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COLORECTAL CANCER AND DETOXIFICATION ENZYMES

with emphasis on enzyme modulation and genetic polymorphisms

COLORECTAL CANCER AND DETOXIFICATION ENZYMES

with emphasis on enzyme modulation and genetic polymorphisms

Een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

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Introduction and aim of the thesis

Epidemiology and aetiology of colorectal cancer

Colorectal cancer (CRC) is one of the most common forms of cancer in Western societies and shows a 20-fold geographic variation in incidence worldwide (1). Despite recent advances in diagnosis and treatment, the mortality from CRC remains high. In The Netherlands, CRC is the second cause of death from malignant disease in women after breast cancer and the third cause of death in men after lung- and prostate cancer (2).

It is now generally accepted that a time-dependent accumulation of multiple mutations in tumour suppressor genes (e.g. APC, p53, DCC), oncogenes (i.e. K-ras) and genes involved in DNA mismatch repair (e.g. MLH-1, MSH-2) results in the transformation of normal colonic epithelium into hyperproliferative tissue, adenoma, and finally eventually in carcinoma (3, 4). Accumulation of these mutations results in disturbance of the balanced epithelial cell turnover, as determined by rates of cell proliferation and apoptosis (4).

Up to 10% of all CRC cases are due to hereditary factors of high penetrance; the most frequent being hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP; refs. 5, 6). These syndromes may result in the development of tumours at a relatively early age. The other 90% of CRC, so called sporadic CRC, may be attributed to diet or lifestyle factors, eventually in combination with genetic factors of low penetrance. The incidence of sporadic CRC is strongly age-related, with the majority of cases diagnosed above 50 years of age. Ultimately, at the age of 75 years, up to 4.7% of men and 3.2% of women will have developed CRC in The Netherlands (7).

Though it is recognized that genetic factors are important determinants for the genesis of part of the CRC cases (8), epidemiological studies have revealed that the worldwide variation in CRC incidence is strongly related to differences in dietary habits and lifestyle factors (9). This is supported by migrant studies, which have shown that individuals migrating from low- to high-risk areas rapidly adopted the incidence rates of the high-risk areas (10, 11). Moreover, several epidemiological studies have shown that the consumption of a typical Western-style diet, which is characterized by high intake of red meat, (saturated) fat and alcohol, and a low intake of fresh vegetables, fruits and calcium is associated with a high risk for CRC (9, 12).

In addition, humans may be exposed daily to a large variety of toxic or even carcinogenic compounds, present in food (13, 14) or as a result of lifestyle habits such as smoking of cigarettes or drinking alcohol (15-17). However, humans also possess a highly efficient system of defence against such compounds, and an important role is reserved for the various detoxification enzymes (18). The levels and activities of these enzymes strongly vary between the various organs and between individuals (19-21). Variations between individuals may be due in part to the presence of genetic polymorphisms (genetic variations between individuals), which may contribute to the inter-individual differences in expression levels and enzyme activities of these enzymes (22-24). This might also influence the conversion rate of toxic or carcinogenic compounds in the gastrointestinal tract and consequently, may partly determine the levels of toxins and carcinogens in the colon. Therefore, polymorphisms in detoxification enzymes could contribute to individual susceptibility to CRC. In summary, tumour formation in the colon may result from interaction between external (environmental carcinogen exposure) and internal (genetic) factors.

Detoxification enzymes

Humans possess highly efficient defence systems against harmful compounds; one of these is the detoxification enzyme system (18). The main purpose of the detoxification enzymes is to facilitate the elimination of potentially toxic or carcinogenic compounds from the body. This process usually involves the two-stage route of phase I and phase II reactions. Phase I reactions include oxidation, reduction, dealkylation or hydrolysis of chemical compounds and provide the molecules with hydroxyl- or amino-groups. This however, often leads to bioactivation of chemicals into more reactive compounds that even might be carcinogenic. Phase II reactions comprise conjugation of these chemical compounds with glucuronic acid, glutathione, sulphate, acetyl groups or amino acids. These cofactors react with functional groups that are either present or are introduced by the phase I reactions. In general, phase II reactions result in less biologically active compounds that are more water soluble, thereby facilitating their excretion *via* urine or bile. Phase I reactions are mainly catalyzed by a variety of cytochromes P450 monooxygenases (CYP; ref. 25) or epoxide hydrolases (EPHX; ref. 26), whereas glutathione S-transferases (GSTs; ref. 27) and UDP-glucuronosyltransferases (UGTs; ref. 28) play an important role in the phase II metabolism. However, many other

detoxification enzymes including alcohol dehydrogenases (ADH; ref. 29), NAD(P)H: quinone oxidoreductases (NQO; ref. 30) or paraoxonases (PON; ref. 31) have their own specific role in detoxification.

Anticarcinogens and enzyme modulation

In Western countries, environmental factors may account for up to 65% of the risk for CRC (32) and it has been estimated that ~50% of this risk is attributable to dietary factors (33). This makes CRC a potentially preventable disease to a large extent. Epidemiological studies have shown that regular consumption of vegetables and fruits may result in significant protection against cancer of the gastrointestinal tract (9, 12). In the face of these epidemiological associations, an impressive and still growing number of dietary non-nutritive constituents, which may inhibit mutagenesis or carcinogenesis, have been identified (34-37). Information on the anticarcinogenic potential of these dietary components mainly comes from studies on single crude dietary components (such as cruciferous vegetables, citrus fruits or garlic) and from studies on specific purified non-nutritive constituents of vegetables and fruits (such as glucosinolates, flavonoids, phenolic acids and organosulfides). Many of these dietary components have been shown to inhibit chemical-induced carcinogenesis in a variety of animal studies (14, 38-41) and therefore are called anticarcinogens or chemopreventive agents. According to the stage in the carcinogenic process at which they are effective, i.e. initiation, promotion and progression, they can be classified into different categories. Some anticarcinogens act as blocking agents by preventing carcinogens to be formed or to reach and react with critical targets. Others anticarcinogens act as suppressing agents by preventing the transformation of initiated cells, which have been previously exposed to carcinogens, into tumours (42).

Though the exact mechanism(s) leading to the anticarcinogenic action of the potential anticarcinogenic dietary compounds is not fully known, there are strong indications that their chemopreventive capacities may be due to the modulation of activity or composition of phase II detoxification enzyme systems, such as GSTs and UGTs (34-36, 43-45). Changes in detoxification capacity may play a key role in the altered susceptibility to carcinogens we are exposed to daily, and thus may influence the risk for developing cancer. The biological

control of detoxification enzymes is complex, as they exhibit sex-, age-, tissue-, species-, and tumour-specific patterns of expression (27, 28). A diverse range of dietary components regulates detoxification enzyme systems. Many of the compounds that induce detoxification enzymes, themselves are substrates of these enzymes. This suggests that induction of detoxification enzymes represents part of an adaptive response mechanism to carcinogen-induced cellular stress.

Previous research at our institution demonstrated that several dietary naturally occurring or synthetic anticarcinogens increase rat hepatic and intestinal GST enzyme activity (46-48). In addition, a dose-dependent induction of rat hepatic and intestinal GST enzyme activity was observed after supplementation with the anticarcinogens α-angelicalactone or flavone in varying concentrations, either alone or in combination (49). High intake of fruits or vegetables in humans was associated with high gastrointestinal isoenzyme levels of the GST detoxification system (50, 51). Furthermore, an intervention study in 10 healthy controls revealed that daily consumption of 300 g Brussels sprouts for one week was able to increase rectal GSTA and GSTP1 levels (52). O'Dwyer *et al.* (53) found that a single dosage of oltipraz (125 or 250 mg/m^2), a synthetic dithiolethione, resulted in an enhancement of glutathione S-transferase activity of ± 21% in the sigmoid of patients at increased risk for CRC. However, Clapper *et al.* (54) found no significant difference in GST-activity in the sigmoid of 29 patients at increased risk for CRC, who consumed 3g/day of broccoli components for 2 weeks.
At present little information on the effects of dietary anticarcinogens on the UGT activity of the digestive tract is available. Recent data, mostly obtained from animal studies, have indicated that dietary agents may be able to elevate UGT activity (44, 55-58).

The majority of malignant tumours in the digestive tract do develop in the colon, which has a detoxification capacity that is critically low, and this may contribute to the susceptibility of colon cancer in humans (19, 59-61). Enhancement of the activity of detoxification enzymes, such as GSTs and UGTs, potentially could increase the capacity to withstand the burden of toxic agents and (pre)carcinogens humans are exposed to daily (13, 14, 43, 45, 52), and knowledge of the exact protection mechanism(s) of the non-nutritive anticarcinogens may be of importance for the prevention of CRC.

Genetic polymorphisms

Genetic polymorphisms are defined as commonly occurring variations in genes and are characterized by the presence of several distinct forms of a gene within a population. The genetic polymorphisms may be caused by single nucleotide substitutions, repetitive sequences, insertions or deletions of nucleotides. Polymorphisms in low-penetrance genes, such as those encoding for the detoxification enzymes, may account in part for the inter-individual differences in the sensitivity to cancer-inducing or cancer-promoting compounds (22-24). Functional polymorphisms may result in enzymes with enhanced or reduced activity, or even in complete absence of enzyme activity. This might influence the conversion rates of toxic or carcinogenic compounds in the colon. Therefore, functional polymorphisms in detoxification enzymes could contribute to individual susceptibility to CRC and indeed some of these genetic polymorphisms have already been associated with altered CRC susceptibility (62-65).

Reactive oxygen species and colorectal cancer

It is suggested that reactive oxygen species (ROS) may also play a role in human cancer development (66-68). Sources of ROS may be both exogenous (drugs, ozone, radiation) and endogenous (NO, phagocytes, leakage from mitochondria; ref. 68). In a recent review, Klauning and Kamendulis (68) described the involvement of ROS in the various stages (initiation, promotion, progression) of the process of carcinogenesis.

In healthy individuals, the generation of ROS appears to be counterbalanced by the antioxidant defence, which is recruited either from endogenous sources (glutathione, cysteine, uric acid, bilirubin, etc.) or from the diet (vitamins A, C and E; refs. 69, 70). An imbalance between ROS and antioxidant defences in favour of the former creates oxidative stress (70, 71). This may occur when the antioxidant levels are low/depleted and when the formation of ROS is high. Severe oxidative stress affects a large variety of metabolic processes. For example, ROS can cause structural alterations in DNA and may affect cytoplasmic and nuclear signal transduction pathways (67). Furthermore, ROS can modulate the activity of

proteins that respond to stress, and which regulate genes that are involved in cell proliferation, differentiation and apoptosis (67).

While it is clear that ROS may induce cellular changes, similar to those produced by known carcinogens (67), the precise mechanisms remain unclear. It is suggested that the overproduction of ROS, such as superoxide and hydrogen peroxide, by polymorphonuclear leukocytes (PNMs), might sometimes also play a role in initiation of carcinogenesis (72). Phagocytosis by PNMs results in the release of ROS, referred to as the respiratory burst, which plays an important role in host defence against certain micro-organisms. Excessive generation of ROS by these phagocytes may cause harm to surrounding tissue (72). When key genes or proteins responsible for intestinal cell homeostasis are targeted, dysplasia and subsequent development of adenoma or carcinoma may occur.
Data on a role of ROS in the pathogenesis of CRC is accumulating. Recently, Schmielau and Finn (73) reported that patients with advanced cancer of the colon, pancreas or breast, showed signs of extensive granulocyte activation with release of ROS. In addition, Gackowski *et al.* (74) observed that the levels of 8-oxo-2'-deoxyguanosine, a typical product of ROS-induced DNA base modification, were significantly higher in lymphocytes of patients with CRC in comparison to those of control subjects. These data show that production of ROS may be higher in patients with CRC, but do not reveal whether this is a result of the disease itself. However, Keshavarzian *et al.* (75) found that normal-appearing rectal mucosa of patients with a history of CRC was able to produce significantly higher levels of luminol-enhanced chemiluminescence than corresponding mucosa of control subjects, which may indicate that overproduction of ROS could contribute to development of the disease.

Aim of this thesis

The main objectives of this thesis are to investigate: 1) the modulation of rat hepatic and intestinal UGTs by anticarcinogens, 2) whether genetic polymorphisms in detoxification enzymes may modulate the risk for CRC, and 3) whether neutrophil oxygen radical production may contribute to the aetiology of CRC.

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Induction of rat hepatic and intestinal UDP-glucuronosyltransferases by naturally occurring dietary anticarcinogens

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Abstract

Gastrointestinal tumours are among the most common malignancies in Western society, the majority of which are associated with dietary and lifestyle factors. Many dietary or lifestyle factors have been identified which may have toxic or carcinogenic properties. However, also several dietary compounds able to reduce gastrointestinal cancer rates both in humans and animals have been characterized. Though the exact mechanism leading to the anticarcinogenic action of these compounds is not fully known, it has been demonstrated that this chemopreventive capacity may be due to elevation of the glutathione S-transferase detoxification enzymes. Here we have investigated the effect of several anticarcinogens on the gastrointestinal UDP-glucuronosyltransferase (UGT) enzymes. Diets of male Wistar rats were supplemented with ellagic acid, ferulic acid, Brussels sprouts, quercetin, α-angelicalactone, tannic acid, coumarin, fumaric acid, curcumin and flavone separately, and combinations of α-angelicalactone and flavone. Hepatic and intestinal (proximal, mid and distal small intestine and colon) UGT enzyme activities were quantified using 4-nitrophenol and 4-methylumbelliferone as substrates. All anticarcinogens tested increased UGT enzyme activity with both substrates, at one at least of the five different sites investigated. α-Angelicalactone, coumarin and curcumin showed enhanced UGT enzyme activities at all five sites. Both small and large intestinal UGT enzyme activities were increased by quercetin, α-angelicalactone, coumarin, curcumin and flavone. Except for tannic acid, all agents induced hepatic UGT enzyme activity. Furthermore, dietary administration of α-angelicalactone and flavone, given individually or in combination, enhanced the UGT detoxification system in the liver and, to a lesser extent, in intestine. In conclusion, induction of gastrointestinal UGT enzyme activities after consumption of dietary anticarcinogens may contribute to a better detoxification of potentially carcinogenic compounds and subsequently to the prevention of gastrointestinal cancer.

Introduction

Gastrointestinal tumours are among the most common malignancies in Western society. Epidemiological studies have shown the importance of dietary habits in the risk for gastrointestinal tumours in general and for colon cancer more particularly. Diets rich in vegetables and fruit are associated with a lower risk (1-3). In the face of these epidemiological associations many dietary non-nutritive constituents with anticarcinogenic properties have been identified (4-9). Though the exact mechanism(s) leading to the anticarcinogenic action of these compounds is not fully known, it has been suggested that their chemopreventive capacities may be due to elevation of detoxification enzymes (4, 6-8, 10, 11). Important detoxification or drug-metabolizing enzymes are UDP-glucuronosyltransferases (UGTs) and glutathione S-transferases (GSTs). UGTs catalyze the conjugation of exogenous and endogenous, mainly lipophilic compounds with glucuronic acid, while GSTs catalyze the reaction of glutathione with mainly exogenous electrophiles and endogenous products of oxidative stress. Some overlap in substrate specificity may occur between these detoxification enzymes. Glucuronidation or conjugation with glutathione in general results in less biologically active molecules and enhances the water solubility of the conjugated products, which facilitates excretion from the body *via* bile or urine (12). UGTs have been divided into two families, termed UGT1 and UGT2 (13). UGT1 enzymes mainly catalyze glucuronidation of exogenous agents (drugs, pesticides, benzo[*a*]pyrene, etc.) whilst UGT2 enzymes glucuronidate endogenous agents (steroid hormones and bile acids), however this classification is not that strict since, for instance, human UGT1A1 is the main enzyme catalyzing conjugation of the endogenous substrate bilirubin, whereas nonsteroidal anti-inflammatory drugs are conjugated by rat UGT2B1 (14).

In humans, glucuronidation capacity is prominently present in the liver, where most of the various UGT isoforms have been identified at relatively high levels. However, different isoenzymes of UGT1 have also been distinguished in extrahepatic tissues, including skin, kidney, oesophagus, stomach, small intestine, colon and many other organs (15, 16). Since the gastrointestinal tract is in direct contact with potentially toxic or (pre)carcinogenic agents, ingested by food, medication, drugs, etc. the intestinal mucosa acts as a first line barrier. A striking observation is the significantly lower glucuronidation rate in colon compared with those in liver and small intestine (15, 17, 18). Tissue-specific expression of the different UGT

genes encoding for the various isoenzymes in colon, liver and small intestine could partially contribute to the differences in UGT activity between these tissues (15, 18). However, because of the magnitude of the UGT enzyme activity differences between colon and liver or small intestine (15, 18), it cannot be ruled out that additional modifiers of UGT activity are responsible for the dramatic differences in functional UGT activities between these tissues.

The majority of malignant tumours in the digestive tract develop in the colon, where UGT enzyme activity is low, and this may contribute to the susceptibility of colon cancer in humans (13, 15, 17). Enhancement of the activity of such enzymes could potentially increase the capacity to withstand the burden of toxic agents and (pre)carcinogens we are exposed to daily (7, 19 20). Knowledge of the exact protection mechanism(s) of anticarcinogenic compounds present in food may be of importance for the prevention of colon cancer. Previous research demonstrated that several dietary agents increase GST enzyme activity in liver and intestine of male Wistar rats (10). Therefore, we have now investigated the effects of several naturally occurring dietary anticarcinogens on rat hepatic and intestinal UGT enzyme activity. In an earlier study we showed a dose-dependent induction of rat hepatic and intestinal GST enzyme activity after supplementation with the anticarcinogens α-angelicalactone or flavone in varying concentrations, either alone or in combination (21). Now we have also investigated the effects of this dose-dependent and combination treatment on gastrointestinal UGT enzyme activities.

Materials and methods

Materials

Ellagic acid, ferulic acid, quercetin, coumarin, curcumin, flavone, bovine serum albumin, dithiothreitol, 4-methylumbelliferone (4-MUB), UDP-glucuronic acid (UDPGA) and D-saccharic acid 1,4-lactone were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol and 4-nitrophenol (4-NP) were from Merck (Darmstadt, Germany). α-Angelicalactone, tannic acid and fumaric acid were obtained from Aldrich Chemie (Zwijndrecht, The Netherlands). Brussels sprouts were collected from local shops.

Study design

Hepatic and intestinal microsomes were prepared from Wistar rats, kept and treated as described in previous studies performed by Nijhoff *et al.* (10, 21). In short, male Wistar rats (Central Laboratory Animal Centre, University of Nijmegen, The Netherlands) were housed individually on wooden shavings in macrolon cages maintained at 20-25°C and 30-60% relative humidity. A ventilation rate of 7 air changes/h and a 12 h light/dark cycle were used. The rats were randomly assigned to one of the dietary treatment groups. All groups were fed powdered RMH-TM laboratory chow (Hope Farms, Woerden, The Netherlands). After acclimatization for 3 days, the animals were fed either the basal diet (control group) or one of the experimental diets.

Diets

Ellagic acid, ferulic acid, quercetin, tannic acid, fumaric acid and curcumin were incorporated at the level of 1% (w/w) in the basal diet. α-Angelicalactone and flavone were provided at the level of 0.5% (w/w) and coumarin at the level of 0.25% (w/w). Brussels sprouts were incorporated at the level of 20% (w/w) (10). α-Angelicalactone and flavone were incorporated into the diet, either separately or as a combination of both compounds, at 0.01, 0.05, 0.1 and 0.5% (w/w), respectively (21). Food and water were available *ad libitum*. Food cups were replenished with freshly prepared diet every 2-3 days. Food consumption and gain in body weight was recorded daily. After 2 weeks the animals were killed by decapitation.

Tissue preparation

Tissue handling, isolation of liver and intestinal mucosa and preparation of microsomal pellet was performed as described previously (22). In short, liver tissue and intestinal mucosal scrapings were homogenized in buffer A (4 ml buffer A/g tissue or mucosal scraping; buffer A = 0.25 M saccharose, 20 mM Tris-HCl, 1 mM dithiothreitol, pH 7.4), followed by centrifugation at 9000 *g* (4°C) for 20 min. The resulting supernatant fraction was spun at 150 000 *g* (4°C) for 50 min, resulting in the sedimentation of the microsomes. The microsomal pellet was resuspended in 5 vol of buffer A.

Assays

Protein concentration was assayed in duplicate by the method of Lowry *et al.* (23) using bovine serum albumin as the standard.

UGT activity with 4-NP and 4-MUB as substrates was measured in the microsomes as described previously (24, 25). Briefly, liver and intestinal microsomes were resuspended in 50 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 1 mM dithiothreitol. Treatment of microsomes with varying concentrations of Triton X-100 failed to activate microsomes and rather inhibited UGT enzyme activity at higher Triton concentrations. Therefore, we did not perform an activation step in the UGT enzyme activity assay.

Conjugation of 4-NP was measured in a final volume of 0.1 ml Tris-HCl, pH 7.4, containing 3.5 mM UDPGA, 10 mM $MgCl_2$, 0.05-0.5 mg microsomal protein, 1mM saccharic acid 1,4-lactone, and 1 mM 4-NP. 4-NP was dissolved in 0.01 M NaOH. After incubation for 1-20 min at 37°C, the reaction was terminated by adding 2 ml of 0.3 M NaOH, followed by centrifugation for 10 min at 10 000 *g*. Absorbance was measured at 405 nm on a Perkin Elmer Lambda 12 spectrophotometer.

Conjugation of 4-MUB was performed in the presence of 4 mM UDPGA, 10 mM $MgCl_2$, 0.025-0.25 mg microsomal protein, 1 mM saccharic acid 1,4-lactone and 0.1 mM 4-MUB in a final volume of 0.1 ml Tris-HCl, pH 7.4. 4-MUB was dissolved in ethanol (50 mM) and diluted with assay medium just before use. After incubation at 37°C for 0.5-20 min, 1 ml of 0.5 M glycine/NaOH, pH 10.35, was added to the reaction mixture to terminate the reaction. Subsequently, samples were centrifuged for 10 min at 10 000 *g*. 4-MUB was determined fluorometrically with a Shimadzu RF-5000 spectrofluorophotometer (excitation 370 nm, emission 450 nm).

All samples were measured in duplicate. In all assays a control sample without UDPGA was run simultaneously. The absorbance difference between the control sample and the sample incubated in the presence of UDPGA represents the amount of glucuronidated 4-NP or 4-MUB. Effects of anticarcinogens on UGT enzyme activity are presented as ratios of treated to control.

<u>Statistical analysis</u>

Wilcoxon rank sum test was used to assess statistical significance of differences between control and treatment groups. Correlation analyses between enzyme activities were performed using Spearman rank correlation. $P < 0.05$ was considered to be significant.

Results

The effects of the anticarcinogens on 4-NP UGT activity in the five different parts of the gastrointestinal tract investigated (proximal, mid and distal small intestine, large intestine and liver) are shown in Table I. All compounds tested, except fumaric acid, increased 4-NP UGT activity at one or more sites. The most striking statistically significant enhancement was seen in small intestine by curcumin (5.4×, 6.7× and 7.2× in the proximal-, mid- and distal part, respectively), in large intestine by curcumin (3.1×) and in liver by flavone (7.6×). α-Angelicalactone and coumarin gave an enhancement in 4-NP UGT enzyme activity at all five sites investigated.

Table I. Effects of naturally occurring anticarcinogens on intestinal and hepatic 4-NP UGT enzyme activity

Treatment group	*n*	4-NP UGT activity (ratio treated/control)				
		Small intestine			Large intestine	Liver
		Proximal	Mid	Distal		
Ellagic acid	5	1.1 (0.5-11.0)	1.4 (0.6-7.0)	2.0 (1.5-9.0)	1.4 (0.6-3.7)	2.7 (1.2-3.4) [a]
Ferulic acid	6	2.2 (0.4-10.3)	2.0 (0.7-4.1)	3.3 (0.7-4.2)	1.2 (0.5-1.8)	2.5 (1.8-2.8) [b]
Brussels sprouts	3	0.4 (0.2-0.4) [c]	0.9 (0.8-1.1)	1.1 (0.5-1.5)	1.0 (0.7-1.4)	1.9 (1.4-2.6) [c]
Quercetin	6	4.0 (0.4-13.3)	2.8 (0.9-6.6)	4.0 (1.4-8.3) [c]	3.8 (0.6-5.2)	1.5 (1.2-2.0) [c]
α-Angelicalactone	6	2.8 (1.2-6.7) [c]	2.1 (1.5-3.0) [c]	3.9 (3.1-4.8) [b]	2.3 (1.7-3.0) [b]	3.4 (2.7-4.5) [b]
Tannic acid	3	1.6 (0.7-4.9)	2.1 (1.2-3.1)	3.4 (1.6-3.6) [c]	1.4 (0.6-2.8)	1.2 (1.2-1.7)
Coumarin	6	3.4 (1.8-6.2) [c]	3.0 (1.0-7.0) [c]	4.3 (2.2-5.5) [a]	3.0 (1.8-7.7) [b]	5.6 (3.7-6.3) [b]
Fumaric acid	3	3.5 (0.6-4.7)	1.6 (1.4-2.5)	1.4 (0.8-1.9)	1.4 (1.4-1.5)	1.1 (1.1-1.6)
Curcumin	6	5.4 (2.4-25.2) [c]	6.7 (2.0-8.9) [a]	7.2 (3.8-8.3) [b]	3.1 (1.3-6.1) [b]	1.5 (1.0-2.0)
Flavone	6	1.2 (0.8-1.4)	1.5 (0.8-1.8)	1.5 (0.8-3.4)	1.9 (1.4-3.0) [a]	7.6 (6.4-10.3) [b]

4-NP UGT activity was measured in duplicate as described in Materials and methods.

n, number of rats used in each group.

Results are given as ratios of treated to control (median, range). In the control group ($n = 9$) 4-NP UGT activities (median, range) were 0.21 (0.06-1.18), 0.38 (0.12-1.59), 0.33 (0.14-0.90), 1.8 (0.51-4.4) and 30 (16-55) nmol/min·mg protein for proximal, mid and distal small intestine, large intestine and liver, respectively.

The Wilcoxon rank sum test was used for statistical evaluation; $^aP < 0.01$, $^bP < 0.005$, $^cP < 0.05$.

The effects of the anticarcinogens on 4-MUB UGT activity are more or less the same as observed for 4-NP UGT activity. However, quercetin was able to induce 4-MUB UGT

activity in the proximal small intestine (2.7×) and large intestine (2.2×), α-angelicalactone had no effect on mid small intestinal activity and tannic acid did not have any effect. On the other hand flavone induced distal small intestinal 4-MUB UGT activity (2.2×), whereas fumaric acid and curcumin increased hepatic activity (2.2× and 2.6×, respectively).

The effects on 4-NP UGT activity in animals fed diets with α-angelicalactone, flavone or their combination are summarized in Table II. In general, 4-NP UGT activity was increased most in rats that were fed the highest dose of α-angelicalactone or flavone or the combination of both compounds. In large intestine and liver, treatment with the highest concentration of α-angelicalactone resulted in enhancement of 4-NP UGT activity.

