GSTM, GSTT and *p53* Polymorphisms as Modifiers of Clinical Outcome in Colorectal Cancer

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Abstract. *Background: Cancer of the colorectal region is the second most frequent cause of death among malignant diseases. The influence of two allelic polymorphisms of GSTM1 and GSTT1, and that of p53 gene codon 72 on colon cancer was investigated. Patients and Methods: Intraoperatively removed tissue samples were processed from colorectal cancer patients. Cancer-free human samples were used as matched controls. Samples were digested with proteinase-K. DNA solution was used for PCR amplification. Results: No significant difference was found between tumor patients and controls in the investigated polymorphisms. A significant association was found in Dukes' B stage patients between the GSTM1 and p53 gene variants and survival. In patients with GSTM1 null genotype and p53 Arg/Pro heterozygotes or Pro/Pro homozygotes the chance of survival is significantly lower than in the case of GSTM1+ and p53 Arg/Arg variants (p=0.009 and p=0.008, respectively). Conclusion: The significance of the investigated polymorphisms in prognosis is dependent on the tumor stage. These parameters might be used in certain cases as prognostic biomarkers in clinical diagnostics and in the planning of individual therapy.*

Malignancies of the gastrointestinal tract are among the most frequent malignant diseases in Hungary (1). Cancer of the colorectal region is the second most frequent cause of death from malignant diseases (2). Traditional gastronomical habits which include fatty and dietary fiber deficient foods and the popularity of meats preserved by smoke explain the high prevalence of these diseases (3).

In our present study we aim to examine the relationship between certain allelic polymorphisms and related cancer risk, and its influence on the survival of inflected patients. Our area of interest is in two allelic polymorphisms of glutathione-S-transferases, the GSTM1 and GSTT1 metabolizing enzymes. Glutathione-S-transferases (GSTs) are involved in the metabolism of endogenous and exogenous carcinogenic substances, such as environmental carcinogens, reactive oxygen species and chemotherapeutic agents, by catalyzing reactions between glutathione and electrophilic compounds and is an important means of cellular protection against mutagenic factors (4). In humans, GST enzymes are divided into five subclasses: alpha (α), mu (μ), pi (π), theta (θ) and zeta (ζ). *GSTM1*, belonging to the μ class is located on chromosome 1, while *GSTT1*, belonging to the θ class, is located on chromosome 22 (5, 6). *GSTM1* products catalyze the conjugation of glutathione to epoxide derivatives of polycyclic aromatic hydrocarbons, which are found in tobacco smoke and in smoked meats in high concentration (7). *GSTT1* products are involved in activation and detoxification reactions and catalyze the conjugation of industrial chemicals with glutathione (8, 9).

The null genotype of these genes (deletions of both paternal and maternal alleles) causes the lack of GSTM1 and GSTT1 proteins which results in an increased risk of development of certain types of cancers (10, 11). Polymorphisms in *GSTM1* and *GSTT1* may modify the risk of colorectal cancer, and may be important in determining an individual's susceptibility to colorectal cancer.

In the first part of our study, we tested the effect of allelic polymorphisms *GSTM1* and *GSTT1* on the risk of colorectal cancer in the Hungarian population.

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The second target of our study was to investigate the influence of p53 on colon cancer. *p53* is a tumor-supressor gene that plays an important role in controlling cell cycle regulation and inhibiting clonal expansion. *p53* is one of most frequently mutated genes in malignant diseases. Several polymorphisms have been registered in the *p53* gene locus. One of the most important polymorphisms of this area is in codon 72 of exon 4 coding for the Arg (72Arg: CGC), or Pro (72Pro: CCC) variant. The Arg variants have been shown to induce apoptosis with more efficacy than the Pro variants (12). Several studies found a relationship between *p53* aberration and malignancies, including colon carcinoma (13, 14).

In our present study, we investigated whether these polymorphisms are related with the survival time of the patients.

Patients and Methods

Formalin-fixed, paraffin-embedded tissue samples of intraoperatively resected colorectal malignancies were used in this study. Samples were collected in the Markusovszki Hospital (Szombathely, Hungary), and Baranya County Hospital (Pécs, Hungary) between 1998-2003. All cases were diagnosed as adenocarcinomas. One hundred and two patients were enrolled in our study (65 males, 37 females; mean age 62±5.5 years). Pathological and follow-up reports were collected from the participating hospitals. Pathological variables were determined by each hospital's Department of Pathology. For tumor staging, Dukes' classification (15) was used: 14 tumor cases were Dukes' A, 34 were Dukes' B, 46 were Dukes' C, and 8 Dukes' D. All patients were followed up. Survival data were taken from the oncological departments' databases. Patients were followed up for 45 months or until the date of death.

