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Polymorphisms of Glutathione-S-Transferase and Arylamine N-Acetyltransferase Enzymes and Susceptibility to Colorectal Cancer

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Abstract. *Background: Glutathione-S-transferases (GSTs) and N-acetyltransferases (NATs) are involved in the metabolism of a wide range of carcinogenic chemicals. Allelic polymorphism of these enzymes is associated with variations in enzyme activity, hence it may affect the concentration of activated carcinogenic chemicals in the body. Previous studies suggest a possible cancer risk-modifying effect of these allelic polymorphisms, but the results are still controversial. We evaluated the effect of GSTM1, GSTT1, GSTP1, NAT1 and NAT2 enzymes on individual susceptibility to colorectal cancer, with particular attention to possible interactions between the studied genotypes. Materials and Methods: Five hundred colorectal cancer patients and 500 matched cancer-free controls were included in the study. The allelic polymorphisms of GSTM1, GSTT1 and GSTP1, NAT1 and NAT2 enzymes were determined by PCR-based methods, from peripheral blood leukocytes, and allelic distributions were compared between colorectal cancer patients and controls. Results: The GSTM1 0 allele (OR: 1.48, 95% CI: 1.15-1.92) and rapid acetylator genotypes of NAT2 (OR: 1.52, 95% CI: 1.17-1.98) were associated with an elevated risk. No statistically significant correlation between NAT1, GSTT1, GSTP1 genotypes and colorectal cancer was found. Remarkably increased risk was associated with the GSTM1 0 allele – NAT2 rapid acetylator genotype combination (OR: 2.39, 95% CI: 1.75-3.26) and with the GSTM1 0 allele – NAT2 and NAT1 rapid acetylator triple combination (OR: 3.28, 95% CI: 2.06-5.23). Carrying 4 or 5 putative "high-risk" alleles substantially increased the risk of colorectal cancer (OR: 3.69, 95% CI: 2.33-5.86). Conclusion: The genotype of certain metabolizing enzymes affects the risk for colorectal cancer. This effect is particularly important when certain allelic combinations are studied. In the near future, individual level risk assessment may be reached by further increasing the number of studied polymorphisms, combining them with traditional epidemiological risk factors.*

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It is generally accepted that cancer risk is determined by the interaction of environmental and genetic factors. Except for hereditary tumors, external carcinogenic exposure is involved in human tumorigenesis. Carcinogenic chemicals, however, undergo a complicated process of metabolism in the human body. Typically, these chemicals are activated by the so-called phase I metabolizing enzymes, which results in the formation of electrophilic, reactive compounds (1). The amount of active carcinogens is in good correlation with the risk of DNA damage and cancer formation. Detoxifying enzymes – phase II enzymes – help in the removal of carcinogens from the body (2). Most of these enzymes conjugate the carcinogenic chemical with a small molecule, making it less toxic and more water soluble. Therefore, it seems to be a logical assumption that the detoxifying capacity to a certain extent determines the individual susceptibility to cancer.

The activity of detoxifying enzymes in humans is basically determined by the genotype of the enzyme (2). Most of our metabolizing enzymes are genetically polymorphic, encoding proteins with different activities (2). Among the phase II enzymes, the glutathione-S-transferase (GST) superfamily and the N-acetyltransferases (NATs) have long been suspected to have an influence on cancer susceptibility (3-9).

Table I. *Allelic distributions of the studied GST and NAT enzymes in the control group.*

	+	0	Ile/Ile	Heterozygous	Val/Val	Slow	Rapid
GSTM1	258	242	-	-	-	-	-
GSTT1	392	108	-	-	-	-	
GSTP1	-	-	214	212	74	-	-
NAT1	-	-	-	-	-	305	195
NAT2	-	-	-	-	-	318	182

Table II. *Allelic distributions of the studied GST and NAT enzymes among colorectal cancer patients.*

	+	0	Ile/Ile	Heterozygous	Val/Val	Slow	Rapid
GSTM1	209	291	-	-	-	-	-
GSTT1	369	131	-	-	-	-	
GSTP1	-	-	200	212	88	-	-
NAT1	-	-	-	-	-	289	211
NAT2	-	-	-	-	-	267	233