Table II. Effects of dietary α-angelicalactone and flavone, individually and in combination, on rat intestinal and hepatic 4-NP UGT enzyme activity

Treatment group	Per cent w/w	4-NP UGT activity (ratio treated/control)		
		Mid small intestine	Large intestine	Liver
α-Angelicalactone	0.01	1.0 (0.6-1.1)	1.0 (0.7-1.2)	0.8 (0.6-0.9)
	0.05	0.9 (0.7-1.4)	1.0 (0.9-1.3)	1.0 (0.7-1.8)
	0.1	1.0 (0.9-1.4)	0.9 (0.7-1.2)	1.4 (1.3-1.8) [a]
	0.5	1.0 (0.8-1.6)	1.1 (1.1-1.3) [a]	3.7 (3.2-5.0) [b]
Flavone	0.01	0.8 (0.6-1.1)	1.1 (0.9-1.3)	1.6 (0.9-2.2) [a]
	0.05	1.2 (0.8-1.4)	1.0 (0.7-1.3)	1.7 (1.3-2.7) [b]
	0.1	1.2 (1.0-1.4)	1.2 (0.9-1.3)	3.3 (2.4-4.8) [b]
	0.5	1.2 (1.1-1.3) [a]	1.4 (1.0-1.7) [a]	11.0 (6.4-14.2) [b]
Combination	0.01/0.01	1.1 (0.9-1.3)	0.9 (0.7-1.3)	1.6 (1.4-2.1) [b]
	0.05/0.05	1.2 (1.1-1.3)	0.9 (0.7-1.3)	3.1 (2.1-5.7) [b]
	0.1/0.1	0.8 (0.7-1.4)	1.2 (0.9-1.7)	7.2 (5.8-8.8) [b, c]
	0.5/0.5	1.7 (1.3-2.0) [b]	2.2 (1.5-3.0) [a]	10.7 (2.9-15.1) [b]

4-NP UGT activity was measured in duplicate as described in Materials and methods.
Number of animals: control group, $n = 9$; treatment groups, $n = 6$.
Results are given as ratios of treated to control (median, range).
The Wilcoxon rank sum test was used for statistical evaluation; [a]$P < 0.05$, [b]$P < 0.005$.
[c] Synergistic induction of 4-NP UGT activity, $P < 0.05$.

Treatment with the highest dose of flavone or the combination of both agents increased 4-NP UGT activity at all sites investigated. Furthermore, treatment with flavone resulted in a larger increase in 4-NP UGT activity as compared with α-angelicalactone treatment. With increasing concentrations of α-angelicalactone, flavone or the combination of both agents, hepatic 4-NP UGT activity was gradually increased. Even the lowest concentration (0.01% w/w) of flavone and the combination treatment resulted in increased 4-NP UGT activity. Treatment with the mix of α-angelicalactone and flavone (0.1% w/w) resulted in a synergistic induction of 4-NP UGT enzyme activity. In contrast to effects on 4-NP UGT activity, enhancement of 4-MUB UGT activity occurred after treatment with 0.01 and 0.05% w/w α-angelicalactone and the 0.1% w/w combination treatment resulted in induction of the mid small and large intestinal enzyme activity.

Discussion

It is well known that environmental factors affect the development of human cancers. The human diet may contain a large number of (pre)carcinogens (19, 20). However, apart from carcinogens, our diet may also contain a wide variety of compounds which inhibit mutagenesis and/or carcinogenesis, as tested in laboratory models (19, 20, 26-28). These anticarcinogens are very diverse in chemical structure and their protective mechanisms are generally unclear. However, there are strong indications that anticarcinogens are effective by virtue of enhancing detoxification systems (4, 8, 10). In a recent study in humans by Hoensch *et al.* (11) high intake of fruits or vegetables was associated with high upper gastrointestinal levels of isoenzymes of the GST system. Detoxification systems such as UGTs and GSTs can minimize carcinogenicity by conjugation reactions, which add functional groups to the carcinogen, thereby lowering their biological activity and increasing their excretion. Although in a few studies induction of hepatic UGT enzyme activity has been suggested as a protective mechanism of some dietary anticarcinogens (29, 30), little is known about the effects of anticarcinogens on UGT enzyme activity in the small intestine or colon, the latter being the organ where most gastrointestinal tumours are formed.

Flavone has been shown to induce hepatic 4-NP UGT activity (30, 31), which is in comparison with the results observed here. In our study, quercetin significantly increased

UGT enzyme activity in liver and proximal and distal small intestine. In contrast, Siess *et al.*, Brouard *et al.* and Canivenc-Lavier *et al.* (30-32) found no effect on hepatic 4-NP UGT activity. This difference may be caused by variations in dietary treatment, since Siess *et al.* and Canivenc-Lavier *et al.* used a lower dose of quercetin (0.3 versus 1.0% w/w in our study). However, Brouard *et al.* also used a dose of 1% w/w quercetin for 2 weeks, suggesting that other factors influence the level of UGT activity. For example, starvation may affect UGT activity, since rats were fasted 18 h before collecting tissues in the study of Brouard *et al.* (31), whereas in our study animals had free access to food until decapitation. Since starvation is known to rapidly decrease phase II enzyme activity (33), an initial increase in UGT enzyme activity may have disappeared during this starvation period.

After treatment with quercetin, increased UGT activity in proximal small intestine was only seen with 4-MUB as substrate. In distal small intestine this treatment induced both 4-NP and 4-MUB UGT activity. In contrast, Brouard *et al.* (31) found no induction of intestinal 4-NP UGT activity after feeding quercetin to rats. This difference may be explained as follows: Brouard *et al.* studied the overall 4-NP UGT activity of the intestine, while we measured the UGT activity in three different parts of the intestine, revealing an increase in 4-NP UGT activity in the distal part of the intestine only. Furthermore, as mentioned above, due to a starvation period in the study of Brouard *et al.*, eventual effects may have disappeared. Like Brouard *et al.* (31), we found no effect of flavone on intestinal 4-NP UGT activity, whereas dietary ellagic acid was shown by Ahn *et al.* (29) to increase hepatic 4-NP UGT activity, which was confirmed by us. However, we used a higher dose of ellagic acid, which resulted in a more elevated UGT activity.

For all other compounds studied no effects on UGT enzyme activity have been reported before. All agents had an enhancing effect on UGT activity at one or more sites investigated, except for Brussels sprouts, which had an inhibitory effect on proximal small intestinal UGT activity. Nevertheless, Brussels sprouts were able to enhance hepatic UGT activity. This discrepancy might be explained by the fact that certain metabolites of glucosinolates present in Brussels sprouts may be responsible for induction of hepatic UGT activity, whereas these metabolites may not yet have been formed in the intestine. Colonic 4-NP UGT enzyme activity was significantly increased by α-angelicalactone, coumarin, curcumin and flavone, whereas the same agents as well as quercetin significantly induced colonic 4-MUB UGT

activity. In general, the same anticarcinogens enhanced both small intestinal as well as large intestinal UGT enzyme activities.

A study on the dose-dependency effects of dietary flavone on hepatic 4-NP UGT activity was performed by Siess *et al.* (34). Increased 4-NP UGT activity was found after treatment with flavone at 0.002, 0.005, 0.02, 0.05 and 0.2% w/w. In accordance with these results, we found that both 4-NP and 4-MUB hepatic UGT activity was significantly induced by 0.01, 0.05, 0.1 and 0.5% w/w flavone. In the intestinal tissues studied here, only the highest dose of dietary flavone resulted in enhancement of UGT activity. For α-angelicalactone, for which no data on dose-response effects have been reported before, a dose dependency for both 4-NP and 4-MUB UGT activity was only seen in the liver. In conclusion, increasing concentrations of α-angelicalactone or flavone did show a gradual increase of hepatic UGT activities.

Since humans may be exposed to mixtures of anticarcinogens in their diets, it may be worthwhile to study combinations of such compounds in laboratory animals in order to learn more on possible additional effects. In general, combination treatment with α-angelicalactone and flavone showed only additive effects. However, in small intestine the 4-MUB UGT activity was significantly enhanced in the 0.1 and 0.5% w/w combination groups (1.6- and 1.8-fold, respectively), whereas in the corresponding flavone or α-angelicalactone groups no significant induction was noticed. In large intestine 4-MUB UGT activity was significantly induced in the 0.1% w/w combination group, whereas treatment with flavone or α-angelicalactone alone did not result in a significant increase. In addition, a synergistic effect on hepatic UGT activity was seen after the 0.1% w/w combination treatment. Thus, exposure to mixtures of anticarcinogens can have additive or even synergistic effects on the activities of biotransformation enzymes.

We observed higher UGT activities as measured with 4-NP, in rat large intestine as compared to small intestinal values. This is quite remarkable since in human studies phase II biotransformation enzymes were shown to be much more active in small intestine as compared with colon (17, 35). Contradictory data about rat large intestinal versus small intestinal UGT activities have been reported. Hänninen *et al.* (36), using mucosal scrapings, found that 4-NP UGT activity decreased along the small intestine, but except for the first part of the small intestine, large intestinal activity was found to be higher. However, Koster and

Noordhoek (37), measuring 1-naphthol or morphine glucuronidation capacity in intestinal cells isolated by vibration, measured much lower activities in cecum and colon as compared with the small intestine.

It has often been reported that the specific activities of GSTs and UGTs in rats gradually decrease down the small intestine, when mucosal scrapings or isolated cells were analyzed (36-39). However, in studies with *in situ* perfused segments of the rat intestine no significant difference in the total glucuronidation capacity of proximal, intermediate and distal small intestine could be found (40, 41). In the study described here, the 4-NP or 4MUB UGT enzyme activity also remained constant in different parts of the rat small intestine. The apparent discrepancy in distribution of UGTs along rat small intestine may thus be explained by either the methods used for studying the intestinal tissue, as well as by the different UGT substrates used for measurement of enzyme activity. The latter is further supported by an earlier study by us (42), in which the distribution of UGT enzyme activities along the human small intestine showed various patterns, depending on the substrates used. The activity for bilirubin UGT declined, whereas the activity for 4-NP or 4-MUB UGT seemed to increase and remain constant, respectively. This may indicate that the different UGT isoenzymes may have different expression patterns along the small intestine.

Finally, we examined the associations between 4-NP and 4-MUB UGT enzyme activities and GST activities, as measured in our earlier studies (10, 21). Like Bock *et al.* (43) a strong significant correlation was observed between 4-NP and 4-MUB UGT enzyme activity in all organs investigated. The correlation coefficient varied from 0.24 ($P < 0.05$) to 0.91 ($P < 0.0001$) for mid small intestine and liver, respectively. This may be due to the fact that glucuronidation of both substrates may be mainly catalyzed by the same isoforms of UGT (12, 16). In rats, UGT1A1, 1A6 and 1A7 are highly expressed in liver and intestine (44) and therefore total UGT enzyme activity in these organs may be mainly covered by these UGT isoenyzmes. In liver there is also a strong association between GST and UGT enzyme activities (GST, 4-NP UGT, $r_s = 0.85$, $P < 0.0001$; GST, 4-MUB UGT, $r_s = 0.84$, $P < 0.0001$), which may be explained by the fact that both GST and UGT genes are regulated by the transcription factor Nrf2 (45). Surprisingly, no or only weak correlations were found between GST and UGT enzyme activities in the intestine. An explanation could be the different expression patterns of GST and UGT isoenzymes in rat intestine and liver, in combination with the different substrate specificities of the GST or UGT isoenzymes (35, 38). Since

expression of UGT or GST isoenzymes may differ considerably from one organ to another and since UGT or GST isoenzymes may have different substrate specificities, an association between UGT and GST enzyme activities as found in one organ (liver) does not necessarily mean that such an association also exists in another organ.

In conclusion, most naturally occurring dietary anticarcinogens tested, and in particular α-angelicalactone, coumarin and curcumin, were able to increase UGT enzyme activity in liver and, to a lesser extent, in small and large intestine. Furthermore, dose-dependent effects of α-angelicalactone, flavone or a combination of both compounds on the UGT detoxification system of the liver, and to a lesser extent the intestine, was demonstrated. Such high detoxification capacity may account, at least in part, for the observed chemopreventive action of these compounds. However, one should realise that the dose of anticarcinogens applied here is unlikely to be reached in the human diet, but since the human diet may contain many of such compounds and since effects on UGTs may be additive or even synergistic, enhancement of UGT activity by these minor compounds could still play a significant role in the prevention of gastrointestinal tumours in humans.

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Effects of dietary anticarcinogens and nonsteroidal anti-inflammatory drugs on rat gastrointestinal UDP-glucuronosyltransferases

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Abstract

Dietary compounds or nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce cancer rates. Elevation of phase II detoxification enzymes might be one of the mechanisms leading to cancer prevention. We investigated the effects of dietary anticarcinogens and NSAIDs on rat gastrointestinal UDP-glucuronosyltransferases (UGT). Diets of Wistar rats were supplemented with oltipraz, α-tocopherol, β-carotene, phenethylisothiocyanate (PEITC), sulforaphane analogue compound-30, indole-3-carbinol, D-limonene, relafen, indomethacin, ibuprofen, piroxicam, acetyl salicylic acid or sulindac. Hepatic and intestinal UGT enzyme activities were quantified using 4-nitrophenol and 4-methylumbelliferone as substrates. Compound-30, D-limonene, indomethacin, ibuprofen or sulindac enhanced proximal small intestinal UGT activities. Only compound-30 was able to induce mid and distal small intestinal UGT activities. Large intestinal UGT activities were increased by ibuprofen and sulindac, whereas oltipraz, PEITC and D-limonene gave enhanced hepatic UGT activities. In conclusion, mainly rat proximal small intestinal and hepatic UGT enzyme activities were induced by dietary anticarcinogens or NSAIDs. Enhanced UGT activities might lead to a more efficient detoxification of carcinogenic compounds and thus could contribute to prevention of gastrointestinal cancer.

Introduction

There is considerable interest in identifying dietary or synthetic compounds with anticarcinogenic properties. From epidemiological studies there is growing evidence that diets containing abundant vegetables and fruit may reduce the risk of cancers, especially cancers of the gastrointestinal tract (1, 2). The human diet may contain a large number of both (pre)carcinogens as well as a variety of compounds with potential anticarcinogenic properties (3, 4).

The carotenoids, α-tocopherol and β-carotene protected rodents against development of chemically-induced tumours in the gastrointestinal tract (5-7), whereas epidemiological data on their anticarcinogenic capacity are somewhat contradictory. A case-control study showed that α-tocopherol levels in blood were inversely correlated with cancer risk (8). Subsequently, β-carotene intake was also associated with a decreased risk for developing colorectal adenomas in a case-control study (9). In addition, strong evidence was provided for a protective role of α-tocopherol or β-carotene against oesophageal, but not gastric cancer risk (10). However, in a prospective cohort study no significant association between dietary α-tocopherol or β-carotene and risk for colorectal cancer was found (11). Phenethylisothiocyanate (PEITC) and indole-3-carbinol, breakdown products of glucosinolate precursors present in cruciferous vegetables, posses anticarcinogenic properties. PEITC was shown to inhibit chemically-induced carcinogenesis in oesophagus and colon of rats (12, 13), while indole-3-carbinol was able to inhibit chemically-induced tumours in forestomach (14), colon (15, 16) and liver (17) in rodents. In contrast, indole-3-carbinol treatment for 25 weeks strongly induced glutathione S-transferase Pi (GST-Pi) foci in the liver of rats (16). The monoterpene D-limonene inhibited chemically-induced gastric (18), colonic (19) or hepatic cancer (20). Compound-30 is a structural analogue of sulforaphane (21) and a component of broccoli. Sulforaphane was demonstrated to have anticarcinogenic properties in colon of rats (13). Oltipraz is a substituted dithiolthione, which was used in humans as an antischistozomal drug. Dithiolthiones occur in cruciferous vegetables. Oltipraz was found to inhibit chemically-induced carcinogenesis in stomach (22), colon (23) and liver (24) of rats. The first clinical trial with oltipraz to investigate the effects on aflatoxin biomarkers was conducted in 1995 in 234 residents of Qidong, who were at high risk for exposure to aflatoxin and development of hepatocellular carcinoma (25). Intermittent, high-dose oltipraz was shown to

inhibit phase I activation of aflatoxins, while sustained low-dose oltipraz increased phase II conjugation of aflatoxin (26).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed drugs worldwide and have anti-inflammatory, analgesic and antipyretic activities. In addition to their therapeutic use, there is strong evidence that NSAIDs may have anticarcinogenic effects in humans. Epidemiological, animal and clinical studies suggest that NSAIDs may reduce the risk for development of and mortality from gastrointestinal cancer (27).

Relafen inhibited development of aberrant crypt foci (ACF) in azyxomethane-treated rats and suppressed development of intestinal tumours in adenomatosis polyposis coli (APC) Min mice (28). Indomethacin inhibited chemically-induced carcinogenesis in forestomach (29), colon (15) and liver (30). Ibuprofen also inhibited colon carcinogenesis (15, 27). Furthermore, ibuprofen reduced tumour multiplicity and incidence in the forestomach (31). Piroxicam inhibited carcinogen-induced ACF in colon of rats (27, 32). In APC Min mice piroxicam reduced tumour number and multiplicity (33). Chemically-induced carcinogenesis was inhibited by acetyl salicylic acid in colon (27, 32) and liver (34). Recently, a clinical trail with aspirin reported significant reduction in incidence of colorectal adenomas in patients with previous colorectal cancer (35). Sulindac suppressed the development of colonic preneoplastic lesions induced by azoxymethane (27, 36). In addition, sulindac reduced the relative risk of development of oesophageal cancer (37) and reduced tumour multiplicity and incidence in the forestomach (31). In patients treated with sulindac a reduced polyp number in the rectal segment was observed (38). However, this was not confirmed by Giardiello *et al.* (39).

The exact mechanisms of action of above-mentioned inhibitors of carcinogenesis have not been clearly defined yet. Although the chemopreventive potential of these dietary and synthetic compounds may be due to multiple mechanisms, one mode of action may be enhancement of phase II detoxification enzymes, such as UDP-glucuronosyltransferases (UGTs; ref. 40) and glutathione S-transferases (GSTs; ref. 41). UGTs conjugate a wide variety of compounds to UDP-glucuronic acid (UDPGA), while GSTs catalyze the conjugation with glutathione. Conjugation with glucuronic acid or glutathione in general results in less biologically active molecules and enhances the water solubility of the conjugated products, which facilitates excretion from the body *via* bile or urine (42). In the

digestive tract, the colon is the site where the majority of malignant tumours do develop, whereas the detoxification capacity in the colon may be critically low (43). Therefore, enhancement of the activity of such enzymes could potentially increase the capacity to withstand the burden of toxic agents and (pre)carcinogens we are exposed to daily (3, 4). Knowledge of the exact protection mechanism(s) of dietary anticarcinogens and NSAIDs may be of importance for the prevention of gastrointestinal cancer. Previous research demonstrated that several dietary agents and NSAIDs increased GST enzyme activities in liver and intestine of male Wistar rats (44-46). Therefore, we here investigated the effects of naturally occurring or synthetic anticarcinogens and NSAIDs on rat hepatic and intestinal UGT enzyme activities.

Materials and methods

Materials

Oltipraz was from Rhone Poulenc Rorer (France). Bovine serum albumin, dithiothreitol, 4-methylumbelliferone (4-MUB), UDPGA and D-saccharic acid 1,4-lactone, α-tocopherol, β-carotene, phenethylisothiocyanate (PEITC), indole-3-carbinol, relafen, indomethacin, ibuprofen, piroxicam, acetyl salicylic acid and sulindac were purchased from the Sigma Chemical Company (USA). D-limonene was obtained from Aldrich Chemie (Germany). 4-Nitrophenol (4-NP) was from Merck (Germany). Sulforaphane analogue compound-30 was synthesized as described before (21). All dietary anticarcinogens and NSAIDs used were of the highest grade purity commercially available.

Study design

Hepatic and intestinal microsomes were prepared from Wistar rats, kept and treated as described in previous studies performed by Van Lieshout *et al.* (44-46). In short, male Wistar rats (Central Laboratory Animal Centre, University of Nijmegen, The Netherlands) were housed in pairs on wooden shavings in macrolon cages maintained at 20-25°C and 30-60% relative humidity. A ventilation rate of 7 air cycles/h and a 12 h light/dark cycle were used. The rats were randomly assigned to one of the dietary treatment groups. All groups were fed powdered RMH-TM laboratory chow (Hope Farms, Woerden, The Netherlands). After acclimatization for seven days, the animals were fed either the basal diet (control group) or one of the experimental diets.

Diets

The diets were prepared by supplementation with either one of the dietary compounds: 0.03% (w/w) oltipraz, 0.02% (w/w) α-tocopherol, 0.02% (w/w) β-carotene, 0.045% (w/w) PEITC, 0.145% (w/w) sulforaphane analogue compound-30, 0.025% (w/w) indole-3-carbinol and 1.0% (w/w) D-limonene, or with one of the following NSAIDs: 0.02% (w/w) relafen, 0.0025% (w/w) indomethacin, 0.04% (w/w) ibuprofen, 0.04% (w/w) piroxicam, 0.04% (w/w) acetyl salicylic acid and 0.032% (w/w) sulindac. Food and water were available *ad libitum*. Food cups were replenished every 2-3 days. Food consumption and gain in body weight was recorded daily. After 2 weeks the animals were killed by decapitation.

Tissue preparation

Tissue handling, isolation of liver and intestinal mucosa and preparation of microsomal pellet was performed as described previously (44). In short, liver tissue and intestinal mucosa were homogenised in buffer A (4 ml buffer A/g tissue (liver) or mucosal scraping (intestine); buffer A = 0.25 M saccharose, 20 mM Tris-HCl, 1 mM dithiothreitol, pH 7.4), followed by centrifugation at 9000 *g* (4°C) for 30 min. The resulting supernatant fraction was spun at 150 000 *g* (4°C) for 60 min, resulting in the sedimentation of the microsomes. The microsomal pellet was resuspended in 5 vol. of buffer A.

Assays

Protein concentration was assayed in duplicate by the method of Lowry *et al.* (47) using bovine serum albumin as the standard.

UGT activity with 4-MUB or 4-NP as substrates was measured in the microsomes as described previously (48). Briefly, liver and intestinal microsomes were resuspended in 50 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 1 mM dithiothreitol.

Conjugation of 4-MUB was performed in the presence of 4 mM UDPGA, 10 mM $MgCl_2$, 0.025-0.25 mg microsomal protein, 1 mM saccharic acid 1,4-lactone and 0.1 mM 4-MUB in a final volume of 0.1 ml Tris-HCl, pH 7.4. 4-MUB was dissolved in ethanol (50 mM) and diluted with assay medium just before use. After incubation at 37°C for 0.5-20 min, 1 ml 0.5 M glycine/NaOH, pH 10.35, was added to the reaction mixture to terminate the reaction. Subsequently, samples were centrifuged for 10 min at 10 000 *g*. 4-MUB was determined fluorometrically with a Shimadzu RF-5000 spectrofluorophotometer (excitation 370 nm, emission 450 nm).

Conjugation of 4-NP was measured in a final volume of 0.1 ml Tris-HCl, pH 7.4, containing 3.5 mM UDPGA, 10 mM $MgCl_2$, 0.05-0.5 mg microsomal protein, 1mM saccharic acid 1,4-lactone, and 1 mM 4-NP. 4-NP was dissolved in 0.01 M NaOH. After incubation for 1-20 min at 37°C, the reaction was terminated by adding 2 ml of 0.3 M NaOH, followed by centrifugation for 10 min at 10 000 *g*. Absorbance was measured at 405 nm on a Perkin Elmer Lambda 12 spectrophotometer. All samples were measured in duplicate. In all assays a control sample without UDPGA was run simultaneously. The absorbance difference between the control sample and the sample incubated in the presence of UDPGA represents the amount of 4-MUB or 4-NP that was conjugated. Effects of anticarcinogens on UGT enzyme activity are presented as ratios of treated to control.

Statistical analysis

Wilcoxon rank sum test was used to assess statistical significance of differences between control and treatment groups. Correlation analyses between enzyme activities were performed using Spearman rank correlation. $P < 0.05$ was considered to be significant.

Results

The effects of feeding the dietary agents and NSAIDs on intestinal and hepatic 4-MUB UGT enzyme activities are summarized in Table I. Compound-30 was able to induce 4-MUB UGT activity in all studied parts of the small intestine (1.4×, 1.5× and 1.4×, respectively). Proximal small intestinal 4-MUB UGT activity was also enhanced by D-limonene (1.2×), indomethacin, ibuprofen and sulindac (1.1×, 1.2×, and 1.2×, respectively). Large intestinal 4-MUB UGT activity was only increased by ibuprofen (1.4×). Furthermore, hepatic 4-MUB UGT activity was enhanced after treatment with oltipraz (2.4×), PEITC (2.1×) and D-limonene (3.2×). No statistically significant changes in 4-MUB UGT activity were found with α-tocopherol, β-carotene, indole-3-carbinol, relafen and acetyl salicylic acid in all organs investigated. The effects of the different anticarcinogens on intestinal and hepatic 4-NP UGT enzyme activities are the same as observed for 4-MUB UGT activity, except for acetyl salicylic acid which also induced 4-NP UGT activity in the proximal small intestine (1.3×). However, sulindac was not able to enhance 4-NP UGT activity in the proximal small intestine, though it increased large intestinal 4-NP UGT enzyme activity (1.7×).

Table I. Effects of dietary anticarcinogens or NSAIDs on rat intestinal- and hepatic 4-MUB UGT enzyme activities

Treatment group	*n*	4-MUB UGT activity (ratio treated/control)				
		Small intestine			Large intestine	Liver
		Proximal	Mid	Distal		
Oltipraz	8	1.3 (0.8-1.8)	0.69 (0.6-2.9)	0.90 (0.6-2.8)	1.1 (0.6-1.8)	2.4 (1.0-4.9) [a]
α-Tocopherol	8	1.1 (0.8-2.0)	0.88 (0.4-1.8)	1.4 (0.6-2.7)	0.81 (0.5-2.7)	1.1 (0.4-1.9)
β-Carotene	8	0.89 (0.8-1.0)	0.66 (0.5-0.9)	1.4 (0.7-3.3)	0.73 (0.4-1.8)	1.4 (0.6-2.4)
PEITC	8	0.86 (0.8-1.1)	0.69 (0.5-1.3)	1.3 (1.0-3.1)	0.87 (0.4-1.6)	2.1 (0.8-3.4) [a]
Compound-30	8	1.4 (1.0-2.1) [b]	1.5 (1.3-2.0) [a]	1.4 (1.2-1.8) [a]	1.0 (0.9-1.5)	1.0 (0.6-3.1)
Indole-3-carbinol	8	1.1 (0.7-1.8)	1.1 (0.8-2.6)	0.87 (0.5-1.5)	1.1 (0.6-1.5)	1.1 (0.7-4.7)
D-Limonene	8	1.2 (1.0-2.0) [a]	1.0 (0.8-1.4)	1.3 (0.7-2.1)	0.84 (0.4-1.0)	3.2 (2.2-4.9) [b]
Relafen	8	1.0 (0.5-1.3)	1.1 (0.9-1.5)	0.86 (0.71-1.4)	0.86 (0.7-1.0)	1.5 (0.5-2.3)
Indomethacin	8	1.1 (1.0-1.6) [a]	1.1 (0.8-1.6)	1.2 (1.0-1.4)	1.1 (0.4-1.6)	1.0 (0.6-1.9)
Ibuprofen	8	1.2 (1.0-1.7) [a]	1.0 (0.7-1.4)	1.1 (0.7-1.2)	1.4 (1.0-2.2) [a]	1.0 (0.5-2.1)
Piroxicam	8	1.0 (0.8-1.4)	0.95 (0.8-1.4)	0.98 (0.7-1.3)	0.83 (0.6-1.2)	0.85 (0.6-2.9)
Acetyl salicylic acid	8	1.1 (0.7-1.6)	1.1 (1.0-1.3)	0.95 (0.8-1.3)	1.0 (0.6-1.3)	1.0 (0.8-1.8)
Sulindac	8	1.2 (1.0-1.4) [a]	1.0 (0.8-1.2)	0.90 (0.6-1.1)	1.1 (0.4-1.9)	0.91 (0.7-2.0)

4-MUB UGT activity was measured in duplicate as described in Materials and methods.
n, number of rats used in each group.
Results are given as median ratios of treated to control (range). In the control group ($n = 8$) median 4-MUB UGT activities (range) were 0.25 (0.17-0.29), 0.29 (0.21-0.41), 0.31 (0.20-0.49), 0.24 (0.10-0.33) and 10 (6-15) nmol/min·mg protein for proximal-, mid- and distal
small intestine, large intestine and liver, respectively.
The Wilcoxon rank sum test was used for statistical evaluation; $^{a}P < 0.05$, $^{b}P < 0.01$.

Discussion

It is well known that environmental factors affect the development of human cancers. The human diet contains a large number of both (pre)carcinogens as well as a variety of compounds that may inhibit mutagenesis and/or carcinogenesis as tested in laboratory models (3, 4). Anticarcinogens are very diverse in chemical structure and their protective mechanisms are generally unclear. Although prevention of cancer may be due to multiple mechanisms, one mode of action of anticarcinogens may be enhancement of the carcinogen detoxification systems, such as UGTs and GSTs (40, 41). These detoxification systems can minimize

carcinogenicity by conjugation reactions, which add functional groups to the carcinogen, thereby lowering their biological activity and increasing their excretion.

