Association between Allelic Polymorphisms of Metabolizing Enzymes (CYP 1A1, CYP 1A2, CYP 2E1, mEH) and Occurrence of Colorectal Cancer in Hungary

ISTVÁN KISS[1], ZSUZSA ORSÓS[1], KATALIN GOMBOS[1], BARNA BOGNER[2], ANDRÁS CSEJTEI[3], ANTAL TIBOLD[1], ZSUZSA VARGA[4], EMESE PÁZSIT[5], INGRID MAGDA[1], ANNAMÁRIA ZÓLYOMI[1] and ISTVÁN EMBER[1]

[1]*Department of Preventive Medicine, Faculty of Medicine, Pécs University of Sciences, Pécs;*
[2]*Department of Pathology, Baranya County Hospital, Pécs;*
[3]*Department of Oncoradiology, Vas County "Markusovszky" Hospital, Szombathely;*
[4]*Department of Oncology, Baranya County Hospital, Pécs;*
[5]*Department of Gynecology, Diósgyor Hospital, Miskolc, Hungary*

Abstract. *Background: Genetic polymorphisms of metabolizing enzymes may affect the risk of cancer formation in humans. Since the diet can contain polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HAs), the relationship between polymorphisms of enzymes involved in PAH and HA metabolism and the occurrence of sporadic colorectal cancer was studied. Patients and Methods: Five hundred colorectal cancer patients and 500 controls were genotyped for cytochrome P450 enzymes (CYP) 1A1 Ile/Val, CYP 1A2*1F, CYP 2E1 c1/c2, microsomal epoxy hydrolase (mEH) exon 3 Tyr113His and exon 4 His139Arg polymorphisms by allele-specific polymerase chain reaction (PCR) or PCR-restriction fragmenth length polymorphism (RFLP). Results: The presence of CYP 1A1 Val, CYP 2E1 c2 and mEH exon 3 His alleles was statistically significantly associated with the occurrence of colorectal cancer (OR: 1.44 95% CI: 1.04-2.00; OR: 1.74 95% CI: 1.15-2.65; OR: 1.79 95% CI: 1.10-2.92, respectively). Conclusion: These findings suggest that allelic polymorphism of metabolizing enzymes play an important role in human colorectal carcinogenesis by affecting the metabolism of dietary carcinogens.*

Colorectal cancer is a common tumor in developed countries, ranking generally among the three most important cancer types, in both sexes (1). Colorectal cancer formation is influenced by numerous environmental and genetic factors (2). The hereditary colorectal cancer syndromes are more or less well characterized and the responsible genes have been identified (2). However, convincing data is lacking concerning the effect of certain low penetrance genes. Studying single nucleotide polymorphisms (SNPs) of these low penetrance genes may explain the differences of individual susceptibility to sporadic colorectal cancers.

The cytochrome P450 enzymes (CYPs) take part in the metabolism of carcinogenic compounds in the human body (3). Procarcinogens are typically activated by CYPs (phase I reaction) and subsequently the reactive intermediate compounds are further metabolized (inactivated) by phase II enzymes.

CYP 1A1 plays an important role in the phase I metabolism of carcinogenic polycyclic aromatic hydrocarbons (PAHs) (4). It is responsible for the activation of benzo[a]pyrene, a known human carcinogen, occurring in cigarette smoke or smoked-grilled food items. Two polymorphisms of the enzyme have been reported to be associated with certain tumors, particularly with lung cancer (5-7). One of them is located to exon 7, causing an *Ile→Val* amino acid change in the protein, while the *Msp*I polymorphism, at the 3'-flanking region of the gene may affect the inducibility of the enzyme (8). Studying the effect of the *Msp*I polymorphism is easier, because its variant allele frequency is higher than is the case for the *Ile/Val* polymorphism. Probably this is the reason why the possible effect of the *Ile/Val* polymorphism on human carcinogenesis is still not fully explored.