The GST enzymes have a relatively wide range of substrates, *e.g.* polycyclic aromatic hydrocarbons, monohalomethanes, ethylene oxide, different solvents, pesticides (10). The superfamily consists of 6 families: α, μ, π, σ, θ and ξ. Probably, from a carcinogenetic point of view, GSTM1, GSTT1 and GSTP1 are the most important enzymes, from the μ, θ and π families, respectively. In Caucasian populations, almost half of the individuals have no functional GSTM1 enzyme, due to a homozygous deletion in the gene (0 genotype) (11, 12). The situation is similar in the case of the T1 enzyme, but the ratio of persons with 0 genotype is lower (13). The GSTP1 enzyme possesses two single base polymorphisms, both resulting in an amino acid change in the protein (*Ile105Val*, *Ala114Val*) (14). In the case of the more frequently studied *Ile105Val* polymorphism, the Val allele encoded enzyme exhibits lower activity and, in accordance with this finding, certain tumors (*e.g.* lung, bladder) appeared to occur at higher rates among the carriers of the *Val* allele than among persons with the *Ile* genotype (15, 16).

The N-acetyltransferases are able to catalyze N- and O-acetylation, the former considered to be a detoxifying and the latter an activating reaction (17). Among their substrates, known carcinogenic compounds – like aromatic and heterocyclic amines – can be found (17). In the NAT family, polymorphisms of NAT2 are well characterized, but the NAT1 enzyme has only been recently studied from this point of view. Both NATs have several alleles; in the case of NAT2, the association of genotypes with enzyme activity is also well-established (usually people are categorized as rapid or slow acetylators) (18). The relationship between NAT1 alleles and acetylation speed is not so clear, but certain alleles also seem to be associated with the phenotype (19, 20).

Previous studies tried to find an association between the risk of different cancer types and the allelic polymorphism of GST and NAT enzymes. Regarding colorectal tumors, most of the studies suggested an elevated risk for individuals with the GSTM1 0 genotype (21-24). Based on theoretical considerations (because of O-acetylation of heterocyclic amines present in the GI system), rapid acetylators should also be at higher risk, but the results are controversial (25-28).

In the present case-control study, we tried to characterize the role of GSTM1, GSTT1, GSTP1, NAT1 and NAT2 polymorphisms in determining susceptibility to colorectal cancer. Since carriers of 0 alleles for the GST enzymes have a decreased detoxifying capacity, if this is combined with the rapid formation of metabolites of heterocyclic amines ensured by being a rapid acetylator, individuals with certain allelic combinations might be at a particularly high risk. Earlier, we demonstrated similar interactions between cytochrome P450 1A1 (CYP 1A1), cytochrome P450 2E1 (CYP 2E1) and GSTM1 alleles (29). The most important goal of the present study was to find such allelic combinations, and quantitatively assess their effect on colorectal cancer risk.

Materials and Methods

Five hundred colorectal cancer patients from the Central Hospital of the Ministry of Internal Affairs and from the area of Baranya and Vas County, Hungary, were included in the study. The diagnosis of tumors was always confirmed histologically. Patients with conditions affecting colorectal cancer risk (familial adenomatous polyposis, hereditary non-polyposis colorectal cancer, ulcerative colitis, *etc.*) were excluded from the study. Five hundred cancer-free controls from the same regions (non cancer patients from in- or outpatient wards and volunteers for health status examination) were matched to the cases according to age, sex, smoking habits, and red meat consumption. Ten ml peripheral blood was drawn from the participants, white blood cells were isolated by repeated centrifugation with 0.84% ammonium chloride and DNA was isolated (30).

GSTM1 and GSTT1 genotyping (31) was performed by a simultaneous amplification in the presence of an internal control (a 268 base length fragment of β-globin gene), with the following primers: GSTM1-F: GAACTCCCTGAAAAGCTAAAGC, GSTM1-R: GTTGGGCTCAAATATACGGTGG, GSTT1-F: TTCCTTACTGGTCCTCACATCTC, GSTT1-R: TCACCGGATC ATGGCCAGCA, β-globin-F: CAACTTCATCCACGTTCACC, β-globin-R: GAAGAGCCAAGGACAGGTAC. The reaction was performed in 20 μl volume: 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH=8.3), 2 mg/ml bovine serum albumin, 4 x 0.25 mM dNTP, 2 U Taq DNS-polymerase, 30-30 pmol GSTT1-F and GSTT1-R primers, 50-50 pmol GSTM1-F and GSTM1-R primers, 20-20 pmol β-globin-F and β-globin-R primers, 13 μl DNS-template. After a 7-min denaturation at 94˚C, 35 PCR cycles were performed: 60 sec 94˚C, 60 sec 60˚C, 60 sec 72˚C, followed by 5 min at 72˚C.