In human organs at high risk for cancer development, low UGT levels were measured (43). At present little information on the effects of dietary and synthetic anticarcinogens on the UGT activity of the digestive tract is available. Recent data, mostly obtained from animal studies, have indicated that naturally occurring dietary anticarcinogens may be able to elevate UGT activity (49, 50).

Oltipraz has been shown to elevate hepatic 4-NP UGT enzyme activity (23, 49, 51), which is similar to the results observed here. Rao *et al.* (23) also described a significant induction of colonic 4-NP UGT activity after oltipraz treatment in F344 rats, whereas we found no effect on colonic UGT activities in Wistar rats. Furthermore, oltipraz treatment did not significantly change UGT activities in rat proximal small intestine (51) and in other parts of the small intestine investigated here.

In agreement with the results reported by Astorg *et al.* (52), we found no effect of β-carotene on hepatic 4-NP UGT enzyme activity.

For PEITC, we found hepatic 4-NP UGT enzyme activity to be significantly increased, whereas a somewhat lower enhancement was also observed by Guo *et al.* (50), which may be explained by monitoring 48 h after treatment.

Except for one recent report on changes in UGT1A1 mRNA and protein levels in HepG2 and HT29 cells (53), induction of UGT enzyme activity by sulforaphane or one of its analogues has not been reported before. Here we now demonstrate that the sulforaphane analogue compound-30 significantly enhances UGT enzyme activities in small intestine, but not in large intestine and liver.

In the past, effect of indole-3-carbinol on hepatic UGT enzyme activity was only examined using 1-naphthol as substrate, and no effect could be observed [54]. This is in accordance with our results since we found no effect of indole-3-carbinol on either hepatic or intestinal UGT enzyme activity with both 4-NP and 4-MUB as substrates.

Like Elegbede *et al.* (55), we investigated the effects of D-limonene treatment on hepatic UGT enzyme activity. We found that 1% D-limonene significantly elevated 4-NP and 4-MUB UGT activities, whereas Elegbede *et al.* only observed an inducing effect with 5% and not 1% D-limonene on α-naphthol UGT activity. Such variations in results may be either caused by

the use of different substrates (4-NP versus α-naphthol) or by the different strains of rats. With regard to the effects of NSAIDs, Falzon *et al.* (56) described that 8.5 mg/kg indomethacin given intraperitoneally for 3 days, significantly decreased hepatic 4-NP UGT activity by 22%. Furthermore, pallor of the liver and severe intestinal lesions were observed. We found no macroscopic signs of toxicity of indomethacin in all organs studied. In addition, we observed no inhibitory effects of indomethacin on 4-NP or 4-MUB UGT enzyme activities.

No earlier reports on the effects of α-tocopherol, relafen, ibuprofen, piroxicam, acetyl salicylic acid and sulindac on hepatic or intestinal UGT enzyme activity were found.

Until recently, the tissue distribution of only the UGT1A family had been examined in liver and gastrointestinal tract of rats (57). Now, Shelby *et al.* (58) reported on the mRNA expression of the different members of the UGT1 and UGT2 family in the rat gastrointestinal tract. In the intestine many UGT1 and only few UGT2B mRNAs were expressed, in contrast with the liver where many UGT2B mRNAs were predominantly expressed. The glucuronidation of NSAIDs may be mainly catalyzed by the isoenzymes UGT1A6 (intestine) and UGT2B (liver; ref. 59). In this study we measured UGT enzyme activity with 4-NP and 4-MUB as substrates: both substrates mainly react with the isoenzymes of UGT1 family (40, 42). This might explain why we did not find any effect of NSAIDs on the UGT enzyme activity in the liver.

Finally, we examined the associations between 4-MUB and 4-NP UGT, and GST enzyme activities, as measured in our earlier studies (44-46), and the data are presented in Table II. In general, strong correlations were observed between 4-MUB and 4-NP UGT enzyme activities in liver and, to a much lesser extent in small and large intestine. Bock *et al.* (60) also found a strong association between 4-MUB and 4-NP UGT activities in the liver. This may be due to the fact that glucuronidation of both substrates may be mainly catalyzed by the same UGT isoforms, and that by far the highest activities are present in liver (40, 42). In rats UGT1A1, 1A6 and 1A7 are highly expressed in liver and intestine (57), and therefore total UGT enzyme activity in these organs may be mainly covered by these UGT isoforms. Furthermore, significant associations between GST and UGT enzyme activities are also observed, however, the correlation coefficients are low. The associations found may be explained by the fact that both GST and UGT genes are regulated by the same transcription factor Nrf2 (61).

Table II. Correlations between UGT and GST enzyme activities

Study (ref.)	Associations studied	Correlation coefficients (rs)				
		Small intestine			Large intestine	Liver
		Proximal	Mid	Distal		
Van Lieshout 1996 (44)	GST, 4-NP UGT	ND	ND	ND	0.35 [a]	0.35 [a]
	GST, 4-MUB UGT	ND	ND	ND	0.28	0.31 [a]
	4-NP UGT, 4-MUB UGT	ND	ND	ND	0.43 [a]	0.94 [b]
Van Lieshout 1998 (46)	GST, 4-NP UGT	0.49 [c]	0.22	0.29	0.072	0.067
	GST, 4-MUB UGT	0.41 [a]	0.23	0.28	0.20	0.066
	4-NP UGT, 4-MUB UGT	0.66 [b]	0.54 [c]	0.63 [b]	0.084	0.70 [b]
Van Lieshout 1997 (45)	GST, 4-NP UGT	0.35 [a]	0.58 [b]	0.29	0.075	0.26
	GST, 4-MUB UGT	0.40 [a]	0.53 [c]	0.17	0.23	0.23
	4-NP UGT, 4-MUB UGT	0.31 [a]	0.071	0.31 [a]	0.48 [c]	0.69 [b]

Correlation analyses between GST and 4-NP UGT, GST and 4-MUB UGT, 4-NP and 4-MUB UGT enzyme activities were performed using values from individual animals.
GST data are from earlier studies by us (44-46).
ND: no GST and 4-NP UGT data available.
Spearman rank correlation was used for statistical evaluation; [a]$P < 0.05$, [b]$P < 0.0001$, [c]$P < 0.005$.

In the present study we demonstrated that dietary anticarcinogens or NSAIDs are capable of inducing UGT enzyme activities in the rat gastrointestinal tract, particularly in the proximal small intestine or liver. This may be of direct significance in the protection against cancer in the particular organ. However, organs such as the colon could also benefit from a more efficient detoxification in the proximal part of the digestive tract, since lower levels of carcinogens may reach the colon.

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Chapter 3.1

Genetic polymorphisms in UDP-glucuronosyltransferases and glutathione S-transferases and colorectal cancer risk

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Abstract

Colorectal cancer (CRC) is one of the most common malignancies in the Western world showing an increasing incidence, and has been associated with genetic and lifestyle factors. Individual susceptibility to CRC may be due partly to variations in detoxification capacity in the gastrointestinal tract. Genetic polymorphisms in detoxification enzymes may result in variations in detoxification activities, which subsequently might influence the levels of toxic/carcinogenic compounds, and this may influence the risk for CRC. To determine whether genetic polymorphisms in detoxification enzymes predispose to the development of CRC, 371 patients with sporadic CRC and 415 healthy controls were genotyped for polymorphisms in the important detoxification enzymes UDP-glucuronosyltransferase *UGT1A1*, *UGT1A6*, *UGT1A7* and *UGT1A8*, and glutathione S-transferase *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1*. Patients and controls were all of Caucasian origin. DNA was isolated from either blood or tissue and tested by polymerase chain reaction followed by restriction fragment length polymorphism analyses. Logistic regression analyses showed significant age- and gender-adjusted risks for CRC associated with variant genotypes of *UGT1A6* [OR 1.5, 95% confidence interval (CI) 1.03-2.3] and *UGT1A7* (OR 2.4, 95% CI 1.3-4.6), whereas no associations were found between CRC and the other polymorphic genes as mentioned above. In conclusion, these data suggest that the presence of variant *UGT1A6* and *UGT1A7* genotypes with expected reduced enzyme activities, might enhance susceptibility to CRC.

Introduction

Colorectal cancer (CRC) is an important cause of death in Western countries. In The Netherlands, it is the second cause of death from malignant disease in women, and the third cause of death in men (1). It is estimated that up to 10% of CRC cases can be attributed to hereditary factors of high penetrance (2) leaving ~90%, so called sporadic CRC cases, which may be attributed to diet, lifestyle factors and genetic factors of low penetrance. Epidemiological studies have shown that diets low in fruit and vegetables, and high in red meat and fat are associated with an increased risk of CRC (3, 4). Humans may be exposed daily to a large variety of toxic or even carcinogenic compounds, present in food (5) or as a result of lifestyle habits such as smoking of cigarettes (6, 7). However, humans also possess a highly efficient system of defence against such harmful compounds, and the detoxification enzymes are a main part of this.

Detoxification enzymes such as UDP-glucuronosyltransferases (UGTs; ref. 8) and glutathione S-transferases (GSTs; 9) are responsible for the efficient modification of harmful molecules, making them less biologically active and facilitating their excretion. These enzymes are present predominantly in the gastrointestinal tract, especially in the liver, where detoxification enzymes have been identified at very high levels. However, these enzymes have also been distinguished in extra-hepatic tissues, including skin, kidney, intestine and many other organs (8-11). Since the gastrointestinal tract is in direct contact with potentially carcinogenic agents, ingested by food, medication, drugs, etc. the intestinal mucosa acts as a first line barrier. Tissue-specific expression of the various isoforms of detoxification enzymes in colon and liver was shown to contribute to the differences in enzyme activities observed in these tissues (10-13). In addition, variations due to the presence of genetic polymorphisms may contribute to the inter-individual differences in expression levels and enzyme activities of these enzymes (9, 14). These genetic polymorphisms may result in variations in detoxification activities, which also might influence the levels of toxic/carcinogenic compounds in the colon. Therefore, polymorphisms in the detoxification enzymes could contribute to individual susceptibility to CRC.

UGTs and GSTs are important families of detoxification enzymes. UGTs catalyze the conjugation of a wide variety of exogenous (e.g. drugs, pesticides, components of tobacco

smoke) and endogenous (e.g. bilirubin, bile acids, steroid hormones) compounds to glucuronic acid (8), while GSTs catalyze the reaction of glutathione with exogenous electrophiles (e.g. polycyclic aromatic hydrocarbons, heterocyclic amines) and endogenous products of oxidative stress (9, 10). The metabolites formed by these reactions are generally less toxic and more water soluble, which facilitates their biliary and renal excretion. Polymorphic variations in these detoxification enzymes may influence the rates of conversion of toxic or carcinogenic compounds. Many genetic polymorphisms in UGTs or GSTs have been described and some have been associated with increased CRC susceptibility (15-18).

In humans, two UGT families have been classified: UGT1A and UGT2 (19). So far, nine functional UGT1A isoenzymes (UGT1A1, UGT1A3-UGT1A10) have been characterized, all derived from a single gene locus on chromosome 2 (8, 20). UGT1A enzymes are mainly involved in the metabolism of exogenous compounds, this is not strictly the case however as bilirubin and steroid hormones are important endogenous substrates. UGT2 isoenzymes 2A1, 2B4, 2B7, 2B10, 2B11, 2B15 and 2B17 are involved mainly in the glucuronidation of endogenous compounds and therefore were not studied further here. Most UGT1A family members are expressed at low levels in the colon. UGT1A7, UGT1A8 and UGT1A10 are expressed only extra-hepatically and may be highly relevant for colonic detoxification (21). Several functional polymorphisms in UGT1A family isoenzymes have been identified (8, 14, 22, 23). In Caucasians homozygosity for the *UGT1A1*28* polymorphism in the *UGT1A1* promoter results in significantly reduced hepatic bilirubin UGT enzyme activity (24, 25), leading to a mild form of hyperbilirubinemia, known as Gilbert's syndrome (26, 27). Two missense mutations in exon 1 of *UGT1A6* have been described, which results in T181A and R184S amino acid changes (28). These polymorphisms are usually linked on one allele (*UGT1A6*2*), although alleles carrying only the R184S polymorphism (*UGT1A6*3*) are found occasionally. Metabolism rates of phenols by recombinant *UGT1A6*2* were lower than those of the most common enzyme. For *UGT1A7*, eight allelic variants of the most common *UGT1A7*1* allele have been described (29, 30); however, only *UGT1A7*1* to **4* have been identified in Caucasians. Complete loss, or a very strong reduction, of activity was reported for *UGT1A7*3* (29), whereas substantial reduction of activity was demonstrated for *UGT1A7*2* and *UGT1A7*4*. In *UGT1A8*, two missense mutations in exon 1 were identified, resulting in an A173G substitution with little impact on catalytic activity, in contrast to the substitution of C277Y yielding an inactive enzyme (22).

In humans, the GST family comprises four main classes (alpha, mu, pi, theta) which genes are mapped on different chromosomes (9). Coles *et al.* described a polymorphism at nucleotide 69 in 5'-regulatory region of the *GSTA1* gene (31). Homozygotes for this polymorphism (GSTA1*B) have reduced enzyme activity compared with GSTA1*A homozygotes. Five GST Mu class genes (*M1* to *M5*) have been identified clustered on chromosome 1 (32). For intestine GSTM1 seems most important, however 40 to 60% of the Caucasians do not express GSTM1 due to the *GSTM1 null* genotype (16, 33). GSTP1, the only member of the GST Pi class, appears to be the most widely distributed GST isoenzyme (10). A functional polymorphism has been described for *GSTP1* resulting in an I105V substitution (34) and leading to a lower enzyme activity (35, 36). Two GST Theta class genes, *GSTT1* and *GSTT2*, have been characterized and in humans a *GSTT1 null* genotype may be present at a frequency of ~10-20% in Caucasians (16, 37).

Aim

To determine whether genetic polymorphisms in several important isoenzymes of the UGT and GST family predispose to the development of CRC, 371 Caucasian patients with sporadic CRC and 415 Caucasian healthy controls were genotyped for polymorphisms in *UGT1A1*, *UGT1A6*, *UGT1A7*, *UGT1A8*, *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1*.

Materials and Methods

Patients and control subjects

A total of 371 patients (212 males, 159 females; mean age 64 ± 11 yrs) with sporadic CRC were recruited at the Departments of Gastroenterology and General Surgery, University Medical Centre St Radboud, Nijmegen, The Netherlands. 415 healthy subjects (168 males, 247 females; mean age 42 ± 12 yrs) served as controls and were recruited by advertisement in a local paper. All subjects were of Caucasian origin.

DNA isolation

Whole blood from 280 CRC patients and 415 healthy controls was obtained by venapuncture in sterile vacutainer tubes anti-coagulated with EDTA and stored at –20ºC until use. Most blood samples from CRC patients were collected at the Department of Clinical Chemistry.

DNA was isolated from whole blood using the Pure Gene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the instructions of the manufacturer and stored at 4ºC. For 91 patients with CRC no blood samples were available and here DNA was isolated from resected normal colorectal tissue obtained at the Department of Surgery. After surgical resection, tissue specimens were immediately frozen in liquid nitrogen and stored at –80ºC until use. DNA was extracted from normal colorectal mucosa using phenol-chloroform-isoamylalcohol extraction according to Maniatis *et al.* (38). Cancer diagnosis was confirmed by histopathological investigation of tissue specimens by a pathologist. Cases were classified according to Dukes' stages (A, B, C, D) and according to location of the tumour in the large intestine as either proximal (cecum, ascending or transverse) or distal (descending, sigmoid, rectosigmoid junction or rectum).

Genotyping

UGT1A1. The number of TA repeats in the promoter region of the *UGT1A1* gene was analyzed using the polymerase chain reaction (PCR) conditions and primers (Table I) as described by Monaghan et al. (24). Amplification was confirmed by agarose electrophoresis before fragments were resolved on 12% polyacrylamide gels (19:1 acrylamide/bisacrylamide; Biorad) in Tris-borate-EDTA buffer. Gels (20 × 20 × 0.075 cm) were run at 400 V for 3 h and stained with ethidium bromide for 30 min (25). Fragments of 98 bp indicate the *UGT1A1*1* allele, containing six TA repeats, and fragments of 100 bp indicate the *UGT1A1*28* allele, containing seven TA repeats.

GSTM1 and GSTT1. The *GSTM1* genetic polymorphism was determined by PCR according to the method of Brockmöller *et al.* (33). As an internal positive control, the β-globin gene was co-amplified, whereas sterile H_2O was substituted for genomic DNA and served as a negative control. PCR primers are given in Table I. PCR products were subjected to electrophoresis on 1% agarose gels and analyzed for the presence of a 650-bp product, which is indicative of at least one functional *GSTM1* allele. The lack of an amplification product is consistent with the *null* genotype. The procedure followed to detect the *GSTT1 null* polymorphism is similar to that of *GSTM1* and it is based on the method of Pemble *et al.* (37). PCR reactions and electrophoretic analyses were performed under the same conditions as described for *GSTM1*. Amplifications were performed in duplicate. The visualization of a 480-bp product indicates the presence of a least one functional *GSTT1* allele.

Other investigated genes. The polymorphisms in all other investigated genes were studied using PCRs followed by restriction fragment length polymorphism (RFLP) analyses. The primers and restriction enzymes used for PCR-RFLP are shown in Table I.

Table I. Primers and restriction enzymes used for genotyping analyses

Gene	Primers (5'-3'; F = forward, R = reverse)	Restriction enzyme
UGT1A1	F: GTC ACG TGA CAC AGT CAA AC	-
	R: TTT GCT CCT GCC AGA GGT T	
UGT1A6 codon 181 and 184	F: GGA AAA TAC CTA GGA GCC CTG TGA	T181A: *Nsi*I
	R: AGG AGC CAA ATG AGT GAG GGA G	R184S: *Fnu*4HI
UGT1A7 codon 129 and 131	F1: AAT TGC AGG AGT TTG **a**TT AA [a]	*Vsp*I
	F2: AAT TGC AGG AGT TTG **a**TT A [a]	*Vsp*I
	R1: TTC AGA GGC TAT TTC TAA GA	
UGT1A7 codon 208	F3: ATG CTC GCT GGA CGG CAC CAT TG	*Rsa*I
	R2: TGC CGT GAC AGG GGT TTG GAG A	
UGT1A7 allele specific	F4: ATT GCA GGA GTT TGT TTA AGG ACA	*Rsa*I
	R1: TTC AGA GGC TAT TTC TAA GA	
UGT1A8 codon 173	F: CAG TTC TCT CAT GGC TCG CA	*Alu*I
	R: GTG TGG CTG TAG AGA TCA TAT GCT	
UGT1A8 codon 277	F: TCT TCA TTG GTG GTA TCA **G**CT [a]	*Pvu*II
	R: AAA ATT TGA TAA CTG ATG AGT ACA TA	
GSTA1	F: TGT TGA TTG TTT GCC TGA AAT T	*Ear*I
	R: GTT AAA CGC TGT CAC CGT CC	
GSTM1	F: CTC CTG ATT ATG ACA GAA GCC	-
	R: CTG GAT TGT AGC AGA TCA TGC	
β-Globin	F: CAA CTT CAT CCA CGT TCA CC	-
	R: GAA GAG CCA AGG ACA GGT AC	
GSTP1 codon 105	F: GTA GTT TGC CCA AGG TCA AG	*Alw*26I
	R: AGC CAC CTG AGG GGT AAG	
GSTT1	F: TTC CTT ACT GGT CCT CAC ATC TC	-
	R: TCA CCG GAT CAT GGC CAG CA	

[a] Bold 'a' in the primer sequence denotes site-directed mutagenesis for introduction of a *Vsp*I restriction site in the wild-type allele; bold 'G' in the primer sequence creates a restriction site for *Pvu*II in the wild-type allele.

PCR-RFLP assays were adapted from methods described earlier and were used to identify the polymorphic variants of the following genes: *UGT1A6* (28, 39), *GSTA1* (31) and *GSTP1* (35). Polymorphic variants in the *UGT1A7* and *UGT1A8* genes were identified by PCR-RFLP

methods developed in our laboratory. In short, to detect the variations at *UGT1A7* codons 129/131, we used the forward primers F1 and F2 (the 'a' in the primer sequence denotes site-directed mutagenesis for introduction of a *Vsp*I restriction site in the wild-type allele) and the reverse primer R1 (Table I). F1 only detects the N129K/R131K polymorphism; F2 detects both the N129K/R131K and N129R/R131K polymorphisms (see Figures 1A and 1B).

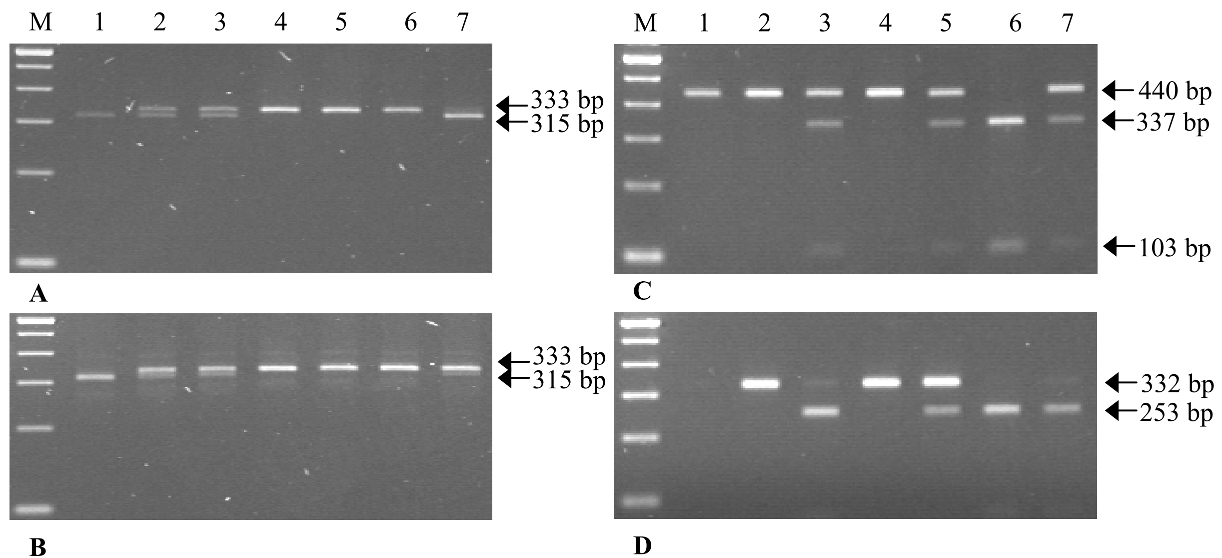

Fig. 1. Genotyping of *UGT1A7* 129/131 and 208 polymorphisms with PCR-RFLP. (**A**) N129K/R131K PCR fragment digested with *Vsp*I: most common: 315 + 18 bp; heterozygous: 333 + 315 + 18 bp; homozygous 333 bp. (**B**) N129K/R131K or N129R/R131K PCR fragment digested with *Vsp*I: most common: 315 + 18 bp; heterozygous: 333 + 315 + 18 bp; homozygous 333 bp. (**C**) W208R PCR fragment digested with *Rsa*I: most common: 440 bp; heterozygous: 440 + 337 + 103 bp; homozygous: 337 + 103 bp. (**D**) Allele specific PCR for the detection of N129K/R131K or N129R/R131K variations, digested with *Rsa*I: no product: the 129/131 mutations are not present; 332 bp product: only the 129/131 mutations are present or the 129/131 mutations are not in the same allele as the 208 mutation; 253 + 79 bp products: the 129/131 mutations are in the same allele as the 208 mutation; 332 + 253 + 79 bp products: the 129/131 mutations are present in one allele and in the other allele the 129/131 mutations and the 208 mutation are present. Lane M, 100 bp marker; lane 1, *UGT1A7*1*1*; lane 2, *UGT1A7*1*2*; lane 3, *UGT1A7*1*3*; lane 4, *UGT1A7*2*2*; lane 5, *UGT1A7*2*3*; lane 6, *UGT1A7*3*3*; lane 7, *UGT1A7*1*10* (genotype *UGT1A7*2*4* was not identified; alleles carrying only the W208R polymorphism were not found). The sizes of the PCR fragments are indicated.

To detect the W208R alteration, we used the forward primer F3 and the reverse primer R2 (Table I and Figure 1C). To determine whether the N129K/R131K or N129R/R131K and W208R occur *cis* or *trans*, we use the primers F4 and R1 (Table I and Figure 1D). PCR conditions were 5 min at 95ºC, then 37 cycles of 30 s at 95ºC, 30 s at 55ºC (codons 129/131) / 65ºC (codon 208) / 56ºC (allele specific), and 30 s at 72ºC, and finally an elongation step at 72ºC for 5 min. Aliquots of 10 µl of the PCR product were digested with the restriction

enzyme *Vsp*I (codons 129/131) or *Rsa*I (codon 208 / allele specific) for at least 1 h at 37ºC (see Figure 1).

The polymorphisms in the *UGT1A8* gene corresponding with amino acid substitutions at position 173 and 277 were analyzed with two separate PCRs followed by RFLP analyses. The primers used for the PCR to detect the A173G substitution are shown in Table I. The PCR conditions were 4 min at 95ºC, then 35 cycles of 30 s at 95ºC, 1 min at 58ºC, and 1 min at 72ºC, and finally an elongation step at 72ºC for 7 min. A 750-bp product was amplified and aliquots of 5 μl of the PCR mixture were digested for 1 h at 37°C with the restriction enzyme *Alu*I, followed by electrophoresis on 3% agarose gel, containing ethidium bromide. The *UGT1A8*2* allele (A173G) contains only one restriction site for *Alu*I, instead of two restriction sites for the *UGT1A8*1* and *UGT1A8*3* alleles (see Figure 2A).

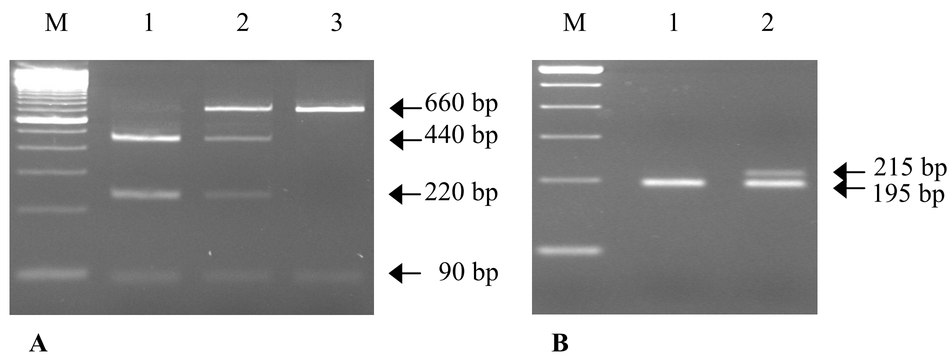

Fig. 2. Genotyping of *UGT1A8* 173 and 277 polymorphisms with PCR-RFLP. (**A**) Electrophoresis patterns of PCR fragments after digestion with *Alu*I for detection of the A173G polymorphism and (**B**) after digestion with *Pvu*II for the C277Y polymorphism. Lane M, 100 bp marker; lane 1, homozygosity for the common allele; lane 2, heterozygosity; lane 3, homozygosity for the variant allele (homozygosity for the C277Y polymorphism was not found). The sizes of the PCR fragments are indicated.

The primers used to detect the C277Y substitution by PCR are shown in Table I (the last G of the forward primer creates a restriction site for *Pvu*II in the wild-type allele). Except for the annealing temperature, which was 1 min at 49ºC, similar PCR conditions were used as described above for detection of the A173G substitution. The digestion of the 215-bp PCR product with *Pvu*II was carried out under similar conditions as described above. The *UGT1A8*3* allele (C277Y) contains no restriction site for *Pvu*II, distinct from the *UGT1A8*1* and *UGT1A8*2* alleles, which have one *Pvu*II restriction site (Figure 2B). During each PCR analysis, sterile H_2O was added instead of genomic DNA in several wells of the 96-wells PCR

plate, which served as negative control for amplification. All genotypes analyzed are summarized in Table II.

