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Polymorphisms of biotransforming enzymes (GSTs) and their association with colorectal cancer in the Slovak population

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The aim of present study was to summarize the results of a case-control study focused on genetic polymorphisms of selected Phase II metabolizing enzymes (GSTM1, T1, P1) and to investigate the association of these polymorphisms with the colorectal cancer risk among the Slovak population.

A case-control study with 183 colorectal cancer cases and 422 controls was conducted. DNA was extracted from peripheral blood leukocytes, and the polymorphisms of GSTM1, GSTT1 and GSTP1 enzymes were determined by PCR-based methods. Association between specific genotypes and the development of colorectal cancer were examined using logistic regression analysis to calculate odds ratios (OR) and 95% confidence intervals (CI).

The GSTP1 val/val genotype (OR=2.1, 95%CI: 1.1 - 4.0, $\chi^2 = 0.28$ and P = 0.0025) was associated with an elevated risk. The statistically significant correlation was found also for the combined genotypes of GSTM1 null and GSTP1 valine homozygosity (OR = 2.7, 95% CI: 1.1–6.1, $\chi^2 = 4.5$ and P = 0.03).

The genotype of certain metabolising enzymes affects the risk for colorectal cancer. This effect is also important when certain allelic combinations are studied. In the near future, individual risk assessment may be reached by further increasing the number of studies of polymorphisms, combining them with the traditional epidemiological risk factor.

Key words: *colorectal cancer; Glutathione S-transferase T1, M1, P1; polymorphism*

Primary colorectal cancers (CRC) are tumors that occur at high frequency in developed countries, and in all European countries, including Slovakia. Each year more than 2700 new cases of colorectal cancers are diagnosed in Slovakia. Despite the progress in early diagnosis and the improvement of treatment modalities, more than 1600 cancer-related deaths continue to occur each year. CRC occurs in three specific settings: (a) sporadic form that accounts for over 85% of all cases, (b) familial form that constitutes less than 10% and (c) inherited form with a clear Mendelian transmission, observed in 5% cases, which include familial adenomatous polyposis (FAP), and hereditary non-polyposis colorectal cancer (HNPCC) syndromes [1]. It is estimated that common CRCs account for 5-10%, with the remainder comprising of sporadic colorectal carcinoma. Risk of the disease is determined by the interaction between genetic and environmental factors. In this regard, genetically-determined interindividual differences in the host ability to detoxify diet-derived carcinogens may be important [2, 3]. Glutathione S-transferases (GSTs) are among the various candidate genes implicated in many malignant neoplasm, including colorectal tumors.

Glutathione S-transferase constitutes a super family of ubiquitous, multifunctional enzymes that play a key role in cellular detoxification. The GSTs catalyze the conjugation of tripeptide glutathione (GSH) to a wide variety of exogenous and endogenous chemicals with an electrophilic functional group (e.g., products of oxidative stress, environmental pollutants, and carcinogens), thereby neutralizing their electrophilic sites rendering the products more water-soluble [4]. Because electrophiles can bind to DNA, forming adducts and potentially DNA mutations, GSTs play a critical role in protecting cells against the cytotoxic and mutagenic effects of these reactive compounds. GSTs are divided into two distinct

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super-family members: the membrane bound microsomal and cytosolic family members. On the base of sequence homology and immunological cross reactivity, human cytosolic GSTs have been grouped into eight classes, designated GST-alpha (α), mu (μ), pi (π), sigma (σ), omega (ω), theta (θ), kappa (κ) and zeta (ζ) [5, 6]. One major reason of individual variation of GST activity is due to the existence of polymorphism in these genes. The most extensively studied to date are GSTM1, GSTT1 and GSTP1.

