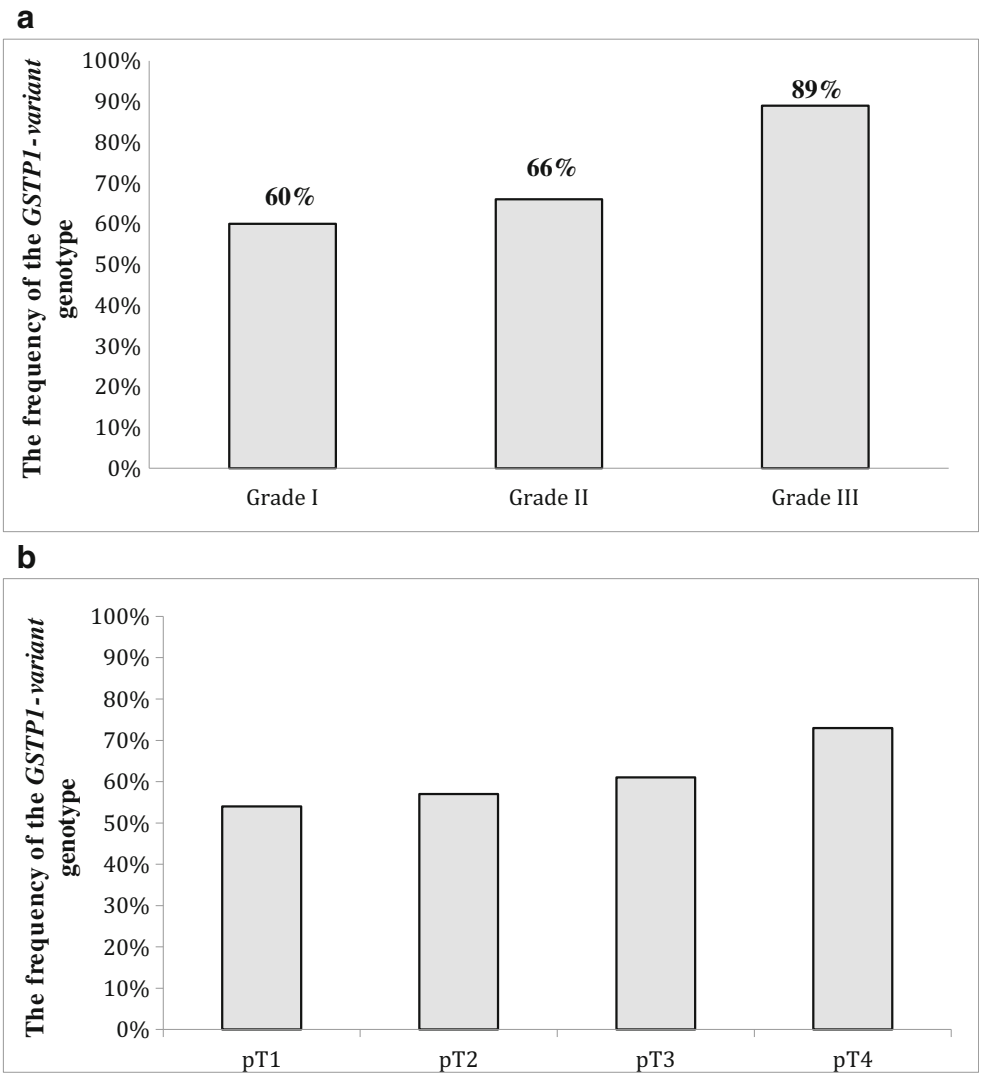

Fig. 2 The frequency of *GSTP1* risk-associated genotype in CRC patients stratified according to: **a** tumor grade; **b** tumor stage

elevated CRC risk. Results of our study, on 1.35-fold increased CRC risk in carriers of *GSTT1-null* genotype, are consistent with mentioned reports. This might be of a particular importance in the case of *GSTT1-null* individuals exposed to compounds recognized as the GSTT1 substrates, that are readily recognized as contributing factors to the CRC development, such as ethylene oxide derived from ethane, abundant in cigarrete smoke [28].