Table II. Polymorphic variants of the detoxification enzymes investigated

Polymorphic variant	Allelic variation [a]
*UGT1A1*1*	$(TA)_6TAA$
*UGT1A1*28*	$(TA)_7TAA$
*UGT1A6*1*	$T^{181}R^{184}$
*UGT1A6*2*	$\mathbf{A}^{181}\mathbf{S}^{184}$
*UGT1A6*3*	$T^{181}\mathbf{S}^{184}$
*UGT1A7*1*	$N^{129}R^{131}W^{208}$
*UGT1A7*2*	$\mathbf{K}^{129}\mathbf{K}^{131}W^{208}$
*UGT1A7*3*	$\mathbf{K}^{129}\mathbf{K}^{131}\mathbf{R}^{208}$
*UGT1A7*10*	$\mathbf{R}^{129}\mathbf{K}^{131}\mathbf{R}^{208}$
*UGT1A8*1*	$A^{173}C^{277}$
*UGT1A8*2*	$\mathbf{G}^{173}C^{277}$
*UGT1A8*3*	$A^{173}\mathbf{Y}^{277}$
*GSTA1*A*	-69C
*GSTA1*B*	-69**T**
GSTM1 +	At least one functioning allele
GSTM1 null	Deletion in both alleles
GSTP1 Ile 105	I^{105}
GSTP1 Val 105	$\mathbf{V}^{105}$
GSTT1 +	At least one functioning allele
GSTT1 null	Deletion in both alleles

[a] Bold characters indicate nucleotide or amino acid changes as compared to the most common allele.

Statistical analyses

Differences between characteristics of patients and controls were analyzed with χ^2 test and *t*-test. All genotypes investigated among controls and patients were tested whether they were distributed according to the Hardy-Weinberg equilibrium. Furthermore, χ^2 statistic was used to test for differences in the distribution of the genotypes between the two study groups, or to estimate differences in allele frequencies. In total, four genetic polymorphisms of the UGT1A family were analyzed. Because the different UGT1A isoforms are derived from one single gene locus, we corrected for multiple testing with Bonferroni, meaning that a *P*-value of < 0.013 instead of 0.05 was considered to represent statistical significance. Odds ratios (OR)

with 95% confidence interval (95% CI) were calculated by logistic regression analyses for genotypes associated with normal enzyme activity versus genotypes associated with expected reduced enzyme activity (variant genotypes). All statistical analyses were performed with SAS (version 8.0; SAS Institute, Cary, NC).

Results

Characteristics of patients and controls

Descriptive statistics of CRC patients and controls are given in Table III. The mean age of CRC patients (64 ± 11 years) is significantly higher compared with that of the control group (42 ± 12 years; $P < 0.0001$).

Table III. Clinical characteristics of patients with sporadic CRC and controls

Characteristics	Group	Controls (%)	CRC patients (%)
Number of subjects		415	371
Age (years; mean ± SD)		42 ± 12	64 ± 11 [a]
Gender	Male	168 (40.5)	212 (57.1) [a]
	Female	247 (59.5)	159 (42.9)
Location tumour [b]	Cecum		40/310 (12.9)
	Ascending colon		22/310 (7.1)
	Transverse colon		17/310 (5.5)
	Proximal		89/324 (27.5) [c]
	Descending colon		8/310 (2.6)
	Sigmoid colon		85/310 (27.4)
	Rectosigmoid junction		40/310 (12.9)
	Rectum		98/310 (31.6)
	Distal		235/324 (72.5) [c]
Stage tumour [d]	Dukes A		7/293 (2.4)
	Dukes B		103/293 (35.2)
	Dukes A/B		112/299 (37.5) [e]
	Dukes C		81/293 (27.6)
	Dukes D		102/293 (34.8)
	Dukes C/D		187/299 (62.5) [e]

[a] Controls versus CRC patients, $P < 0.0001$.

[b] For 61 CRC cases, no information on the exact location of the tumour is available.

[c] For some patients only proximal or distal location was reported in the medical files.

[d] For 78 cases, no information on the exact stage of the tumour is available.

[e] For some patients only stage Dukes A/B or C/D was reported in the medical files.

There is also a statistically significant difference in gender between CRC patients and healthy controls, with more female subjects in the control group ($P < 0.0001$).

Polymorphisms in genes of biotransformation enzymes

Genotype distributions of the UGT and GST biotransformation enzymes investigated are summarized in Table IV, and corresponding allele frequencies are shown in Table V. The genotype distributions and allele frequencies in patients are based on PCR-RFLP, using DNA extracted from either whole blood or normal colorectal mucosa. All genotype distributions tested here fulfilled the Hardy-Weinberg criteria. χ^2 analyses revealed no significant differences for the investigated polymorphisms between CRC patients and controls.

Calculation of odds ratios of genotypes associated with normal enzyme activity versus genotypes associated with expected reduced enzyme activity (variant genotypes) with logistic regression analyses showed significant age- and gender-adjusted risks for CRC associated with variant genotypes of *UGT1A6* (OR 1.5, 95% CI 1.03-2.3) and *UGT1A7* (OR 2.4, 95% CI 1.3-4.6). There were no statistically significant differences between cases and controls for the other genotype distributions investigated in this study (Table IV).

χ^2 analyses revealed no significant differences in the presence of variant alleles between CRC patients and controls, for all enzymes investigated. When combining alleles that provide normal catalytic properties and alleles that may yield lower enzyme activity we calculated the odds ratios for CRC risk (Table V). Logistic regression analyses showed significant age- and gender-adjusted risks for CRC associated with variant *UGT1A7* alleles (OR 1.5, 95% CI 1.1-2.0).

Table IV. Distribution of genotypes of UGT and GST detoxification enzymes in patients with CRC and controls

Gene	Genotype	Controls (%) [a]	CRC patients (%) [a]	Crude OR (95%CI) [b]	Adjusted OR (95%CI) [c]
UGT1A1	*1*1	183/399 (45.9)	175/371 (47.2)		
	*1*28	169 (42.4)	159 (42.9)		
	*28*28	47 (11.8)	37 (10.0)	0.95 (0.71-1.3)	1.2 (0.83-1.8)
UGT1A6	*1*1	171/413 (41.4)	142/363 (39.1)		
	*1*2	175 (42.4)	160 (44.1)		
	*2*2	54 (13.1)	38 (10.5)		
	*1*3	9 (2.2)	15 (4.1)		
	*2*3	4 (1.0)	8 (2.2)	1.1 (0.82-1.5)	1.5 (1.03-2.3) [d]
UGT1A7	*1*1	51/405 (12.6)	35/367 (9.5)		
	*1*2	71 (17.5)	63 (17.2)		
	*2*2	27 (6.7)	28 (7.6)		
	*1*3	102 (25.2)	105 (28.6)		
	*2*3	79 (19.5)	75 (20.4)		
	*3*3	70 (17.3)	60 (16.3)		
	*1*10	5 (1.2)	1 (0.3)	1.4 (0.87-2.2)	2.4 (1.3-4.6) [d]
UGT1A8	*1*1	216/404 (53.5)	186/370 (50.3)		
	*1*2	143 (35.4)	138 (37.3)		
	*2*2	25 (6.2)	29 (7.8)		
	*1*3	14 (3.5)	15 (4.1)		
	*2*3	6 (1.5)	2 (0.5)	0.92 (0.48-1.8)	0.69 (0.30-1.6)
GSTA1	*A*A	168/411 (40.9)	158/371 (42.6)		
	*A*B	184 (44.8)	160 (43.1)		
	*B*B	59 (14.4)	53 (14.3)	0.93 (0.70-1.2)	0.80 (0.54-1.2)
GSTM1	+	212/415 (51.1)	186/370 (50.3)		
	null	203 (48.9)	184 (49.7)	1.0 (0.78-1.4)	0.81 (0.54-1.2)
GSTP1	Ile/Ile	174/414 (42.0)	155/369 (42.0)		
	Ile/Val	185 (44.7)	175 (47.4)		
	Val/Val	55 (13.3)	39 (10.6)	1.0 (0.75-1.3)	0.93 (0.62-1.4)
GSTT1	+	346/415 (83.4)	299/371 (80.6)		
	null	69 (16.6)	72 (19.4)	1.2 (0.84-1.7)	1.0 (0.64-1.7)

[a] In both the CRC and control group there are some missing data because of insufficient amount of DNA or unsuccessful PCR.

[b] Genotypes were combined on the basis of an expected phenotype-genotype relationship (variant genotypes with reduced enzyme activity versus genotypes with normal enzyme activity) and crude OR were calculated.

[c] OR adjusted by age and gender were calculated.

[d] Statistically significant.

Table V. Allele frequencies of UGT and GST genes investigated in patients with CRC and controls

Alleles	Controls (number/total) [a]	CRC patients (number/total) [a]	Crude OR (95% CI) [b]	Adjusted OR (95% CI) [c]
*UGT1A1*1*	0.67 (535/798)	0.69 (509/742)		
*UGT1A1*28*	0.33	0.31	0.93 (0.75-1.2)	1.1 (0.84-1.5)
*UGT1A6*1*	0.64 (526/826)	0.63 (459/726)		
*UGT1A6*2*	0.35	0.34		
*UGT1A6*3*	0.016	0.032	1.0 (0.83-1.3)	1.2 (0.94-1.7)
*UGT1A7*1*	0.35 (280/810)	0.33 (239/734)		
*UGT1A7*2*	0.25	0.26		
*UGT1A7*3*	0.40	0.41		
*UGT1A7*10*	0.0062	0.0014	1.1 (0.89-1.4)	1.5 (1.1-2.0) [d]
*UGT1A8*1*	0.73 (589/808)	0.71 (525/740)		
*UGT1A8*2*	0.25	0.27		
*UGT1A8*3*	0.025	0.023	0.95 (0.49-1.8)	0.98 (0.72-1.3)
*GSTA1*A*	0.63 (520/822)	0.64 (476/742)		
*GSTA1*B*	0.37	0.36	0.96 (0.78-1.2)	0.87 (0.66-1.2)
GSTM1 +	0.51 (424/830)	0.50 (372/740)		
GSTM1 null	0.49	0.50	1.0 (0.85-1.3)	0.81 (0.61-1.1)
GSTP1 Ile	0.64 (533/828)	0.66 (485/738)		
GSTP1 Val	0.36	0.34	0.94 (0.77-1.2)	0.87 (0.65-1.2)
GSTT1 +	0.83 (692/830)	0.81 (598/742)		
GSTT1 null	0.17	0.19	1.2 (0.93-1.6)	1.0 (0.74-1.5)

[a] In both controls and cases there are missing data because of insufficient amount of DNA or unsuccessful PCR.

[b] Alleles were combined on the basis of an expected phenotype-genotype relationship (variant alleles with reduced enzyme activity versus alleles with normal enzyme activity) and crude OR were calculated.

[c] OR adjusted by age and gender were calculated.

[d] Statistically significant.

Possible associations of genotype distributions of the UGT and GST biotransformation enzymes and clinical characteristics, such as tumour location and tumour stage were also investigated and the results are summarized in Table VI. These analyses revealed an association of *UGT1A6* variant genotypes with proximal CRC (adjusted OR 2.1 95% CI 1.1-4.1), whereas *UGT1A7* variant genotypes were associated with distal CRC (adjusted OR 3.0, 95% CI 1.5-6.2).

Table VI. Distribution of genotypes of UGT and GST detoxification enzymes with respect to tumour location and tumour stage in patients with CRC

Gene	Genotype	Proximal CRC (%) [a]	Distal CRC (%) [a]	CRC Dukes A/B (%) [a]	CRC Dukes C/D (%) [a]
UGT1A1	*1*1	39/89 (42.8)	112/235 (45.9)	52/112 (46.4)	84/187 (44.9)
	*1*28	41 (46.1)	98 (42.3)	51 (45.5)	83 (44.3)
	*28*28	9 (10.1)	25 (11.8)	9 (8.0)	20 (10.7)
UGT1A6	*1*1	31/88 (35.2) [b, c]	92/230 (40.0)	51/112 (45.5)	62/183 (33.9) [b, c]
	*1*2	40 (45.4)	99 (43.0)	42 (37.5)	92 (50.3)
	*2*2	10 (11.4)	25 (10.9)	10 (8.9)	21 (11.5)
	*1*3	5 (5.7)	9 (3.9)	3 (2.7)	7 (3.8)
	*2*3	2 (2.3)	5 (2.2)	6 (5.4)	1 (0.55)
UGT1A7	*1*1	8/88 (9.1)	23/232 (9.9) [b, c]	13/111 (11.7) [b, c]	15/184 (8.2) [b, c]
	*1*2	13 (14.8)	43 (18.5)	28 (25.2)	22 (12.0)
	*2*2	7 (8.0)	17 (6.9)	9 (8.1)	14 (7.6)
	*1*3	23 (26.1)	64 (26.1)	27 (24.3)	58 (31.5)
	*2*3	19 (21.6)	47 (19.8)	17 (15.3)	45 (24.5)
	*3*3	18 (20.4)	37 (16.8)	17 (15.3)	30 (16.3)
	*1*10	0 (0)	1 (0.94)	0 (0)	0 (0)
UGT1A8	*1*1	46/89 (51.7)	118/234 (50.4)	51/112 (45.5)	97/186 (52.2)
	*1*2	34 (38.2)	84 (35.9)	46 (41.0)	67 (36.0)
	*2*2	7 (7.9)	19 (8.1)	10 (8.9)	16 (8.6)
	*1*3	2 (2.3)	11 (4.7)	4 (3.6)	5 (2.7)
	*2*3	0 (0)	2 (0.85)	1 (0.89)	1 (0.54)
GSTA1	*A*A	41/89 (46.1)	95/235 (40.4)	48/112 (42.9)	80/187 (42.8)
	*A*B	36 (40.4)	106 (45.1)	52 (46.4)	74 (39.6)
	*B*B	12 (13.5)	34 (14.5)	12 (10.7)	33 (17.7)
GSTM1	+	47/89 (52.8)	118/234 (50.4)	63/112 (56.3) [b, c]	90/186 (48.4)
	null	42 (47.2)	116 (49.6)	49 (43.8)	96 (51.6)
GSTP1	Ile/Ile	35/87 (40.2)	99/235 (42.1)	52/112 (46.4)	78/185 (42.2)
	Ile/Val	44 (50.6)	110 (46.8)	59 (43.8)	85 (46.0)
	Val/Val	8 (9.2)	26 (11.1)	11 (9.8)	22 (11.9)
GSTT1	+	76/89 (85.4)	187/235 (79.6)	93/112 (83.0)	149/187 (79.7)
	null	13 (14.6)	48 (20.4)	19 (17.0)	38 (20.3)

[a] (%: number/total); Among cases there are some missing data because of insufficient amount of DNA or unsuccessful PCR.

[b] Genotypes were combined on the basis of an expected phenotype-genotype relationship (variant genotypes with reduced enzyme activity versus genotypes with normal enzyme activity) and statistically significant OR adjusted by age and gender were calculated.

UGT1A6: proximal CRC 2.1 (1.1-4.1); CRC Dukes C/D 2.0 (1.2-3.2);

UGT1A7: distal CRC 3.0 (1.5-6.2); CRC Dukes A/B 2.5 (1.1-6.3); CRC Dukes C/D 2.8 (1.4-6.0);

GSTM1: CRC Dukes A/B 0.47 (0.25-0.83).

[c] Statistically significant.

In addition, logistic regression analyses showed significant age- and gender-adjusted risks for Dukes C/D CRC associated with variant genotypes of *UGT1A6* (OR 2.0, 95% CI 1.2-3.2), whereas presence of *GSTM1* was associated with a reduced risk for Dukes A/B CRC (OR 0.47, 95% CI 0.25-0.83). Risk for CRC associated with variant *UGT1A7* genotypes was independent of tumour stage (Dukes A/B: OR 2.5 (1.1-6.3); Dukes C/D: OR 2.8 (1.4-6.0)).

Discussion

The risk of sporadic CRC is associated mainly with lifestyle factors and may be further modulated by several genetic factors of low penetrance (1-6). Since the gastrointestinal tract is in direct contact with potentially toxic or (pre)carcinogenic agents, the intestinal mucosa acts as a first-line barrier. In humans, detoxification enzymes are present predominantly in the liver. However, these enzymes have also been distinguished in extra-hepatic tissues of the gastrointestinal tract (8-11). Polymorphic variations in the detoxification enzymes may modulate the rate of conversion of toxic or carcinogenic compounds in the epithelium lining the lumen of the gastrointestinal tract. Several polymorphisms of genes encoding for the detoxification enzymes have been described and have sometimes been associated with increased CRC susceptibility (15-17). In the present study we investigated the relationship between sporadic CRC and polymorphisms in *UGT* and *GST* genes, which are associated with functional changes in enzyme activity.

In this study, a significant age- and gender-adjusted risk for CRC associated with variant genotypes of *UGT1A6* was revealed. In particular, variant genotypes of *UGT1A6*, with an expected reduction of the corresponding enzyme activities, were associated with tumours of the proximal colon and with Dukes C/D tumour stages. The frequency of the *UGT1A6*2* allele in healthy subjects was estimated at 35%, which is higher than the 16.8% reported by Ciotti *et al.* (28), but comparable to 30.7% and 32.5% found by Lampe *et al.* (40) and Köhle *et al.* (41), respectively. Earlier, Bigler *et al.* (42) described an inverse association with intake of aspirin and colon adenoma risk, for individuals who carried the variant *UGT1A6* alleles. The low activity alleles were protected against adenomas by aspirin intake whereas the individuals bearing only high activity alleles were not. UGT1A6 primarily metabolizes simple phenols (43) and planar heterocyclic amines (HCAs; ref. 44). HCAs are formed in protein-

rich foods, such as meat, as a result of pyrolysis during cooking. Meat intake, specifically red meat, has been associated with higher CRC risk (45). Recently, Butler *et al.* (46) examined the association between CRC and meat intake categorized by the stage of the cooking process, cooking method, and estimated levels of HCAs. They reported moderate, dose-dependent associations between CRC and red meat intake, in particular for pan-fried and well-done red meat, which showed the strongest correlations with the investigated HCAs. Individuals bearing variant *UGT1A6* genotypes, associated with reduced enzyme activity to detoxify these meat-derived HCAs sufficiently, may therefore be at an increased risk for CRC.

Strassburg *et al.* (18) showed a significant association of CRC and the presence of the variant *UGT1A7*3* allele (OR 2.8, 95% CI 1.6-4.7). We found only a weak association; presence of the *UGT1A7*3* allele yielded an OR of 1.2 (95% CI 0.91-1.6), which was not statistically significant. However, the combination of all variant alleles revealed a significant association with CRC (OR 1.5, 95% CI 1.1-2.0). Furthermore, an increased risk for CRC was specifically observed among patients with distal CRC who had the variant *UGT1A7* genotypes compared with subjects who had the most common genotype (OR 3.0). The differences between our results and those published by Strassburg *et al.* could be explained by the relatively low frequency of the *UGT1A7*3* allele in their control subjects (18). In our study, the frequency of the *UGT1A7*3* allele averaged 40% in healthy controls. This is comparable with frequencies of 32, 36 or 37% in Caucasian control subjects reported by Villeneuve *et al.*, Guillemette *et al.* and Köhle *et al.*, respectively (29, 30, 41), but is much higher than values of 16-21%, as reported by Strassburg *et al.* (18, 47-49). UGT1A7 has been demonstrated to catalyze the glucuronidation of tobacco smoke-derived polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene, as well as dietary-derived heterocyclic amines (21, 50), all of which are known carcinogens. The risk factors for CRC identified here, the polymorphisms *UGT1A7*2* and *UGT1A7*3*, have been shown to result in significant reduction of enzyme activity towards carcinogenic metabolites of benzo[*a*]pyrene in catalytic studies (18). Because these polymorphisms can result in reduced detoxification activities, accumulation of carcinogenic compounds, such as benzo[*a*]pyrene, may occur in individuals bearing variant *UGT1A7* genotypes, which eventually may result in an increased risk for developing CRC. In addition, epidemiological studies showed that smoking of cigarettes is a risk factor for CRC with a long lag time of up to 35-40 years (51). This association between smoking and CRC risk may be due partly to insufficient detoxification of carcinogenic components of cigarette smoke by genetic polymorphisms in *UGT1A7*.

For *UGT1A1* and *UGT1A8* we found no difference in the distributions of the polymorphic variants in healthy controls compared to CRC patients. This suggests that individuals carrying the UGT alleles, encoding for less active enzymes do not have a higher risk for developing CRC. The frequency of the *UGT1A1*28* allele was 33% in healthy controls. This is comparable with the frequencies (29-39%) reported in Caucasian control subjects by several other groups (40, 41, 52-54). The detected frequency of 2.5% for the *UGT1A8*3* allele in healthy controls was similar to 2.2% as reported by Huang *et al.* (22).

The different UGT1A isoforms investigated here are derived from one single gene locus and recently, frequent co-occurrence of variant alleles of *UGT1A1*, *UGT1A6* or *UGT1A7* has been reported (39, 41). Both *UGT1A6* and *UGT1A7* variant genotypes were associated with an increased CRC risk after adjustment for age and gender, as described above. Therefore, we also performed logistic regression analyses with both UGT1A6 and UGT1A7 in the regression model. This revealed an age- and gender adjusted OR of 1.3 (95% CI 0.85-2.1) for *UGT1A6* and an OR of 2.0 (95% CI 0.98-3.9) for *UGT1A7*. This means that the *UGT1A7* polymorphism is the most important risk factor for development of CRC in comparison with the other *UGT1A* polymorphisms investigated here.

In this study, similar frequencies for the homozygous *GSTA1*B* genotype were observed in controls (14%) and cases (14%), which is not consistent with the data of Coles *et al.* (55) that 14% of the control subjects and 24% of the CRC patients bore this genotype. This discrepancy could possibly be explained by the much larger population of controls (415) and CRC patients (371) we examined, as compared to the 226 control and 100 case subjects investigated by Coles *et al.*.

The relationship between CRC risk and *GSTM1* polymorphism has been most extensively studied and recently, two meta-analyses have been published by de Jong *et al.* (16) and Houlston and Tomlinson (17). Both pooled analyses revealed no association of the *GSTM1* polymorphism with CRC. Our results are in accordance with these observations. In contrast, a significant association for *GSTM1 null* genotype carriers and an increased CRC risk was described in a recent study by Sachse *et al.* (15). Earlier, Zhong *et al.* (56) reported that the risk of CRC associated with *GSTM1*-deletion was restricted to proximal disease, but this association was not seen either in our study or in the meta-analyses, mentioned above (16, 17). A striking observation was the association between *GSTM1 null* genotype carriers and a

reduced risk of Dukes A/B CRC. The primary hypothesis is that individuals with the *GSTM1 null* genotypes eventually are at higher cancer risk because of reduced capacity to dispose of carcinogens. However, GSTM1 also plays an important role in the disposition of isothiocyanates (ITC), breakdown products of glucosinolates, which are abundant in cruciferous vegetables, and which are strong inducers of the GSTs and other detoxification enzymes. The *GSTM1 null* polymorphism, associated with reduced enzyme activity, may result in longer circulating half-lives of ITC and potentially greater chemopreventive effects of cruciferous vegetables (57). Some case-control studies provide evidence that presence of *GSTM1* in conjunction with low intake of cruciferous vegetable is an important risk factor for CRC or pre-cancerous lesions (58, 59). Using ITC as biomarker of cruciferous vegetable exposure, Seow *et al.* and London *et al.* (60, 61) further strengthened the understanding of this gene-diet interaction. Seow *et al.* reported that high dietary ITC is associated with a significantly lower risk of CRC among individuals who are both *GSTM1 null* and *GSTT1 null* and London *et al.* observed that men with null genotypes of *GSTM1* or *GSTT1*, who had consumed cruciferous vegetables were at lower risk for lung cancer. A similar explanation as given above may be valid here, and it may be hypothesized that: in early stages of CRC, ITC may be involved in prevention of the development of CRC in *GSTM1 null* or *GSTT1 null* genotype carriers. However we do not have any data on consumption of cruciferous vegetables by the CRC patients or controls investigated here to test this hypothesis.

Several studies reported on the genetic polymorphism in codon 105 of the *GSTP1* gene as a possible risk factor for CRC (15, 62-64). However, pooled analyses by de Jong *et al.* (16) and Houlston and Tomlinson (17) did not show an increased risk of this polymorphism for CRC, which is in accordance with our results.

Conflicting findings have also been reported on the relationship between *GSTT1* status and CRC (15-17). We found no association between the *GSTT1 null* genotype and CRC risk, in agreement with the results found in the meta-analysis by Houlston and Tomlinson (17). However, the meta-analysis by de Jong *et al.* (16) reported the opposite. The overall odds ratio for *GSTT1* calculated by de Jong *et al.* was based on the crude data of the different studies, without corrections for age and gender. This may be crucial, as outlined recently by Butler *et al.* (65); crude data revealed a statistically significant association between *GSTT1 null* genotype and CRC risk, whereas after adjustment for age the increased risk was not observed anymore. Subgroup analysis for tumour location revealed no association between

GSTT1 null and CRC in our study, which is in accordance with the results of the meta-analysis by de Jong *et al.* (16).

Our findings have to be viewed in the perspective of potential limitations. Odds ratios can only be calculated correctly when confounding factors, such as age, gender, diet and lifestyle factors, are taken into consideration. In this study we observed a statistically significant difference in age and gender between CRC patients and controls. By including both age and gender in the logistic regression analyses we corrected the calculated odds ratios for differences in these factors. This is necessary because younger control subjects in comparison to CRC patients, have a shorter time of exposure to carcinogens and thus at the moment they reach the age of the CRC patients, some of them may also have developed CRC. It would be preferable to better match control and patient populations implicating that no afterwards corrections is needed, but in practice it appeared very difficult to realize. In this study no information was available on the dietary habits, alcohol use and smoking patterns of both patients and controls, which may also be confounding factors. Possibly some of the low-penetrance genes investigated here only contribute to CRC in combination with (some of) these dietary or lifestyle factors.

Polymorphic variations in detoxification enzymes may determine in part the rates of conversion of toxic or carcinogenic compounds, and thus may influence their levels in the gastrointestinal tract. We conclude that individuals carrying the low enzyme activity associated genotypes of *UGT1A6* and *UGT1A7* might be more prone to develop CRC. It is hard to estimate the actual impact of a respective 1.5- and 2.4-fold increased risk, however these findings may guide the research to the search of relevant substrates of these enzymes, as important risk factors for CRC. Such factors may be components of cigarette smoke and heterocyclic amines, present in protein rich food such as meat. However, other UGT family members may compensate reduced conversion rates because detoxification enzymes possess overlapping substrate-specificity. Furthermore, dietary anticarcinogens are able to enhance the activity of detoxification enzymes. Therefore, it is also a challenge to investigate whether such anticarcinogens can compensate for the reduced enzyme activities caused by the genetic polymorphisms studied here.

