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Polymorphisms of the human *UDP-glucuronosyltransferase (UGT) 1A7* gene in colorectal cancer

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Background: Genetic polymorphisms in the human *UDP-glucuronosyltransferase-1A7 (UGT1A7)* gene are detected and significantly correlated with sporadic colorectal carcinoma. *UGT1A7*, which has recently been demonstrated to glucuronidate environmental carcinogens, is now implicated as a cancer risk gene. A silent mutation at codon 11 and missense mutations at codons 129, 131, and 208 lead to the description of three polymorphic alleles designated *UGT1A7*2*, *UGT1A7*3*, and *UGT1A7*4*.

Methods: *UGT1A7* polymorphisms were analysed by polymerase chain reaction amplification and sequencing, as well as temperature gradient gel electrophoresis in 210 healthy blood donors and 78 subjects with colorectal cancer.

Results: Homozygous wild-type *UGT1A7* alleles were present in 20% of normal controls but were only detected in 9% of patients with colorectal carcinoma (odds ratio (OR) 0.39 (95% confidence interval (CI) 0.17–0.92); $p=0.03$). Analysis of individual polymorphic alleles identified a highly significant association between the presence of *UGT1A7*3* alleles and colorectal cancer (OR 2.75 (95% CI 1.6–4.71); $p<0.001$). Recombinant expression of *UGT1A7* polymorphic cDNA in eukaryotic cell culture showed reduced carcinogen glucuronidation activity in comparison with wild-type *UGT1A7*. *UGT1A7* may therefore represent a modifier gene in colorectal carcinogenesis.

Conclusion: We have identified a potential novel risk factor in sporadic colorectal cancer which may contribute to the identification of risk groups and to the elucidation of factors involved in colon carcinogenesis.

The human *UDP-glucuronosyltransferase (UGT) 1A7*^{1–3} belongs to a family of nine proteins encoded at the human *UGT1A* gene locus on chromosome 2q37, which is highly conserved in vertebrate organisms.⁴ UGTs catalyse the conversion of hydrophobic substrates to inactive hydrophobic β -D-glucopyranosiduronic acids (glucuronides) which can undergo facilitated renal or biliary elimination. Target substrates for glucuronidation consist of compounds from many divergent chemical classes, including dietary byproducts, endogenous metabolites such as steroids and bilirubin, many therapeutic drugs, and many environmental mutagens, such as polycyclic and heterocyclic aromatic hydrocarbons as well as heterocyclic amines.⁴ These enzymes play a critical role in cellular defence and are considered to be a major class of proteins involved in cellular detoxification.

In humans, tissue specific expression of the *UGT1A* locus gene products has been demonstrated.⁵ *UGT1A7*, *UGT1A8*, and *UGT1A10* are not expressed in hepatic tissues and have been shown to be differentially regulated in gastrointestinal organs such as the oesophagus,⁶ stomach,³ small intestine,² and colon.⁷ In addition, regulation and function of the *UGT1A* genes has been linked to gastrointestinal carcinogenesis.^{2,4,6,8} Analysis of human cancers has shown differential regulation of UGT expression in tumour tissues with an impact on epithelial glucuronidation activity.^{2,8} In gastric mucosa, *UGT1A* regulation leading to interindividual variations in UGT activity has been implicated as a cancer risk factor² while in oesophageal cancer, downregulation of *UGT1A7* transcripts is correlated with a decrease in overall oesophageal benzo(a)pyrene metabolite glucuronidation.⁶ This is significant as the cloning and characterisation of *UGT1A7* has demonstrated its capacity to glucuronidate polycyclic aromatic hydrocarbons as well as dietary derived heterocyclic amines,^{3,6} both of which are known carcinogens. These findings prompted us to predict that alterations in *UGT1A7* gene expression or in the

functional properties of *UGT1A7* may be an indicator of potential cancer risk. Hence we examined *UGT1A7* as a target gene for the evaluation of polymorphisms and their relation to gastrointestinal cancer.

Colorectal cancer (CRC) accounts for 9.7% of all newly diagnosed carcinomas.⁹ Pedigree and genetic analyses have identified a heritable aetiology in 15–20% of colorectal cancers. This has been linked to mismatch repair genes which have thus been indicated as colon cancer susceptibility markers.^{10–16} For a proportion of the remaining sporadic cancers, carcinogenesis based on mutagen mediated genotoxicity capable of initiating neoplastic transformation represents a potential mechanisms.¹⁷ The combined risk of carcinogenesis may therefore be determined by the outcome of an interaction between genetically defined biochemical defence mechanisms of the cell, such as those provided by the well characterised UGT super gene family, and the environmentally defined exposure to carcinogens.^{18,19}

In this study we show the significant association of CRC with polymorphisms of the *UGT1A7* carcinogen metabolising *UGT* gene.

