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Glutathione-S-Transferases in Development, Progression and Therapy of Colorectal Cancer

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1. Introduction

Etiologically, sporadic colorectal cancer (CRC) is a complex, multifactorial disease that is linked to both exogenic and endogenic factors. Accumulating evidence indicates that susceptibility to cancer in general, and to CRC in particular, is mediated by genetically determined differences in the effectiveness of detoxification of potential carcinogens and reactive oxygen species. The antioxidant enzymes and phase I and II biotransformation enzymes are important candidates for involvement in susceptibility to sporadic CRC, due to their ability to regulate the metabolism of a wide range of environmental exposures (Perera, 1997; Potter, 1999; McIlwain et al., 2006; Di Pietro et al., 2010). In addition to carcinogens and reactive oxygen species, the majority of anticancer drugs applied in the chemotherapy are also substrates and are biotransformed by xenobiotic-metabolizing enzymes, leading to their activation and/or detoxification (O'Brien &Tew, 1996; Eaton &Bammler, 1999; Townsend &Tew, 2003; Hayes et al., 2005; Michael &Doherty, 2005; Townsend et al., 2005). In this respect, great efforts have been focused to clarify the effects of genetic variations, expression and activity of xenobiotic-metabolizing enzymes in development, progression and therapy of cancers with different histological origin, including CRC (Ranganathan &Tew, 1991; Tew &Ronai, 1999; Welfare et al., 1999; Cotton et al., 2000; de Jong et al., 2002; Dogru-Abbasoglu et al., 2002; Stoehlmacher et al., 2002; Ates et al., 2005; Romero et al., 2006; Liao et al., 2007; Pistorius et al., 2007; Koutros et al., 2009; Di Pietro et al., 2010; Economopoulos & Sergentanis, 2010).

2. Role of GSTs in cell processes

Glutathione-S-transferase (GST, EC. 2.5.1.18) isoemzymes are involved in phase II xenobiotic biotransformation. GSTs belong to a large superfamily of dimeric enzymes, which play an important role in cell defense system. So far, 24 isoenzymes have been described in humans, classified into 11 classes: 7 cytosolic - alpha (α, A), mu (μ, M), pi (π, P), sigma (σ, S), theta (θ, T), zeta (ζ, Z), and omega (ω, O), one mitochondrial - kappa (κ, K), and three microsomal classes, also referred to as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Sheehan et al., 2001; Hayes et al., 2005; McIlwain et al., 2006; Laborde, 2010) The most abundant mammalian GST enzymes belong to cytosolic classes alpha, mu, and pi, and their regulation has been studied in details (Hayes &Pulford, 1995). Most of the cytosolic GST classes are coded by several genes, gathered in clusters and thus these enzymes have several subunits, which form a number of homo- and/or heterodimeric isoenzymes (Table 1) (McIlwain et al., 2006; Laborde, 2010).

GST classes	Subunits	Gene (locus) designation	Chromosome location of the genes/gene clusters
Cytosolic			
GST-alpha (GSTα, GSTA)	1,2,3,4,5	GSTA1, GSTA2, GSTA3, GSTA4, GSTA5	6p12
GST-mu (GSTμ, GSTM)	1,2,3,4,5	GSTM1, GSTM2, GSTM3, GSTM4, GSTM5	1p13
GST-omega (GSTω, GSTO)	1,2	GSTO1, GSTO2,	10q25.1
GST-pi (GSTπ, GSTP)	1	GSTP1	11q13
GST-sigma (GSTσ, GSTS)	1	GSTS ([a] HPGDS; PGDS)	4q22.3
GST-theta (GSTθ, GSTT)	1,2	GSTT1, GSTT2	22q11.2
GST-zeta (GSTξ, GSTZ)	1	GSTZ1	14q24.3
Mitochondrial			
GST-kappa (GSTκ, GSTK)	1	GSTK1	7q34
Microsomal			
[b]MAPEG		[c] MGST1, [c] MGST2, [d] ALOX5AP (FLAP) [e] LTC_4S [c] MGST3 [f] PGES (PTGES)	12p12.3-p12.1 4q28.3 13q12 5q35 1q23 9q34.3

[a]HPGDS - hematopoietic prostaglandin D synthase (PGDS - prostaglandin D synthase)
[b]MAPEG - membrane-associated proteins in eicosanoid and glutathione metabolism
[c]MGST - microsomal glutathione S-transferase
[d]ALOX5AP (FLAP) - arachidonate 5-lipoxygenase-activating protein
[e]LTC4S - leukotriene C4 synthase
[f]PGES - prostaglandin E synthase

Table 1. Classes, subunits and gene location of human GSTs

GSTs catalyze the conjugation of reduced glutathione with a variety of endogenic and exogenic electrophilic compounds, including several carcinogens and antineoplastics (Hayes &Strange, 1995; Hayes et al., 2005; Michael &Doherty, 2005). This process results in alteration, usually a reduction, of the reactivity of the compounds and makes them more water soluble and favors their elimination.

GSTs can also function as peroxidases and isomerases (Hayes &Pulford, 1995; Cho et al., 2001). Thus GSTA1-1 and GSTA2-2 efficiently catalyze the reduction of fatty acid and phospholipid hydroperoxides (Zhao et al., 1999). Moreover, it has been shown that GSTA3-3 is essential in obligatory double-bond isomerizations of precursors of testosterone and progesterone in steroid hormone biosynthesis (Johansson &Mannervik, 2001). Although the exact physiological function of omega-class GSTs remains undefined (Board et al., 2000; Board, 2011), it has been demonstrated that they can catalyze a range of thiol transferase and reduction reactions that are not catalyzed by members of the other classes: GSTO1 has GSH-dependent reductive activity to dehydroascorbate and to monomethylarsenic acid (V) (Board, 2011). GSTZ1 has isomerase activity and catalyzes the conversion of maleylacetoacetate to fumarylacetoacetate in the catabolic pathway of phenylalanine and tyrosine and also catalyzes the GSH-dependent transformation of α-halogenated acids (McIlwain et al., 2006).

There are six MAPEG (membrane associated proteins in eicosanoid and glutathione metabolism) subfamily members localized to the endoplasmic reticulum and outer mitochondrial membrane. Three of them are involved in the production of leukotrienes and prostaglandin E, whereas the other three have glutathione S-transferase and peroxidase activities, thus implicated in the protection of membranes from oxidative stress (Morgenstern et al., 2011).

In addition to their catalytic functions GSTs have several complementary functions. Some of the GSTs can serve as nonenzymatic binding proteins (known as ligandins) interacting with various lipophilic compounds including steroid and thyroid hormones (Litwack et al., 1971; Ishigaki et al., 1989; Cho et al., 2001; Vasieva, 2011). Moreover, GST isoenzymes can play a regulatory role in cellular signaling by forming protein:protein interactions with key signaling tyrosine kinases, involved in controlling stress response, apoptosis, inflammation, cellular differentiation and proliferation (Adler et al., 1999; Cho et al., 2001; Wang et al., 2001; Townsend &Tew, 2003; Townsend et al., 2005; McIlwain et al., 2006; Laborde, 2010; Vasieva, 2011).