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Role of epoxide hydrolase, NAD(P)H:quinone oxidoreductase, cytochrome P450 2E1 or alcohol dehydrogenase genotypes in susceptibility to colorectal cancer

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Mutation Research: in press

Abstract

Colorectal cancer (CRC) is one of the most common forms of cancer in Western countries. CRC has been associated with genetic and lifestyle factors. Individual susceptibility to CRC may be due partly to variations in detoxification capacity in the gastrointestinal tract. Genetic polymorphisms in detoxification enzymes may result in variations in detoxification activities, which subsequently might influence the levels of toxic/carcinogenic compounds, and this may influence the risk for CRC. Therefore, we determined whether polymorphisms in the genes coding for microsomal epoxide hydrolase (*EPHX1*), NAD(P)H:quinone oxidoreductase (*NQO1*), cytochrome P450 2E1 (*CYP2E1*) and alcohol dehydrogenase (*ADH3*) predispose to the development of CRC. DNA samples were obtained from 371 patients with sporadic CRC and 415 healthy controls. Patients and controls were all of Caucasian origin. All genetic polymorphisms were determined by polymerase chain reaction, eventually followed by restriction-fragment-length-polymorphism analyses, except for the *EPHX1* codon 113 polymorphism, which was genotyped by an allele-specific discrimination assay. Calculation of crude Odds Ratios (ORs) revealed an increased risk for CRC associated with variant *NQO1* (OR 1.5, 95% CI 1.1-2.0) and *CYP2E1* intron 6 genotypes (OR 2.2, 95% CI 1.3-3.8). However, after adjustment for age and gender, logistic regression analyses only showed a statistically significant risk for CRC associated with variant *NQO1* genotypes (OR 1.6, 95% CI 1.03-2.4). No associations were found between CRC and the other polymorphic genes as mentioned above. In conclusion, these data suggest that the presence of variant *NQO1* genotypes, with expected reduced enzyme activities might enhance susceptibility to CRC.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the Western world showing an increasing incidence. In the Netherlands, cancer incidence rates in women are highest for breast cancer followed by CRC, whereas in men CRC cancer incidence rates are third highest after lung and prostate cancer (1). The risk of sporadic CRC is mainly associated with lifestyle factors and may be modulated by several genetic factors of low penetrance (1-6). Epidemiological studies have shown the importance of dietary habits in the risk for CRC. Diets low in fruit and vegetables, and high in red meat and fat are associated with an increased risk of CRC (3, 4). Humans may be daily exposed to a large variety of toxic or even carcinogenic compounds, present in food (7) or as a result of lifestyle habits such as smoking of cigarettes and drinking alcohol (5, 6, 8). Tumour formation may result from interaction between external (environmental carcinogen exposure) and internal (genetic) factors. Phase I and II detoxification enzymes play an important role in this process.

In humans, detoxification enzymes are present at high levels in the liver. However, these enzymes have also been distinguished in large amounts in extra-hepatic tissues of the gastrointestinal tract (9-15). Variations due to genetic polymorphisms may play an important role in the inter-individual differences in expression levels and enzyme activities of separate members of these detoxification enzymes (16-18). Furthermore, genetic polymorphisms may result in an imbalance between Phase I and II reactions, which may lead to accumulation of toxic/carcinogenic compounds in the colonic lumen or mucosa. Therefore, individual susceptibility to CRC could be due partly to polymorphic variations in detoxification enzymes.

The phase I enzymes, epoxide hydrolase, NAD(P)H:quinone oxidoreductase 1 (NQO1), cytochrome P450 (CYP) and alcohol dehydrogenase (ADH) play an important role in the metabolism of toxic or potentially carcinogenic compounds. In general, the metabolic pathways ultimately leading to detoxification consist of a number of successive reactions, catalyzed by phase I and phase II enzymes, respectively. Phase I enzymes hydrolyze, reduce or oxidize ingested chemical compounds, often leading to bioactivation into ultimate toxins or carcinogens. The products of phase I reactions in general however are rapidly conjugated

(phase II), leading to more water-soluble and biologically less active molecules that can be excreted.
Microsomal epoxide hydrolases are an important group of hydrolases covering a broad substrate specificity, including epoxide derivates of polycyclic aromatic hydrocarbons (e.g. derived from tobacco smoke), anticonvulsant drugs or steroids (19). They catalyze the hydrolysis of highly reactive epoxides (e.g. tobacco smoke-derived benzo[a]pyrene) to dihydrodiols, which however, can be metabolized into highly carcinogenic polycyclic hydrocarbon diol epoxides. Therefore, epoxide hydrolases may play a dual role in the (de)toxification of procarcinogens (20).
NAD(P)H:quinone oxidoreductase 1 (NQO1), catalyzes the two-electron reduction of quinones (e.g. derived from tobacco smoke or red meat), quinine-imines or azo- and nitro compounds to hydroquinones and thereby prevents the generation of semiquinone free radicals and reactive oxygen species. However, not all hydroquinones are chemically stable and sometimes NQO1 activity may lead to more active products, and eventually this may result in the production of reactive oxygen species (17).
Cytochrome P450-mediated oxidation of pre-carcinogens to their ultimate reactive metabolites is a well known mechanism in chemical-induced cancer. For example, CYP2E1 catalyzes the oxidation of ethanol to its far more toxic metabolite acetaldehyde. CYP2E1 is also involved in the metabolism of benzene, aniline and several components of cigarette smoke, such as nitrosamines (21).
The ADH gene family (ADH1-5) encodes enzymes that metabolize a wide variety of substrates, including ethanol, retinol, hydroxysteroids, and lipid peroxidation products (18). ADH3 plays a prominent role in the oxidation of ethanol to acetaldehyde in addition to the above-mentioned CYP2E1. At least 80% of the ingested ethanol is metabolized by ADH3 (18).
Overall, the enzymes mentioned above play an important role in the toxification and detoxification of tobacco smoke-derived compounds and alcohol, substrates that are relevant in colon carcinogenesis. Epidemiological studies showed that smoking of cigarettes is a risk factor for CRC with a long lag time of up to 35-40 years (22). Furthermore, a pooled analysis of 8 cohort studies revealed that that the consumption of alcohol was associated with an elevated risk for CRC, mainly at the highest levels of alcohol intake (6).

Polymorphic variations in these detoxification enzymes, at least in part, determine the rates of conversion of toxic or carcinogenic compounds. In the genes encoding for the enzymes mentioned above polymorphisms have been described (23-28) and some have been associated with increased CRC susceptibility (29). In Table I the genetic polymorphisms studied here, as well as their functional consequences and associations with CRC as published so far (30-36) are summarized.

Table I. Polymorphic variants of the biotransformation enzymes investigated here and their association with CRC as found in earlier studies

Gene	Polymorphic variation [a]	Effect on function (ref.)	Association with CRC (ref.)
EPHX1 exon 3	Y113**H**	40% ↓ activity (23)	↑ risk (30); ↓ risk (31)
EPHX1 exon 4	H139**R**	25% ↑ activity (23)	No association (30, 31)
NQO1 exon 6	P187**S**	3× ↓ or no activity (24)	↑ risk (32); no association (31, 33)
CYP2E1 5'-flanking region	-1293G;-1053C/-1293**C**;-1053**T** (c1/**c2**)	↑ activity (25, 26)	↑ risk (34); no association (35)
CYP2E1 intron 6	7632T/7632**A** (D/**C**)	↑ activity (27)	No association (35)
ADH3 exon 8	I349**V**	2.5× ↓ activity (28)	No association (36)

[a] Bold characters indicate nucleotide or amino acid changes with respect to the most common allele.

Overall, for the genetic polymorphisms studied here only a few studies were performed so far in association with CRC, and there was little consensus. Therefore, we studied the association between CRC and the above-mentioned genetic polymorphisms in a large study population of Dutch Caucasian CRC patients.

Aim

To determine whether genetic polymorphisms in *EPHX1*, *NQO1*, *CYP2E1* or *ADH3* may modulate the development of CRC, we genotyped 371 Caucasian patients with sporadic CRC and 415 Caucasian healthy controls.

Materials and Methods

Patients and control subjects

Selection of patients and controls has been described previously (37). Briefly, the sporadic CRC group consists of 371 patients and for comparison a control group of 415 healthy subjects was recruited in the Nijmegen area, The Netherlands, by advertisement in a local paper. All subjects studied were Caucasians of Dutch origin.

Table II. Clinical characteristics of patients with sporadic CRC and controls

Characteristics	Group	Controls (%)	CRC patients (%)
Number of subjects		415	371
Age (years; mean ± SD)		42 ± 12	64 ± 11 [a]
Gender	Male	168 (40.5)	212 (57.1) [a]
	Female	247 (59.5)	159 (42.9)
Location tumour [b]	Cecum		40/310 (12.9)
	Ascending colon		22/310 (7.1)
	Transverse colon		17/310 (5.5)
	Proximal		89/324 (27.5) [c]
	Descending colon		8/310 (2.6)
	Sigmoid colon		85/310 (27.4)
	Rectosigmoid junction		40/310 (12.9)
	Rectum		98/310 (31.6)
	Distal		235/324 (72.5) [c]
Stage tumour [d]	Dukes A		7/293 (2.4)
	Dukes B		103/293 (35.2)
	Dukes A/B		112/299 (37.5) [e]
	Dukes C		81/293 (27.6)
	Dukes D		102/293 (34.8)
	Dukes C/D		187/299 (62.5) [e]

[a] Controls versus CRC patients, $P < 0.0001$.

[b] For 61 CRC cases, no information on the exact location of the tumour is available.

[c] For some patients only proximal or distal location was reported in the medical files.

[d] For 78 cases of CRC, no information about the exact stage of the tumour is available.

[e] For some patients only stage Dukes A/B or C/D was reported in the medical files.

The diagnosis of colorectal cancer was based on histopathological investigation of tissue specimens by a pathologist. Cases were classified according to Dukes' stages (A, B, C, D)

and according to location of the tumour in the large intestine as either proximal (cecum, ascending or transverse colon) or distal (descending colon, sigmoid, rectosigmoid junction or rectum). Patients had no (family) history of CRC and suspected cases of hereditary CRC syndromes (i.e. hereditary non-polyposis colorectal cancer or familial adenomatous polyposis) were excluded. Controls had to be at least 18 years of age and were excluded when they had a (family) history of CRC. The study was approved by the local medical ethical review committee and all subjects gave their written informed consent. Relevant data of patients and controls are summarized in Table II.

Genotyping

DNA was extracted from either whole blood (280 cases and 415 controls) or from normal colorectal mucosa (91 cases) as was described previously (37). Genetic polymorphisms were studied using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analyses, except for the polymorphism at codon 113 of *EPHX1*. The primers and restriction enzymes used for PCR-RFLP are shown in Table III. PCR-RFLP assays were adapted from methods described earlier and were used to identify the polymorphic variants of the following genes: *EHPX1* exon 4 (30), *NQO1* (38), *CYP2E1* 5'-flanking region and intron 6 (39) and *ADH3* (40).

Table III. Primers, beacons and restriction enzymes used for genotyping analyses

Gene	Primers/beacons (5'-3'; F = forward, R = reverse) [a]	Restriction enzyme
EPHX1 codon 113	F: CAA CTC CAA CTA CCT GAA G-3'	-
	R: TGA CAT ACA TCC CTC TCT G-3'	
	FAM: **cgc gat** GAT TCT CAA CAG ATA CCC TCA CTT CAA **tcg cg**	
	HEX: **cgc gat** ATT CTC AAC AGA CAC CCT CAC TTC AA**t cgc g**	
EPHX1 codon 139	F: ACA TCC ACT TCA TCC ACG T	*Rsa*I
	R: ATG CCT CTG AGA AGC CAT	
NQO1 codon 187	F: GAG ACG CTA GCT CTG AAC TGA T	*Hinf*I
	R: ATT TGA ATT CGG GCG TCT GCT G	
CYP2EI 5'-flanking region	F: CCA GTC GAG TCT ACA TTG TCA	*Pst*I / *Rsa*I
	R: TCA TTC TGT CTT CTA ACT GGC A	
CYP2EI intron 6	F: GCT CGT CAG TTC TGA AAG CAG	*Dra*I
	R: GAG CTC TGA TGC AAG TAT CGC A	
ADH3 codon 349	F: GCT TTA AGA GTA AAT ATT CTG TCC CC	*Ssp*I
	R: AAT CTA CCT CTT TCC GAA GC	

[a] Bold characters indicate linkers attached to the beacons.

Yoshikawa *et al.* reported a silent substitution polymorphism (G to A) at codon 119 of the *EPHX1* gene (41). This polymorphism may affect the accuracy of the codon 113 genotyping by PCR-RFLP (30), a method also used by us to analyse our samples. Therefore, we now also developed a dual-colour allele-specific discrimination assay for genotyping the polymorphism at codon 113 of the *EPHX1* gene. *EPHX1* genotypes were detected using the iCycler iQ Multicolour Real-Time Detection System (Bio-Rad Laboratories) using molecular beacons. PCR was performed with the forward primer 5'-CAA CTC CAA CTA CCT GAA G-3' and the reverse primer 5'-TGA CAT ACA TCC CTC TCT G-3' in the presence of the FAM-labeled wild-type beacon (5'-cgc gat GAT TCT CAA CAG ATA CCC TCA CTT CAA tcg cg-3') and the HEX-labeled mutant beacon (5'-cgc gat ATT CTC AAC AGA CAC CCT CAC TTC AAt cgc g-3'; see Table III). The 25 µl reaction mixture contained 200 ng of genomic DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 4 mM $MgCl_2$, 0.25 mM dNTPs, 50 ng of each primer, 200 nM of each beacon and 2.5 U Taq-DNA-polymerase. The PCR conditions were 3 min at 95°C, then 40 cycles of 30 s at 95°C, 30 s at 59°C and 30 s at 72°C. Fluorescent signals were measured at 59°C. Genotypes were assigned using the iCycler iQ Optical System Software version 3.1. At each PCR run, sterile H_2O was substituted for genomic DNA and served as a negative control for amplification.

Statistical analyses

Differences between characteristics of patients and controls were analyzed with χ^2 test and Student's t-test. A *P*-value below 0.05 was considered significant. Among the control group, each polymorphism was tested whether it fitted the Hardy-Weinberg equilibrium. χ^2 statistics were used to test for differences in the distribution of the genotypes between the two study groups. When one of the genotypes had expected counts of less then five, we used Fisher's exact test. Odds ratios (OR) with 95% confidence interval (95% CI) were calculated by logistic regression analyses for genotypes associated with normal enzyme activity (common genotype) versus genotypes associated with expected reduced or enhanced enzyme activity (heterozygous and homozygous variant genotypes). Finally, the Spearman rank coefficient of correlation was calculated for the association between the two *EPHX1* polymorphisms and the *CYP2E1* polymorphisms, respectively. All statistical analyses were performed with SAS (version 8.0; SAS Institute Inc., Cary, NC, USA.).

Results

Characteristics of patients and controls

Descriptive statistics of CRC patients and controls are given in Table II. The variables age and gender differed statistically significant among CRC patients and controls. The mean age for cases was 64 years and for controls 42 years ($P < 0.0001$), and 57.1% of cases was male versus 40.5% of controls ($P < 0.0001$).

Polymorphisms in genes of detoxification enzymes

Genotype distributions of the different detoxification enzymes investigated are summarized in Table IV. All polymorphisms investigated were in Hardy-Weinberg equilibrium. χ^2- or Fisher's exact test analyses revealed a significant difference for the investigated polymorphisms in *NQO1* ($P = 0.02$) and *CYP2E1* intron 6 ($P = 0.004$) between CRC patients and controls.

In addition, genotypes were combined on the basis of an expected phenotype-genotype relationship (variant genotypes with reduced or enhanced enzyme activity versus genotypes with normal enzyme activity) and crude ORs were calculated. In comparison with healthy controls, CRC patients with variant *NQO1* or *CYP2E1* intron 6 genotypes were more common (1.5- or 2.2-fold, respectively), which was statistically significant. After adjustment for age and gender, the risk for CRC associated with variant *NQO1* or *CYP2E1* intron 6 genotypes was 1.6 (95% CI 1.03-2.4) or 1.8 (95% CI 0.88-3.8), respectively, implicating that only the association of CRC with the variant *NQO1* genotypes was statistically significant.
There were no statistically significant differences between cases and controls for the other genotype distributions investigated here (Table IV).

In addition, the co-occurrence of the two polymorphisms in *EPHX1* was investigated and no correlation was found. For the 5'-flanking region- and intron 6 *CYP2E1* polymorphisms a correlation was observed in CRC patients ($r_s = 0.36$, $P < 0.01$) as well as in controls ($r_s = 0.32$, $P < 0.01$), meaning that these polymorphisms are usually linked on one allele.

Table IV. Distribution of genotypes of biotransformation enzymes in patients with CRC and controls

Gene	Genotype	Controls (%) [a]	CRC patients (%) [a]	Crude OR (95%CI) [b]	Adjusted OR (95%CI) [c]
EPHX1 codon 113	Tyr/Tyr	194/391 (49.6)	185/365 (50.7)		
	Tyr/His	165 (42.2)	141 (38.6)		
	His/His	32 (8.2)	39 (10.7)	0.96 (0.72-1.3)	1.0 (0.70-1.5)
EPHX1 codon 139	His/His	269/414 (65.0)	241/371 (65.0)		
	His/Arg	128 (30.9)	106 (28.6)		
	Arg/Arg	17 (4.1)	24 (6.5)	1.0 (0.75-1.3)	0.99 (0.66-1.5)
NQO1 codon 187	Pro/Pro	292/415 (70.4)	225/369 (61.0) [d]		
	Pro/Ser	112 (27.0)	134 (36.3)		
	Ser/Ser	11 (2.7)	10 (2.7)	1.5 (1.1-2.0) [e]	1.6 (1.03-2.4) [e]
CYP2E1 5'-flanking region	c1c1	389/412 (94.4)	333/357 (93.3)		
	c1c2	21 (5.1)	23 (6.4)		
	c2c2	2 (0.5)	1 (0.3)	1.2 (0.68-2.2)	1.1 (0.48-2.4)
CYP2E1 intron 6	DD	388/410 (94.6)	324/365 (88.8) [f]		
	DC	19 (4.7)	38 (10.4)		
	CC	3 (0.7)	3 (0.8)	2.2 (1.3-3.8) [e]	1.8 (0.88-3.8)
ADH3 codon 349	Ile/Ile	144/385 (37.4)	113/320 (35.3)		
	Ile/Val	186 (48.3)	149 (46.6)		
	Val/Val	55 (14.3)	58 (18.1)	1.1 (0.80-1.5)	0.83 (0.54-1.3)

[a] In both the CRC and control group there are some missing data because of insufficient amount of DNA or unsuccessful PCR.

[b] Genotypes were combined on the basis of an expected phenotype-genotype relationship (heterozygous and homozygous variant genotypes with reduced or enhanced enzyme activity versus common genotypes with normal enzyme activity) and crude ORs were calculated.

[c] OR adjusted for age and gender were calculated.

[d] $P = 0.02$.

[e] Statistically significant.

[f] $P = 0.004$.

Next, the occurrence of various combinations of *EPHX1* codon 113 and codon 139 genotypes or imputed EPHX1 phenotypes were considered in CRC patients and controls (see Table V; classification of expected phenotypes was performed according to Ulrich *et al.* (42). Only the combination of homozygous variant codon 139 with any genotype of codon 113 of *EPHX1* revealed a statistically significant increased risk for CRC (OR 1.3, 95% CI 1.04-1.5). In addition, the codon 139 homozygous variant genotype, compared to most common genotype showed a slightly increased risk for CRC, although results were not statistically significant (OR 1.5, 95% CI 0.97-2.4).

Table V. Risk of CRC associated with *EHPX1* genotypes and imputed EPHX1 phenotypes

Gene [a]	Cases/Controls [b]	Adjusted OR (95%CI) [c]
EPHX1 codon 113		
Tyr/Tyr (MC)	185/194	1.0 (reference)
Tyr/His (heterozygous)	141/165	1.0 (0.69-1.6)
His/His (homozygous variant)	39/32	0.99 (0.71-1.4)
EPHX1 codon 139		
His/His (MC)	241/269	1.0 (reference)
His/Arg (heterozygous)	106/128	0.85 (0.56-1.3)
Arg/Arg (homozygous variant)	24/17	1.5 (0.97-2.4)
Combined *EPHX1* genotypes		
MC/MC	119/130	1.0 (reference)
MC codon 113 / heterozygous codon 139	51/55	1.2 (0.64-2.2)
MC codon 139 / heterozygous codon 113	94/110	1.2 (0.90-1.5)
Double heterozygous	42/49	0.96 (0.77-1.2)
Homozygous variant codon 113 / MC or heterozygous codon 139	36/30	1.0 (0.85-1.2)
Homozygous variant codon 139 / any genotype codon 113	23/16	1.3 (1.04-1.5) [d]
Imputed EPHX1 phenotypes		
Rapid	67/63	1.5 (0.86-2.6)
Normal	160/178	1.0 (reference)
Slow	94/109	1.4 (0.86-2.4)
Very slow	25/14	1.2 (0.73-1.9)

MC; most common.

[a] Genotypes and phenotypes as classified by Ulrich *et al.* (42).

[b] In both the CRC and control group there are some missing data because of insufficient amount of DNA or unsuccessful PCR.

[c] OR adjusted for age and gender were calculated.

[d] Statistically significant.

We also investigated potential associations of genotype distributions of the different detoxification enzymes with clinical characteristics, such as tumour location and tumour stage (Table VI). These data revealed statistically significant associations of variant *NQO1* genotypes with distal CRC (adjusted OR 1.6, 95% CI 1.03-2.6) and of variant *CYP2E1* intron 6 genotypes and Dukes C/D CRC (adjusted OR 2.2, 95% CI 1.01-5.0).

Table VI. Distribution of genotypes of different biotransformation enzymes with respect to tumour location and tumour stage in patients with CRC

Gene	Genotype	Proximal CRC (%) [a]	Distal CRC (%) [a]	CRC Dukes A/B (%) [a]	CRC Dukes C/D (%) [a]
EPHX1 codon 113	Tyr/Tyr	43/87 (49.4)	121/232 (52.2)	61/110 (55.5)	83/184 (45.1)
	Tyr/His	33 (37.9)	89 (38.4)	37 (33.6)	82 (44.6)
	His/His	11 (12.6)	22 (9.5)	12 (10.9)	19 (10.3)
EPHX1 codon 139	His/His	52/89 (58.4)	160/235 (68.0)	76/112 (67.9)	125/187 (66.8)
	His/Arg	34 (38.2)	59 (25.1)	28 (25.0)	53 (28.3)
	Arg/Arg	3 (3.4)	16 (6.8)	8 (7.1)	9 (4.8)
NQO1 codon 187	Pro/Pro	51/89 (57.3) [b]	146/233 (62.7) [c, d]	67/112 (59.8)	117/185 (63.2)
	Pro/Ser	37 (41.6)	79 (33.9)	39 (34.8)	65 (35.1)
	Ser/Ser	1 (1.1)	8 (3.4)	6 (5.4)	3 (1.6)
CYP2E1 5'-flanking region	c1c1	78/85 (91.8)	212/228 (93.0)	103/109 (94.5)	168/179 (93.8)
	c1c2	6 (7.1)	16 (7.0)	5 (4.6)	11 (6.2)
	c2c2	1 (1.1)	0 (0)	1 (0.9)	0 (0)
CYP2E1 intron 6	DD	72/88 (81.8) [e]	210/231 (90.9)	95/111 (85.6) [f]	165/184 (89.7) [c, d, g]
	DC	15 (17.1)	20 (8.7)	15 (13.5)	17 (9.2)
	CC	1 (1.1)	1 (0.4)	1 (0.9)	2 (1.1)
ADH3 codon 349	Ile/Ile	25/71 (35.2)	72/209 (34.4)	39/102 (38.2)	56/157 (35.7)
	Ile/Val	29 (40.9)	103 (49.3)	48 (47.1)	72 (45.8)
	Val/Val	17 (23.9)	34 (16.3)	15 (14.7)	29 (18.5)

[a] (%: number/total); Among cases there are some missing data because of insufficient amount of DNA or unsuccessful PCR.

[b] $P = 0.02$.

[c] Genotypes were combined on the basis of an expected phenotype-genotype relationship (variant genotypes with reduced or enhanced enzyme activity versus genotypes with normal enzyme activity) and statistically significant ORs adjusted for age and gender were calculated. *NQO1*: Distal CRC 1.6 (1.03-2.6); *CYP2E1* intron 6: CRC Dukes C/D 2.2 (1.01-5.0).

[d] Statistically significant.

[e] $P < 0.0001$.

[f] $P = 0.003$.

[g] $P = 0.04$.

Discussion

In the present study we investigated the relationship between sporadic CRC and polymorphisms in genes coding for *EPHX1*, *NQO1*, *CYP2E1* and *ADH3* that are all associated with functional changes in enzyme activity for substrates relevant in colon carcinogenesis. Up till now, controversial data have been provided by different studies investigating the *EPHX1 Y113H* polymorphism in relation to CRC (30, 31; see Table I). In the present study, no association between variant genotypes for the *EPHX1* codon 113 polymorphism and CRC risk was found. In accordance, three recent studies (43-45) found a similar association between this polymorphism and occurrence of colorectal adenomas. With respect to the *EPHX1* codon 139 polymorphism, our results are similar to those of Harrison *et*

al. (30) and Sachse *et al.* (31). Furthermore, no association with tumour location or Dukes' stage was found for both *EPHX1* polymorphisms studied here, whereas Harrison *et al.* (30) showed the His113 allele to be present significantly more often in the left-sided colon cancer group compared with controls.

It is unclear, whether the reported risk estimates by Harrison *et al.* were adjusted for any covariates, which may explain in part the discrepancy found for the codon 113 polymorphism in the various studies as discussed above. In addition, the genotype distribution for this polymorphism in the control subjects of Harrison *et al.* as calculated by us, was not in Hardy-Weinberg equilibrium ($\chi^2 = 4.2$, $P = 0.04$). This deviation may be explained by a genotyping error, since Yoshikawa *et al.* (41) reported that the accuracy of the genotyping method for the *EPHX1* codon 113 polymorphism as used by Harrison *et al.*, might be influenced by a silent polymorphism in codon 119 (AAG119AAA). The reverse primer used for genotyping of the codon 113 polymorphism by the PCR-RFLP assay used by Harrison *et al.* includes this area. In Tyr113/His113 heterozygotes this primer mismatch may result in failure of the Tyr113 allele to amplify, leading to a false genotype classification of His113/His113. Baxter *et al.* (46) and Gsur *et al.* (47) confirmed the existence of this codon 119 polymorphism in Caucasians. The presence of this polymorphism has serious consequences for the reliability of the conventional PCR-RFLP method. Baxter *et al.* and Gsur *et al.* reported that 37.8% and 43.8%, respectively, of their heterozygotic controls were falsely classified as His113 homozygotes. We therefore genotyped our patients and controls for the *EPHX1* codon 113 polymorphism by the allele-specific discrimination assay as described under Materials and Methods, as well as by the PCR-RFLP assay as described by Harrison *et al.* (30), and found that 50.7% of the heterozygotic patients and 64.8% of the heterozygotic controls were misclassified as His113 homozygotes (data not shown). In the present study, the genotype frequency of His113 homozygotes among controls was 8.2%, which is similar to frequencies reported in other studies on Caucasian populations that used allele-specific discrimination assays (42, 46-49). To our knowledge, the majority of previous studies on the *EPHX1* codon 113 polymorphism rates have been conducted using the PCR-RFLP assay (30, 31, 43, 50) and consequently the validity of these studies must be questioned, especially when deviations from the Hardy-Weinberg equilibrium are present. To date, some studies have been published that used allele-specific discrimination assays for genotyping the *EPHX1* codon 113 polymorphism, and most studies contain data that obey the Hardy-Weinberg equilibrium, except for the recent study by Tranah *et al.* (45). Here, the reverse primer covers the silent polymorphism in codon 119, and similar to the conventional PCR-RFLP assay by Harrison *et*

al. (30), this may result in a primer mismatch and subsequently lead to a falsely high His113/His113 genotype rate of 17.1%.
Based on the *in vitro* studies of Hassett et al. (23), we assumed that the alleles corresponding with the protein sequences *EPHX1 113Y* and *139R* are associated with a higher total EPHX1 enzyme activity. However, other polymorphic variants in the 5'-flanking promoter region of *EPHX1* have been identified, which may also influence enzyme activity (51).

Earlier studies (31-33; see Table I) reported contrary results on an association of CRC with the *NQO1 P187S* polymorphism. When combining genotypes with an expected reduced enzyme activity (heterozygous and homozygous Ser187 variants; ref. 24), we found an increased CRC risk (OR 1.6, 95% CI 1.03-2.4) associated with these variant genotypes, which was comparable with the OR reported by Lafuente *et al.* (OR 1.4, 95% CI 1.02-1.9; ref. 32). Furthermore, in contrast to Lafuente *et al.*, we showed that this increased risk was seen particularly in patients with distal CRC (adjusted OR 1.6, 95% CI 1.03-2.6). Although a significant association between *NQO1* genotypes and CRC risk was found by us and Lafuente *et al.* (32), two other studies did not find such an association (31, 33). Therefore, the contribution of the *NQO1* polymorphism to the individual susceptibility to CRC remains unclear.