CYP 1A2 is a major drug-metabolizing enzyme, with a wide range of substrates (9). Its role in the metabolism of

Correspondence to: István Kiss, Department of Preventive Medicine, Faculty of Medicine, Pécs University of Sciences, Szigeti Str. 12. H-7624 Pécs, Hungary. Tel: +36 72 536 394, Fax: +36 72 536 395, e-mail: istvan.kiss@aok.pte.hu

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Table I. *Characteristics of cases and controls.*

	Mean age (years)	Gender (males/females)	Tumor location (proximal colon/ distal colon/rectal)	Response rate (%)	Ratio of smokers (%)	Red meat consumption frequency (times/week)
Cases	64.1	222/278	207/142/151	95.6	46.1	13.1
Controls	63.8	222/278	-	91.2	45.7	13.4

PAHs and heterocyclic amines (HAs) underlines its possible significance in carcinogenesis. Since both PAHs and HAs are present in food, the activity of the CYP 1A2 enzyme may affect the formation of their activated forms after absorption from the large bowel, and thus influence on the risk of colorectal cancer. Investigations of the phenotypical enzyme activity variations have revealed allelic polymorphisms of the encoding gene. The *CYP 1A2* gene has several allelic polymorphisms, most of them SNPs, located in the 5' noncoding region or within the exons (10, 11). SNPs at both locations may have an influence on the activity of the enzyme. The exonic polymorphisms can result in an amino acid replacement in the encoded protein, causing a minor change in the structure of the enzyme (12). SNPs located in the promoter region might affect the transcription of the gene. Unfortunately, the genotype-phenotype (enzyme activity) correlation for the different alleles is still not clear. A frequently occurring polymorphic form is the *CYP 1A2*1F* allele, which is the most frequent variant in the promoter region. It is caused by an A→C polymorphism at the -164 position (12).

CYP 2E1, another member of the CYP family, is also involved in the metabolism of several potentially carcinogenic chemicals such as benzene, N-nitrosamine and ethanol (13). It is an alcohol-inducible enzyme, exhibiting a high degree of interindividual variation in its activity and inducibility (14). Several polymorphisms have been characterized at the *CYP 2E1* locus (15). The most frequently studied SNP is a *Pst*I polymorphism, caused by a G→C change in the gene at position 1259 (16). The enzyme in *c2/c2* homozygotes is characterized by better alcohol inducibility than in individuals carrying the *c1* allele (17).

Microsomal epoxide hydrolase (mEH) is considered to be a phase II enzyme, but, depending on its substrate, it may also be involved in the activation of certain PAHs (18). Two major polymorphisms of the gene have been identified, a *Tyr113His* (due to a T→C substitution) in exon 3, resulting in a lower enzymatic activity in homozygous *His/His* individuals, and a *His139Arg* in exon 4 (A→G substitution), associated with a higher activity, also in homozygotes (19). Their role has been extensively studied in lung carcinogenesis, based on the fact that tobacco smoke is a rich source of PAHs (18). Publications concerning their effect on colorectal carcinogenesis are much rarer, and there is no unambiguous viewpoint on this question (20, 21).

Table II. *Primer sequences for the PCR reactions.*

	CYP 1A1
Primer 1	GAAAGGCTGGGTCCACCCTCT
Primer 2	
tube 1	AAGACCTCCCAGCGGGCAAT
tube 2	AAGACCTCCCAGCGGGCAAC
	CYP 1A2*1F
Primer 1	TGAGGCTCCTTTCCAGCTCTCA
Primer 2	AGAAGCTCTGTGGCCGAGAAGG
CYP 2E1	
Primer 1	CCAGTCGAGTCTACATTGTCAG
Primer 2	TTCATTCTGTCTTCTAACTGG
	mEH Tyr113His
Primer 1	GATCGATAAGTTCCGTTTCACC
Primer 2	ATCCTTAGTCTTGAAGTGAGGAT
	mEH His139Arg
Primer 1	ACATCCACTTCATCCACGT
Primer 2	ATGCCTCTGAGAAGCCAT

In our case-control study the effect of *CYP 1A1 Ile/Val, CYP 1A2*1F, CYP 2E1 c1/c2, mEH Tyr113His* and *His139Arg* alleles on the risk of colon carcinogenesis was evaluated. Occurrence of the studied alleles were described and compared between a group of colorectal cancer patients (n=500) and cancer-free controls (n=500). Possible interaction between the allelic polymorphisms was also analyzed.