There is strong evidence that GST-pi can bind by protein:protein interaction, sequester and inhibit c-Jun N-terminal kinase (JNK)/stress-activated protein kinases (SAPKs). JNK is a MAP kinase that phosphorylates c-Jun, a component of the activator protein-1 (AP-1) transcriptional factor, resulting in the induction of AP-1-dependent target genes which play role in cell survival and apoptosis. Thus JNK is implicated in pro-apoptotic/survival signaling pathways and may be required for induced cytotoxicity of a variety of antitumor drugs (Adler et al., 1999; Wang et al., 2001; Townsend &Tew, 2003; Townsend et al., 2005; McIlwain et al., 2006; Laborde, 2010; Vasieva, 2011).

Recently, GST-pi was shown to affect the apoptosis pathways also by physical association with TNF receptor associated factor 2 (TRAF2), an adaptor protein which mediates the signal transduction of different receptors and is required for the activation of ASK1 (apoptosis signal-regulating kinase 1) (Wu et al., 2006; Laborde, 2010; Sau et al., 2010;

Vasieva, 2011). ASK1 is a MAP kinase kinase kinase (MAP3 kinase, MAPKKK) that can phosphorylate MKK4/7 and MKK3/6 (MAP kinase kinases, MAP2Ks, MAPKK) which are involved in stress-induced activation of JNK- and p38 signaling pathways, respectively (Dorion et al., 2002; Wu et al., 2006; Sau et al., 2010).

Isoenzymes of the alpha and mu classes have also been shown in vitro to bind to JNK-Jun complexes and inhibit the activation of c-Jun by JNK, however their inhibitory activity was weaker than GST-pi (Villafania et al., 2000; Laborde, 2010). In addition, it has been noted that GST-mu interacts physically with N-terminal portion of ASK1, thus inhibiting its activity and the ASK1-elicited MKK4/7–JNK and MKK3/6–p38 signaling pathways (Dorion et al., 2002).

Another binding partner of GST-pi is the antioxidant enzyme 1-cys peroxiredoxin (1-cysPrx, Prx VI), which is a member of the peroxiredoxin superfamily and is able to protect cells from membrane peroxidation via GSH-dependent peroxidase activity on phospholipid hydroperoxides. The process of heterodimerization of 1-cysPrx with GST-pi leads to activation involving also the S-glutathionylation of 1-cysPrx (Manevich et al., 2004; Vasieva, 2011).

GST-pi has also been found to function in the S-glutathionylation of oxidized cysteine residues of several target proteins following oxidative and nitrosative stress thus playing a direct role in the control of posttranslational S-glutathionylation reactions (McIlwain et al., 2006; Townsend et al., 2006; Townsend et al., 2009; Tew et al., 2011). S-glutathionylation occurs on cysteine moieties located in relatively basic environment in response to oxidative (ROS) or nitrosative stress (RNS) signaling events. Glutathiolylation is reversible process that can occur spontaneously by GSH or catalytically by thioredoxin (Trx), glutaredoxin (Grx) or sylphoredoxin (Srx). Thus besides the phosphorylation/dephosphorylation, the cells are provided with additional dynamic system of controlling the protein activity (Townsend et al., 2009). Proteins sensitive to modification by S-glutathionylation are variety of enzymes with thiols in the active centers, cytoskeleton proteins, signaling proteins – particularly kinases and phosphatases, transcriptional factors, Ras oncogenic proteins, heat shock proteins, ion channels, and calcium pumps (Tew et al., 2011). Since a number of proteins that are S-glutathionylated are involved in growth regulatory pathways, the over-expression of GST-pi in cancers may account for the impaired balance between cell death, proliferation and differentiation and could contribute to tumor development, progression and treatment response (Townsend et al., 2009; Tew et al., 2011).

GST-pi was also shown to bind proteins and compounds containing iron and nitric oxide and thus may influence the NO metabolism and NO signaling (Vasieva, 2011). It has been shown that the natural low molecular mass NO carriers, dinitrosyl-iron complexes (DNIC) and S-nitrosoglutathion (GSNO) bind with high affinity to one active site of the dimeric GST-pi enzyme, while the enzyme maintains its detoxification activity (Lo Bello et al., 2001; Townsend et al., 2006; Vasieva, 2011). Hence, GST-pi (GSTP1-1) may act as a NO carrier, which determines it as a player of a number of processes as formation of nitrothiols, nitrosylation of proteins, NO mediated iron mobilization from cells, and Zn-homeostasis (Vasieva, 2011).

It has also been reported that certain GSTs play novel roles implicated in cell defense: GST-theta was suggested to inhibit the pro-apoptotic action of Bax (Kampranis et al., 2000), and GST-omega (GSTO1-1) was shown to modulate ryanodine receptors (RyR), which are

calcium release channels in skeletal and cardiac sarcoplasmic reticulum, suggesting protective functions of GSTO1-1 in mammalian cells from radiation damage and Ca^{2+} induced apoptosis (Dulhunty et al., 2001)

Thereby, these multiple functionalities of the members of GST family, in addition to the well-characterized catalytic activities, could contribute and be of importance in GST-highly expressing tumors for development and progression of cancers and for acquisition of resistance to applied chemotherapeutics.

3. Polymorphic variants of GSTs

Numerous polymorphisms have been described in the genes encoding GSTs as most of them have been associated with a lack or an alteration of enzymatic activity toward several substrates (Ali-Osman et al., 1997; Whyatt et al., 2000; Hayes et al., 2005; McIlwain et al., 2006).

3.1 *GSTP* class

The GST-pi class is encoded by a single gene spanning approximately 3 kb and located on chromosome 11 (11q13). Two *GSTP1* single nucleotide polymorphisms (SNPs) have been identified. They are characterized by transitions at $A^{1578}G$ (exon 5, $A^{313}G$) and $C^{2293}T$ (exon 6, $C^{341}T$), resulting in amino acid substitutions $Ile^{105}Val$ and $Ala^{114}Val$, respectively, which appear to be within the active site of the GST-pi protein (Ali-Osman et al., 1997; Watson et al., 1998; Hayes et al., 2005; McIlwain et al., 2006). These two SNPs lead to the following four alleles: *GSTP1*A* (105Ile, 114Ala), *GSTP1*B* (105Val, 114Ala), *GSTP1*C* (105Val, 114Val), and *GSTP1*D* (105Ile, 114Val).

It has been proven that the substitutions due to SNPs in *GSTP1* are functional: the substitution of Ile to Val at position 105 (*GSTP1* $Ile^{105}Val$) results in altered enzyme activity to variety of electrophilic molecules (Hayes et al., 2005; McIlwain et al., 2006). Thus, there is a strong experimental evidence that the two proteins, encoded by the allelic variants, 105Ile and 105Val of the human *GSTP1* gene, differ significantly in their catalytic activities toward a model substrate; the GST-pi 105Val variant has lower activity toward 1-chloro-2,4-dinitrobenzene, a standard substrate, than its 105Ile counterpart (Ali-Osman et al., 1997; Townsend &Tew, 2003, Coles, 2000 #47). On the other hand, the same variant (105Val) displays greater activity toward polycyclic aromatic hydrocarbon (PAH) diol epoxides (Sundberg et al., 1998; Coles et al., 2000; Bostrom et al., 2002). The GST-pi 105Val enzyme variant is found to be more active than 105Ile variant in conjugation reactions with the bulky diol epoxides of PAHs, being up to 3-fold as active toward the *anti*- and *syn*-diol epoxide enantiomers with R-absolute configuration at the benzylic oxiranyl carbon (Sundberg et al., 1998; Coles et al., 2000). The bay-region diol epoxides of PAHs are known to be ultimate mutagenic and carcinogenic metabolites (Sundberg et al., 1998; Bostrom et al., 2002).