Thus far, two studies investigated the *CYP2E1* 5'-flanking region polymorphisms in association with CRC (34, 35; see Table I). Like Butler *et al.* (35), we found no difference in genotype distribution in healthy controls compared to CRC patients. This suggests that individuals carrying the *c2* alleles, encoding for more active enzymes do not have a higher risk for developing CRC. In the present study, the frequency of the *c2* allele in healthy controls was 3%, which is similar to the frequencies reported in Caucasian control subjects of several other studies (35, 52-54). However, Kiss *et al.* (34) reported much higher *c2* allele frequency in Hungarian control subjects (14%), which may explain the statistically significant difference in genotype distribution between cases and controls in this study. Inheritance of variant *CYP2E1* intron 6 genotypes conferred a 2.2-fold risk for CRC, which was statistically significant when applying the crude data, but lost significance after adjustment for age and gender. This confirmed the results reported by Butler *et al.* (35; see Table I). However, the variant *CYP2E1* intron 6 genotypes were associated significantly with Dukes C/D tumour stages (adjusted OR 2.2 95% CI 1.01-5.0) in this study.

Yet, only three studies on the relationship between *ADH3* polymorphisms and colorectal neoplasia, adenomas (55, 56) or carcinomas (36; see Table I) have been published. Overall, these data suggest that the *ADH3* polymorphisms do not confer a significant risk for colorectal neoplasia. We obtained similar results for this polymorphism and CRC risk. Frequencies of the Ile349 and Val349 alleles among controls were 62 and 38%, respectively, which are similar to frequencies reported in other Caucasian populations (36, 53, 55, 56).

Our findings have to be viewed in the perspective of potential limitations. First, odds ratios can only be calculated correctly, when confounding factors, such as age, gender, diet and lifestyle factors, are taken into consideration. In this study we observed a statistically significant difference in age and gender between CRC patients and controls. By including both age and gender in the logistic regression analyses we corrected the calculated odds ratios for differences in these factors. This is necessary because the younger control subjects, in comparison to the CRC patients, have a shorter time of exposure to carcinogens and thus at the moment they reach the age of the CRC patients, some of them may also have developed CRC. It would be preferable to better match control and patient populations, but in daily practice this appeared very difficult to realize. Secondly, despite the fact that the present study is one of the largest studies in its sort so far, we realize that the sample size may be too small to perform extensive subgroup analyses, and we realize that the multiple comparisons we performed may increase the risk of chance findings. Therefore, it is important to verify results by other studies. Finally, for the participants of our study, no information was available on dietary habits, alcohol use and smoking patterns, which may also be confounding factors. Possibly some of the low-penetrance genes investigated here only contribute to CRC in combination with (some of) these dietary or lifestyle factors.

Polymorphic variations in the detoxification enzymes may determine in part the conversion rates of toxic or carcinogenic compounds and thus may influence their levels in the gastrointestinal tract. We conclude that carriers of the low enzyme activity associated genotypes of *NQO1* might be more prone to develop CRC. It is hard to estimate the actual impact of a respective 1.6-fold higher risk, because members of other detoxification enzymes may compensate reduced conversion rates. In addition, the combination of genetic polymorphisms in different enzymes may result in higher CRC risk as compared to a single polymorphism. Recently, Hou *et al.* (57) observed that polymorphisms in *CYP1A1* (I462V) or *NQO1* (P187S) were weakly associated with risk for colorectal adenoma. However,

individuals carrying the *CYP1A1 Val462* and *NQO1 Ser187* alleles showed an increased risk (OR 2.2, 95% CI 1.1-4.5), particularly among recent and heavy cigarette smokers. This finding points to involvement of several tobacco smoke-derived benzo[*a*]pyrene metabolic pathways in colorectal carcinogenesis. It is well known that phase II enzymes such as glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases are also involved in the metabolism of tobacco smoke-derived benzo[*a*]pyrene. For example, GSTs catalyze the conjugation of glutathione to epoxides and diol-epoxide, which are formed in phase I reactions catalyzed by the enzymes under study here. Finally, (dietary) anticarcinogens are able to enhance the activity of detoxification enzymes and it is a challenge to investigate whether such anticarcinogens can reduce the number of patients with CRC in future.

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No association between genetic polymorphisms in NAD(P)H oxidase $p22^{phox}$ and paraoxonase 1 and colorectal cancer risk

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Abstract

Impaired metabolism of ingested toxic or carcinogenic compounds is a postulated mechanism underlying colorectal cancer (CRC). Furthermore, it is suggested that reactive oxygen species (ROS) may play a role in human cancer development. Polymorphic variations in NAD(P)H oxidase p22^{phox} and paraoxonase 1 (PON1) enzyme activities may alter superoxide production or the rate of chemical metabolism, respectively and this may influence the risk for CRC. Therefore, this study was designed to determine whether distribution of polymorphisms in *NAD(P)H oxidase p22^{phox}* and *PON1* genes was different in sporadic CRC patients versus healthy controls. The study participants (365 cases and 354 controls) were all of Caucasian origin. *NAD(P)H oxidase p22^{phox} H72Y*, and *PON1 L55M* and *Q192R* polymorphisms were genotyped by polymerase chain reaction, eventually followed by restriction-fragment-length-polymorphism analyses. Comparison of CRC patients and controls revealed no significant differences in genotype distributions or allele frequencies for polymorphisms in the *NAD(P)H oxidase p22^{phox}* and *PON1* genes. Investigation of potential associations between the variant *NAD(P)H oxidase p22^{phox}* or *PON1* alleles and the clinical characteristics, tumour location or tumour stage, also did not reveal statistically significant associations. In conclusion, variant genotypes of *NAD(P)H oxidase p22^{phox}* and *PON1* do not contribute to the susceptibility of CRC.

Introduction

The aetiology of colorectal cancer (CRC) remains elusive. It is estimated that up to 10% of CRC cases can be attributed to hereditary factors of high penetrance (1) leaving approximately 90% so-called sporadic CRC cases, which may be attributed to diet (2), lifestyle factors (3, 4) and genetic factors of low penetrance (5). Genetic predisposition to CRC may involve polymorphic variations in genes encoding for detoxification enzymes. Genetic variations in these enzymes may alter the conversion rate of toxic/carcinogenic compounds ingested by food, medication or lifestyle habits (e.g. smoking), which subsequently might influence the levels of these compounds in the colonic lumen or mucosa, possibly altering the risk for CRC. In addition, it is suggested that reactive oxygen species (ROS) may also play a role in human cancer development (6-8). ROS may induce cellular changes characteristic of those produced by known carcinogens (7). Phagocytosis by polymorphonuclear leukocytes results in the release of ROS, referred to as the respiratory burst, which plays an important role in host defence against certain micro-organisms. However, excessive generation of ROS by these phagocytes may cause harm to surrounding tissue and this may influence the risk for CRC (9).

The NAD(P)H oxidase enzyme is involved in the production of large quantities of superoxide during the respiratory burst of activated phagocytes. However, not only phagocytes, but also non-phagocytic cells, such as endothelial cells, vascular smooth muscle cells, cultures of transformed colonic epithelial cells or primary colonic epithelial cells, may produce superoxide (10, 11). In non-phagocytic cells, superoxide has been suggested to act as a regulator of genes involved in proliferation, apoptosis and inflammation (12). NAD(P)H oxidase is a membrane bound enzyme complex, which consists of a transmembrane electron transporting component, comprising a catalytic subunit cytochrome b_{558}, consisting of $gp91^{phox}$ and $p22^{phox}$ (13). In the *p22*^{phox} gene a C242T polymorphism has been identified that substitutes histidine by tyrosine at codon 72 (11). This may result in 72H homozygotes with normal enzyme activity, and heterozygotes or 72Y homozygotes both with diminished enzyme activity (14). These variations in *NAD(P)H oxidase p22*^{phox} genotypes eventually could lead to different amounts of superoxide in the colonic lumen or mucosa, which actually may influence the risk for CRC.

Paraoxonase 1 (PON1) is an esterase that is widely distributed among tissues such as liver, kidney and intestine, but also is present in blood plasma, where it is associated with high-density lipoproteins (HDL; refs. 15, 16). The PON family (PON1-3) enzymes are hydrolases with a broad substrate specificity (17). The PON1 protein was identified first and, therefore, has been most studied. PON1 is a phase I detoxification enzyme that hydrolyzes organophosphates, such as the insecticides paraoxon and chlorpyriphos, and nerve agents such as sarin or soman (18). It also hydrolyzes aliphatic lactones, like dihydrocoumarin and homocysteine-thiolactone (19, 20). Furthermore, PON1 inactivates lipoxidation derivatives of low-density lipoprotein (LDL; refs. 15, 21, 22). In summary, PON1 can offer protection against toxic environmental agents, as well as endogenous products of oxidative stress. In the *PON1* gene two common functional polymorphisms, L55M and Q192R, have been described, both of which may affect serum paraoxonase activity. *PON1-55L* is correlated with higher PON1 activity and mRNA levels than *PON1-55M* (23, 24), possibly caused by a decreased stability of the PON1-55M protein (25). The Q192R polymorphism results in substrate-dependent differences in the kinetics of hydrolysis of various substrates. The *PON1-192R* allele is associated with a higher activity to hydrolyze paraoxon, whereas the efficiency to hydrolyze sarin, diazoxon or lactones was lower compared with the *PON1-192Q* allele (18, 26). In addition, the Q192R polymorphism also alters the ability of the enzyme to protect LDL from oxidation, the *PON1-192Q* allele being most protective (27). Since PON is a HDL-associated enzyme many studies have investigated the relationship between *PON1* polymorphisms and coronary heart disease (28) and atherosclerosis (29). Although PON is also known to play a role in the detoxification of toxic/carcinogenic compounds and, therefore, may influence susceptibility to cancer, data on its association with cancer are rare. Kerridge *et al.* (30) were the first to demonstrate an association between *PON1* polymorphisms and cancer in humans: the homozygous variant *PON1-192R* genotype was significantly more often present in non-Hodgkin's lymphoma cases compared with control subjects. Furthermore, Akcay *et al.* found lower PON serum levels in patients with pancreatic (31) or gastric cancer (32) than in healthy controls, which suggests that PON may play a role in the aetiology of gastrointestinal cancer.

Polymorphic variations in NAD(P)H oxidase $p22^{phox}$ and PON1 enzymes may alter superoxide production or conversion rates of toxic/carcinogenic compounds, respectively, and this may influence the risk for CRC. Therefore, this study was designed to determine whether

sporadic CRC patients have another distribution of polymorphisms in *NAD(P)H oxidase p22^{phox}* and *PON1* genes as compared to healthy controls.

Materials and Methods

Patients and control subjects

Selection of patients and controls has been described previously (33). Briefly, the sporadic CRC group consists of 365 patients (209 males, 156 females; mean age 64 ± 11 years) and for comparison a control group of 354 healthy subjects (144 males, 210 females; mean age 43 ± 13 years) was recruited by advertisement in a local paper. All subjects studied were Caucasians of Dutch origin. Relevant data of patients and controls are summarized in Table I.

DNA isolation

DNA was extracted from either whole blood (276 cases and 354 controls) or normal colorectal mucosa (89 cases), as described previously (33). Cases were classified according to Dukes' stages (A, B, C, D) and according to location of the tumour in the large intestine as either proximal (cecum, ascending or transverse) or distal (descending, sigmoid, rectosigmoid junction or rectum).

Genotyping

The genetic polymorphisms in *p22^{phox}* and *PON1* genes were analyzed using polymerase chain reaction (PCR) followed by restriction-fragment-length-polymorphism (RFLP) analyses. Detection of the H72Y substitution in the *p22^{phox}* gene was based on the method described by Inoue *et al.* (11). In short, the forward and reverse primers used were: 5'-TGC TTG TGG GTA AAC CAA GGC CGG TG-3' and 5'-AAC ACT GAG GTA AGT GGG GGT GGC TCC TGT-3', respectively. The PCR conditions were 5 min at 95ºC, then 35 cycles of 30 s at 95ºC, 40 s at 58ºC, and 40 s at 72ºC, and finally an elongation step at 72ºC for 5 min. Digestion of the PCR product with the restriction enzyme *Rsa*I was followed by separation on 2% agarose. A 348-bp fragment indicates the *72H*-allele, whereas fragments of 188- and 160-bp are indicative for the *72Y*-allele.

Table I. Clinical characteristics of patients with sporadic CRC and controls

Characteristics	Group	Controls (%)	CRC patients (%)
Number of subjects		354	365
Age (years; mean ± SD)		43 ± 13	64 ± 11 [a]
Gender	Male	144 (40.7)	209 (57.3) [a]
	Female	210 (59.3)	156 (42.7)
Location tumour [b]	Cecum		40/307 (13.0)
	Ascending colon		22/307 (7.2)
	Transverse colon		17/307 (5.5)
	Proximal		88/319 (27.6) [c]
	Descending colon		8/307 (2.6)
	Sigmoid colon		84/307 (27.4)
	Rectosigmoid junction		41/307 (13.4)
	Rectum		95/307 (30.9)
	Distal		231/319 (72.4) [c]
Stage tumour [d]	Dukes A		7/287 (2.4)
	Dukes B		101/287 (35.2)
	Dukes A/B		110/293 (37.5) [e]
	Dukes C		79/287 (27.5)
	Dukes D		100/287 (34.8)
	Dukes C/D		183/293 (62.5) [e]

[a] Controls versus CRC patients, $P < 0.0001$.

[b] For 58 CRC cases, no information on the exact location of the tumour is available.

[c] For some patients only proximal or distal location was reported in the medical files.

[d] For 78 cases of CRC, no information about the exact stage of the tumour is available.

[e] For some patients only stage Dukes A/B or C/D was reported in the medical files.

The polymorphisms in the *PON1* gene corresponding with amino acid substitutions at the codons 55 and 192 were determined according to Humbert *et al.* (34). The forward and reverse primers used for detection of the L55M substitution were: 5’-GAA GAG TGA TGT ATA GCC CCA-3’ and 5’-TTT AAT CCA GAG CTA ATG AAA GCC-3’, respectively. The PCR conditions were 4 min at 95ºC, then 35 cycles of 30 s at 95ºC, 1 min at 56ºC, and 1 min at 72ºC, and finally an elongation step at 72ºC for 7 min. Digestion with *Nla*III resulted in a

non-digested 170-bp PCR product indicative for the *55L*-allele and 126- and 44-bp fragments for the *55M*-allele.

For detection of the Q192R substitution, we used the forward primer: 5'-TAT TGT TGC TGT GGG ACC TGA G-3' and reverse primer: 5'-CAC GCT AAA CCC AAA TAC ATC TC-3'. Except for the annealing temperature, which was 54ºC for 1 min, similar PCR conditions were used as described for detection of the L55M substitution. Digestion with *Alw*I yielded a non-digested 99-bp fragment indicative for the *192Q*-allele and 66- and 33-bp fragments for the *192R*-allele. Digested PCR products of the two *PON1* polymorphisms were separated on 3% agarose and visualized using ethidium bromide. At each PCR run, sterile H_2O was run in parallel with genomic DNA samples and served as negative control for amplification.

Statistical analyses

Differences between characteristics of patients and controls were analyzed with χ^2 test and *t*-test. A *P*-value below 0.05 was considered significant. All genotypes investigated among controls were tested to find out whether they were distributed according to the Hardy-Weinberg equilibrium. χ^2 statistic was used to test for differences in genotype distribution and allele frequencies between the two study groups. Odds ratios (OR) with 95% confidence interval (95% CI) were calculated by logistic regression analyses, taking into account confounding factors such as age and gender. Finally, the Spearman rank coefficient of correlation was calculated for the association between the two *PON1* polymorphisms. All statistical analyses were performed using SPSS (version 12.0; SPSS Inc., Chicago, USA).

Results

Descriptive statistics of CRC patients and controls are given in Table I. The mean age of CRC patients (64 ± 11 years) was higher compared to that of the control group (43 ± 13 years; $P < 0.0001$). Subsequently, there was a statistically significant difference in gender between CRC patients and healthy controls, with more female subjects in the control group ($P < 0.0001$). For all polymorphisms investigated, the distribution of the allele frequencies among the control subjects was tested and found to be in Hardy-Weinberg equilibrium.

No differences in genotype distribution were observed for the three polymorphisms investigated between CRC patients and controls (Table II). The allele frequencies also showed no statistical differences between the two study groups (Table III).

Table II. Distribution of *p22^{phox}* and *PON1* genotypes in patients with CRC and controls

Gene	Genotype	Controls (%) [a]	CRC patients (%) [a]	Crude OR (95%CI) [b]	Adjusted OR (95%CI) [c]
p22^{phox}	HH	169/336 (50.3)	177/365 (48.5)		
	HY	137 (40.8)	148 (40.5)		
	YY	30 (8.9)	40 (11.0)	1.1 (0.80-1.4)	1.2 (0.80-1.8)
PON1-55	LL	140/352 (39.8)	139/364 (38.2)		
	LM	162 (46.0)	166 (45.6)		
	MM	50 (14.2)	59 (16.2)	1.1 (0.79-1.4)	1.1 (0.73-1.6)
PON1-192	QQ	158/295 (53.6)	180/354 (50.8)		
	QR	120 (40.7)	150 (42.4)		
	RR	17 (5.8)	24 (6.8)	1.1 (0.82-1.5)	1.1 (0.76-1.7)

[a] In both the CRC and control group there are some missing data because of insufficient amount of DNA or unsuccessful PCR.

[b] Genotypes were combined on the basis of an expected phenotype-genotype relationship (variant genotypes with reduced or enhanced enzyme activity versus genotypes with normal enzyme activity) and crude OR were calculated. Starting from paraoxon as substrate, phenotype-genotype relationships for the PON1 polymorphisms were estimated (23).

[c] OR adjusted by age and gender were calculated.

Table III. Allele frequencies of *p22^{phox}* and *PON1* in patients with CRC and controls

Alleles	Controls (number/total) [a]	CRC patients (number/total) [a]	Crude OR (95% CI) [b]	Adjusted OR (95% CI) [c]
p22^{phox} 72H	0.71 (475/672)	0.69 (502/730)		
p22^{phox} 72Y	0.29	0.31	1.1 (0.87-1.4)	1.1 (0.93-1.3)
PON1 55L	0.63 (442/704)	0.61 (444/728)		
PON1 55M	0.37	0.39	1.1 (0.87-1.3)	1.1 (0.94-1.2)
PON1 192Q	0.74 (436/590)	0.73 (510/708)		
PON1 192R	0.26	0.27	1.1 (0.86-1.4)	1.1 (0.91-1.3)

[a] In both the CRC and control group there are some missing data because of insufficient amount of DNA or unsuccessful PCR.

[b] Crude OR were calculated.

[c] OR adjusted by age and gender were calculated.

We also investigated potential associations between allele frequencies of the detoxification enzymes and clinical characteristics of the patients, such as tumour location and tumour stage, and results are summarized in Table IV. These data only reveal a borderline association of the variant *PON1-55M* allele with distal CRC, age- and gender-adjusted OR 1.1, 95% CI 0.98-1.3. No relationship was found between allele frequencies of the investigated enzymes and tumour stage.

Table IV. Allele frequencies of *p22*^{phox} and *PON1* with respect to tumour location and tumour stage in patients with CRC

Gene	Proximal CRC (number/total) [a]	Distal CRC (number/total) [a]	CRC Dukes A/B (number/total) [a]	CRC Dukes C/D (number/total) [a]
p22phox 72H	0.67 (117/176)	0.70 (321/462)	0.70 (154/220)	0.68 (250/366)
p22phox 72Y	0.33	0.30	0.30	0.32
PON1 55L	0.63 (111/176)	0.59 (273/460) [b]	0.61 (134/220)	0.64 (235/366)
PON1 55M	0.37	0.41	0.39	0.36
PON1 192Q	0.71 (120/168)	0.74 (331/450)	0.72 (157/218)	0.73 (257/354)
PON1 192R	0.29	0.26	0.28	0.27

[a] There are some missing data because of insufficient amount of DNA or unsuccessful PCR.

[b] OR adjusted by age and gender were calculated. *PON1-55*: Distal CRC 1.1 (0.98-1.3).

Finally, the co-occurrence of the *PON1-55* and *PON1-192* polymorphisms was investigated. A negative correlation was found in both patients (r_s = -0.29, $P < 0.01$) and controls (r_s = -0.37, $P < 0.01$). Thus, the *PON1-55M* and *PON1-192Q* polymorphisms, both associated with low enzyme activity, are usually linked on one allele. This combination of *PON1* polymorphisms was not distributed differently between CRC patients and healthy controls.

Discussion

It is generally accepted that inter-individual differences in genetic factors of low penetrance and environmental exposures may influence the risk for CRC (35). Polymorphisms in genes encoding for detoxification enzymes may be of importance in susceptibility to toxic or carcinogenic environmental chemicals (5, 36). In addition, there is increasing evidence that ROS may also play a role in human cancer development (6-8). To investigate the role of

NAD(P)H oxidase and paraoxonase enzymes in colorectal carcinogenesis, we determined the frequencies of *NAD(P)H oxidase p22^{phox}* and *PON1* gene polymorphisms in healthy controls and patients with sporadic CRC. The observed frequencies of the variants of *p22^{phox}* and *PON1* in our control population are in full agreement with the corresponding data from several other studies (14, 17, 30, 37, 38).

Perner *et al.* (10) concluded that superoxide, released in epithelial cells isolated from the normal human colon by NAD(P)H oxidases expressing the p22^{phox} subunit, can contribute to the maintenance of normal colonic barrier. Knowing this, it may be hypothesized that polymorphisms in *NAD(P)H oxidase p22^{phox}* can contribute to an increased risk of colon cancer, but the results of this study do not confirm this hypothesis. The *p22^{phox}-72Y* homozygotes, however, may have only diminished and not a complete loss of enzyme activity (14), and this reduced enzyme activity can still be sufficient to maintain the normal colonic barrier.

Since, it has been demonstrated that polymorphisms affecting amino acid substitutions at positions 55 and 192 are associated with marked alterations of PON1 serum concentrations (23, 24) and PON1 serum levels were reported to modulate susceptibility towards gastrointestinal cancers (31, 32), we investigated the relationship between *PON1* polymorphisms and CRC. No differences were observed for genotype distributions and allele frequencies between patients and controls, except for a trend towards a slightly increased risk for distal CRC in individuals bearing the *PON1-55M* allele. The *PON1-55M* variant is associated with a reduced enzyme activity (23, 24), which could mean that the conversion rate of carcinogenic compounds is decreased and that these compounds may accumulate in the colonic lumen. Nevin *et al.* (16) reported that the *PON1* genotype accounts for 76% of the variation in serum PON enzyme activity level. In addition, PON1 serum levels are modulated by disease state, dietary-, lifestyle- and environmental factors and, therefore, may vary up to 13-fold between individuals (17, 39). Moreover, human studies characterizing the *PON1* polymorphisms have indicated the importance of estimating the PON1 status (*i.e.* genotype and phenotype taken together) rather than genotyping alone (40). Unfortunately, no serum was available from the majority of our patients, so we were unable to measure serum PON1 enzyme activities.

Our findings have to be viewed in the perspective of potential limitations. Odds ratios can only be calculated correctly when confounding factors, such as age, gender, diet and other lifestyle factors are taken into consideration. In this study we observed a statistically significant difference in age and gender between CRC patients and controls. By including both age and gender in the logistic regression analyses, we corrected the calculated odds ratios for differences in these factors. This is necessary because the younger control subjects, in comparison to the CRC patients, have a shorter time of exposure to carcinogens and, thus, at the moment they reach the age of the CRC patients, some of them may also have developed CRC. It would be preferable to better match control and patient populations, but in practice this appeared very difficult to realize. In this study, no information was available on the dietary habits, alcohol use and smoking patterns of both patients and controls, which may also be confounding factors. Possibly some of the low-penetrance genes investigated here only contribute to CRC in combination with (some of) these dietary or lifestyle factors.
In conclusion, no association was found between polymorphic variations in *NAD(P)H oxidase* $p22^{phox}$ and *PON1* genes and the risk for sporadic CRC.

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Multiple polymorphisms in phase I and phase II detoxification enzymes and susceptibility to colorectal cancer

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Each day the colon is exposed to a large variety of ingested toxic or carcinogenic compounds. Metabolism of these compounds often requires modification by detoxification enzymes before they can be excreted from the body. Genetic polymorphisms in detoxification enzymes may result in altered enzyme activities, which may have consequences for detoxification (1). Apart from the diet, variations in enzyme activities might influence the levels of toxic or carcinogenic compounds in the colon. Therefore, genetic polymorphisms in detoxification enzymes could contribute to individual susceptibility to colorectal cancer (CRC).

Recently, we evaluated the effects of genetic polymorphisms in different families of detoxification enzymes. We found that the risk for sporadic CRC may be modulated by UDP-glucuronosyltransferase 1A6 (*UGT1A6)*, *UGT1A7* or NAD(P)H:quinone oxidoreductase 1 (*NQO1*) polymorphisms. No associations were found for genetic polymorphisms in *UGT1A1*, *UGT1A8*, glutathione S-transferases (*GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*), microsomal epoxide hydrolase (exon 3 or exon 4 of *EPHX1*)*,* NAD(P)H oxidase p22^{phox}, paraoxonase 1 (codon 55 or 192 of *PON1*), alcohol dehydrogenase (*ADH3*) and cytochrome P450 2E1 (5'-flanking region or intron 6 of *CYP2E1*; refs. 2-4).

Two meta-analyses (5, 6) summarized the inconsistent results that have been reported by previous case-control studies on genetic polymorphisms in detoxification enzymes and sporadic CRC susceptibility. For example, most studies investigating the relationship between the *GSTM1* polymorphism and CRC found no association, however two studies revealed a strong statistically significant association between the *GSTM1 null* genotype and CRC risk. A possible reason for this inconsistency is that a single polymorphism may only have a weak effect, while CRC susceptibility may be strongly associated with a combination of polymorphisms at multiple gene loci. In our earlier studies we investigated the effect of a single polymorphism (2-4). We now studied whether the simultaneous occurrence of different genetic polymorphisms in the detoxification enzymes as mentioned above, might alter the risk for sporadic CRC. Therefore, we assessed whether combinations of polymorphisms in detoxification enzymes could be found more often in patients with sporadic CRC than in healthy controls. Individuals were classified according to the number of variant high-risk genotypes as follows: 0, reference group with no high-risk genotypes; 1, individuals with one of the high-risk genotypes; 2, individuals with two of the high-risk genotypes; etcetera. Age and gender adjusted odds ratios (OR) with 95% confidence intervals (CI) were estimated by

logistic regression analysis. All statistical analyses were performed with SPSS (version 12.0; SPSS Inc., Chicago, USA).

So far, just a few studies have evaluated combinations of genetic polymorphisms in detoxification enzymes for a possible association with sporadic CRC. These studies only report on the combination 2 or 3 genetic polymorphisms. In a study by Kiss *et al.* (7), combined analysis of *CYP1A1*, *CYP2E1* (5'-flanking region) and *GSTM1* polymorphisms showed that individuals carrying all the three high-risk alleles have an increased risk for sporadic CRC (OR = 4.6, 1.2-25.7). In the present study, evaluation of the combination of *CYP2E1* (5'-flanking region) and *GSTM1* polymorphisms did not reveal an association between high-risk genotypes and sporadic CRC (see Table I). We also tested different combinations of polymorphisms in GST-isoenzymes (see Table I). No significant associations were found for any of the GST-combinations, which confirmed the results from previous studies on *GSTM1*/*GSTP1*- (8, 9), *GSTM1*/*GSTT1*- (9-12) and *GSTP1*/*GSTT1*-combinations in Caucasian CRC patients and control subjects (9).