Five GST mu class genes (M1-M5) have been identified clustered on chromosome 1 [7]. The frequency of the GSTM1 null genotype varies significantly among ethnic populations, and 38% - 67% Caucasians do not express GSTM1 due to the GSTM1 null genotype [8]. GSTP1 appears to be the most widely distributed GST isoenzyme [9]. Two polymorphisms have been described in GSTP1 gene, one in codon 105 and one in codon 114. The codon 114 variant allele is only found in combination with the codon 105 variant allele. Codon 105 polymorphism modifies the enzyme's specific activity [10]. Functional polymorphism has been described for GSTP1 resulting in an I105V substitution and leading to a lower enzyme activity. Two GST theta class genes, GSTT1 and GSTT2, have been characterized and in humans, a GSTT1 null genotype may be present at frequency of ~10-20% in Caucasians [8].

Materials and methods

Study population. Blood samples were obtained from 183 incident primary colorectal cancer patients . This group comprised patients who attended the Surgery Clinic and Oncology Centre of Martin's Faculty Hospital in Martin in the period of November 2005 - December 2007. The following data on the cases were retrieved from medical records: age, date of diagnosis of colorectal cancer, personal history, family history (number of relatives affected by colorectal cancer, or other malignant diseases), clinical stage, TNM classification according to UICC, tumor size, histological grade and type of tumor. The main criterion for inclusion of patients into the study was histologically verified colorectal cancer malignancy. This group of 183 patients included 88 (48.1%) women and 95 (51.9%) men. the median age was - cases 63±10 years, controls 61±12 years. The control group comprised of 422 healthy volunteers from the same geographic region (middle Slovakia). Samples from the control subjects were collected during the same period as the cases. No cancer controls were included into the study. The composition of the control group was comparable to the cases in terms of age, gender, and ethnicity (Caucasian only). Patients and controls were asked to read and sign an informed consent in accordance with the requirements of the Ethical Commission for Research.

Genotype analysis Genomic DNA was isolated using standard techniques (proteinase K digestion, the phenol /chloroform extraction and ethanol precipitation, dissolved in TE buffer (pH = 7.5) from blood drawn into 4.5 ml EDTA tubes and stored at -20°C until use. The concentration of DNA was adjusted to 100 μg/mL, and the DNA was stored at -20°C. All genotyping analyses were PCR-based, with a total volume of 25 μl for each reaction containing a PCR buffer (16.6 mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, pH=8.8, 1.2 μl DMSO, 1.2 μl DTT), 0.2mM deoxynucleotide triphosphates, 0.5 U Taq polymerase, 25 pM primers and 100ng DNA. Digests were electrophoresed on 3% agarose gel and photographed. In all reactions, a positive and negative control is included. As a quality control, 10-20% of all samples are repeated as blinded duplicates.

GSTM1 and GSTT1. The GSTM1 and GSTT1 genetic polymorphisms are determined simultaneously by multiplex PCR [11], with the following modification. Primer sequences used were: 5'-GAACTCCCTGAAAAGCTAAAGC-3' and 5'-GTTGGGCTCAAATATACGGTGG-3' for GSTM1; and 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCA-3' for GSTT1; and 5'-CAACTTCATCCAC GTTCACC-3' and 5'-GAA-GAGCCAAGGACAGGTAC-3' for β- globin. After initial denaturation at 95°C for 1 min, the samples underwent 35 cycles of 30s at 94°C, 30s at 64°C and 1 min at 72°C, followed by the final extension at 72°C for 5 min. The absence of the GSTM1- and/or GSTT1-specific PCR-product indicated the corresponding null genotype. β-globin was amplified in the same reaction as an internal positive control. PCR-reaction products are analyzed by gel electrophoresis (3% agarose) and ethidium bromide staining for the presence of a 273 bp GSTM1 product, a 480 bp GSTT1 product, and a 320 bp β-globin product. The lack of a GSTM1 or GSTT1 amplification product in the presence of an actin amplification product is consistent with the homozygous null genotype.