Another examined GST polymorphism in our study was *GSTA1*. Interestingly, this polymorphism has been investigated to a much lesser degree ili extent compared to other GSTs. *Martinez* et al. [17] were the first to investigate GSTA1, amoung other examined classes of GSTs in CRC patients. Results of this study suggest that GSTA1 does not affect the predisposition for CRC, although deficiency of GSTA1 enzyme activity might influence carcinogenesis in persons previously exposed to environmental hazards, considering its known role in the process of detoxification. In previously mentioned meta-analysis of *Economopoulos* et al. [14] individual *GSTA1* polymorphism was also not associated to CRC risk. Likewise in the results of *Hezova* et al. [19] there was no association of *GSTA1* polymorphism and CRC. In this line, recent meta-analysis of *Deng* et al. [28] also suggested that GSTA1 polymorphism has not been recognized as CRC risk factor in majority of included studies [29]. Results of our study are consistent with all reported data. One of the probably most widely investigated *GST* polymorphisms is *GSTP1*. *GSTP1* is shown to be often excessively expressed in various tumors, including CRC [30], suggesting it's involvement in metabolism of various carcinogenic substances [19]. Similarly to other GSTs, the distribution of *GSTP1* genotypes differs among populations with various geographical and ethnical origin, which significantly contributes to conflicting results regarding the role of *GSTP1* polymorphism in CRC development and progression. Recent analysis by *Tan* et al. [31] and *Economopoulos* et al. [15] did not show an association between *GSTP1-variant* genotype and increased risk for CRC, which is not in accordance with our results. However, results of *Matakova* et al. [32], *Wang* et al. [33] and *Kassab* et al. [34], as well as, meta-analysis by *Ramsey* et al. [6], were all consistent regarding the significant role of *GSTP1* polymorphism is susceptibility to CRC development, what our results agree with.

Aside from analyzing the association of independent *GST* polymorphisms with CRC risk, we have assessed the presence of the combined effects of two, three and all four putative risk genotypes of *GST* gene variants with respect to CRC risk.

Firstly, we have assessed the combined effects of two presumed risk genotypes with respect to low-risk GST genotypes. Of all enrolled CRC patients, 40% had the combination of *GSTT1-null* with *GSTP1-variant*, with a 1.95-fold increased CRC risk. The combination of *GSTM1-null* and *GSTP1-variant* was present in 64% CRC patients, whereas *GSTA1 low-activity* and *GSTP1-variant* in 76% of all patients, with increased CRC risk 1.53-fold and 1.83-fold, respectively. Majority of the data on combined *GST* genotypes are related to combination of *GSTM1-null* and *GSTT1-null* [23, 33, 34]. The study of *Matakova* et al. [32] suggested that combination of *GSTM1-null* and *GSTP1- variant genotypes* is a predisposing factor for CRC development. Combined *GSTT1-null/GSTP1-variant* genotype indicated 1.89-fold increased risk for CRC development in the study of *Wang* et al. showed, which is rather similar to the results of our study. Combined *GSTA1 low-activity/GSTP1-variant* genotype was of interest in only one study related to colorectal adenoma and cruciferous vegetables [20], whereas this study is the first to asses this gene-gene interaction alone.

Later, we have analyzed three putative risk genotypes in respect to low risk GST genotypes in patients with CRC. Combination of *GSTM-null, GSTT1-active* and *GSTP1-variant* genotype showed 2.07-fold increased risk for CRC, which is in agreement with the results of Ates et al. [35] and Wang et al. [33]. However, for the first time to our knowledge, our study has demonstrated an increased risk of CRC development in individuals carrying combined *GSTM1-null/ GSTT1-null/ GSTA1 low-activity / GSTP1-variant* genotype, since no data regarding the combined effect of all four GST genotypes are available in the literature.

Several limitations of this study need to be addressed. First of all, in order to estimate the association between GST genotypes and the risk of CRC we used a case-control design and therefore the selection bias might influence the results. Additionally, our controls were hospital-based instead of population based, comprising white subjects only, therefore the possible effect of ethnicity could not be assessed. A recall bias regarding the questions from the questionnaire might have influenced the results as well. Furthermore, the data on dietary habits were not validated, hence not used in the adjusted analysis of the obtained results.

Conclusion

This study supports hypothesis that *GST* polymorphisms might have an important role in the process of the CRC development. Additionally, combined*GSTM1-null*,/ *GSTT1-null/GSTA1 low-activity/GSTP1-variant* genotype could be potential combination of four common GST genotypes making its carriers more prone to CRC development. Prospectively, further case-control studies assessing the association of *GST* gene variants and CRC risk should be preformed on patients with individual-level data being collected especially regarding dietary habits, which might further contribute to the adjusted risk analysis.

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Compliance with Ethical Standards

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest Authors have no conflict of interest to declare.

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