Table I. Combinations of genetic polymorphisms in detoxification enzymes that have been studied previously among sporadic CRC patients and controls: results of our recent study

Number of polymorphisms	Controls	Sporadic CRC	Adjusted OR	95% CI
No. of *CYP2E1 5'-flanking region* and *GSTM1* variants (ref. 7)				
0	202/412	167/356	1	Reference
1	195	180	0.86	(0.57-1.3)
2	15	9	0.53	(0.17-1.6)
No. of *GSTM1* and *GSTP1* variants (refs. 8, 9)				
0	90/414	79/368	1	Reference
1	205	182	1.0	(0.61-1.8)
2	119	107	0.89	(0.67-1.2)
No. of *GSTM1* and *GSTT1* variants (refs. 9-12)				
0	179/415	150/370	1	Reference
1	200	184	1.1	(0.70-1.6)
2	36	36	0.79	(0.55-1.1)
No. of *GSTP1* and *GSTT1* variants (ref. 9)				
0	148/414	123/369	1	Reference
1	223	206	1.2	(0.81-1.9)
2	43	40	0.85	(0.60-1.2)

Next, we considered the polymorphisms in the genes of detoxification enzymes that catalyze the same type of metabolic reaction to evaluate the combined effects. CYP2E1 and ADH3

both catalyze the oxidation of ethanol. The combination of genetic polymorphisms in these enzymes revealed that with an increasing number of variant genotypes the risk for sporadic CRC also increased in comparison with individuals that carry no variant genotypes (see Table II). After evaluation of combinations of *EPHX1* and *PON1* polymorphisms, enzymes that both catalyze hydrolysis reactions, no significant associations between variant genotypes and the CRC risk were observed (see Table II).

Table II. Combination of genetic polymorphisms in phase I detoxification enzymes among sporadic CRC patients and controls

Number of polymorphisms	Controls	Sporadic CRC	Adjusted OR	95% CI
No. of *CYP2E1 5'-flanking region* or *intron 6* and *AHD3* variants				
0	216/383	168/319	1	Reference
1	151	129	1.3	(0.86-2.0)
2	13	18	2.7	(0.97-7.4)
3	3	4	-	-
No. of *EPHX1 exon 3* or *exon 4* and *PON1-55* or *-192* variants				
0	18/191	13/223	1	Reference
1	47	58	0.97	(0.32-2.9)
2	67	78	0.76	(0.26-2.3)
3	51	60	0.95	(0.31-3.0)
4	8	14	1.1	(0.22-5.5)

-, Not enough data to calculate an odds ratio

Table III shows the combined effects of polymorphisms in genes encoding for different GST-isoenzymes, which catalyze the conjugation of glutathione to ingested agents. No association was found for the number of genetic polymorphisms and CRC risk.

Table III. Combination of genetic polymorphisms in phase II detoxification enzymes among sporadic CRC patients and controls

Number of polymorphisms	Controls	Sporadic CRC	Adjusted OR	95% CI
No. of *GSTA1*, *GSTM1*, *GSTP1* and *GSTT1* variants				
0	27/410	28/368	1	Reference
1	132	102	1.3	(0.51-3.3)
2	147	151	1.2	(0.76-1.8)
3	93	72	0.95	(0.72-1.3)
4	11	15	0.93	(0.65-1.3)
No. of *UGT1A1*, *UGT1A6*, *UGT1A7* and *UGT1A8* variants				
0	42/390	27/359	1	Reference
1	90	85	1.9	(0.93-4.0)
2	63	74	1.9	(0.90-4.1)
3	191	170	2.6	(1.3-5.5)
4	4	3	-	-

-, Not enough data to calculate an odds ratio

UGTs catalyze the conjugation of glucuronic acid to ingested compounds. We found that the risk for CRC increased with the number of variant *UGT* genotypes. The ORs were 1.9, 1.9 and 2.6 in individuals with one, two or three variant genotypes, respectively (see Table III). However, note that only individuals carrying three variant genotypes have a statistically higher risk for CRC.

From our recent studies (2-4), in which we investigated single polymorphisms, we concluded that *UGT1A6*, *UGT1A7* or *NQO1* polymorphisms might modulate the risk for sporadic CRC. Now, we also examined the combination of these three polymorphisms (see Table IV). Individuals carrying all three variant genotypes had a statistically increased risk for CRC (OR 3.0, 1.3-6.8) compared with the reference group (no variant genotypes).

Table IV. Combination of genetic polymorphisms in detoxification enzymes among patients with sporadic CRC and controls

Number of polymorphisms	Controls	Sporadic CRC	Adjusted OR	95% CI
No. of *UGT1A6*, *UGT1A7* and *NQO1* variants				
0	29/403	27/359	1	Reference
1	104	74	1.1	(0.51-2.3)
2	203	164	1.7	(0.78-3.6)
3	67	94	3.0	(1.3-6.8)
Combination of all polymorphisms investigated; genetic polymorphisms that contribute the most to CRC risk				
NQO1			1.5	(0.95-2.5)
UGT1A7			2.1	(0.96-4.6)
CYP2E1 intron 6			3.2	(1.3-7.9)

-, Not enough data to calculate an odds ratio

Finally, all investigated genetic polymorphisms as well as age and gender, were simultanenously entered in a backward conditional logistic regression analysis, to determine which polymorphisms contribute most to CRC risk. The *NQO1*, *UGT1A7* and *CYP2E1* (intron 6) polymorphisms appeared in the last step model, corresponding odds ratios are presented in Table IV.

These data indicate that these polymorphisms contributed the most to sporadic CRC risk. In contrast with the results obtained from our recent study (2), in which we investigated single polymorphisms, the *UGT1A6* polymorphism does not seem to modulate the risk for sporadic CRC, whereas the contribution of the *CYP2E1* (intron 6) polymorphism was highly

significant now. Earlier (3), the *CYP2E1* (intron 6) polymorphism did not show an association with overall sporadic CRC (adjusted OR 1.8, 0.88-3.8), however it showed a statistically significant association with Dukes C/D stages of CRC (adjusted OR 2.2, 1.01-5.0).

Some limitations have to be considered in this study, including false positive results arising from multiple comparisons and the great difference in mean age of the CRC patients and controls.

Table V. Genetic polymorphisms in detoxification enzymes and sporadic CRC risk; whole study versus age- and gender-matched group

	Controls / CRC patients	Age and gender matched controls and CRC patients
Number of subjects	415 / 371	140 / 140
Mean age (years)	42 / 64	55 / 55
Gender (%)	Male: 40.5 / 57	48 / 48
	Female: 59.5 / 43	52 / 52
Gene	Adjusted OR (95%CI) for age and gender	OR (95%CI) for age and gender matched controls and patients
UGT1A1	1.2 (0.83-1.8)	1.5 (0.91-2.4)
UGT1A6	1.5 (1.03-2.3) [a]	1.6 (1.01-2.6) [a]
UGT1A7	2.4 (1.3-4.6) [a]	2.5 (1.2-5.2) [a]
UGT1A8	0.69 (0.30-1.6)	0.47 (0.14-1.6)
GSTA1	0.80 (0.54-1.2)	0.67 (0.41-1.1)
GSTM1	0.81 (0.54-1.2)	0.66 (0.41-1.1)
GSTP1	0.93 (0.62-1.4)	0.95 (0.59-1.5)
GSTT1	1.0 (0.64-1.7)	0.73 (0.40-1.3)
EPHX1 codon 113	1.0 (0.70-1.5)	0.86 (0.53-1.4)
EPHX1 codon 139	0.99 (0.66-1.5)	1.0 (0.64-1.7)
NQO1	1.6 (1.03-2.4) [a]	1.6 (0.97-2.7)
CYP2E1 5'-flanking region	1.1 (0.48-2.4)	1.4 (0.56-3.7)
CYP2E1 intron 6	1.8 (0.88-3.8)	1.8 (0.80-4.4)
ADH3	0.83 (0.54-1.3)	0.77 (0.46-1.3)
$p22^{phox}$	1.2 (0.80-1.8)	1.2 (0.73-1.9)
PON1 codon 55	1.1 (0.73-1.6)	1.1 (0.67-1.8)
PON1 codon 192	1.1 (0.76-1.7)	0.99 (0.58-1.7)

[a] Statistically significant.

By including age in the logistic regression analyses we corrected the calculated odds ratios for this factor. This is necessary because the younger control subjects, in comparison to the CRC patients, have a shorter time of exposure to carcinogens and thus at the moment they reach the age of the CRC patients, some of them may also have developed CRC. However, we also compared the genotype distributions of the investigated polymorphism in 140 age- and gender-matched case-control pairs, which were selected from the whole study group. Highly similar odds ratios with 95% CI were found between these two study groups (see Table V).

In conclusion, these findings suggest that *UGT1A6*, *UGT1A7*, *NQO1* and *CYP2E1* (intron 6) polymorphisms, which are associated with altered detoxification capacity, may modulate the susceptibility of sporadic CRC. In future, studies should be performed that examine the exact role of the enzymes involved (UGT1A6, UGT1A7, NQO1 and CYP2E1) and try to define the substrates of these enzymes, which are contributing to development of CRC. In this way the mechanism of colorectal carcinogenesis could be unravelled. In addition, studies on enhancement of the disturbed enzyme activity of the above enzymes could lead to reduction of the CRC cases.

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High oxygen radical production in patients with sporadic colorectal cancer

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Abstract

It is hypothesized that excessive generation of reactive oxygen species (ROS) by phagocytes or leakage from mitochondria may harm key genes or proteins responsible for intestinal cell homeostasis. This may initiate the multistage process of colon cancer development. The present study investigates whether ROS production by whole blood may contribute to the aetiology of colorectal cancer (CRC). Whole blood oxygen radical production was measured by luminol-enhanced chemiluminescence and performed in fourfold with and without the stimuli phorbol 12-myristate 13-acetate (PMA) and serum-treated zymosan (STZ). We evaluated patients (i) with a history of sporadic CRC at least 3 month after surgery, (ii) who are hereditary non-polyposis colorectal cancer (HNPCC) gene carriers and (iii) with familial adenomatous polyposis (FAP). For each patient group (n = 20) an age- and gender-matched healthy control group was measured. Unstimulated and PMA-stimulated values for maximal oxygen radical production were significantly higher in patients with sporadic CRC in comparison to controls (P = 0.01 and P = 0.04, respectively). Furthermore, trends toward higher unstimulated and PMA-stimulated area under the curve chemiluminescence were seen in CRC patients compared with controls (P = 0.08 and P = 0.09, respectively). In patients with HNPCC or FAP, unstimulated, PMA- or STZ-stimulated chemiluminescence did not differ as compared to their control groups. In conclusion, whole blood oxygen radical production was higher in patients with a history of sporadic CRC, in comparison with age- and gender-matched controls, which indicates that ROS may play a role in the aetiology of sporadic CRC.

Introduction

Cancer development is a multistage process. The cascade of molecular and cellular changes that occurs during the process of carcinogenesis can be mediated by a variety of endogenous and environmental factors (1, 2). It is suggested that reactive oxygen species (ROS) may play a role in human cancer development (1, 3, 4). Sources of ROS may be both exogenous (drugs, ozone, radiation) and endogenous (NO, phagocytes, leakage from mitochondria; ref. 1). In a recent review, Klaunig and Kamendulis (1) described the involvement of ROS in the various stages (initiation, promotion, progression) of the process of carcinogenesis.

In healthy individuals, the generation of ROS seems to be counterbalanced by the antioxidant defence, which is recruited either from endogenous sources (glutathione, cysteine, uric acid, bilirubin, etc.) or from the diet (vitamins A, C and E; refs. 5, 6). An imbalance between ROS and antioxidant defences in favour of the former creates oxidative stress (6, 7). This may occur when the antioxidant levels are low/depleted and when the formation of ROS is high. Severe oxidative stress may affect a variety of metabolic processes. For example, ROS can cause structural alteration in DNA and may affect cytoplasmic and nuclear signal transduction pathways (4). Furthermore, ROS can modulate the activity of the proteins that respond to stress and which regulate genes that are involved in cell proliferation, differentiation, and apoptosis (4).

Although it is clear that ROS may induce cellular changes similar to those produced by known carcinogens (4), the precise mechanisms remain unclear. For instance it is suggested that overproduction of ROS, such as superoxide and hydrogen peroxide, by polymorphonuclear leukocytes might sometimes initiate carcinogenesis (8). Phagocytosis by polymorphonuclear leukocytes results in the release of ROS, referred to as the respiratory burst, which plays an important role in host defence against certain micro-organisms. Excessive generation of ROS by these phagocytes may cause harm to surrounding tissue (8). When key genes or proteins responsible for intestinal cell homeostasis are targeted, dysplasia and subsequent development of adenoma or carcinoma may occur.

Data on a role for ROS in the pathogenesis of colorectal cancer (CRC) are accumulating. Recently, Schmielau and Finn (9) reported that patients with advanced cancer of the colon,

pancreas, or breast showed signs of extensive granulocyte activation with release of ROS. In addition, these patients had elevated plasma levels of isoprostane, a product of lipid oxidation and a marker for oxidative stress. In addition, Gackowski *et al.* (10) observed that the levels of 8-oxo-2'-deoxyguanosine, a typical product of ROS-induced DNA base modification, were significantly higher in lymphocytes of patients with CRC in comparison to control subjects. Furthermore, they also found lower antioxidant plasma levels in patients with CRC in this study. They hypothesized that severe oxidative stress resulting in the production of ROS is responsible for this consumption of the antioxidant vitamins, creating a pro-oxidant environment in the blood of CRC patients. However, it is also possible that this pro-oxidant environment is characteristic for advanced stages of colon cancer and that oxidative stress is a result of the disease itself. Oldenburg *et al.* (11) demonstrated that whole blood ROS production was significantly higher in patients with inflammatory bowel disease compared to healthy volunteers. Additionally, inflammatory bowel disease has been associated with increased risk for colon cancer (12, 13). Furthermore, Keshavarzian *et al.* (14) found that normal-appearing rectal mucosa of patients with a history of CRC was able to produce higher levels of luminol-enhanced chemiluminescence than corresponding mucosa of control subjects.

Aim

The aim of this study was to investigate whether ROS production by whole blood plays a role in the aetiology of CRC. Oxygen radical production can be measured by means of the luminol-enhanced chemiluminescence technique and can be performed on isolated neutrophils as well as in whole blood, the latter due to the high sensitivity of this technique (15-17). We measured oxygen radical production using luminol-enhanced chemiluminescence with or without the stimuli phorbol 12-myristate 13-acetate (PMA) and serum treated zymosan (STZ), in whole blood from patients with a history of sporadic CRC or at risk for CRC (hereditary non-polyposis colorectal cancer (HNPCC) gene carriers or familial adenomatous polyposis (FAP)) and healthy control subjects.

Materials and methods

Patients and control subjects

Patients with a history of sporadic CRC (*n* = 20), gene carriers for HNPCC (*n* = 20), and patients with FAP (*n* = 20) were recruited at the Departments of Gastroenterology and General Surgery, University Medical Centre, Nijmegen, The Netherlands, from July 2003 until July 2004. In the patients with a history of sporadic CRC (*n* = 20) tumours were located in the cecum (*n* = 7), in the descending colon (*n* = 3), in the sigmoid (*n* = 4), in the rectosigmoid junction (*n* = 1), or in the rectum (*n* = 5). Cancer diagnosis was confirmed after histopathological investigation by a pathologist, and cases were classified according to Dukes' stages (A = 1, B = 11, C = 7, D = 1). These patients underwent surgery at least 3 months before blood sampling, and patients did not receive any adjuvant chemotherapy at the time of blood sampling. None of the CRC patients has had a previous tumour elsewhere. HNPCC patients were classified according to the Amsterdam Criteria (18). All HNPCC patients were mismatch repair gene mutation carriers (10 MLH-1, 8 MSH-2, 2 MSH-6). FAP patients were diagnosed as having more than 100 colorectal adenomatous polyps and belonged to typical FAP families or were gene carriers. Healthy volunteers, who served as controls, were recruited from the Nijmegen area, by advertisement in a local paper. Controls had to be at least 18 years of age and were excluded when they had a (family) history of CRC. Patients and controls were matched for age and gender in order of inclusion in the study. All subjects were Caucasians of Dutch origin. Data on smoking habits and alcohol consumption for patients and controls were obtained from the medical files and a questionnaire, respectively. It is known that antioxidants may influence ROS levels. Unfortunately, no information on the use of antioxidants could be obtained from the medical file of most of the patients and therefore we were not able to account for this factor. The local medical ethical review committee approved the study and all subjects gave their written informed consent.

Materials

Reagents were from Sigma Chemicals (St Louis, MO), unless stated otherwise. Stock solutions of PMA (0.1 mg/ml DMSO) and ammonium persulfate (APS; 10% in phosphate-buffered saline (PBS)) were stored at -20ºC until use. STZ (10 mg/ml 0.9% NaCl) was prepared as described (19) and stored at -80ºC until use. Hank's balanced salt solution (HBSS) was from Life Technologies Ltd. (Paisley, Scotland). PBS (pH 7.4) contained Na^+

163.9 mmol/l, Cl^- 140.3 mmol/l, HPO_4^{2-} 10.9 mmol/l, and H_2PO_{4-} 1.8 mmol/l. A stock solution of luminol (10 mmol/l in DMSO) was kept in the dark at room temperature.

Procedures and assays

Whole blood was obtained from each subject by venapuncture in sterile vacutainer tubes anticoagulated with lithium-heparin (chemiluminescence) or EDTA (leukocyte count and differentiation). Within 1 h after blood sample collection an *ex vivo* whole blood chemiluminescence assay was performed in 96-wells plates at 37ºC using an automated LB96V Microlumat Plus Luminometer (EG&G Berthold, Vilvoorde, Belgium). Briefly, heparin blood was diluted 1:100 in HBSS and 200 µl of this diluted blood was added to each well. Luminol-enhanced chemiluminescence was measured with or without the stimuli PMA (receptor-independent) or STZ (receptor-dependent). Twenty microlitres of PMA (0.5 µg/ml final) or STZ (1 mg/ml final) was added to each well. As internal standard 0.1% APS in PBS (stock 1:100 diluted) was run simultaneously. Each measurement was performed in fourfold. To each well, containing either whole blood or APS, 20 µl of 1 mmol/l luminol (stock 1:10 diluted in HBSS supplemented with 0.5% (w/v) bovine serum albumin) was added. Chemiluminescence was monitored every 145 s for 1 h and maximal oxygen radical production (relative light units (RLU)/s) and area under the curve (AUC) in this period (RLU) were calculated. Data were analyzed with Winglow (EG&G Berthold) software. Afterwards, these data were corrected for the unstimulated chemoluminescence and values were expressed as RLU per neutrophil.

Statistical analyses

Differences between characteristics of patients and controls were analyzed with the Wilcoxon rank-sum test or χ^2 test. A *P*-value below 0.05 was considered significant. The Wilcoxon rank-sum test was used to assess statistical significance of differences between control and treatment groups. In addition, Kruskal-Wallis ANOVA was performed to assess statistical significance in chemiluminescence values between the three control groups. Values are given as median and range unless stated otherwise. All statistical analyses were performed using SPSS (version 12.0; SPSS Inc., Chicago, USA).

Results

Characteristics of the three study and control groups are given in Table I. Age, gender, and leukocyte and neutrophil counts did not differ significantly between each patient and corresponding control group.

Table I. Characteristics of patients and controls

Characteristic	Group	Sporadic CRC		HNPCC gene-carrier		FAP	
		Controls	Cases [a]	Controls	Cases [a]	Controls	Cases [a]
Age [years]		55	61	43	42	44	42
(range)		(46-71)	(46-76)	(26-56)	(27-55)	(21-64)	(19-64)
Gender (%)	Female	9 (45)	8 (40)	11 (55)	12 (60)	8 (40)	8 (40)
	Male	11 (55)	12 (60)	9 (45)	8 (40)	12 (60)	12 (60)
Smoking (%)	No	7 (35)	8 (40)	9 (45)	5 (25)	10 (50)	4 (20)
	Yes	5 (25)	4 (20)	5 (25)	2 (10)	7 (35)	4 (20)
	Stopped	8 (40)	3 (15)	6 (30)	-	3 (15)	1 (5)
	Unknown	-	5 (25)	-	13 (65)	-	11 (55)
Alcohol (%)	No	11 (55)	5 (25)	7 (35)	5 (25)	8 (40)	3 (15)
	Yes [b]	9 (45)	8 (40)	13 (65)	-	12 (60)	5 (25)
	Stopped	-	2 (10)	-	1 (5)	-	-
	Unknown	-	5 (25)	-	14 (70)	-	12 (60)
Leukocyte count [$*10^9$/l]		6.7	6.1	5.9	6.3	7.0	6.3
(range)		(5.1-8.9)	(4.0-10.0)	(4.0-9.6)	(3.3-10.0)	(4.0-10.0)	(3.1-11.0)
Neutrophil count [$*10^9$/l]		4.1	4.0	3.6	4.0	4.4	4.2
(range)		(3.0-6.2)	(2.0-7.3)	(2.0-5.8)	(1.7-7.7)	(2.0-7.0)	(1.9-8.5)

[a] In all patient groups there were missing data on smoking and/or alcohol consumption, because lack of information on these lifestyle habits in the medical files.

[b] Alcohol consumption less than 60 g (females) or 80 g (males) of ethanol per day.

Data on whole blood oxygen radical production are summarized in Table II. Unstimulated and PMA-stimulated values for maximal oxygen radical production were significantly higher in patients with sporadic CRC in comparison to controls (P = 0.01 and P = 0.04, respectively). Furthermore, trends toward higher unstimulated and PMA-stimulated AUC chemiluminescence were seen in the CRC patient group compared with the control group (P = 0.08 and P = 0.09, respectively). After stimulation with STZ no differences in either maximal or AUC chemiluminescence were observed in sporadic CRC patients compared to control subjects.

Table II. Whole blood oxygen radical production in patients with a history of/at risk for CRC and controls

Chemiluminescence	Stimulus	Sporadic CRC		HNPCC gene-carrier		FAP	
		Controls	Cases	Controls	Cases	Controls	Cases
Maximal	-	60 (25-100)	75 [b] (33-180)	60 (25-105)	70 (28-155)	60 (29-105)	65 (35-150)
	PMA [a]	75 (3-180)	115 [c] (20-195)	100 (25-475)	90 (26-195)	70 (2-475)	85 (47-195)
	STZ [a]	225 (34-440)	235 (70-420)	240 (55-415)	265 (155-330)	220 (38-440)	235 (155-446)
AUC	-	160 (70-270)	195 [d] (100-350)	180 (70-310)	185 (75-375)	170 (85-290)	165 (80-365)
	PMA [a]	145 (10-435)	185 [e] (45-290)	155 (40-435)	190 (105-305)	160 (5-325)	155 (100-295)
	STZ [a]	555 (70-1065)	605 (185-1070)	545 (105-980)	645 (430-890)	535 (80-1065)	600 (395-940)

Data were measured in fourfold and are given as median (range) in $*10^{-6}$ RLU/s/neutrophil or $*10^{-3}$ RLU/neutrophil for maximal and AUC values, respectively.
[a] Data were corrected for unstimulated measurements. Controls versus patients with a history of CRC: [b] $P = 0.01$, [c] $P = 0.04$, [d] $P = 0.08$, [e] $P = 0.09$.

Unstimulated, PMA-stimulated or STZ-stimulated maximal and AUC chemiluminescence did not differ between HNPCC gene carriers or FAP patient groups and their corresponding control groups. In addition, Kruskal-Wallis analyses revealed no statistically significant differences between the chemiluminescence values (unstimulated, PMA and STZ) of the three control groups (maximal, $P = 0.60$, 0.62 and 0.98, respectively; AUC, $P = 0.38$, 0.84 and 1.0, respectively). Chemiluminescence parameters showed no significant differences between females and males in each patient and control group. Within-run coefficients of variation for the internal standard (APS) were 3.3% for maximal and 1.8% for AUC chemiluminescence. Day-to-day coefficients of variations for the APS internal standard were 13.5% for maximal and 8.5% for AUC chemiluminescence.

Discussion

Evidence for a role of ROS in the pathogenesis of CRC is accumulating (9-11, 13, 14). The aim of this study was to investigate whether oxygen radical production by peripheral whole blood may contribute to the aetiology of CRC. By measuring luminol-enhanced

chemiluminescence in whole blood of healthy controls and patients with a history of CRC, HNPCC gene carriers, or patients with FAP, whole blood oxygen radical production could be calculated. Both unstimulated and PMA-stimulated oxygen radical production was higher in patients with a history of CRC, whereas no differences were observed between patients at risk for CRC due to hereditary syndromes like HNPCC and FAP, compared to age- and gender-matched healthy control subjects. These data correspond with the results reported by Keshavarzian *et al.* (14), who found that normal-appearing rectal mucosa of patients with a history of CRC was able to produce higher levels of luminol-enhanced chemiluminescence than corresponding mucosa of healthy control subjects. This finding is more or less similar to our observation that whole blood unstimulated chemiluminescence values were higher in patients with a history of CRC compared to healthy controls. The colon is an organ with an intensive blood supply and therefore ROS produced by mucosal biopsies may originate partly from the neutrophils present in the blood vessels. However, Perner *et al.* (20) observed that primary colonic epithelial cells, isolated from mucosal biopsies, produced superoxide. Furthermore, they found that mRNA coding for the catalytic subunits of NAD(P)H oxidase, Nox1 and p22phox, was detected in these cells. This finding was confirmed by Geiszt *et al.*, who observed high Nox1 expression in the entire colon and rectum (21). They also showed that Nox1 interacts with phagocytic oxidase components, such as $p22^{phox}$, $p47^{phox}$, or $p67^{phox}$, and observed a significant dependence on PMA stimulation for superoxide production (21). This raises the possibility of a functional homology between the phagocytic and intestinal oxidase enzymes (22). Phagocytic NAD(P)H oxidase ($gp91^{phox}$) plays an important role in the host defence against a broad range of micro-organisms by production of ROS, whereas intestinal NAD(P)H oxidase (Nox1) may provide an oxidative host defence barrier against intestinal pathogens in particular.

Phagocyte activation is necessary to elicit its effector functions and can be induced by several agents in a distinctive manner. The phorbol ester PMA and STZ, i.e. yeast particles opsonized with both IgG and C3 complement fragments, are the most frequently used agents in studies assessing neutrophil functions. PMA crosses the cellular membrane and binds directly to and activates protein kinase C (receptor-independent). This results in phosphorylation and translocation of the cytosolic NAD(P)H oxidase components, which in turn mediates the reduction of oxygen to form several ROS (23, 24). STZ comprises a particulate stimulus that binds to membrane complement receptors or Fcγ receptors on neutrophils. After binding to these receptors, the STZ particles are ingested and concomitantly this leads to respiratory

burst and degranulation (25). The present study showed a higher PMA-stimulated oxygen radical production in patients with a history of sporadic CRC, compared to healthy controls. This suggests that the receptor-independent activation of neutrophils differs between these groups. In patients with a history of CRC, PMA homologous stimuli may provoke a reaction that results in a higher production of ROS; eventually this may be harmful to the surrounding tissue and may have contributed to the development of CRC.

Next, the question may arise as to why patients eventually do develop colon cancer as a result of overproduction of ROS and not all other kinds of cancer. This could be explained by the characteristics of the colonic mucosa, being a highly proliferative tissue with a high load of toxic or carcinogenic compounds and low levels of protecting enzymes (26, 27).

Wyche et al. (28) reported that a functional polymorphism in codon 72 of the gene encoding for the NAD(P)H subunit $p22^{phox}$ results in a 30% reduction in respiratory burst after PMA stimulation, suggesting decreased activity of the NAD(P)H oxidase. These variations in *NAD(P)H oxidase* $p22^{phox}$ genotypes eventually could lead to different amounts of ROS in the colonic mucosa, which actually may influence the risk for CRC. However, in a case-control study performed by us, genotyping of this $p22^{phox}$ codon 72 polymorphism in 365 sporadic CRC cases and 354 healthy controls revealed no association between this polymorphism and risk for CRC (29).