MATERIALS AND METHODS

Patients

Patient samples were collected from consecutive patients attending the Department of Gastroenterology and Hepatology, and Department of Transfusion Medicine, Hannover Medical School in 1999 and 2000. All patients were of German Caucasoid ancestry. Written consent was obtained from all

Abbreviations: UGT, UDP-glucuronosyltransferase; CRC, colorectal cancer; TGGE, temperature gradient gel electrophoresis; PhIP, amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine.

Figure 1 Graphic representation of the three polymorphic sites identified on the human *UGT1A7* exon 1 sequence. The mutation of CCC/A at codon 11 is silent. At codons 129, 131, and 208, mutations lead to amino acid substitutions. Sequence analysis identified the presence of three different polymorphisms designated UGT1A7*2, UGT1A7*3, and UGT1A7*4. Exchanges at codons 11 and 208 (UGT1A7*4) as well as the polymorphisms at codons 129 and 131 (UGT1A7*2) did not occur individually (GenBank Nos AF296226, AF292627, AF292628). Base pair exchanges are shown in bold type and amino acid exchanges in italics.

Amino acid No	aa 7 15	aa 127 134	aa 205 213
UGT1A1	GGRP LVLG	LLHNKELM	QRVKNMLIA
UGT1A3	VP L P W L A T G	LLHNEAL I	QRVKNMLYP
UGT1A4	VP L P Q L A T G	LLHNEAL I	QRVKNMLYP
UGT1A5	VP L P R L A T G	LLHNEAL I	QRVKNMLYP
UGT1A6	R S F Q R I S A G	LLQDRDTL	QRVANFLVN
UGT1A7	TGL L P L Y V C	LFNDRKL V	ERVWNHIMH
UGT1A8	T S P I P L C V S	LFNDRKL V	ERVNRHIMH
UGT1A9	T S P L P L C V C	LFKDKKL V	ERVNRHIMH
UGT1A10	DQ P R S F M C V	LFNDRKL V	ERVWNHIVH
UGT1A7 polymorphisms:		N129K/R131K	W208R

Figure 2 Compared with the region around codon 11 (left box), the identified polymorphisms at positions 129/131 and at position 208 fall within regions of highly conserved sequences (middle and right boxes). The N129K/R131K polymorphism represents the wild-type sequence of UGT1A9 at these positions. W208R represents the wild-type sequence of UGT1A8 and UGT1A9 at this position.

pCDNA3.1 (Invitrogen, San Diego, California, USA). The sequence of the generated polymorphic UGT1A7 cDNAs was determined by automated fluorescence sequencing. In addition, each product was analysed by in vitro transcription/translation (TNT quick Transcription/Translation Systems, Promega, Madison, Wisconsin, USA) to document generation of 55 kDa products for each clone prior to expression in HEK293 cells.

Expression and characterisation of UGT1A7*1, UGT1A7*2, UGT1A7*3, and UGT1A7*4

Human embryonic kidney (HEK293) cells were transfected with pCDNA3.1 plasmids containing the UGT1A7*1, UGT1A7*3, and UGT1A7*4 cDNAs as previously described.³ Cells were harvested after 72 hours and lysed by freeze thawing. Expression of equal amounts of the different recombinant UGT proteins was controlled by β -galactosidase transfection and western blot using a rabbit anti-exon 2 antibody as reported previously.³ The following substrates were resuspended in methanol at a concentration of 5 mM: 4-nitrophenol, 1-naphthol, 4-methylumbelliferone, 4-tert-butylphenol, 7-hydroxybenzo(α)pyrene, 8-hydroxybenzo(α)pyrene, 7/8-hydroxybenzo(α)pyrene, 9-hydroxybenzo(α)pyrene, and amino-1-methyl-6-phenylimidazo-(4,5- β)-pyridine (PhIP). Whole cell extracts (50 μ g) expressing recombinant protein in reaction buffer (50 mM Tris-Cl (pH 7.6), 10 mM MgCl₂) were used for catalytic activity assays in a modification of the method of Bansal and Gessner,²¹ as previously described.⁵ The UDP-glucuronic acid concentration was 250 μ M. The ¹⁴C-labelled glucuronides were detected by thin layer liquid chromatography, visualised by autoradiography, and quantitated using a Fujifilm BAS-1000 phosphorimager (Raytest GmbH, Straubenhardt, Germany) and TINA 2.0 software (Raytest GmbH, Straubenhardt, Germany) for calculation of specific activities that were expressed as pmol glucuronide formed/min/mg of recombinant

protein. Autoradiography hard copies were additionally analysed with a GS-710 calibrated imaging densitometer (BioRad Laboratories, Hercules, California, USA) using the Quantity One software package.