The frequency of *GSTP1* 105Ile allele in different Caucasian groups varied from 0.63 to 0.77, whereas the frequency of the variant *GSTP1* 105Val allele ranged between 0.23 and 0.37 (Table 2) (Katoh et al., 2008). In our previous study we determined the frequency of $Ile^{105}Val$ *GSTP1* genotypes in 126 ethnic Bulgarian individuals from the region of Stara Zagora (0.54 for Ile/Ile, 0.39 for Ile/Val and 0.07 for Val/Val) (Vlaykova et al., 2007). The obtained figures are consistent with those published for the controls in the case-control study of Bulgarian

patients with Balkan endemic nephropathy (Andonova et al., 2004), and for other Caucasian type control cohorts in Finland (Mitrunen et al., 2001), Edinburgh area, Scotland (Harries et al., 1997), Newcastle and North Tyneside, England (Welfare et al., 1999), East Anglia region (Loktionov et al., 2001), etc. (Table 2). Based on these similarities we can conclude that despite the heterogeneous origin ethnic Bulgarians do not differ from other Caucasians in frequency of Ile^{105}Val *GSTP1* genotypes and could be included in larger interinstitutional case-control studies for investigation of the effect of this polymorphism on the susceptibility to different diseases, including cancers.

Country/racial origin	Allele frequencies			Genotype frequencies			
	105Ile (%)	105Val (%)	p-value	105 Ile/Ile (%)	105 Ile/Val (%)	105Val/ Val (%)	p-value
Bulgaria/Caucasian (Vlaykova et al., 2007)	73	27		54	39	7	
Bulgaria/Caucasian (Andonova et al., 2004)	66	34	0.284	47	38	15	0.182
Finland/Caucasian (Mitrunen et al., 2001)	74	26	0.873	55	38	7	0.989
Scotland (UK)/Caucasian (Harries et al., 1997)	72.2	27.8	0.899	51	42.5	6.5	0.906
Surrey, UK/Caucasian (Kote-Jarai et al., 2001)	70.4	29.6	0.684	51.2	38.5	10.3	0.702
Newcastle, UK/Caucasian (Welfare et al., 1999)	66.5	33.5	0.318	45	43	12	0.312
East Anglia, UK/Caucasian (Loktionov et al., 2001)	65.5	34.5	0.252	40	49	11	0.128
Germany/ Caucasian (Steinhoff et al., 2000)	73	27	1.00	55	36	9	0.827
Sweden/ Caucasian (Sorensen et al., 2007)	69	31	0.534	49	40	11	0.564
Austria/ Caucasian (Gsur et al., 2001)	63.3	36.7	0.142	39.2	48.2	12.6	0.085
Portugal/ Caucasian (Jeronimo et al., 2002)	67	33	0.356	43.3	47.5	9.2	0.315
American non-Hispanic/ Caucasian (Agalliu et al., 2006)	66	34	0.284	43	46	11	0.258

Table 2. Allele and genotype frequencies of the *GSTP1* Ile^{105}Val gene polymorphism in Bulgarians compared to other Caucasian populations.

3.2 *GSTM* class

GSTM1 together with the other four *GSTM* class members (*GSTM2, GSTM3, GSTM4* and *GSTM5*) are mapped to 1p13.3 (Pearson et al., 1993; McIlwain et al., 2006; Laborde, 2010). The close proximity of *GSTM1* and *GSTM2*, as well as the presence of two almost identical 4.2-kb regions flanking the *GSTM1* gene have been suggested to be the reasons for the observed entire *GSTM1* gene deletion resulting in a null *GSTM1* allele (*GSTM1*0*) (Pearson et al., 1993; Bolt &Thier, 2006). Furthermore, a transversion of G with C at position 534 (534G>C, formerly noted as 519G>C) was described leading to a substitution of 172Lys with 172Asn (formerly $Lys^{173}Asn$) (McLellan et al., 1997; Bolt &Thier, 2006; McIlwain et al., 2006; Gao et al., 2010). This SNP results in two new alleles - *GSTM1*A* and *GSTM1*B*, which were reported to be functionally identical (McLellan et al., 1997). In addition, a duplication of *GSTM1* gene has been identified and characterized (*GSTM1*1x2* allele) in people who displayed ultrarapid GSTM1 activity (McLellan et al., 1997).

Thus, four allele loci have been described in the human *GSTM1* - *GSTM1*A, GSTM1*B, GSTM1*0* and *GSTM1*1x2*, which determine several phenotypes. The frequencies of *GSTM1* alleles and genotypes display race and ethnic variations: 42% to 60% of Caucasians, 41% to 63% of Asians and only 16% to 36% of Africans are homozygous for *GSTM1*0* (null *GSTM1* genotype) (O'Brien &Tew, 1996; Cotton et al., 2000; He et al., 2004; Hayes et al., 2005; Bolt &Thier, 2006; McIlwain et al., 2006; Katoh et al., 2008; Gao et al., 2010). Our results showed that the frequency of *GSTM1* genotype in Bulgarian control individuals (36% and 42%) (Figure 1A) (Dimov et al., 2008; Emin et al., 2009; Vlaykova et al., 2009) is commensurable to that reported for some other European populations (Cotton et al., 2000; Ates et al., 2005; Katoh et al., 2008; Gao et al., 2010).

Polymorphic variants have been described for the other GSTM members: *GSTM2, GSTM3, GSTM4* and *GSTM5* (Inskip et al., 1995; Mitrunen et al., 2001; Reszka &Wasowicz, 2001; Hayes et al., 2005; Reszka et al., 2007; Yu et al., 2009; Moyer et al., 2010). The most extensive studies have been performed on *GSTM3* polymorphisms. This gene has an insertion/deletion polymorphism (rs1799735, *GSTM3*A/*B*) with a wild-type *GSTM3*A* allele and a variant one, *GSTM3*B*, which differ in the rate of expression. The variant *GSTM3*B* allele has 3 bp deletion in intron 6, which introduces a recognition site for YY1 transcriptional factor and results in enhanced expression of the enzyme protein. (Inskip et al., 1995; Loktionov et al., 2001; McIlwain et al., 2006; Reszka et al., 2007). Recently, several SNPs in *GSTM3* have been identified and studied for their functional activity and in association with variety of diseases. These are the rare $Gln^{174}Trp$ ($G^{174}W$), the more common $Val^{224}Ile$ ($V^{224}I$) substitutions, and the transversion of A with C at -63 position in promoter region of *GSTM3* (-62A>C) (Liu et al., 2005; McIlwain et al., 2006). The variant 174Trp allele, as well as the wild-type 224Val allele, were reported to exhibit decreased catalytic activity, whereas the variant -63C allele was associated with increased expression of the gene (Liu et al., 2005; McIlwain et al., 2006).