In patients with HNPCC or FAP, unstimulated, PMA-stimulated or STZ-stimulated chemiluminescence did not differ as compared to the corresponding control groups, whereas unstimulated or PMA-stimulated values were significantly higher in sporadic CRC patients in comparison to their controls. This difference between patients with HNPCC or FAP versus patients with sporadic CRC could be explained by the fact that a genetic factor of high penetrance is responsible in HNPCC or FAP, whereas such a driving factor is missing in sporadic CRC patients. However, in the latter group, ROS could be a factor of importance in the process of carcinogenesis, as revealed by our findings. In contrast to the sporadic CRC group, HNPCC or FAP patients did not have had a resection for a tumour in the colon, and it would be worthwhile to also investigate the ROS production in patients with HNPCC or FAP after development of a colon tumour; however such patients are not available to us at this moment.

In our three patient groups there were some missing data on smoking and/or alcohol consumption, because no information on these lifestyle habits was reported in the medical files. Therefore, and also because of the small groups tested, no estimation could be made of the influence of these smoking and drinking habits on the oxygen radical production. Kopprasch *et al.* (16) reported that smoking of 10 or more cigarettes per day had no effect on phagocyte oxygen radical production. In addition, Lafuente *et al.* (15) found no difference in basal chemiluminescence values between smokers and non-smokers; however, healthy smokers showed a significantly higher PMA-stimulated chemiluminescence when compared with non-smokers. Furthermore, the use of antioxidants could contribute to the differences between the groups. Unfortunately, however, we do not have information on the use of antioxidants. It seems not very likely, however, that individuals in one study group (sporadic CRC) are far more deficient in antioxidants as compared to the individuals in the other study groups.

Luminol-enhanced chemiluminescence is an accepted and reliable means of estimating oxygen radical production, and its measurement can be performed in isolated neutrophils as well as in whole blood (15-17). Kopprasch *et al.* (16) compared both methods and enumerated their advantages and disadvantages. In short, the time required to separate neutrophils, the risk of cell priming during the separation procedure, and the relatively large amounts of blood needed are considered major limitations of chemiluminescence measurements with isolated neutrophils. The whole blood assay has the advantage of avoiding neutrophil pro-activation during the purification process. Furthermore, measurement in whole blood allows monitoring of cellular responses in an environment more closely resembling the *in vivo* situation than analysis of isolated neutrophils. On the other hand, the whole blood chemiluminescence assay is flawed due to light quenching by erythrocytes or plasma proteins (decreased sensitivity), whereas production of ROS by other cells than neutrophils, like monocytes and platelets, may occur. Egger *et al.* (30) investigated the changes in neutrophil function in whole blood induced by storage time, temperature, and agitation. They recommend that blood samples should be processed carefully without mechanical stress, however blood samples can be handled at room temperature. These criteria were taken into consideration before performing our whole blood chemiluminescence assay.

In conclusion, whole blood oxygen radical production was higher in patients with a history of CRC, in comparison with age- and gender-matched healthy controls, which may indicate a role for ROS in the aetiology of sporadic CRC.

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Summary and conclusions

Colorectal cancer (CRC) is one of the most common forms of cancer in Western societies and shows a 20-fold geographic variation in incidence worldwide. In The Netherlands, cancer incidence rates in women are highest for breast cancer followed by CRC, whereas in men CRC cancer incidence rates are third highest after lung and prostate cancer. The aetiology of CRC is complex and involves genetic (high- and low-penetrance) as well as lifestyle factors (dietary habits, alcohol use, smoking). It is now generally accepted that a time-dependent accumulation of mutations in genes involved in the regulation of cell proliferation and cell death (apoptosis) results in the transformation of normal colonic epithelium into hyperproliferative tissue, adenoma, and finally eventually in carcinoma. Epidemiological studies have revealed that the worldwide variation in CRC incidence is strongly related to differences in dietary habits and lifestyle factors. Consumption of a typical Western-style diet, which is characterized by high intake of red meat, (saturated) fat and alcohol, and a low intake of fresh vegetables, fruits and calcium, is associated with a high risk for CRC. It is assumed that the Western-style diet contains more harmful and carcinogenic compounds as compared to the diets containing more fruits and vegetables. Humans however, possess a highly efficient system of defence against such harmful compounds and detoxification enzymes, such as glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs), play an important role in detoxification. However, when the detoxification capacity of the colon is insufficient to deal with the daily load of toxic or carcinogenic compounds, this may lead to DNA mutations and damage of cells and ultimately to the development of CRC.

In **Chapter 1** a general introduction is presented and the objectives of the thesis are summarized as follows: 1) to investigate the modulation of rat hepatic and intestinal UGTs by anticarcinogens, 2) to study whether genetic polymorphisms in detoxification enzymes may modulate the risk for CRC, and 3) to elucidate whether neutrophil oxygen radical production may contribute to the aetiology of CRC.

In the **Chapters 2.1 and 2.2** the effects of potential anticarcinogens on rat hepatic and intestinal UGT enzyme activities are described. As a representative of cruciferous vegetables we supplemented the standard diet with Brussels sprouts. In addition, the following compounds, present in the digestive tract after consumption of cruciferous vegetables (e.g. Brussels sprouts and broccoli), were studied: indole-3-carbinol, compound 30 (a sulforaphane analog), phenethylisothiocyanate (PEITC; all glucosinolate hydrolysis products) and oltipraz (a substituted dithiolthione). Furthermore, we studied the following

non-nutritive dietary anticarcinogens: ellagic acid, ferulic acid, quercetin, α-angelicalactone, tannic acid, coumarin, fumaric acid, curcumin, flavone, D-limonene and the carotenoids α-tocopherol and β-carotene (all naturally occurring component of vegetables and fruits). Most anticarcinogens studied were shown to enhance UGT enzyme activity at one or more sites in the gastrointestinal tract. In the liver, the most striking enhancement of 4-methylumbelliferone (MUB) UGT enzyme activity was seen after supplementation of the diet with flavone (10.6×), coumarin (6.2×), α-angelicalactone (4.2×) or D-limonene (3.2×). Curcumin was the most potent compound in the colon, enhancing 4-MUB UGT enzyme activity 3.1-fold, followed by coumarin (2.7×), quercetin and α-angelicalactone (both 2.2×). In the small intestine, curcumin showed the largest effect on 4-MUB UGT enzyme activity (3.2×, 4.3× and 6.1× in the proximal, mid and distal part, respectively). No statistically significant changes in 4-MUB UGT enzyme activities were found with tannic acid, α-tocopherol, β-carotene and indole-3-carbinol in all organs investigated. Since humans may be exposed to mixtures of anticarcinogens in their diets, it may be worthwhile to study combinations of such compounds in laboratory animals in order to learn more on possible additional effects. Therefore, we conducted a dose-dependent combination study with α-angelicalactone and flavone in rats, as described in **Chapter 2.1**. UGT enzyme activity was increased most in rats that were fed the highest dose of α-angelicalactone, flavone or the combination of both compounds. In addition, dietary administration of α-angelicalactone and flavone, even at relatively low concentrations, may exert anticarcinogenic effects in the liver and to a lesser extent in the small- and large intestine by enhancing UGT detoxification activity. Exposure to the combination of both compounds showed additional effects on UGT enzyme activity. From these studies it may be concluded that dietary anticarcinogens may exert their chemopreventive effects in the gastrointestinal tract of the rat by enhancing UGT enzyme activity. Enhancement of UGT enzyme activity might lead to a more efficient elimination of carcinogens. The combined effect of dietary anticarcinogens may therefore contribute to the prevention of cancer in the gastrointestinal tract. In **Chapter 2.2** also another class of anticarcinogens was studied; the nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs have been shown to inhibit the process of carcinogenesis in laboratory animals as well as in humans. After supplying NSAIDs to the diet of rats, enhancement of small intestinal UGT enzyme activity was mainly observed in the proximal part by ibuprofen and indomethacin. In addition, ibuprofen was also able to enhance large intestinal UGT enzyme activity. However, none of the NSAIDs had a significant effect on hepatic UGT enzyme

activity. The enhancement of UGT activity in the upper part of the gastrointestinal tract, resulting in a more efficient detoxification, may directly contribute to the tumor preventing properties of NSAIDs in this part of the gastrointestinal tract. However, organs such as the colon could also benefit from a more efficient detoxification in the proximal part of the gastrointestinal tract, since lower concentrations of carcinogens may reach the colon.

The **Chapters 3.1 to 3.4** are focussed on the possible role of genetic polymorphisms in detoxification enzymes in the aetiology of sporadic CRC. Therefore, the possible association between genetic polymorphisms in detoxification enzymes and predisposition to sporadic CRC are studied. UDP-glucuronosyltransferases (UGTs) and glutathione S-transferases (GSTs) are important detoxification enzymes (**Chapter 3.1**). The genetic polymorphisms in the different UGT- and GST-isoenzymes (*UGT1A1*, *UGT1A6*, *UGT1A7*, *UGT1A8*, *GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*) as studied here, may result in lower enzyme activities and subsequent less efficient conversion of toxic or carcinogenic compounds. After adjustment for age and gender, polymorphic variants in the *UGT1A6* and *UGT1A7* genes were found significantly more often in patients with CRC (n = 371) as compared to healthy controls (n = 415), indicating that the presence of these polymorphic variants may contribute to development of sporadic CRC. In **Chapter 3.2** the contribution of genetic polymorphisms in other enzymes that may be involved in detoxification of CRC related carcinogens, being: microsomal epoxide hydrolase (*EPHX1*), NAD(P)H:quinone oxidoreductase (*NQO1*), cytochrome P450 2E1 (*CYP2E1*) and alcohol dehydrogenase (*ADH3*) is described. Genetic polymorphisms in *EPHX1*, *CYP2E1* and *ADH3* occurred equally frequent in patient and control groups. Polymorphic *NQO1* variants however, were found significantly more often in patients with sporadic CRC. The presence of variant *NQO1* alleles may result in a reduced or complete loss of enzyme activity and consequently to a partly deficient detoxification. Therefore, the presence of these polymorphic variants may contribute to the development of sporadic CRC. The study in **Chapter 3.3** was performed to investigate the possible association between genetic polymorphisms in NAD(P)H oxidase $p22^{phox}$ or paraoxonase 1 (*PON1*) and sporadic CRC. Similar distributions of the $p22^{phox}$ and *PON1* genotypes are present in healthy control subjects and patients with sporadic CRC, suggesting that these polymorphisms are not associated with sporadic CRC. Based on the results of these studies, we conclude that individuals carrying the low enzyme activity associated genotypes of *UGT1A6*, *UGT1A7* or *NQO1* might be more prone to develop sporadic CRC. **Chapter 3.4** describes the results of combinations of polymorphisms in the genes of detoxification

enzymes as studied in the **Chapters 3.1 to 3.3**. The number of genetic polymorphisms in detoxification enzymes may influence the occurrence of sporadic CRC. When considering all investigated polymorphisms, the *NQO1*, *UGT1A7* and *CYP2E1* (intron 6) polymorphisms contributed most to the risk for sporadic CRC. Summarizing, this suggests that impairment in detoxification of toxic or carcinogenic compounds as a result of genetic polymorphisms in *NQO1*, *UGT1A6*, *UGT1A7* and *CYP2E1* may contribute to the aetiology of sporadic CRC.

Chapter 4 reports on a study on neutrophil oxygen radical production in individuals at risk for CRC. Oxygen radical production was measured in whole blood from patients with (i) a history of sporadic CRC, (ii) who are hereditary non-polyposis colorectal cancer (HNPCC) gene carriers and (iii) with familial adenomatous polyposis (FAP), and also in age- and gender-matched controls. Whole blood oxygen radical production was significantly higher in patients with a history of sporadic CRC in comparison with the age- and gender-matched controls, whereas oxygen radical production did not differ between HNPCC or FAP patients and their corresponding control groups. These data indicate that ROS might play a role in the aetiology of sporadic CRC.

In summary, we may conclude that anticarcinogens, components of fruit and vegetables, and to a lesser extent NSAIDs can induce rat hepatic and intestinal UGT enzyme activities. This enhancement in UGT enzyme activity may lead to a more efficient detoxification of toxins and carcinogens and subsequently this might inhibit carcinogenesis. It also seems worthwhile to study the combined effect of dietary anticarcinogens, since additional or even synergistic effects may occur. The significance of the results in animal studies should be verified in human intervention studies.
Furthermore, a possible modulating effect on the risk for sporadic CRC might be due to polymorphic variations in detoxification enzymes, in particular *NQO1*, *UGT1A6, UGT1A7* and *CYP2E1* (intron 6), which may result in an activation or impaired deactivation of toxic compounds.
At last, we showed by measuring whole blood oxygen radical production in patients with a history or at risk for CRC and age- and gender-matched controls, that oxidative stress might be involved in the aetiology of sporadic CRC.

Samenvatting en conclusies

Dikke darmkanker is een van de meest voorkomende vormen van kanker in de Westerse wereld en wereldwijd is er een 20-voudige geografische variatie in incidentie te zien. In Nederland, is de incidentie van kanker bij vrouwen het hoogst voor borstkanker, gevolgd door dikke darmkanker, terwijl bij mannen dikke darmkanker het op drie na vaakst voorkomt, na long- en prostaatkanker. De etiologie van dikke darmkanker is complex en betreft zowel genetische- als leefstijl factoren (voedingsgewoonten, alcohol consumptie, roken). Algemeen wordt erkend dat een tijdsafhankelijke opeenhoping van mutaties in genen betrokken bij de regulatie van celproliferatie en celdood (apoptose) resulteert in de overgang van normaal dikke darmepitheel naar hyper-proliferatief weefsel, adenoom en uiteindelijk carcinoom. Epidemiologische studies hebben laten zien dat de wereldwijde variatie in de incidentie van dikke darmkanker sterk gerelateerd is aan verschillen in voedingsgewoonten en leefstijl factoren. De consumptie van een typisch Westers dieet, dat wordt gekarakteriseerd door een hoge inname van rood vlees, (verzadigd) vet en alcohol en een lage inname van verse groeten, fruit en calcium, wordt geassocieerd met een hoog risico op dikke darmkanker. Verder wordt verondersteld dat het Westers dieet meer schadelijke en kankerverwekkende (carcinogene) stoffen bevat in vergelijking met voeding die meer groeten en fruit bevat. Mensen bezitten echter een zeer efficiënt afweersysteem tegen zulke schadelijke stoffen en vooral ontgiftingsenzymen zoals glutathion S-transferasen (GST) en UDP-glucuronosyltransferasen (UGT) spelen een belangrijke rol in de ontgifting. Echter, wanneer de ontgiftingscapaciteit van de dikke darm tekortschiet om de dagelijkse belasting van toxische en kankerverwekkende stoffen te kunnen opvangen, kan dit leiden tot celschade en uiteindelijk tot het ontstaan van dikke darmkanker.

In **Hoofdstuk 1** wordt een algemene introductie gegeven en worden de doelen van dit proefschrift als volgt samengevat: 1) onderzoek naar de modulering van lever- en darm UGT door anticarcinogenen in de rat, 2) bestuderen of genetisch polymorfismen in ontgiftingsenzymen het risico op dikke darmkanker kunnen beïnvloeden, en 3) onderzoek naar de rol van zuurstof radicaal productie door neutrofielen in de etiologie van dikke darmkanker.

In de **Hoofdstukken 2.1 en 2.2** worden de effecten van potentiële anticarcinogenen op de lever- en darm UGT enzymactiviteit van ratten beschreven. Als modeldieet voor crucifere (kruisbloemige) groenten hebben we aan het standaard ratten dieet spruitjes toegevoegd. Daarnaast werden de volgende stoffen bestudeerd, die ontstaan in het spijsverteringskanaal na

consumptie van crucifere groenten zoals spruitjes, bloemkool, broccoli etc.: indole-3-carbinol, compound 30 (een synthetisch analoog van sulforaphaan), phenethylisothiocyanaat (PEITC) en oltipraz (een gesubstitueerde dithiolthion). Bovendien bestudeerden we de volgende non-nutriënten en potentiële anticarcinogenen in voeding: de carotenoïden α-tocopherol (vitamine E) en β-caroteen (een voorloper van vitamine A), ellaginezuur, ferulinezuur, quercetine, α-angelicalacton, tanninezuur, coumarine, fumaarzuur, curcumine, flavon en D-limoneen (allemaal natuurlijk voorkomende componenten uit groenten en fruit). De meeste anticarcinogenen lieten op een of meer plaatsen van het maag-darmkanaal een verhoging van UGT enzymactiviteit zien. In de lever was de meest opvallende verhoging van 4-methylumbelliferon (MUB) UGT enzymactiviteit te zien na toevoeging van flavon (10,6×), coumarine (6,2×), α-angelicalacton (4,2×) of D-limoneen (3,2×) aan het dieet. Curcumine was de meest potente component in de dikke darm en verhoogde de 4-MUB UGT enzymactiviteit 3,1 keer, gevolgd door coumarine (2,7×), quercetine en α-angelicalacton (beide 2,2×). In de dunne darm liet curcumine het grootste effect op 4-MUB UGT enzymactiviteit zien (3,2×, 4,3× en 6,1× respectievelijk in het proximale, middelste en distale deel). Er werden geen statistisch significante veranderingen gezien in de 4-MUB UGT enzymactiviteit met tanninezuur, α-tocopherol, β-caroteen en indole-3-carbinol in alle bestudeerde organen. Aangezien mensen aan een mix van anticarcinogenen (aanwezig in hun voeding) kunnen worden blootgesteld, is het de moeite waard om de combinatie van zulke stoffen te bestuderen in proefdieren, zodat meer inzicht wordt verkregen in mogelijk elkaar versterkende effecten van de afzonderlijke componenten. Daarom hebben we een dosis-afhankelijke combinatiestudie met α-angelicalacton en flavon uitgevoerd in ratten, zoals beschreven staat in **Hoofdstuk 2.1**. De UGT enzymactiviteit werd het meest verhoogd in ratten die de hoogste dosis α-angelicalacton, flavon of de combinatie van beide stoffen gevoerd kregen. Bovendien kon toediening van α-angelicalacton en flavon via de voeding, zelfs bij relatief lage concentraties, anticarcinogene effecten teweegbrengen in de lever en in mindere mate in de dunne- en dikke darm, door een verhoging van de UGT ontgiftingsactiviteit. Blootstelling aan de combinatie van beide stoffen liet additionele effecten zien op de UGT enzymactiviteit. Uit deze studies kan geconcludeerd worden dat anticarcinogenen in voeding hun beschermende effecten op het maag-darmkanaal van de rat kunnen uitoefenen door verhoging van de UGT enzymactiviteit. De verhoging van de UGT enzymactiviteit zou kunnen leiden tot een efficiëntere afbraak en uitscheiding van carcinogenen. Het gecombineerde effect van anticarcinogenen in voeding kan derhalve bijdragen aan de preventie van kanker van het

maag-darmkanaal. In **Hoofdstuk 2.2** werd ook een andere klasse van anticarcinogenen bestudeerd, de niet-steroïde anti-ontstekingsmiddelen (nonsteroidal anti-inflammatory drugs, NSAIDs). NSAIDs hebben laten zien dat ze zowel in proefdieren als mensen het proces van kankervorming (carcinogenese) kunnen remmen. Na het toevoegen van NSAIDs aan het dieet van ratten werd met name in het proximale deel van de dunne darm een verhoging van de UGT enzymactiviteit gezien bij ibuprofen en indomethacine. Bovendien was ibuprofen ook in staat om de UGT enzymactiviteit in de dikke darm te verhogen. Echter, geen enkel NSAID had een significant effect op de UGT enzymactiviteit in de lever. De verhoging van de UGT enzymactiviteit in het bovenste gedeelte van het maag-darmkanaal, resulterend in een efficiëntere ontgifting, kan direct bijdragen aan de tumor preventieve eigenschappen van NSAIDs in dit deel van het maag-darmkanaal. Echter, organen zoals de dikke darm, zouden ook van een efficiëntere ontgifting in het proximale deel van het maag-darmkanaal kunnen profiteren, aangezien lagere concentraties aan carcinogenen de dikke darm zouden kunnen bereiken.

De **Hoofdstukken 3.1 tot en met 3.4** concentreren zich op de mogelijke rol van genetische polymorfismen in ontgiftingsenzymen bij het ontstaan van sporadische dikke darmkanker. Daarom wordt de mogelijke associatie tussen genetische polymorfismen in ontgiftingsenzymen en aanleg voor sporadische dikke darmkanker bestudeerd. UDP-glucuronosyltransferases (UGTs) en glutathion S-transferases (GSTs) zijn belangrijke ontgiftingsenzymen (**Hoofdstuk 3.1**). De genetische polymorfismen in de verschillende UGT- en GST-isoenzymen (*UGT1A1*, *UGT1A6*, *UGT1A7*, *UGT1A8*, *GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*) zoals hier bestudeerd werden, kunnen resulteren in een lagere enzymactiviteit en vervolgens in een minder efficiënte omzetting van toxische of carcinogene stoffen. Na correctie voor leeftijd en geslacht, werden polymorfe varianten in de *UGT1A6* en *UGT1A7* genen significant vaker gevonden in patiënten met dikke darmkanker (n = 371) in vergelijking met gezonde controles (n = 415), wat er op wijst dat de aanwezigheid van deze polymorfe varianten kan bijdragen aan de ontwikkeling van sporadische dikke darmkanker. In **Hoofdstuk 3.2** wordt de bijdrage van genetische polymorfismen in andere enzymen, die bij ontgifting van dikke darmkanker-gerelateerde carcinogenen betrokken kunnen zijn, zoals microsomaal epoxide hydrolase (*EPHX1*), NAD(P)H:quinone oxidoreductase (*NQO1*), cytochroom P450 2E1 (*CYP2E1*) en alcohol dehydrogenase (*ADH3*) beschreven. Genetische polymorfismen in *EPHX1*, *CYP2E1* en *ADH3* kwamen even vaak voor in de patiënten en controles. Echter, de polymorfe *NQO1* varianten werden significant vaker gevonden in

patiënten met sporadische dikke darmkanker. De aanwezigheid van variante *NQO1* allelen kan resulteren in verlaagde of compleet verlies van enzymactiviteit en dus in een gedeeltelijk deficiënte ontgifting en de aanwezigheid van deze polymorfe varianten zou dus kunnen bijdragen aan de ontwikkeling van sporadische dikke darmkanker. De studie in **Hoofdstuk 3.3** werd uitgevoerd om de mogelijke associatie tussen genetische polymorfismen in NAD(P)H oxidase $p22^{phox}$ of paraoxonase 1 (*PON1*) en sporadische dikke darmkanker te onderzoeken. Vergelijkbare verdelingen van de $p22^{phox}$ en *PON1* genotypen waren aanwezig in gezonde controle personen en patiënten met sporadische dikke darmkanker, dit suggereert dat deze polymorfismen geen rol spelen bij het ontstaan van sporadische dikke darmkanker. Gebaseerd op de resultaten van deze studies kunnen we concluderen dat personen, die de met lagere enzymactiviteit geassocieerde genotypen van *UGT1A6*, *UGT1A7* of *NQO1* dragen meer aanleg zouden kunnen hebben voor het ontwikkelen van sporadische dikke darmkanker. **Hoofdstuk 3.4** beschrijft de resultaten van combinaties van polymorfismen in de genen van ontgiftingsenzymen zoals bestudeerd in de **Hoofdstukken 3.1 tot en met 3.3**. Het aantal genetische polymorfismen in ontgiftingsenzymen zou het voorkomen van sporadische dikke darmkanker kunnen beïnvloeden. Wanneer we met alle onderzochte polymorfismen rekening houden, dragen de *NQO1*, *UGT1A7* en *CYP2E1* (intron 6) polymorfismen het meest bij aan het risico op sporadische dikke darmkanker. Samenvattend suggereert dit dat de insufficiënte ontgifting van toxische of carcinogene stoffen ten gevolge van genetische polymorfismen in *NQO1*, *UGT1A6*, *UGT1A7* en *CYP2E1* kan bijdragen aan de etiologie van sporadische dikke darmkanker.

Hoofdstuk 4 rapporteert over een studie naar neutrofiel zuurstof radicaal productie in personen met een hoger risico op dikke darmkanker. Zuurstof radicaal productie werd gemeten in volbloed van patiënten i) met een geschiedenis van sporadische dikke darmkanker, ii) die gendrager zijn van het erfelijk non-polyposis colorectaal kanker (HNPCC) syndroom en iii) met het familiair adenomateuze polyposis syndroom (FAP), en ook in corresponderende, voor leeftijd- en geslacht-gematchte controles. De volbloed zuurstof radicaal productie was significant hoger in patiënten met een geschiedenis van sporadische dikke darmkanker in vergelijking met de voor leeftijd- en geslacht-gematchte controles, terwijl de zuurstof radicaal productie niet verschilde tussen HNPCC of FAP patiënten en hun overeenkomstige controle groepen. Deze data wijzen er op dat reactieve zuurstof radicalen een rol zouden kunnen spelen in de etiologie van sporadische dikke darmkanker.

Samenvattend kunnen we concluderen dat anticarcinogenen, componenten uit groenten en fruit en in mindere mate NSAIDs, de lever- en darm UGT enzymactiviteit kunnen induceren in ratten. Deze verhoging van de UGT enzymactiviteit kan leiden tot een efficiëntere ontgifting van toxische en carcinogene stoffen wat vervolgens de carcinogenese zou kunnen remmen. Het lijkt ook zinvol om de gecombineerde effecten van anticarcinogenen in voeding te bestuderen, aangezien er additionele en zelfs synergistische effecten kunnen optreden. De betekenis van de resultaten uit dierstudies zouden echter geverifieerd moeten worden via humane interventie studies.

Een mogelijk modulerend effect op het risico voor het ontstaan van sporadische dikke darmkanker zou veroorzaakt kunnen worden door variaties in de genen coderend voor ontgiftingsenzymen, in het bijzonder *NQO1*, *UGT1A6, UGT1A7* en *CYP2E1* (intron 6), die kunnen resulteren in een activatie of verminderde de-activatie van toxische en carcinogene verbindingen.

Tenslotte hebben we laten zien, dat de zuurstof radicaal productie in volbloed van patiënten met een geschiedenis van sporadische dikke darmkanker hoger is vergeleken met leeftijd- en geslacht-gematchte controles. Dit zou kunnen betekenen dat oxidatieve stress betrokken zou kunnen zijn bij de etiologie van sporadische dikke darmkanker.

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Curriculum Vitae

Elise van der Logt werd op 23 december 1975 geboren te Lent. In 1993 behaalde zij het HAVO diploma aan de Notre Dame des Anges in Ubbbergen, waarna zij in 1995 het VWO voltooide aan de Nijmeegse Scholengemeenschap Groenewoud. In hetzelfde jaar startte zij met de studie Biomedische Gezondheidswetenschappen aan de Medische Faculteit van de Katholieke Universiteit Nijmegen. Tijdens haar studie werden drie onderzoeksstages verricht. De eerste stage vond plaats in het kader van het bijvak Geneesmiddelenonderzoek op de afdeling Farmacologie (Medische faculteit) onder supervisie van drs. R. Engbersen en prof. dr. F.G.M. Russel. De hoofdvakstage Toxicologie werd uitgevoerd op de afdeling Maag-, Darm-, en Leverziekten van het Universitair Medisch Centrum Nijmegen (UMCN) met als begeleiders drs. D.J. de Jong en dr. W.H.M. Peters. Tenslotte werd voor de hoofdvakstage Pathobiologie onderzoek gedaan op de afdeling Bioactieve Componenten van Numico Research in Wageningen onder begeleiding van ing. B. van ’t Land, dr. R.L. Smeets en dr. L. M’Rabet. In april 2000 werd het doctoraal diploma behaald.

Van april 2000 tot juni 2004 werkte zij onder supervisie van dr. F.M. Nagengast en dr. W.H.M. Peters als junior onderzoeker op de afdeling Maag-, Darm-, en Leverziekten van het UMCN (hoofd: prof. dr. J.B.M.J. Jansen), wat tot dit proefschrift heeft geleid. Dit promotieonderzoek vond plaats in het kader van het project “Ontwikkeling van biotechnologische testsystemen ten behoeve van het formuleren van een voedingstherapie, die de cascade van darmkanker inducerende mechanismen effectief kan blokkeren” en werd financieel ondersteund door een “Bedrijfstechnologische samenwerking” (BTS) subsidie en Numico Research. Tijdens de “12th United European Gastroenterology Week 2004” heeft zij een “Young Investigators Travel Grant” in ontvangst mogen nemen.