The paraffin-embedded tissue blocks were cut into 10 μm thick sections with a microtome-blade. Samples were placed in Eppendorf tubes and deparaffinized in a xylene bath, then washed in ethanol two times. The tissue samples were resuspended in lysis buffer containing 200 μg/ml proteinase K (Invitrogen, Carlsbad, USA) and were incubated at 37˚C for 12 hours. Proteinase K was inactivated at 95˚C for 10 minutes. All tubes were centrifuged for five minutes at 15000 ×g.

A 2 μl DNA solution from the supernatant was used for PCR. For amplification the following primers were used: GSTT1: forward 5'-TT CC T TAC TGG TCC TCA CAT CTC-3', reverse 5'-TCA CCG GAT CAT GGC CAG CA-3'; and GSTM1: forward 5'-GAA CTC CCT GAA AAG CTA AAG C-3', reverse 5'-GTT GGG CTC AAA TAT ACG GTG G-3'.

p53 genotyping (codon 72, Arg/Pro polymorphism) was performed using 3' primer: GCAACTGACCGTGCAAGTCA and 5' primers for Arg variant ATGCCAGAGGCTGCTCCCCG and for the Pro variant ATGCCAGAGGCTGCTCCCCC. For the PCR reaction, PCR Master Mix (Promega, Woods Hollow, USA) was used, according to the manufacturer's instructions.

The results were compared with those obtained from age-and sex-matched healthy controls from our archive data. Statistical association was tested by Chi-square test. Kaplan-Meier survival curves were constructed for survival analysis. All survival analyses and statistical calculations were performed with MedCalc software (Mariakerke, Belgium). *P*-values≤0.05 were considered statistically significant. The interval from the date of operation to the date of last observation or death was used for analysis.

Table I. *Results of GSTM1, and GSTT1 polymorphism analysis: genotype frequencies in patients and controls.*

	Cases (102)	Controls (97)	OR, (95%) CI	*p*-value
GSTM1 +	42 (41.2%)	51 (52.5%)	1.0	
0	60 (58.8%)	46 (47.4%)	1.58 (0.87-2.89)	0.10
GSTT1 +	68 (66.7%)	77 (79.4%)	1.0	
0	34(33.3%)	20 (20.6%)	1.92 (0.97-3.85)	0.43
P53Arg/Arg	66 (64.7%)	62 (63.9%)	1.0	
Arg/Pro	32 (31.4%)	29 (29.9%)	1.04 (0.54-2.00)	0.90
Pro/Pro	4 (3.9%)	6 (6.2%)	0.63 (0.14-2.67)	0.48

Results

The *GSTM1* null allele was found in 60 (58.8%) cases within the tumor group and in 46 (47.4%) cases in controls (OR:1.58, 95% CI: 0.87-2.89). The connection is not significant (p=0.10). The *GSTT1* null allele was more frequent among tumor patients, 34 (33.3%) *versus* 20 (20.6%) in controls. Although the null allele was more frequent in the colon cancer group of patients (OR:1.92, 95% CI:0.97-3.85), the result is statistically not significant (p=0.43) (Table I). The *p53* Arg/Pro status is also shown in Table I. No significant difference was found between patients and controls (p=0.90 in the case of Arg/Pro, and p=0.48 in the case of Pro/Pro variants).

Survival analysis of the tumor patients is demonstrated in Figure 1. The Kaplan-Meier survival curves show the relation of Dukes' stages and survival. In accordance with previous studies, the average survival time was the longest in Dukes' A stage patients, and the shortest in Dukes' D cases. After 45 months of follow-up the rate of surviving patients was 71.4% in Dukes' A, 50% in Duke's B, 34.8% in Dukes' C, and 0% among patients whose malignancy had been discovered in stage Dukes' D.