Patients and Methods

Patients and controls. Five hundred colorectal cancer patients from Baranya and Vas County, Hungary, were genotyped for the allelic polymorphisms of the *CYP 1A1, CYP 1A2, CYP 2E1* and *mEH* genes. The selection was based on a histologically confirmed diagnosis. The patients were recruited from hospitals, after receiving the initial

Table III. *Conditions of the PCR cycles, and the subsequently applied restriction endonucleases.*

	Number of cycles	Denaturing	Annealing	Synthesis	Restriction endonuclease
CYP 1A2	35	30 sec 94˚C	10 sec 58˚C	60 sec 72˚C	*Bsp*120I
CYP 2E1	35	60 sec 94˚C	60 sec 55˚C	60 sec 72˚C	*Pst*I
mEH exon 3	35	30 sec 94˚C	10 sec 51˚C	60 sec 72˚C	*EcoR*V
mEH exon 4	35	30 sec 94˚C	10 sec 51˚C	60 sec 59˚C	*Rsa*I

treatment. Patients with familial cancer syndromes, hereditary tumors or other genetic conditions affecting colorectal cancer risk were excluded from the study. Five hundred cancer free persons from the same regions (non-cancer patients from in- or outpatient wards /312/ and volunteers for health status examination /188/), matched to the cases according to age, sex, smoking habits and red meat consumption, were used as controls. The main characteristics of the cases and their matched controls are shown in Table I. Genotyping was conducted from peripheral blood leukocytes, isolated by repeated centrifugation with 0.84% ammonium chloride from 10 ml peripheral blood. DNA isolation was achieved by a standard protocol (22).

Allele specific polymerase chain reaction – CYP1A1. CYP1A1 genotyping for the *Ile*/*Val* (A/G) polymorphism was performed by an allele specific polymerase chain reaction (PCR) (23). Each sample was processed in two parallel tubes, with the same upstream primer, but the downstream primers differed in the last base (Table III). Amplification occurred in the tube with the exactly matching downstream primer (homozygous individuals), or in both tubes (heterozygotes). The reactions were performed in a total volume of 20 μl, containing 1.5 mM $MgCl_2$, 10 mM TRIS-HCl, pH 8.3, 2 μg/ml bovine serum albumin, 0.2 mM each dNTP, 1 μM each primer, 0.5 U Taq DNA polymerase (Promega, Mannheim, Germany), 0.5 μg template DNA, 35 cycles of 60 sec at 94˚C, 45 sec at 59˚C, 90 sec at 72˚C.

RFLP - CYP1A2, CYP2E1, mEH. Further genotypings were performed by PCR - restriction fragment length polymorphism (RFLP) (modified from references 12, 23, 24). The modification was that the same PCR reaction mix was used as described for the *CYP1A1* genotyping, without bovine serum albumin. The primer sequences are listed in Table II, the parameters of the reactions and the subsequently applied restriction endonucleases are shown in Table III. The amplification products were digested overnight with the appropriate restriction endonuclease at 37˚C, and analyzed on 2% agarose gel. In the case of the *CYP1A2* genotyping, the presence of adenine at the position -164 resulted in a 265 bp fragment (**1A* allele), while the change to cytosine caused the formation of two fragments, with a length of 211 and 54 bp (**1F* allele). For *CYP2E1*, the digestion resulted in a 410 bp product (*c1*/*c1*), or 410-290-120 bp bands (*c1*/*c2*), or 290-120 bp bands (*c2*/*c2*). The *mEH* exon 3 polymorphism showed 140 and 22 bp fragments in the presence of the wild-type allele and a 162 bp fragment for the *His* genotype. For the SNP in exon 4, the wild-type allele was characterized by a 210 bp fragment, and the A→G substitution resulted in 164 and 46 bp fragments.

Statistics. The Chi-square test was used to test the fit of genotype distributions into the Hardy-Weinberg equilibrium. Logistic regression analysis was performed to evaluate the effect of genotypes, in a multivariate model. Odds ratios, with 95% confidence intervals were calculated to express the risk associated with the studied genotypes / SPSS for Windows statistical package (SPSS Inc., Chicago, IL, USA), and Epi Info 6 (CDC, Atlanta, GA, USA).

Table IV. *Distribution of gentoypes among cases and controls.*

Genotypes	Cases	Controls
CYP 1A1		
Ile/*Ile*	386 (71.2%)	415 (83.0%)
Ile/*Val*	110 (22.0%)	83 (16.6%)
Val/*Val*	4 (0.8%)	2 (0.4%)
CYP 1A2		
1A*/1A*	219 (43.8%)	228 (45.6%)
1A*/1F*	212 (42.4%)	207 (41.4%)
1F*/1F*	69 (13.8%)	65 (13.0%)
CYP 2E1		
c1/*c1*	428 (85.6%)	456 (91.2%)
c1/*c2*	65 (13.0%)	42 (8.4%)
c2/*c2*	7 (1.4%)	2 (0.4%)
mEH exon 3		
Tyr/*Tyr*	220 (44.0%)	248 (49.6%)
Tyr/*His*	227 (45.4%)	221 (44.2%)
His/*His*	53 (10.6%)	31 (6.2%)
mEH exon 4		
His/*His*	337 (67.4%)	329 (65.8%)
His/*Arg*	157 (31.4%)	161 (32.0%)
Arg/*Arg*	6 (1.2%)	10 (2.0%)