RESULTS AND DISCUSSION

The risk of cancer development is defined by genetic predisposition and environmental mechanisms such as exposure to cellular mutagens.^{19, 22} The cellular interaction with putative carcinogens is influenced by the activity of detoxification enzymes expressed in tissues with xenobiotic contact.^{23, 24} Therefore, the link between genetic polymorphisms of carcinogen metabolising enzymes and human cancer represents an attractive model combining genetic predisposition and environmental exposure.^{4, 17}

Detection of three polymorphic sites within exon 1 of the *UGT1A7* gene

In this study, UGT1A7 polymorphisms were analysed in 288 Northern German Caucasoid patients, which included 210 healthy controls and 78 patients with CRC. Confirming an earlier report analysing American patients,²⁵ three polymorphic sites with a total of five individual base pair changes leading to three amino acid changes were detected (figs 1, 2). The sequencing data were confirmed by a TGGE assay developed for the detection of the 129K/131K as well as the 208R missense mutations (fig 3). The combined differences are represented on three alleles defined as UGT1A7*2 (GenBank accession No (acn) AF296127), UGT1A7*3 (GenBank acn AF296128), and UGT1A7*4 (GenBank acn AF296126).

The UGT1A7*4 allele is characterised by a silent mutation within the endoplasmic reticulum signal peptide domain^{1, 4, 26} which is present in all UGT1A proteins with the exception of

Figure 3 Detection of UGT1A7 129K/131K polymorphisms by temperature gradient gel electrophoresis. The silver stained 10% acrylamide gel separated over a temperature gradient of 25–30°C is shown. Heteroduplex formation leads to the distinction of two bands which allows for the discrimination of wild-type 129N/131R (lower band, lanes 4, 5, and 11), of the heterozygous 129K/131K (lower and upper bands, lanes 6, 10, and 12–14), and the homozygous mutant 129K/131K (UGT1A7*3/*3) (upper band only, lanes 1–3 and 7–9). Detection of the 208R polymorphism was performed similarly (not shown).

UGT1A10. The second change is represented by the missense mutation at codon 208. Codon 208 falls within a region of sequence conserved not only in the first exons of UGT1A7–10 but in all members of the UGT1A family (fig 2). The presence of an RV-N motif between amino acids 206 and 209 is common to all UGT1A proteins.

UGT1A7*2 combines two missense mutations in close proximity. As found for UGT1A7*4 this polymorphism also affects highly conserved sequence of the UGT protein. Following leucine-127, which is present in all UGT1A proteins, a LHN—L motif (amino acids 128–133) is conserved in UGT1A1, UGT1A3, UGT1A4, and UGT1A5, and a F-D-KLV motif (amino acids 128–134) is conserved in the extrahepatic

UGT1A7, UGT1A8, UGT1A9, and UGT1A10 (fig 2). UGT1A6 is shown to be more divergent at this site (LQDRDTL). The homologies at the polymorphic site are in agreement with the previously described overall homology within the UGT1A3–5 and the UGT1A7–10 clusters of UGT sequences.³

UGT1A7*3 is represented by the combination of all of the changes observed in UGT1A7*4 and UGT1A7*2. In our analysis, none of the 288 individuals demonstrated the single heterozygous or homozygous mutation at any of the individual codons displayed by the three alleles.

Significant association of UGT1A7 polymorphisms with colorectal carcinoma

Following the identification of polymorphic UGT1A7 alleles, the occurrence of the three missense mutations in normal controls as well as in CRC was determined (table 1). We used a control group of 210 healthy blood donors in addition to a cancer group of 78 CRC patients. The number of patients studied resulted in a statistical power of 90% in the present study. In the control population, homozygosity for wild-type UGT1A7 was confirmed in 20%. Of the other UGT1A7 alleles, UGT1A7*2/*1 was found in 25%, UGT1A7*2/*2 in 12%, and UGT1A7*3/*3 in 10% of individuals. The occurrence of at least one allele of UGT1A7*3 was found to be present in 27% and the allelic frequency was 0.17 (table 1). In a previously reported series, 144 healthy blood donors from the Boston area were analysed and found to exhibit an allelic frequency of 0.361 for the UGT1A7*3 polymorphic allele²⁵ which contrasts with our findings in a homogeneous group of 210 German blood donors all of Caucasian descent from Northern Germany. Unfortunately, the study by Guillemette *et al* did not provide data on subjects' genetic backgrounds. It is therefore possible that differences in genetic/ethnic background and immigration patterns in the USA versus a more homogeneous population in Northern Germany contribute to the observed differences. In the present study, we compared a defined control group with patients with manifest CRC who were also of German descent from the same area, and therefore shared the genetic background.