3.3 *GSTT* class

A null polymorphism has also been described in *T1* locus of *GSTT* cluster at 22q11.2. Analogously to *GSTM1, GSTT1* consisting of 5 exons, is flanked by two highly homologous 18 kb regions (HA3 and HA5). The null *GSTT1*0* allele is possibly caused by a homologous recombination resulting in 54 kb deletion containing the entire *GSTT1* gene (Sprenger et al.,

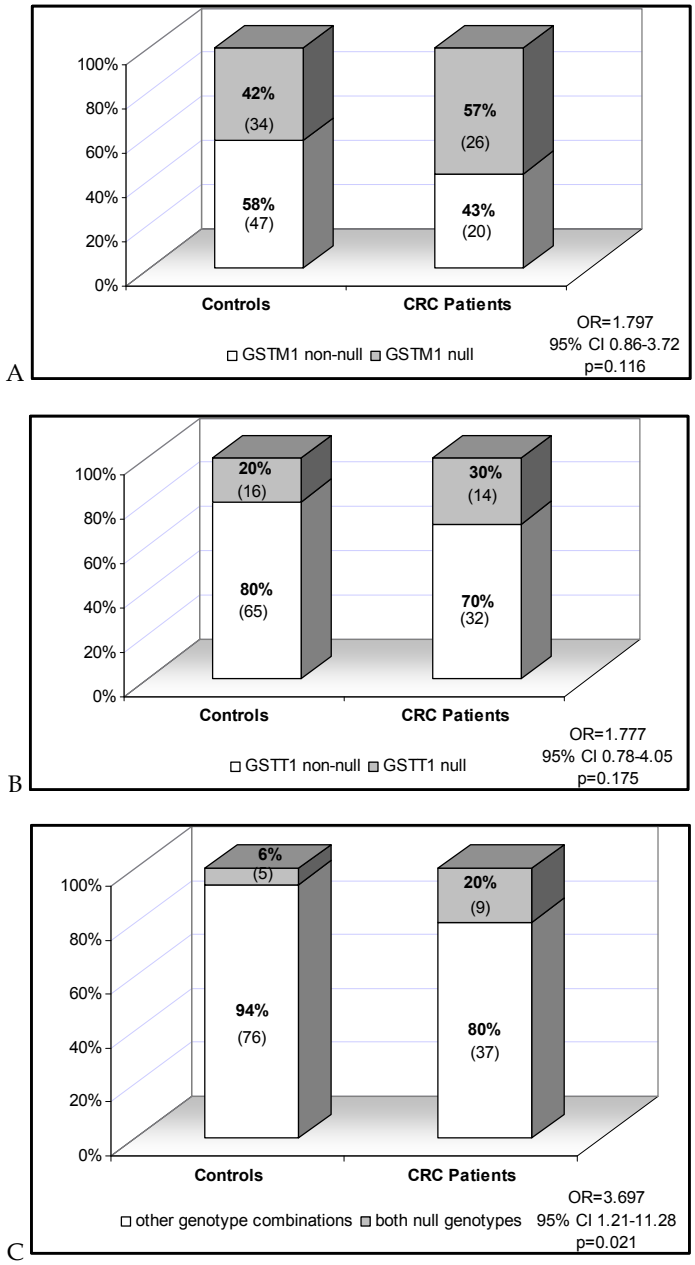


Fig. 1. Distribution of GSTM1 (A) and GSTT1 (B) null and non-null genotypes in Bulgarian patients with CRC and control individuals. Frequency of carriers of GSTM1 and GSTT1 double null genotype among the patients and controls (C). Data are presented in percentages and in real numbers (in brackets); the ORs and the 95% CI are also given.

2000; Bolt &Thier, 2006). A SNP (310A>C) in exon 3 of *GSTT1* is the reason for substitution of Tre104 with Pro104 (Tre^{104}Pro) in GST-theta protein, which was associated with a decrease in the catalytic activity possibly due to a conformational changes of the protein molecule (Alexandrie et al., 2002). The frequency of the null *GSTT1* genotype has also been found to vary significantly between different races and ethnic groups: between 13% and 31% (with some exceptions) in Caucasians in Europe and USA and between 35% and 48% in Asians (O'Brien &Tew, 1996; Cotton et al., 2000; He et al., 2004; Hayes et al., 2005; Bolt &Thier, 2006; McIlwain et al., 2006; Katoh et al., 2008). Our preliminary results concerning a small Bulgarian control group showed homozygosity for *GSTT1*0* (*GSTT1* null genotype) in a rate of only 7% (Dimov et al., 2008; Vlaykova et al., 2009). However, when the control group was extended the frequency of *GSTT1* null genotype turned out to be 20% (Figure 1B) (Emin et al., 2009) which is comparable to other Caucasian populations (Bolt &Thier, 2006; Katoh et al., 2008).

Polymorphic variants have been described also in the second theta-class GST gene, *GSTT2*. Coggan et al. reported a pseudogene (*GSTT2P*), which rises from G to T transition at nt 841 (841G>T) in intron 2 of *GSTT2* and C to T transition at nt 3255 (3255C>T) in exon 5 of *GSTT2P* changing 196Arg to a stop codon. In addition a G to A transition at nt 2732 (2732G>A) in exon 4 of *GSTT2* was defined that results in substitution of 139Met to 139Ile (Met^{139}Ile) (Coggan et al., 1998). However, there is still no clear evidence that the latter SNP may have influence on the enzyme function. In the meantime, the defined promoter polymorphisms in *GSTT2* (-537G>A, -277T>C, -158G>A, and -129T>C) were shown to affect the gene expression (Guy et al., 2004; Jang et al., 2007).

3.4 *GSTA* class

Although, variety of polymorphisms of alpha-class GST genes has been defined, their functional activity has not yet been comprehensively investigated. Nevertheless, it is already proven that the SNPs in the promoter (5′-regulatory) region of *GSTA1* (-567, -69, and -52) and specifically the substitution at -69C>T (determining a variant *GSTA1*B* allele), result in enhanced promoter activity and increased expression (Coles et al., 2001; Sweeney et al., 2002; McIlwain et al., 2006). However, for 10 SNPs in the coding regions (exons) of *GSTA1* and *GSTA2* was shown to have no significant functional effects (Tetlow et al., 2001). In a later study, the new Pro^{110}Ser polymorphism in *GSTA2* was found to affect the catalysis with several substrates, as the Ser containing isoform has significantly diminished enzyme activity (Tetlow &Board, 2004). Similar decrease in the glutathione-conjugating activity was also shown for the Leu containing isoform of Ile^{71}Leu (I^{71}L) polymorphism of *GSTA3* (Tetlow et al., 2004).

3.5 *GSTO* class

The omega-class GSTs are coded by 2 genes (*GSTO1* and *GSTO2*) both composed of six exons and spread by 7.5 kb on chromosome 10q25.1 (Whitbread et al., 2003; Whitbread et al., 2005). A total of 26 putative variants have been identified in the coding region of *GSTO1* in different databases. Among them only 10 have been confirmed candidates and only one GSTO1*A140D (A^{140}D, Ala^{140}Asp, 419C>T) has been found in the ethnic group studies (Whitbread et al., 2003). In addition a 3-bp deletion polymorphism (AGg from the final GAG codone [155E, 155Glu]) has been identified in the boundary of *GSTO1* exon4 and intron 4.