We compared the variant allele's influence on survival (Figures 2-13). In the case of Dukes' A (Figures 2-4) and D stages (Figures 11-13) the number of cases was too low for statistical analysis. In Dukes' B group the Kaplan-Meier curves show that *GSTM1+* genotypes and *p53* Arg variants are associated with significantly longer survival (Figure 5, 6). In the case of GSTM1 polymorphism the *GSTM1+* variant was associated with 0.53 odds ratio (OR), 95% confidence interval (CI):0.10-0.72, p=0.009. In the case of *p53* we divided patients into two groups. Group I. contained the patients with *p53* Arg variant, while group II. contained

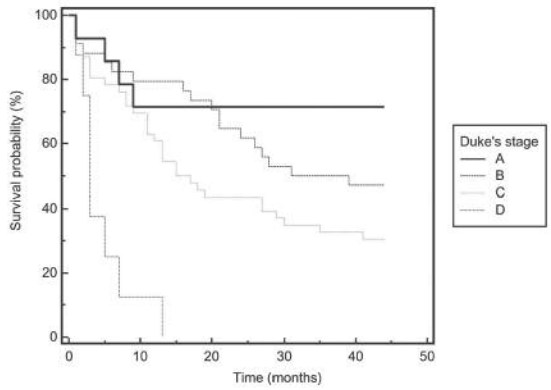

Figure 1. *Kaplan-Meier survival curve for all patients, selected by Duke's stages.*

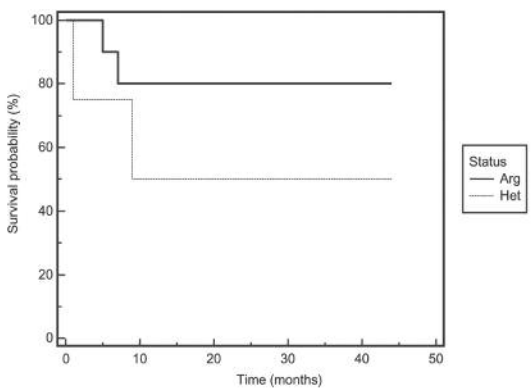

Figure 2. *Dukes' A, p53 polymorphism. Hazard ratio: 0.35, 95% CI: 0.03-2.67, p:0.27.*

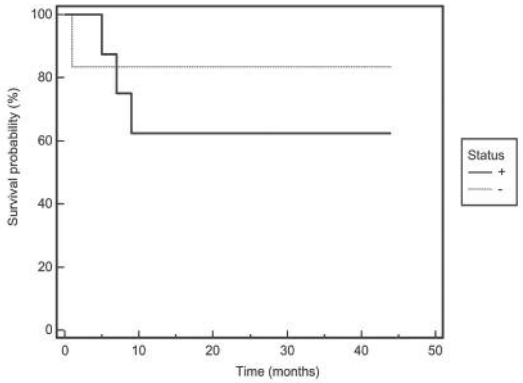

Figure 3. *Dukes' A, GSTM1 polymorphism. Hazard ratio: 2.18, 95% CI: 0.27-14.74, p:0.48.*

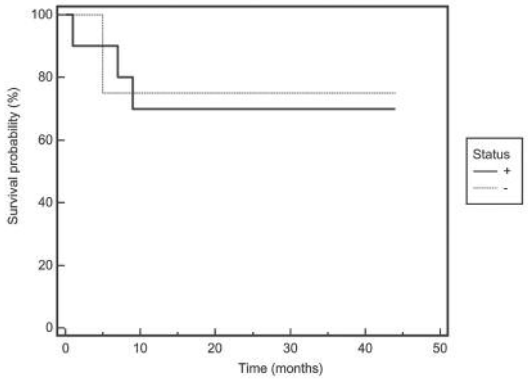

Figure 4. *Dukes' A, GSTT1 polymorphism. Hazard ratio: 1.16, 95% CI: 0.12-10.29, p:0.89.*

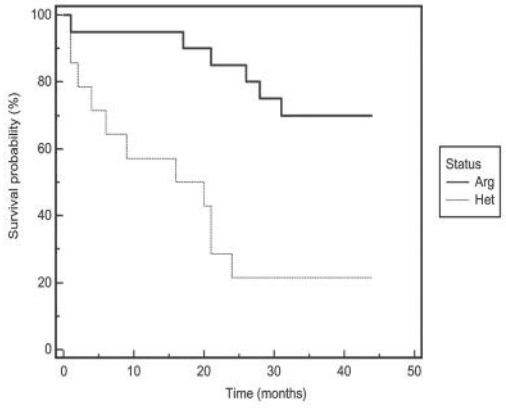

Figure 5. *Dukes' B, p53 polymorphism. Hazard ratio: 0.22, 95% CI: 0.05-0.45, p:0.008.*