For GSTP1 the *Ile105Val* polymorphism was determined by a PCR-RFLP (32). A 176-bp fragment was amplified, with the

Table III. *Risk of colorectal cancer by genotypes of GST and NAT enzymes.*

	Odds ratio	95% confidence interval
GSTM1	1.48	1.15-1.92
GSTT1	1.29	0.95-1.74
GSTP1	1.11	0.85-1.43
NAT1	1.14	0.88-1.48
NAT2	1.52	1.17-1.98

Table IV. *Putative "high-risk" alleles per person in the control and case groups.*

	Controls	Cases
0 "high-risk" allele per person	31	24
1 "high-risk" allele per person	120	119
2 "high-risk" alleles per person	185	131
3 "high-risk" alleles per person	134	135
4 "high-risk" alleles per person	29	75
5 "high-risk" alleles per person	1	16

following primers: 5'-ACCCCAGGGCTCTATGGGAA-3' and 5'-TGAGGGCACAAGAAGCCCCT-3'. The reaction was carried out in 30 μl total volume, containing 50 ng DNA template, 4x200 μM dNTP, 200 ng each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 1 U Taq DNA polymerase. Parameters of the PCR reactions were as follows: 10 min at 95˚C, then 30 cycles of 30 sec at 94˚C, 30 sec at 55˚C, and 30 sec at 72˚C, followed by a final extension step at 72˚C for 10 min.

The NAT2 allelic polymorphism was studied by restriction fragment length polymorphism (33). First, a nested PCR was used to amplify a 547 bp fragment of the gene (outer primer set: 5'-AATTAGTCACACGAGGA-3' and 5'-GCAGAGTGATTCAT GCTAGA-3', inner set: 5'-GCTGGGTCTGGAAGCTCCTC-3', 5'-TTGGGTGATACATACACAAGGG-3', 25 cycles of 30 sec 94˚C, 30 sec 59˚C, 45 sec 72˚C with the outer set was followed by 35 cycles with the inner set with the same parameters). NAT 2*4 (wild-type), NAT2*5, NAT2*6 and NAT2*7 alleles were identified by restriction endonuclease digestion with *Kpn*I, *Taq*I, *Dde*I and *Bam*HI enzymes. Homozygous or any heterozygous carriers of the wild-type allele were characterized as slow acetylators.

NAT1 genotyping was also undertaken using a nested PCR-based RFLP (33), similarly to the NAT2 genotyping, with the following primers: outer: 5'-GATCAAGTTGTGAGAAG AAATCGG-3', 5'-CTAGCATAAATCACCAATTTCCAAG-3', inner: 5'-GACTCTGAGTGAGGTAGAAAT-3', 5'-CCACAGG CCATCTTTAGAA, at the underlined base constructing an additional *Mbo*II restriction site at the amplification of NAT1*4 allele. NAT1*4, NAT1*10 and NAT1*11 alleles were identified by this method, without studying certain rare alleles like NAT1*3 or NAT1*14. The presence of NAT*10 or NAT*11 alleles indicated the rapid acetylators.

Statistical calculations were made by Epi Info 6 (CDC, Atlanta, USA) and SPSS PC+ software. Odds ratios and 95% confidence intervals were used to compare the occurrence of genotypes in the case and control groups. In the case of the GSTM1 and GSTT1 + genotype, at GSTP1 homozygous *Ile* genotype and, in the case of NAT enzymes, slow acetylator genotypes were considered as baseline risk category.