This deletion has the potential to alter the existing splice site, may reform a new splice donor site and causes the deletion of 155Glu (*GSTO1*E155del*) resulting in a loss of heat stability and increased enzyme activity toward 2-hydroxyethyl disulphide (HEDS) and CDNB (Whitbread et al., 2003). Only one variant in GSTO2 has been confirmed and identified in the population studies: this variation results from an A>G transition at nt 424 (424A>G) and causes a substitution of 142Asn to 142Asp (Asn^{142}Asp, N^{142}D) (Whitbread et al., 2003).

3.6 *GSTZ* class

A number of genetic polymorphisms in the gene encoding glutathione S-transferase-zeta (*GSTZ1*) have been defined: G-1002A, Glu^{32}Lys, Gly^{42}Arg, Thr^{82}Met. The latter three SNPs are functional and determine four *GSTZ1* alleles referred to as *GSTZ1*A* (32Lys, 42Arg, 82Thr), *GSTZ1*B* (32Lys, 42Gly, 82Thr), *GSTZ1*C* (32Glu, 42Gly, 82Thr), and *GSTZ1*D* (32Glu, 42Gly, 82Met) (Blackburn et al., 2001). The *B, C* and *D* alleles have been associated with a lower activity to dichloroacetic acid compared to *GSTZ1A* (Blackburn et al., 2001), but non of these SNPs affect significantly the risk of bladder cancer in Spain (Cantor et al., 2010) and breast cancer in Germany (Andonova et al., 2009).

4. Role of GSTs polymorphisms as risk factors for development, progression and therapeutic response of CRC

4.1 *GSTP1*

Epidemiological studies of *GSTP1* (*GSTP1 Ile^{105}Val*) and colorectal cancer risk have suggested a deleterious effect of the low activity genotypes, but findings have been inconsistent (Harries et al., 1997; Welfare et al., 1999; Kiyohara, 2000; Ates et al., 2005; Gao et al., 2009; Economopoulos &Sergentanis, 2010).

The results of our case-control study (Vlaykova et al., 2007) based on 80 patients with primary sporadic CRC and 98 unaffected control individuals showed that the genotype distribution is consistent with those published for other Caucasian type control cohorts. We also found a statistically significant prevalence of heterozygous *GSTP1* genotype by itself (*105Ile/Val* – co-dominant model) and the prevalence of variant allele-containing *GSTP1* genotypes (*105Ile/Val* or *105Val/Val* – dominant model) in control group compared to the CRC cases. This suggests a protective effect of the variant 105*Val* allele lowering the risk for developing of CRC. Based on our observations and on the experimental evidence reported by other research groups for greater activity of the enzyme encoded by the valiant *105Val* allele toward polycyclic aromatic hydrocarbon (PAH) diol epoxides (Sundberg et al., 1998; Coles et al., 2000; Bostrom et al., 2002), we suggest that the heterozygous *GSTP1* genotype may determine a better protection toward GST-pi-metabolized chemical toxins and reactive oxygen species (Vlaykova et al., 2007). This genotype may provide enzyme with an adequate detoxification of some and relatively weak activation of other carcinogens, depending on their characteristics.

Two recent large meta-analyses summarized the results focused on the role of *GSTP1 Ile^{105}Val* from 16 published case-control studies involving a total of 4386 colorectal cancer patients and 7127 controls (Gao et al., 2009) and 19 studies with altogether 5421 cases and 7671 controls (Economopoulos &Sergentanis, 2010) .The results of the meta-analysis

performed by Gao et al. (Gao et al., 2009) sowed no strong evidence that the *105Val* allele conferred increased susceptibility to colorectal cancer compared to *105Ile* allele either in the whole pooled case-controls groups or in the stratified one: by race - Caucasian and Asian descent; by the type of controls - in healthy and hospital controls. They also did not find evidence for an association with colorectal cancer in dominant (OR= 1.02, 95% CI:0.94, 1.10) and co-dominant (OR= 0.88 , 95% CI: 0.77, 1.01) models for the effect of Val. Only a slight, but significant, protective effect of Val allele was observed in the recessive model 0.86 (95% CI: 0.76–0.98). The final conclusion of this large meta-analysis was that *GSTP1 Ile^{105}Val* polymorphism is unlikely to increase considerably the risk of sporadic colorectal cancer (Gao et al., 2009).

Similar are the results and final conclusion of the recent meta-analysis performed by Economopoulos et al. (Economopoulos &Sergentanis, 2010): there were no significant effects of *105Val* allele on the risk of colorectal cancer either in dominant model (OR=1.025, 95% CI: 0.922–1.138), co-dominant model (OR=1.050, 95% CI: 0.945–1.166), or in the recessive model (OR=0.936, 95% CI: 0.823–1.065). Hence, the conclusions confirmed that the *GSTP1 Ile^{105}Val* status did not seem to confer additional risk for colorectal cancer (Economopoulos &Sergentanis, 2010).

4.2 *GSTM1* and *GSTT1*

Because GST-mu and GST-theta are important in the detoxification of carcinogens implicated in colorectal cancer, the absence of these enzymes is assumed to increase the risk of this common malignancy. In this regard a number of epidemiological studies have investigated the association of *GSTM1* and *GSTT1* genetic polymorphisms with colorectal cancer risk, however the results from these studies have also been with quite controversial conclusions (Cotton et al., 2000; Economopoulos &Sergentanis, 2010; Gao et al., 2010). The preliminary results from our study including very limited number of patients and controls (45 and 42), showed 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) (Vlaykova et al., 2009). 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 21.533-fold (95% CI, 3.56-128.71) increased risk associated with the combined null genotypes. The colorectal cancer was diagnosed earlier in patients with *GSTM1* null genotype and those patients had tumors in more advanced stage (III or IV) (p=0.033) and were with more aggressive phenotype, such as presence of lymph vessel invasion (p=0.042) than the patients with non-null genotype.

A slight difference was obtained when the control group was extended to 81 persons (Figure 1A, 1B and 1C): the null *GSTT1* and *GSTM1* genotypes turned out only to tend to associate with an increased risk of colorectal cancer (OR=1.797, 95% CI 0.86-3.72, p=0.116 for *GSTM1*, and OR=1.777, 95% CI 0.78-4.05, p=0.175 for *GSTT1*), however the carriers of *GSTM1* and *GSTT1* double null genotype had significantly higher risk of development of the disease (OR=3.697, 95% CI 1.21-11.28, p=0.021) (Figure 1C). As a conclusion, we suggested that the inherited 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 (Vlaykova et al., 2009).

Our findings are analogous to the one of meta-analyses performed on a large number of published case-control studies. The results of these meta-analyses support the suggestion that *GSTM1* and *GSTT1* null polymorphisms are associated with increased risk of CRC, especially in the Caucasian population (Economopoulos &Sergentanis, 2010; Gao et al., 2010). Economopoulos et al. have summarized the results from 44 studies for *GSTM1* and 34 for *GSTT1* null polymorphisms and concluded that *GSTM1* null genotype carriers exhibited increased colorectal cancer risk in Caucasian population (OR=1.15, 95% CI: 1.06-1.25), but not in Chinese subjects (OR=1.03, 95% CI: 0.90-1.16). They reported similar results for *GSTT1* null polymorphism: OR=1.31, 95% CI:1.12-1.54 for Caucasian population and OR=1.07, 95% CI:0.79-1.45 for Chinese subjects (Economopoulos &Sergentanis, 2010). Gao at al., carried out a meta-analysis of *GSTM1* genotype data from 36 studied including 9149 patients with CRC and 13 916 control individuals (Gao et al., 2010). The results indicated that *GSTM1* null genotype was associated with CRC (OR=1.13, 95% CI: 1.03–1.23) in the pooled cases and controls from a number of different ethnics groups. However, the significance of this association remained for Caucasians, but not for Asians (Gao et al., 2010).