GSTP1. A to G polymorphism at codon 105 is determined by PCR of 176 bp fragment and restriction fragment length polymorphism (RLFP). The reported PCR-RFLP protocol [10] was modified as follows. Primer sequences for GSTP1 were 5'-GTA GTT TGC CCA AGG TCA AG-3' and 5'-AGC CAC CTG AGG GGT AAG-3'. PCR cycles started with initial denaturation at 94°C for 1 min, five cycles were carried out at 94°C for 15 s, 64°C for 30s and 72°C for 1 min with an annealing temperature that decreased by 1°C for each cycle. Then 30 cycles were carried out at 94°C for 15s, 59°C for 30s and 72°C for 1 min, followed by a final extension for 5 min. An amount of 10μl of the amplicon was digested for 4 hours with 5 units of the restriction enzyme Alw 26I at 37°C. Three variants were identified: Ile/Ile, Ile/Val, and Val/Val.

Statistical analysis. The Chi-square (χ^2) test was used to determine the significance of differences from the Hardy-Weinberg equilibrium and the independence of genotype frequency between cases and controls. Odds ratio (OR) and 95% confidence interval (95% CI) were obtained from an unconditional logistic regression model. A level of $P < 0.05$ was accepted as statistically significant. We also analyzed the prevalence of selected combinations of genotypes as follows: GSTM1+GSTT1, GSTM1+GSTP1 and GSTT1+GSTP1. The selection of this combination was based on the hypothesis that the carrier of at least one variant allele in both combined

Table 1: The genotype and allele frequencies in GSTM1, GSTT1 and GSTP1 polymorphisms

Genotype/group	Case [n (%)]	Control [n (%)]	OR	95% CI	P value	χ^2
GSTT1						
positive	142 (76.5)	329 (78)	1.0	(ref.)	0.99#	9.13#
null	41 (22.4)	93 (22)	0.97	0.64-1.48		
GSTM1						
positive	83 (45.4)	202 (47.9)	1.0	(ref.)	0.63#	0.061#
null	100 (54.6)	220 (52.1)	1.1	0.78-1.56		
GSTP1						
Allele (n/%)						
Ile	224 (61.9)	544 (70.5)	1.0	(ref.)	0.004#	7.92#
Val	138 (38.1)	228 (29.5)	1.50	1.13-1.91		
Ile/Ile	63 (34.8)	186 (48.2)	1.0	(ref.)	0.0025*	0.28*
Ile/Val	98 (54.1)	172 (44.6)	1.7	1.15-2.45		
Val/Val	20 (11.1)	28 (7.2)	2.1	1.1-4.0		
Ile/Val or Val/Val	118 (65.2)	200 (51.8)	1.7	1.2-2.5		

(P value, from χ^2 test)
(P value, from χ^2 test for trend)

genes may be at higher risk. All statistical calculations were performed using Microsoft Excel and MedCalc v.5 software for Windows.

Resultes

The frequency of genotypes for GSTM1, GSTT1, and GSTP1 for cases and controls is shown in Table 1. The observed frequencies and genotype distributions in our control group did not differ significantly from data on the majority of other European Caucasian subpopulations [12]. The most interesting result was obtained by the analysis of the distribution of genotypes in GSTP1-exon 5. The difference in distribution of genotypes (χ^2 = 9.13, P = 0.0025) and crude OR analysis were highly significant between cases and controls (OR = 2.1, CI = 1.1 – 4.0 for normal versus variant homozygote).

GSTP1. The overall frequency of GSTP1 alleles and genotypes is shown in Table 1. The frequencies for the Val-105 allele were 29.5% for controls and 38.1 % for cases. There was a significant higher difference between cases and individually matched controls in the frequency of allele Val-105 (OR = 1.5 95% CI = 1.1-1.9 P = 0.0049, χ^2=7.9)

In the controls, 7.2% individuals were homozygous and 44.6% were heterozygous for Val-105, and 48.2% were homozygous for Ile-105. In the patients group figures were 11.1, 54.1, and 34.8%, respectively. We found statistical differences between patients with colorectal cancer and the control group for the homozygous for Ile-105 and homozygous for Val-105 (OR= 2.1, 95%CI ´1.1 – 4.0, p for trend = 0.0025).