Results

The allelic distributions in the control and case groups are shown in Tables I and II, respectively. The found allelic frequencies in the control group were similar to those of other studies in Caucasian populations. As illustrated in Table III, GSTM1 and NAT2 allelic distributions showed statistically significant differences between cases and controls. There were no statistically significant effects of GSTT1, GSTP1 and NAT1 allelic polymorphisms on colorectal cancer risk. Composing subgroups within the NAT1 and NAT2 slow or rapid acetylators by exact genotypes did not give any further statistically significant result (data not shown).

Analyzing the joint effect of allelic combinations, GSTM1 and NAT2 alleles seemed to substantially strengthen each others effect: in the control group there were only 83 people possessing both "high-risk" alleles, while among the cases we found 161 such persons (OR: 2.39, 95% CI: 1.75-3.26). The paired analysis of GSTT1-GSTP1, GSTT1- NAT1 and GSTP1-NAT1 was also performed, but none of these combinations resulted in a statistically significant difference between cases and controls (data not shown). From triple combinations, GSTM1-NAT2-NAT1 caused the most remarkable difference, with an OR of 3.28 (95% CI: 2.06-5.23) for the simultaneous presence of the three "high-risk" alleles, suggesting a further risk-increasing effect by the third allele.

Since the analysis of allelic combinations suggested a possible interaction between the studied polymorphisms, we constructed a table based on the number of putative "high-risk" alleles per person among cases and controls (Table IV). The table clearly shows that persons with several "high-risk" alleles are relatively frequent among cases, while the control group mainly contains persons with fewer "high-risk" genotypes. When comparing the number of individuals with 4 or 5 "high-risk" alleles between cases and controls, the result is significantly different (OR: 3.69, 95% CI: 2.33-5.86). Interestingly, participants with less than 2 "high-risk" alleles were not significantly protected from developing colorectal cancer (OR: 0.93, 95% CI: 0.70-1.23).

Discussion

In our matched case control study, we found that carrying GSTM1 0 alleles or being a rapid acetylator were associated with an elevated risk of colorectal cancer in the studied Hungarian population. Unfortunately, several studies in the

field are not really comparable with each other, because some of them are not matched studies and, when matching is applied, the used variables may differ from each other. Further discrepancies may be caused by the different study populations: the allelic distributions might substantially differ from each other, not only in the studied polymorphism, but also in other genes which may also modify the risk of colorectal tumors.

The described problems can be seen when looking at the previous studies exploring the role of GSTM1 as a cancer risk modifier. The picture is confusing, since some studies suggested an association between 0 genotype and risk increase (34, 35), while others did not find any correlation (13). Our study, with relatively high case numbers, supports the hypothesis that the GSTM1 0 genotype is a risk factor of colorectal cancer susceptibility. This is in accordance with the detoxifying role of GSTM1 in the metabolism of carcinogenic substances.

The effect of GSTT1 polymorphism was not statistically significant, although it was near to that level (OR: 1.29, 95% CI: 0.95-1.74). Such results always raise the question of whether an increased sample size would result in a statistically significant association. Unfortunately, the study of low penetrance genes in human populations is fairly difficult, since existing associations might not be identified because of the presence of several confounding factors and the heterogeneity of the study population. This emphasizes the role of comparing and meta-analysis of different studies. Concerning the effect of GSTT1, it is of interest that, in spite of being near to the level of statistical significance, no effect in double or triple combinations was found, while NAT1, with a weaker effect alone, was part of a triple combination (GSTM1 0 genotype – NAT2 rapid acetylators – NAT1 rapid acetylators) which was associated with substantially elevated risk. In spite of the negative results of our study for the total sample, GSTT1 might be a risk modifier in certain subpopulations with heavy exposure to carcinogenic substances which are substrates of the GSTT1 enzyme.

GSTP1 polymorphisms have not been considered to play an important role in human colorectal carcinogenesis, however, its allelic polymorphism is associated with differences in the activity of the encoded enzymes. Here, we must not forget about the recently explored role of GSTs in cell signaling pathways, independently of their glutathione-S-transferase activity (36). GSTP1 is involved in the regulation of the MAP kinase pathway, by forming a complex with the c-jun N-terminal kinase. In the process of human carcinogenesis, GST enzymes as intracellular regulator proteins have been studied as possible factors with an influence on response to cytostatic treatment. From the cancer risk or cancer prevention point of view, this side of the GSTs has not been studied. Neither do we know whether allelic polymorphisms of GSTs affect their function as intracellular regulators. Answering these questions might give further help in the explanation of the population level effects of GST alleles as cancer risk modifiers.