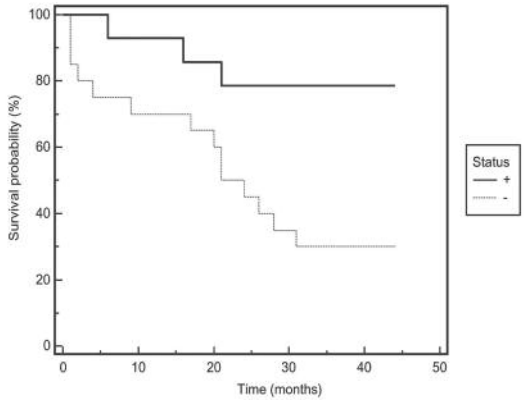

Figure 6. *Dukes' B, GSTM1 polymorphism. Hazard ratio: 0.53, 95% CI: 0.10-0.72, p:0.009.*

the heterozygotes and Pro homozygotes. In stage Dukes' B stage patients, the presence of the *p53* Arg variant was significantly assocated with a longer survival (OR: 0.22, 95% CI:0.05-0.45, p=0.008). The *GSTT1* status had no significant influence on survival in this group (p=0.14). In Dukes' C stage no significant association was found between survival and *p53* (p=0.08), *GSTM1* (p=0.95), and *GSTT1* (p=0.38) polymorphisms (Figures 8-10).

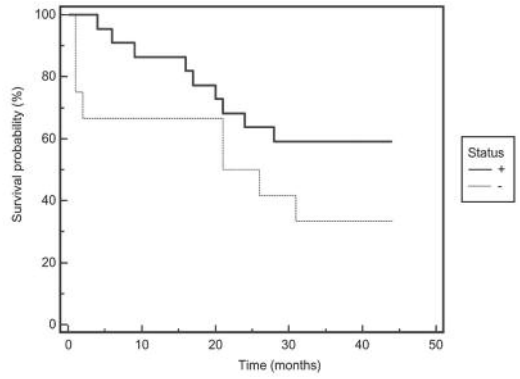

Figure 7. *Dukes' B, GSTT1 polymorphism. Hazard ratio: 0.49, 95% CI: 0.16-1.29, p:0.14.*

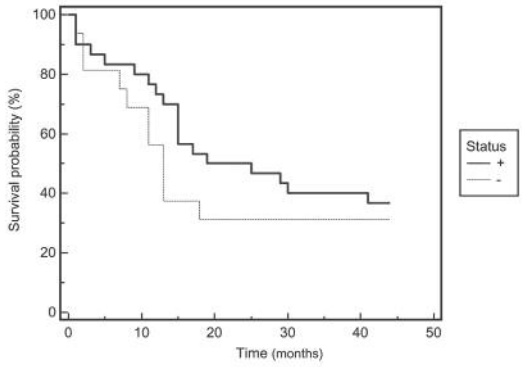

Figure 10. *Dukes' C, GSTT1 polymorphism. Hazard ratio: 0.72, 95% CI: 0.31-1.56, p:0.38.*

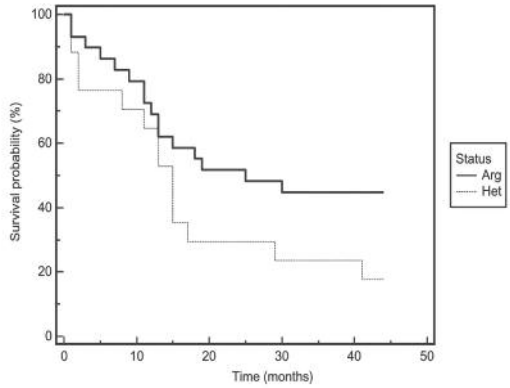

Figure 8. *Dukes' C, p53 polymorphism. Hazard ratio: 0.55, 95% CI: 0.22-1.09, p:0.08.*

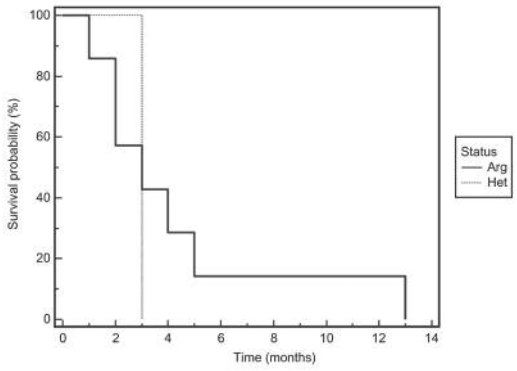

Figure 11. *Dukes' D, p53 polymorphism. Hazard ratio: 0.79, 95% CI: 0.05-9.90, p:0.79.*