4.3 *GSTA1*, *GSTM3*, *GSTO2*

According to our knowledge there are only a limited number of studies aiming to evaluate the possible role of polymorphisms in the genes encoding other GST isoforms as predisposing factors for colorectal cancer. The polymorphisms in *GSTA1* have been explored in colorectal cancer only by four research teams (Sweeney et al., 2002; van der Logt et al., 2004; Martinez et al., 2006; Kury et al., 2008) . The Sweeney at al. have found that the *GSTA1*B/*B* (promoter polymorphisms) genotype is associated with an increased risk of colorectal cancer, particularly among consumers of well-done meat and have suggested that *GSTA1* genotype, in addition to the CYP2A6 phenotype should be evaluated as markers for susceptibility to dietary carcinogens (Sweeney et al., 2002). However, other studies did not find any associations between the *GSTA1* polymorphisms and the risk of CRC (van der Logt et al., 2004; Martinez et al., 2006; Kury et al., 2008).

Kury et al., and Martinez at al, have also attempted to elucidate the influence of *GSTM3* genetic variants on colorectal cancer risk, however no correlation between these polymorphisms and CRC susceptibility was found (Martinez et al., 2006; Kury et al., 2008). Similarly, no effect of *GSTM3* polymorphism was found in a large study investigating the role of single SNPs within 11 genes of phase I and 15 genes of phase II of xenobiotic metabolism (Landi et al., 2005). Opposite results have been reported for GSTM3*A/GSTM3*B alleles (the latter arising from a 3 bp deletion in intron 6): patients who were carriers of genotypes with at least one GSTM3*B allele (GSTM3 AB and GSTM3 BB combined) had advanced tumour T-stage, increasing Dukes' stage, higher frequency of distant metastases and shorter survival (Holley et al., 2006) Thus, the GSTM3 AA genotype was suggested to be associated with improved prognosis of CRC especially in patients with GSTM1 null genotype (Holley et al., 2006). Analogous results have been reported by Loktionov et al. who found associations between GSTM3*B frequency in patients with distal colorectal cancers particularly when combined with the GSTM1 null genotype (Loktionov et al., 2001).

A very recent study investigated the association between *GSTO2 N142D* (*Asn142Asp*) genetic polymorphism and susceptibility to colorectal cancer and reported that ND and DD genotypes were not associated with CRC risk, in comparison with the NN genotype. However subjects with NN genotype and positive family history were at high risk to develop colorectal cancer in comparison with subjects with DD or ND genotypes and negative family history. Thus, GSTO2 NN genotype was suggested to increase the risk of colorectal cancer in persons with positive family history for cancer in the first degree relatives (Masoudi et al., 2010).

The common characteristic of the theta-class GSTs is their high affinity for the organic hydroperoxide species and particularly toward cumene hydroperoxide (GSTT2), underling the importance of GSTT2 activity in protection of cells against toxic ROS and lipid peroxidation products (Tan &Board, 1996), which are a major source of endogenous DNA damage and thus contribute significantly to cancer genesis and progression. In this respect efforts have been done to determine whether *GSTT2* promoter SNPs (-537G>A, -277T>C and -158G>A) are associated with colorectal cancer risk (Jang et al., 2007). Jang at al., reported that -537A allele was associated with colorectal cancer risk, while the -158A allele was protective against colorectal cancer, finally suggesting that SNPs and haplotypes of the *GSTT2* promoter region are associated with colorectal cancer risk in the Korean population (Jang et al., 2007). However, in a Caucasian population there was no such association of GSTT2 polymorphisms with the risk of CRC (Landi et al., 2005)

5. Role of GST-pi in cancer progression

The isoenzyme of class pi, GST-pi, acidic cytosolic protein, possesses unique enzymatic properties: broad substrate specificity (e.g. alkylating antitumor agents such as cisplatin derivatives), glutathione peroxidase activity towards lipid hydroperoxides, and high sensitivity to reactive oxygen species (ROS) (Tsuchida &Sato, 1992; de Bruin et al., 2000; Hoensch et al., 2002). As it was discussed above, GST-pi acts also non-catalytically as intracellular binding protein for a large number of non-substrate molecules of either endogeneous or exogeneous origin, thus contributing to their intracellular transport, sequestration and disposition (Laisney et al., 1984; de Bruin et al., 2000; Hayes et al., 2005). Besides that, GST-pi plays a regulatory role in the MAP kinase pathway that participates in cellular survival and death signals via direct protein:protein interaction with c-Jun-N-terminal Kinase 1 (JNK1) and Apoptosis Signal-regulating Kinase (Ask1) (Adler et al., 1999; Tew &Ronai, 1999; Townsend &Tew, 2003; Hayes et al., 2005; Michael &Doherty, 2005).

Therefore, the increased protein levels and activity of GST-pi found in a variety of neoplastic cancers with different histological origins, including colorectal carcinoma (Moorghen et al., 1991; Ranganathan &Tew, 1991; de Bruin et al., 2000; Dogru-Abbasoglu et al., 2002; Murtagh et al., 2005), are debated as factors responsible, at least partly, for the progression and chemotherapy resistance, observed in many cancers (O'Brien &Tew, 1996; Tew &Ronai, 1999; Townsend &Tew, 2003; Michael &Doherty, 2005).

Earlier we reported our preliminary results concerning the survival of 76 patients with primary CRC according to the level of expression of GST-pi determined by immunohistochemistry (Vlaykova et al., 2005). Further we extended the patient population

to 132 and found that the tumors varied according to their GST-pi immune staining: there were tumors negative for GST-pi, others had weak staining and finally tumors exhibiting strong and very strong immune reaction for GST-pi (Figure 2).