Table V. *Association between allelic polymorphisms and occurrence of colorectal cancer.*

Genotype	OR	95% CI
CYP 1A1 (*Val*/*Val*+*Ile*/*Val*)	**1.44**	**1.04-2.00**
CYP 1A2 (**1F*/**1F*+**1A*/**1F*)	1.08	0.83-1.39
CYP 2E1 (*c2*/*c2*+*c1*/*c2*)	**1.74**	**1.15-2.65**
mEH exon 3 (*His*/*His*)	**1.79**	**1.10-2.92**
mEH exon 4 (*Arg*/*Arg*)	0.60	0.18-1.82
CYP 1A1 + CYP 2E1 + mEH exon 3	**3.35**	**1.61-7.08**

Results

The allelic distribution of the studied polymorphisms is presented in Table IV. The distributions were also analyzed separately for both sexes, but no differences were detected (data not shown). No statistically significant difference was observed between the genotype distributions between the of cases with different tumor locations. The hospitalized and the healthy controls did not differ from each other in the studied genotypes. The allelic frequencies fitted into the Hardy-Weinberg equilibrium.

For the *CYP1A1*, the frequency of the rare *Val*/*Val* homozygous individuals was 4/500 (0.8%) among the cases, while 2/500 (0.4%) such persons were identified in the control group. The number of heterozygotes was 110 (22.0%) and 83 (16.6%), respectively. The presence of the *Val* allele showed a statistically significant ($p=0.042$) association (Table V) with an increased risk of colorectal cancer in our sample (OR: 1.44, 95% CI: 1.04-2.00; *Val*/*Val* homozygotes + heterozygotes *vs. Ile*/*Ile* homozygotes).

In the case of *CYP1A2*, the homozygous carriers of the **1F* allele were present in 69 (13.8%) and 65 (13.0%) of the participants, among cases and controls, respectively. There was no statistically significant difference between cases and controls for this polymorphism.

The prevalence of *CYP2E1 c1*/*c1* homozygotes was 428 (85.6%) in the case group, and 456 (91.2%) in the control group. In comparing the occurrences of the *c1* and *c2* alleles, a statistically significant difference ($p=0.037$) was detected between colorectal cancer patients and controls (OR: 1.74, 95% CI: 1.15-2.65), with the *c2* allele being associated with the higher risk of tumors.

As illustrated in Table IV, the occurrence of the *mEH* variant allele among controls was more common in the exon 3 polymorphism than in the exon 4. With these two polymorphisms only the occurrence of homozygotes for the rare allele were compared between cases and controls (in contrast to the previously described SNPs, where homo- or heterozygous carriers of the putative "high-risk" allele were treated equally), since only the homozygous individuals had been found to have altered enzymatic activity (19). The *Tyr113His* alleles showed a significantly different distribution between the case and the control groups. The occurrence of homozygous *His*/*His* individuals was more frequent among cases than controls (53/10.6%/ *vs.* 31/6.2%, respectively; OR: 1.79, 95% CI: 1.10-2.92). The frequency of *Arg*/*Arg* homozygotes for the *His139Arg* polymorphism were cases 6 (1.2%), controls 10 (2.0%), with no statistically significant difference between cases and controls.

For those polymorphisms which showed a statistically significant association with the risk of colorectal cancer (*CYP1A1 Ile*/*Val, CYP2E1 c1*/*c2, mEH* exon 3), a further, combined analysis was also made. Those alleles which proved to increase the cancer risk were considered to be "high-risk" alleles (in order to ensure statistically evaluable case numbers, the heterozygotes were counted as "high-risk" genotypes also for the *mEH* polymorphism). The number of "high-risk" alleles per person were counted and compared between cases and controls. Thirty five persons who carried the "high-risk" alleles for all three polymorphisms were found among the colorectal cancer patients, while in the control group only eleven such individuals were observed. This statistically significant ($p=0.014$) overall effect (OR: 3.35, 95% CI: 1.61-7.08) illustrates the practical importance of studying low-penetrance genetic factors.