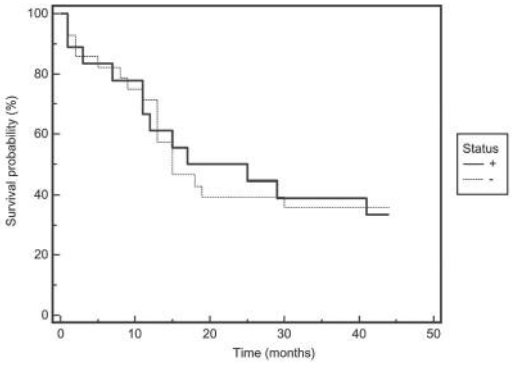

Figure 9. *Dukes' C, GSTM1 polymorphism. Hazard ratio: 1.02, 95% CI: 0.48-2.17, p:0.95.*

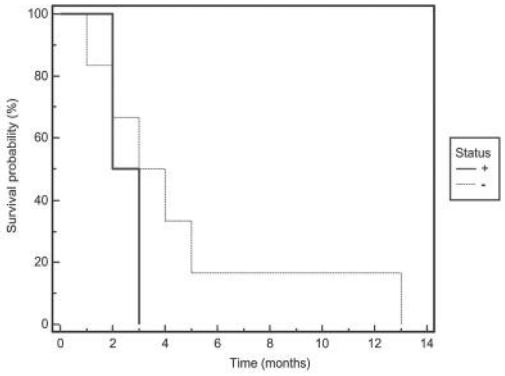

Figure 12. *Dukes' D, GSTM1 polymorphism. Hazard ratio: 1.85, 95% CI: 0.29-25.82, p:0.37.*

Discussion

The consistenently high prevalence and mortality of colorectal malignancies in our geographic region encouraged us to investigate the relationship between genetic background and cancer risk as well as molecular prognostic factors and their influence on clinical parameters and survival. Numerous references have shown that polymorphisms of

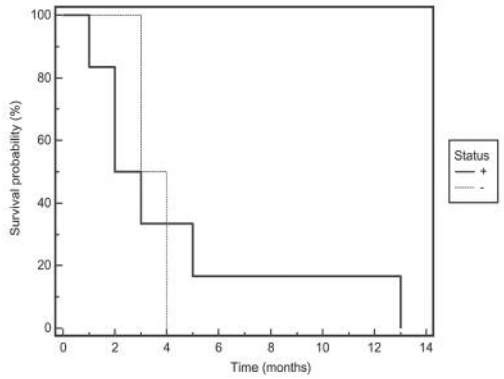

Figure 13. *Dukes' D, GSTT1 polymorphism. Hazard ratio: 0.97, 95% CI: 0.15-6.17, p:0.96.*

various detoxifying enzymes and tumor-supressor genes may influence the risk of malignant disease. Although in this study we did not find a significant difference between the control and the tumor group, this might be due to the relatively low number of cases. Our other aim was to investigate the effect of the Arg/Pro polymorphism of the *p53* codon72 exon4, and *GSTM1* and *GSTT1* polymorphisms on the survival of colorectal cancer patients.

The Arg/Arg form of *p53* is known as the variant that induces more effective apoptosis than the Pro/Pro genotype (12). Numerous studies describe a significant difference in *p53* status between tumor and control groups (16-18), but only a few publications discussed the effects of polymorphisms on survival.

In our present study, we found a significant difference in *p53* status in the subgroup of patients in Dukes' B stage of colorectal carcinoma. The chance of survival of *p53* Arg/Pro heterozygotes or Pro/Pro homozygotes is lower than *p53* Arg/Arg variants (OR:0.22, 95% CI: 0.05-0.45, p=0.008).

The *GSTM1* and *GSTT1* null genotypes are associated with a loss of enzyme function. A few studies showed an association between *GSTT* null variants and an increase in the risk of colorectal carcinomas (19, 20), however, other authors found no association between them (21). We did not find any significant difference in this aspect between the cancer and the control groups. The Kaplan-Meier curves showed a significant difference between the survival of patients with *GSTM1+* and *GSTM1Ø* variants in Dukes' B stage. Survival in patients with *GSTM1Ø* genotype was shorter than in *GSTM1+* cases (OR: 0.53, 95% CI: 0.10-0.72, p=0.009)

From the Kaplan-Meier curves, naturally the Dukes' stage was the most significant independent prognostic indicator of survival. The investigated polymorphisms do not have the same prognostic value in all tumor stages. The best survival is associated with the earliest diagnosis (Dukes' A) without a significant difference between allele variants, and the worst with Dukes' D stage. The early diagnosis of malignant diseases is the most important factor in cureability.

From our results we conclude that the investigated gene polymorphisms may be used in intermediate stages as prognostic markers. These parameters may identify in certain cases patients at higher risk, and might be used as prognostic biomarkers in clinical diagnostics and in the planning of individual therapy.

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