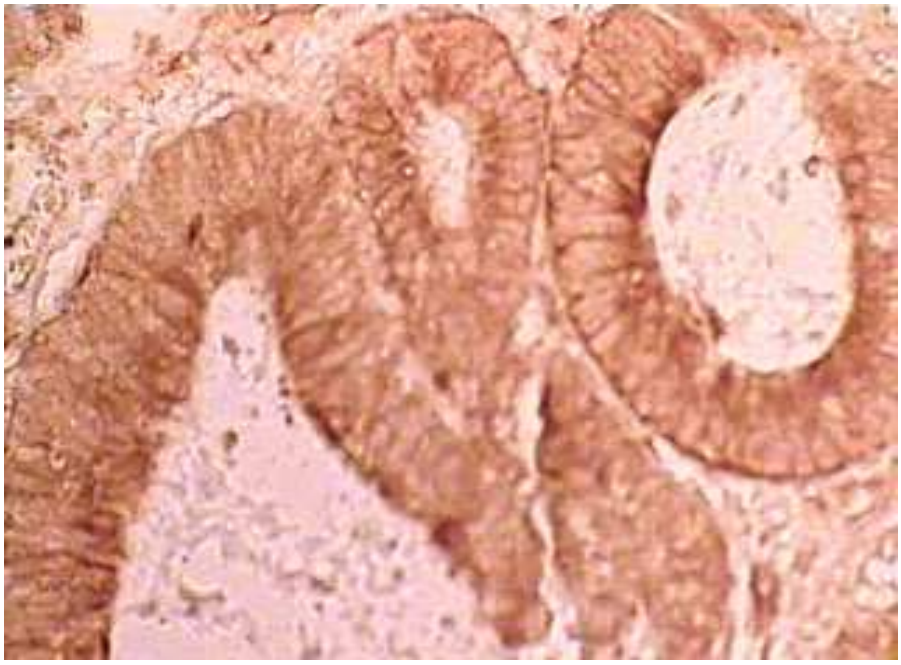
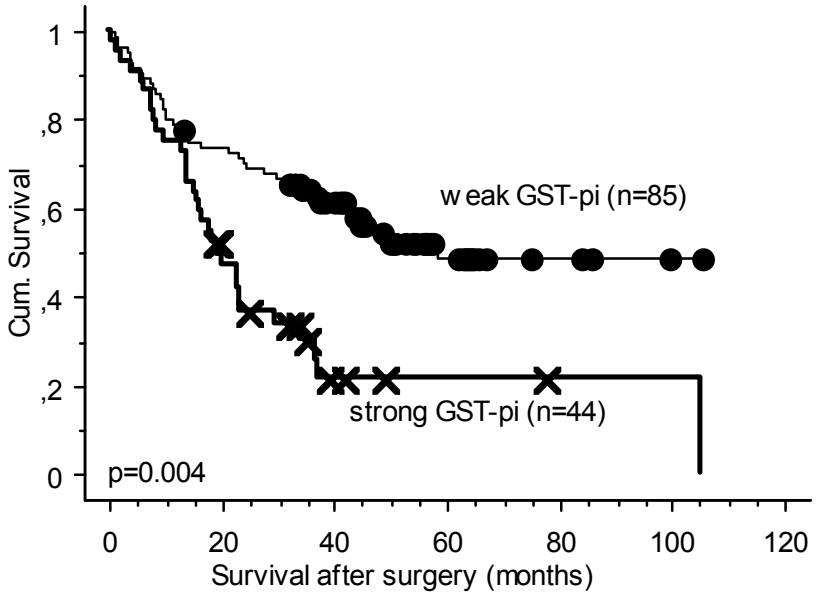
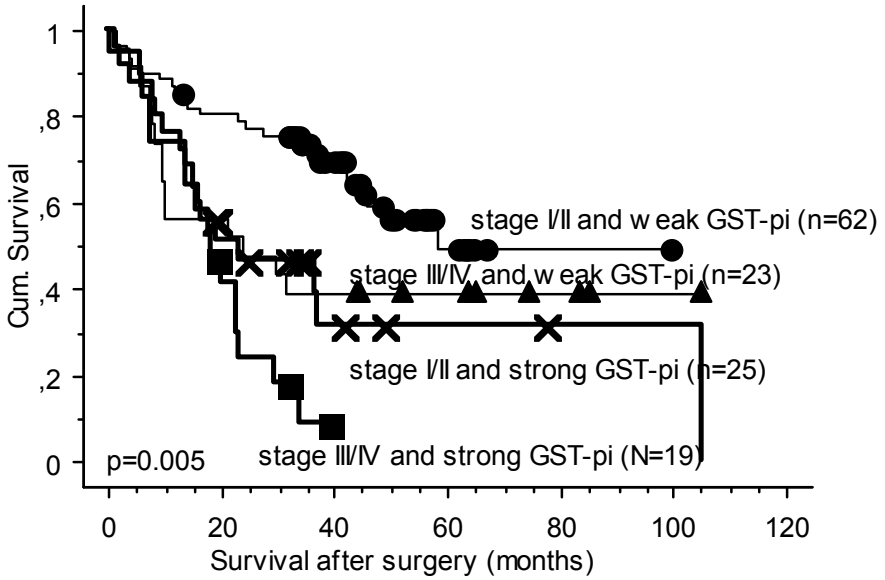


Fig. 2. Intensive cytoplasmic immune reaction for GST-pi in the cells of the tumor glands of a well-differentiated primary colorectal cancer (x 400).

The results concerning survival of the patients with CRC with different level of expression of GST-pi, showed that the higher expression of GST-pi was significantly associated with shorter survival period after surgical therapy (median of 19 months) compared to those negative or with weak GST-pi staining (median of 58 months, p=0.004, Log-rang test) (Figure 3A). This statistically significant association persisted also after stratification for pTNM staging (stage I/II vs. Stage III/IV, p=0.005, Log-rank test) (Figure 3B).
Interestingly, the strong expression of GST-pi retained its impact as unfavorable prognostic factor both for the patients who received an adjuvant chemotherapy (n=63, p=0.008, Log-rank test) (Figure 4A) and for the once without such treatment (n=66, p=0.019, Log-rank test) (Figure 4B). Hence, we suggested that the strong expression of GST-pi may lead to lower effectiveness of the administered anticancer drugs or to inhibiting the apoptosis, thus influencing the survival of the patients.