Discussion

Three of the studied polymorphisms showed a statistically significant association with the occurrence of colorectal cancer in our sample. Since the main goal of our study was to assess the role of genetic factors in colorectal carcinogenesis, further, sophisticated analyses, such as by forming subgroups based on dietary or smoking habits were not performed. The relatively low number of "high-risk" alleles (with the exception of *mEH* exon 4 polymorphism the rare alleles proved to be associated with the increased risk of cancer) or their combinations would have lead to low case numbers and possibly false negative results in such an analysis. The approach of matching cases and controls for these variables was prefered, to increase the case numbers, and to reach a good statistical power for the studied genetic factors. Low-penetrance genetic factors, such as polymorphisms of metabolizing enzymes, are relatively difficult to study in human epidemiological investigations, because of several possible confounding factors like smoking, nutritional habits and alcohol consumption. These confounders often have a much stronger effect on the risk of tumor formation than the studied polymorphisms themselves. In the case of colorectal carcinogenesis, meat consumption (particularly red meat consumption) is the most frequently mentioned nutritional risk factor (25, 26). Smoking is also known to increase the risk of several human tumors, including colorectal cancer (27). These facts led us to choose red meat consumption and smoking as matching variables (beside age and sex).

The CYP enzymes involved in this study are phase I enzymes, activating carcinogenic chemical compounds such as PAHs or HAs. Depending on nutritional habits, the diet can contain high amounts of such substances. High enzyme activity is presumed to produce larger amounts of reactive intermediates, which will then lead to an increased level of DNA damage and an elevated risk of cancer (28). These assumptions were fully supported by the results of our study. Individuals carrying the *CYP1A1 Val* allele were reported to

have a faster metabolism of certain PAHs than heterozygotes or *Ile* homozygotes (8). This is supported by studies investigating the level of PAH metabolites or the amount of DNA-adducts according to *CYP1A1* genotypes (29). In the case of *CYP2E1*, the studied polymorphism does not change the amino acid sequence of the enzyme, but alters the inducibility of the enzyme. Since CYP2E1 is an alcohol-inducible enzyme, the observed association between the c2 genotype and the risk of colorectal cancer may be even stronger among alcoholics. Based on the results of *CYP1A1* and *CYP2E1* the same effect could be expected for the *CYP1A2 *1F* polymorphism. In addition, the substrate specificity of CYP 1A2 to a certain extent overlaps those of the other studied CYP enzymes. The effect of *CYP1A2* genotypes on the transcriptional activity of the gene is, however, not completely described yet. Better understanding of the phenotype-genotype relationship of the *CYP1A2* alleles might provide an explanation for the lack of an association between this allelic polymorphism and the occurrence of colorectal cancer.

Concerning the *CYP1A1* gene, the first article reporting a positive association among Asians between its polymorphisms and colorectal cancer was published in 1994 (30), however the study lacked the necessary power to detect such an association among Caucasians. This was followed by other case-control studies, with different results. Ye *et al.* (31) did not find a statistically significant association between *CYP1A1* alleles and colorectal cancer, in agreement with to our earlier study (32), and to the results of Butler *et al.* (33). Ishibe *et al.* (34) conducted a prospective study, with negative results. However, the sample size of the mentioned studies was around 200, which might be too small to detect the suspected association. In contrast to these results, other case-control studies have found an association between the polymorphism of the *CYP1A1* gene and colorectal cancer (35-37). Our present results suggest that matching cases and controls, with an adequate number of participants, ensures the necessary power to detect an association between carrying the *CYP1A1 Ile* allele and the increased risk of colorectal cancer.

A similar situation arises in the literature in case of the *CYP2E1* gene, unfortunately with fewer, but even more heterogeneous studies. Butler *et al.* (33) did not find a statistically significant connection (no data reported on nutritional habits or smoking) between *CYP2E1* alleles and colorectal tumors, and nor did Landi *et al.* (38), while a Chinese study (39) found the *C2* allele to be a susceptibility factor. A Hawaiian study (40), including participants with Japanese, Hawaiian and Caucasian origin, found that the risk of rectal, but not colon cancer was increased among individuals with the 5' end insert variant allele, particularly if this genotype was associated with high red meat and/or processed meat intake. Our results, confirming the findings of our previous study (32), clearly indicate a positive association between the *c2* allele and the increased risk of colorectal cancer. In contrast to Le Marchand *et al.* (40), no difference with respect to the location of the tumor was found.