GSTT1. For GSTT1 22% of controls and 22.4% of cases were null. There was no difference in the frequency of the null genotype between matched cases and controls (OR = 0.97; 95% CI = 0.64-1.48)

GSTM1. The GSTM1 null genotype was found in 52.1% of all controls and 54.6% of cases (OR = 1.1; 95% CI = 0.78 – 1.56).

Analysis by subgroups of age, and stage or site of the tumor showed no significant differences.

Analysis of combination of genotype. We simultaneously evaluated the association of polymorphisms together with the colorectal cancer risk (Table 2). The combination of GSTP1 homozygous for Val-105 and GSTM1 null was found in 15 from the 381 controls and 14 of the 181 cases (OR = 2.7; 95% CI = 1.1 -6.1). Heterogenetic analysis showed that the interaction between GSTP1 homozygous for Val-105 and GSTM1 null was statistically significant (P = 0.03; χ^2=4.5).

There was no difference in the distribution of the GSTP1 genotypes between the controls or cases according to the GSTT1 genotype.

Examination of the segregation of null and positive genotypes for GSTT1 and GSTM1 showed that they were randomly distributed in the cases. The occurrence of the putative "worst" combination of GSTT1 null and GSTM1 null was found in 13.7% of all controls and 10.4% of cases (not significant). There was also no difference in the frequency of the "best" combination genotype (positive for both GSTT1 and GSTM1) between the controls and cases (39.6% controls and 33.3% cases; not significant).

Discussion

Colorectal cancer is a multifactorial disease, i.e., there are many factors contributing to its development. These include on the one hand dietary and lifestyle habits and on the other hand genetic predispositions. Epidemiological studies indicate that diets high in red meat, diets low in vegetables and fiber,

Table 2: The combination genotype frequencies in GSTM1, GSTT1, GSTP1 polymorphisms

	case [n (%)]	control [n (%)]	OR	95% CI	P value	χ^2	P value	#χ^2	P value	*χ^2
GSTT1 + GSTM1										
present + present	61 (33.3)	167 (39.6)	1.0	ref.			0.17	4.95	0.8	0.061
present + null	83 (45.4)	162 (38.4)	1.4	0.9-2.1	0.1	2.5				
null+ present	20 (10.9)	35 (8.3)	1.6	0.8-2.9	0.2	1.5				
null+null	19 (10.4)	58 (13.7)	0.9	0.5-1.6	0.8	0.04				
GSTT1+GSTP1										
present +Ile/Ile	53 (29.3)	139 (37.5)	1.0	ref.			0.03	8.72	0.45	0.55
present +Ile/Val	75 (41.4)	133 (34.9)	1.5	0.9-2.2	0.08	2.9				
present +Val/Val	16 (8.8)	20 (5.2)	2.1	1.0-4.3	0.06	3.3				
present + Ile/Val or Val/Val	91 (50.2)	153 (40.1)	1.6	1.0-2.3	0.04	4.1				
null + Ile/Ile	11 (6.1)	43 (11.3)	0.6	0.3-1.3	0.3	0.8				
null + Ile/Val	23 (12.7)	38 (10.0)	1.6	0.8-2.9	0.18	1.7				
null + Val/Val	3 (1.7)	8 (2.1)	0.9	0.2-3.8	0.7	0.1				
null+ Ile/Val or Val/Val	26 (14.4)	46 (12.1)	1.5	0.8-2.6	0.2	1.4				
GSTM1+GSTP1										
present +Ile/Ile	30 (16.6)	86 (22.6)	1.0	ref.			0.03	8.9	0.07	3.1
present + Ile/Val	48 (26.5)	83 (21.8)	1.6	0.9-2.8	0.09	2.8				
present + Val/Val	4 (2.2)	13 (3.4)	0.9	0.2-2.9	0.92	0.008				
present + Ile/Val or Val/Val	52 (28.7)	96 (25.2)	1.6	0.9-2.6	0.13	2.1				
null + Ile/Ile	33 (18.2)	96 (25.2)	0.9	0.5-1.7	0.92	0.009				
null + Ile/Val	52 (28.7)	88 (23.1)	1.4	0.9-2.9	0.07	3.2				
null + Val/Val	14 (7.8)	15 (3.9)	2.7	1.1-6.1	0.03	4.5				
null + Ile/Val or Val/Val	66 (36.5)	103 (27)	1.8	1.1-3.0	0.02	4.7				

(χ^2 test)
* (P value, from χ^2 test for trend)

obesity, and smoking are associated with an increased colorectal cancer risk. Diets high in calcium and folate and regular physical activity are associated with a reduced risk. However, some of these associations are still controversial.