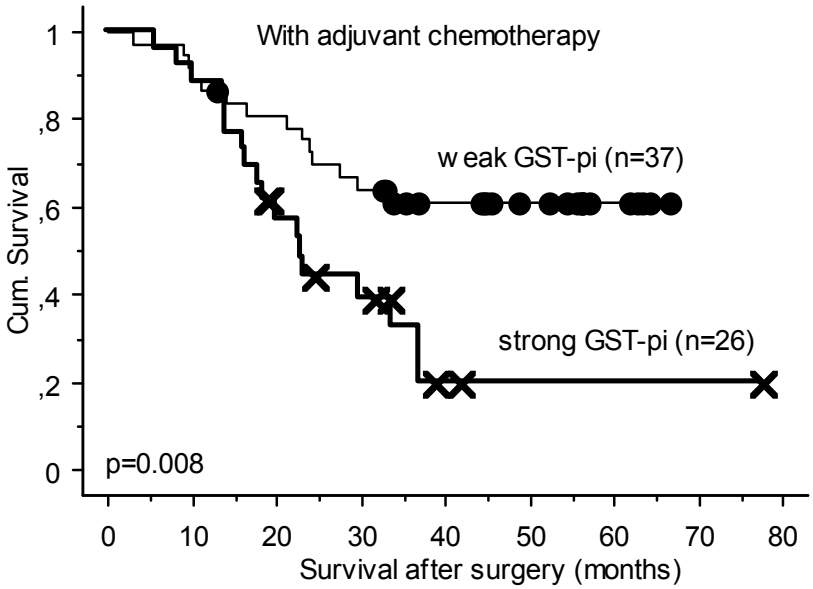


A

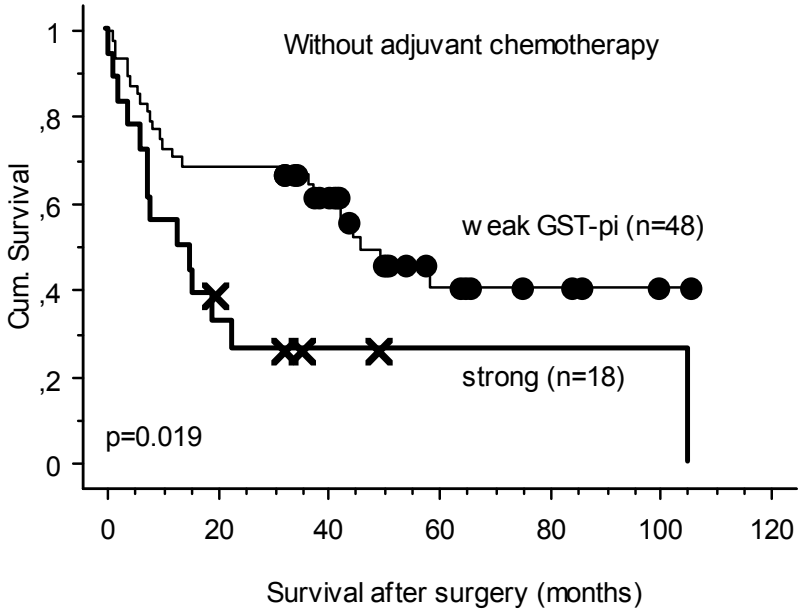


B

Fig. 3. Survival of the whole studied patient population with colorectal carcinoma after surgical treatment according to the level of expression of GST-pi in tumor cells (**A**) and after stratification to pTNM staging (**B**).



A



B

Fig. 4. Survival according to the GST-pi expression of patients with CRC subjected to adjuvant chemotherapy **(A)**, Association between the level of expression of GST-pi and survival of patients, who did not receive adjuvant chemotherapy (**B**).

Previously, we also described expression of GST-pi in chromogranin A-positive endocrine cells in colorectal cancers, which also expressed some other antioxidant enzymes, such as SOD1 and SOD2 (Gulubova &Vlaykova, 2010). Moreover, we found that patients having tumors with GST-pi-positive endocrine cells have an unfavorable prognosis. We suggest that not the neuroendocrine differentiation in general, but the presence of endocrine cells with activated antioxidant defense and probably higher metabolic activity might determine a more aggressive type of cancer leading to worse prognosis for patients (Gulubova &Vlaykova, 2010).

The observed heterogeneous expression of GST-pi in tumor glands could be due to different genetic or epigenetic factors. We suppose that the reactive oxygen species, which are generated in high amount during the metabolism of tumor cells could be such factors resulting in overproduction of GST-pi . These ROS are found to induce the expression of the genes of GST-pi and other phase II xenobiotic-biotransformating enzymes (O'Brien &Tew, 1996; Tew &Ronai, 1999; Hoensch et al., 2002). There is a growing evidence that these genes have regulatory sequences recognized by Nrf2 transcription factor, which in turn is regulated by the antioxidant response element (ARE) (O'Brien &Tew, 1996; Tew &Ronai, 1999; Hoensch et al., 2002). Another Zn-dependent mechanism for ROS-induced expression of genes coding GST-pi and other antioxidant enzymes has been proposed (Chung et al., 2005).

Another factor, resulting in overproduction of GST-pi, could be its gene amplification. Such genetic change has been proven for squamous cell carcinoma of head and neck. *GSTP1* amplification has been shown to be a common event and proposed to be associated with cisplatin resistance and poor clinical outcome in head and neck cancer patients treated with cisplatin-based therapy (Wang et al., 1997; Cullen et al., 2003).

On the other hand, the lack of or the low expression of GST-pi could be due to the somatic inactivation by hypermethylation of promoter sequences of GST-pi gene (Yang et al., 2003; Lasabova et al., 2010). Such hypermethylation is the most common event (about 90%) described in prostate adenocarcinoma (Jeronimo et al., 2002).

The results of our studies demonstrated the association between high expression level of GST-pi and unfavorable prognosis for the patients with colorectal carcinoma. This association was valid both for patients who had received adjuvant chemotherapy and for those without such treatment. We suppose that the shorter survival of patients with higher GST-pi could be due to lowering of the effectiveness of administered antineoplastic agents. The high protein level of GST-pi could contribute to this process either via its direct detoxifying effect towards some of the drugs (oxaliplatin) (O'Brien &Tew, 1996; Michael &Doherty, 2005), or via the inhibitory effect of GST-pi on MAP kinase signal pathways of apoptosis, triggered by 5-FU, mitomicin C, camtothecin or other antitumor drugs included in mono- or polychemotherapeutic regiments (Adler et al., 1999; Townsend &Tew, 2003; Hayes et al., 2005; Michael &Doherty, 2005).

The observed association of high GST-pi level with worse prognosis of the patients, who did not received chemotherapy, could also be explained with the ability of this enzyme protein directly to interact with and inhibit proteins involved in regulation of apoptosis (JNK1 and Ask1) (Adler et al., 1999; Townsend &Tew, 2003; Hayes et al., 2005; Michael &Doherty, 2005). In tumors, the high levels of free radicals, which in general are triggering factors and mediators of apoptosis, probably stimulate the expression of GST-pi that can lead to suppression of apoptosis. As a result, the decreased apoptosis can lead to increased tumor burden, which negatively affects patients survival.

6. Conclusions

Colorectal cancer (CRC) is a neoplasm that occurs at high frequency worldwide, including Bulgaria. CRC is a complex and multifactorial disease, since several environmental and endogenous factors, including personal genetic characteristics, are implicated in its etiology, pathogenesis, progression and outcome. The members of the glutathione-S-tranferase (GST) family are important candidates for involvement in susceptibility to carcinogen-associated CRC and for developing of tumor chemotherapy resistance. In this work we presented a short overview of the main cellular functions of some of the GST isoenzymes, their polymorphic nature, and their role as risk factors for development of CRC and of resistance to chemotherapy. We also presented the results of our studies focused on the role of the null *GSTM1* and *GSTT1* polymorphisms, the *Ile*^{105}*Val* SNP in *GSTP1* and GST-pi expression as risk and prognostic factors in primary CRC. In conclusion, we suggest that the expression level of GST-pi in primary tumors could be a valuable prognostic factor for patients with colorectal carcinoma both treated with adjuvant chemotherapy and those not subjected to such therapy.

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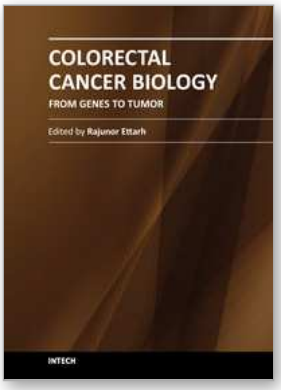

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Colorectal cancer is a common disease, affecting millions worldwide and represents a global health problem. Effective therapeutic solutions and control measures for the disease will come from the collective research efforts of clinicians and scientists worldwide. This book presents the current status of the strides being made to understand the fundamental scientific basis of colorectal cancer. It provides contributions from scientists, clinicians and investigators from 20 different countries. The four sections of this volume examine the evidence and data in relation to genes and various polymorphisms, tumor microenvironment and infections associated with colorectal cancer. An increasingly better appreciation of the complex inter-connected basic biology of colorectal cancer will translate into effective measures for management and treatment of the disease. Research scientists and investigators as well as clinicians searching for a good understanding of the disease will find this book useful.

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