While the GST enzymes are important detoxifiers of metabolites of polycyclic aromatic hydrocarbons, NATs are involved in the metabolism of aromatic and heterocyclic amines. Since these compounds are present in our diet or are formed during food preparation, and NATs are present in the colorectal mucosa, there is a possible mechanistic link to explain the role of NAT polymorphisms in human carcinogenesis.

Allelic polymorphism of the NAT2 enzyme has been known for a long time, first detected phenotypically, based on enzyme activity distribution in healthy subjects, and later these activity differences were bound to an allelic polymorphism (37). Since NAT2 activates heterocyclic amines, rapid acetylators might be at higher risk of colorectal cancer formation. In our study, NAT2 polymorphism proved to be the strongest factor to affect the colorectal cancer risk. During recent years, the role of NAT2 seemed to be clarified by the previously mentioned model, which was also supported by epidemiological and molecular epidemiological facts. Particular importance was attributed to NAT2 in individuals with high red meat and/or well-done meat consumption (38), since these heterocyclic amine-containing dietary constituents served as sources of carcinogenic exposure. Some studies, however, seem to confuse the picture; a meta-analysis of D'Errico *et al.* found the NAT2 polymorphism not to be a significant risk factor (OR: 1.03, 95%CI: 0.93-1.14) (39), while a recent study of Sachse *et al.* did not find an association between NAT2 alleles and colorectal tumorigenesis (OR: 0.82, 95% CI: 0.69-1.12) (40), though still maintaining the connection between red meat consumption and colorectal cancer.

NAT1 was originally believed to be monomorphic, because of the unimodal distribution of its activity in the studied populations. Recently, several alleles have been identified and enzyme activity variations were also demonstrated; however, the phenotypical variations (enzyme activity differences) were lower than those measured in the case of NAT2 (19-20) alleles. Some recent studies also tried to demonstrate an association between NAT1 alleles and cancer risk. The results are controversial. Some studies identified NAT1 variants as risk factors (mainly the NAT1*10 allele was studied) (41, 42), while others did not demonstrate any association at all (43, 44). Further confusion is caused by discrepancies in the genotype-phenotype relationships reported by different authors. The NAT*10 allele is generally considered to be associated with higher activity, but some results seem to contradict these findings (45). Similarly, the activity of the NAT*11 allele is questionable. These contradictory results might be caused by tissue-specific differences in the expression of NAT enzymes,

as suggested by Bruhn *et al.* (45). Since we also performed an allele-specific analysis in the case of NAT1 and NAT2, resulting in the same associations as with large categories (rapid and slow acetylators), misclassification error caused by erroneously putting a genotype into the "slow" or "rapid" acetylator groups can be ruled out in our study.

Probably the most important part of studying the effects of low penetrance genes is the analysis of possible interactions between the investigated alleles. This might bring us to individual level risk assessment by giving a more precise estimation of the risk. From a practical point of view, the question is whether we are able to find such genetic conditions (allelic combinations) which considerably increase the cancer risk of a person. In our study such conditions included a triple combination with an OR of 3.28. A simple but very effective method for risk estimation is the calculation of simultaneously carried "high-risk" alleles. This method has the advantage of taking every existing interaction into consideration, studying actions as they happen, without including further possibilities of errors by introducing complicated mathematical modeling.

Our results (Table IV) support the hypothesis that even those allelic polymorphisms which did not have a significant influence on the risk of colorectal tumors, in certain still unknown circumstances or in not yet determined interactions, also slightly contribute to the modulation of the final risk. In our study, we demonstrated a substantially elevated risk in carriers of 4 or 5 "high-risk" alleles (OR: 3.69), but this still did not reach the "level of intervention". The results, however, allow us to hope that genotyping several polymorphisms simultaneously, together with the analysis of known traditional epidemiological risk factors, will give us the oppurtunity, in the near future, to estimate the individual susceptibility to the most important cancer types, allowing application of individually-shaped preventive strategies, or working out screening programs for identification of "high-risk" individuals.

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