Since the gastrointestinal tract is in direct contact with potentially toxic or (pre-) carcinogenic agents, the intestinal mucosa acts as a first-line barrier. In humans, detoxification enzymes are prominently present in the liver. However, these enzymes have also been distinguished in extra-hepatic tissues of the gastrointestinal tract. GSTM1 is expressed at low levels in the colon. GSTP1, followed by GSTT1 are a more obvious candidate for colorectal cancer susceptibility gene, because they are present at high levels in the colon [13, 14, 15, 16]. Polymorphic variations in the detoxification enzymes may modulate the rate of conversion of toxic or carcinogenic compounds in the epithelium lining the lumen of gastrointestinal tract. Several polymorphisms of genes encoding for detoxification enzymes have been described and have sometimes been associated with increased CRC susceptibility. In the present study we investigated the relationship between sporadic CRC and polymorphisms in GSTs genes, which are associated with functional changes in enzyme activity.

The relationship between CRC risk and GSTM1 polymorphism has been most extensively studied and recently, two meta analyses have been published by de Jong et al. and Houlston and Tomplinson [8, 17]. Both pooled analyses revealed no association of the GSTM1 polymorphism with CRC. Our results are in accordance with these observations. In contrast, a significant association of GSTM1 null genotype carriers and an increased CRC risk was described in a recent study. Some case-control studies provide evidence that the presence of GSTM1 in conjuction with low intake of cruciferous vegetables is an important risk factor for CRC or pre- cancerous lesions [18, 19].

Conflicting findings have also been reported in the relationship between GSTT1 status and CRC. We found no association between GSTT1 status and CRC. A number of studies have shown the GSTT1 null genotype to be associated with increased CRC [4, 20, 21, 22, 23]. However, other studies have failed to replicate this association [24, 25, 26, 27, 28].

Several studies reported on the genetic polymorphism in codon 105 of the GSTP1 as a possible risk factor for CRC. However pooled analyses by de Jong at al. and Houlston did not show an increased risk of this polymorphism for CRC, which is not in accordance with our results [8, 17].

Combinations of GSTM1, GSTT1 and GSTP1.Two studies examined the GSTM1 and GSTP1 (codon 105) polymorphism [9, 29], and five studies examined the GSTM1 and GSTT1 polymorphisms [25, 29, 30, 31, 32]. Pooled analyses showed no association. In one study, the GSTP1 (codon 105) and GSTT1 polymorphisms were studied. Again, no association was observed. There are no studies available that tested the combination of polymorphisms in three or four genes.

Only few studies have demonstrated an association between the GSTT1 or GSTM1 null genotype and colorectal cancer [33], although these have not been confirmed in further studies [25]. Some studies indicate that homozygosis for GSTM1 null is associated with the increased risk of various types of cancer: lung, adenocarcinoma and also colorectal and other types of diseases (chronic bronchitis, arteriosclerosis) [34].

GSTP1, followed by GSTT1, were major izoenzymes in all colon cells [14, 15]. Several studies have indicated an association between GSTP1 polymorphisms and the risk for a variety of cancers as well as various responses to cancer treatment and the susceptibility to some other diseases such as Parkinson□s disease, multiple sclerosis and asthma [35].

In conclusion, our study showed an individual susceptibility to colon cancer estimated by the analysis of GSTs polymorphisms. Further clarification of the puzzled problem brought by this study should help to identify individuals with increased cancer risk.

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