Data have been published concerning the existence of an association between the *CYP1A2* genotypes and the risk of colorectal cancer in humans (38), but other investigators, such Sachse *et al.* (35) concluded that no such association exists. The present study – with relatively large case numbers, was also not able to detect any difference between colorectal cancer patients and controls in relation to the *CYP1A2* genotypes. The results were probably affected by the previously mentioned genotype-phenotype correlation which has also shown a disagreement between the works of other authors. Sachse *et al.* (12) did not find an association between *CYP1A2* genotypes and enzymatic activity (measured by the caffeine metabolizing test), while the *in vivo* CYP1A2 activity was lower in colorectal cancer patients than in controls. Interestingly, Moonen *et al.* in a recent study (41) have suggested a possible genotype-phenotype relationship, recording lower enzymatic activities among *CYP1A2*1F* individuals (the difference only reached the level of statistical significance after removing three participants with extreme values). Le Marchand *et al.* demonstrated a connection between CYP1A2 phenotypes and colorectal carcinogenesis (42). Based on theoretical considerations, supported by experimental evidence, the conclusion that CYP1A2 activity is in an inverse correlation with the risk of human colorectal cancer may be drawn. However, genotyping cannot yet replace the determination of enzyme activity and further studies are required to precisely describe, which factors affect the *CYP1A2* genotype-phenotype connection.

The *mEH* enzyme, is able to catalyze both phase I and phase II reactions, a property which has also been described for N-acetyltransferase 2 (NAT2). The NAT2 performs an N-acetylation which is considered to be a phase II reaction, but it is also capable of catalyzing the O-acetylation of certain substrates (phase I reaction). This explains why rapid acetylators are more frequent among colorectal cancer patients, but the same alleles are protective in occupational bladder carcinogenesis (43).

Theoretically, an increased mEH activity can lead to either elevated or decreased risk of cancer depending on the exon 3 and 4 polymorphisms. If, concerning a particular cancer, the detoxifying (phase II) function of the enzyme is more relevant than its possible PAH activating capacity, the "active" alleles might ensure some protective effect. However, heavy PAH-exposure interacting with the presence of low activity forms of mEH might lead to an increased risk of tumors in sensitive organs. Since cigarette smoke contains high amounts of PAHs, it had been

supposed that high-activity alleles would be associated with an elevated risk of lung cancer. The results of molecular epidemiological studies however were not able to demonstrate this assumption, and the role of *mEH* polymorphisms in the genesis of human lung cancer is still to be explored (24, 44).

Previous studies on *mEH* polymorphisms and colorectal carcinogenesis are somewhat controversial. Some studies (45, 46) did not find any statistically significant association between genotypes and cancer risk, while some others (20, 47-48) reported such associations for certain subpopulations only (based on smoking and/or meat consumption). Harrison *et al.* (21) in a case-control study identified the exon 3 *His* carriers as high risk individuals (with an unusually high odds ratio of 3.84), while no association was found for the exon 4 polymorphism. Huang *et al.* (49) found the high activity variants of both exon 3 and exon 4 to be risk factors for colorectal adenoma. In contrast, however, Sachse *et al.* (35) identified the exon 3 *His* variant as a risk lowering factor, which was explained by possible differences in environmental or dietary factors between the studied populations.

The present study underlines the importance of the phase II activity of mEH, since the low-activity genotype was clearly associated with an increased risk of cancer. Interestingly, the connection between the high-activity genotype (exon 4 polymorphism) and colorectal cancer risk was not statistically significant but based on the results of the exon 3 polymorphism, a possible preventive effect could have been expected. The difference between allelic frequencies of the rare alleles may be the explanation. The occurrence of the exon 3 *His*/*His* homozygotes was 6.2% among controls, while the exon 4 *Arg*/*Arg* individuals formed only 2.0% of the population. Possibly the rare occurrence of the latter would require an even larger sample size to demonstrate its association with cancer.

The combined analysis of the polymorphisms demonstrated the presence of an interaction between their effects. Individuals with all the three high-risk genotypes were found to exhibit a substantially increased risk of colorectal cancer (OR: 3.35). This is of practical importance, because by extension of such analyses with several further polymorphisms, hopefully in the near future high-risk individuals could be identified based on genetic screening for low-penetrance factors. At present, our data suggest that polymorphisms of certain metabolizing enzymes modulate the risk of sporadic colorectal cancer. The magnitude of this effect is moderate, and cannot be used for individual risk assessment. Certain allelic combinations, however, can result in a remarkably increased risk, which may be even more pronounced in people with particularly high exposure to environmental or occupational carcinogens.

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