In the CRC group, a striking difference in the occurrence of UGT1A7 polymorphisms was detected (table 1, fig 4). Only 9% of CRC patients exhibited the UGT1A7 homozygous wild-type ($p=0.03$, OR 0.39 (95% CI 0.17–0.92)). The high incidence of UGT1A7 polymorphisms detected among patients with sporadic CRC patients was remarkable and exceeded that of other individual genetic CRC markers identified to date.^{27,28} In particular, the significance of this novel association differed

Table 1 Association of UGT1A7 polymorphisms with colorectal carcinoma

UGT1A7 alleles	Normal controls† (n=210) (%)	Colorectal cancer† (n=78) (%)	p Value	OR (95% CI)
UGT1A7*1/*1	42 (20)	7 (9)	0.03	0.39 (0.17–0.92)
UGT1A7*2/*1	52 (25)	13 (17)	0.15	
UGT1A7*2/*2	25 (12)	4 (5)	0.12	
UGT1A7*3/*1	20 (10)	15 (19)	0.04	2.26 (1.09–4.68)
UGT1A7*3/*2	19 (9)	15 (19)	0.02	2.39 (1.15–4.99)
UGT1A7*3/*3	17 (8)	8 (10)	0.63	
UGT1A7*4/*1	35 (17)	15 (19)	0.60	
UGT1A7*4/*3	0	1 (1)	NS	
UGT1A7*4/*4	0	0	NS	
Presence of UGT1A7*3	56 (27)	39 (50)	<0.001	2.75 (1.6–4.71)

The wild-type allele of UGT1A7 was significantly more prevalent in the tumour free control group (OR 0.39, 95% CI 0.17–0.92). The association of polymorphic UGT1A7 alleles with colorectal cancer was significant for the UGT1A7*3/*1 and the UGT1A7*3/*2 genotypes. When the data were analysed for the presence of UGT1A7*3 alleles a highly significant association with colorectal cancer (CRC) was found. This analysis identified the presence of the UGT1A7*3 allele as a risk factor for CRC. The frequency of the UGT1A7*3 allele was 0.17 in normal controls and 0.301 in CRC patients.

p values calculated by two tailed Fisher's exact test.

OR, odds ratio; CI, confidence interval; NS, not significant.

†All patients were of Northern German Caucasoid descent.

Figure 4 Graphic representation of the association of polymorphic *UGT1A7* alleles with sporadic colorectal cancer. A highly significant association was apparent for the wild-type alleles in normal control subjects without tumour and for the *UGT1A7**3 allele with sporadic colorectal cancer. All numbers are in per cent (compare with table 1). All controls and colorectal cancer patients were of Northern German Caucasoid descent. Statistical analysis (two tailed Fisher's exact test): * $p < 0.05$, *** $p < 0.001$.

from that reported for other allelic associations of metabolising enzymes and cancer.^{29,30}

When these data were further analysed for the presence of the different defined genotypes (table 1, fig 4), as characterised in fig 1, a statistically significant genotypic association with CRC was observed with *UGT1A7**3/*1 as well as with *UGT1A7**3/*2. This analysis identified the *UGT1A7**3 allele as a risk factor in CRC. The allelic frequency of *UGT1A7**3 in sporadic CRC was 0.30. Thus when the presence of *UGT1A7**3 alleles was compared in normal control subjects and CRC patients, this difference was highly significant ($p < 0.001$, OR 2.75 (95% CI 1.6–4.71)). Additionally, logistical regression analysis demonstrated that tumour occurrence was dependent on the presence of polymorphic *UGT1A7* alleles ($p = 0.025$), in particular *UGT1A7**3 ($p = 0.001$), but was independent of sex ($p = 0.17$).

Modification of catalytic UDP-glucuronosyltransferase 1A7 activity

To our knowledge, these data provide the first evidence of a significant genetic link between the gene of a carcinogen metabolising enzyme and sporadic CRC. These findings raise significant aetiological implications for the disease. In

sporadic CRC, environmental as well as genetic factors have been suggested, yet never directly demonstrated. An attractive hypothesis would suggest that alteration of xenobiotic or carcinogen glucuronidation in individuals expressing polymorphic alleles would affect exposure with these agents to the proximal as well as the downstream intestinal tract which could determine the rate of inflammation as well as cytotoxicity and genotoxicity.^{19,31} To address this issue, polymorphic *UGT1A7* alleles were expressed and characterised for differences in catalytic activity. We demonstrated that glucuronidation of benzo(α)pyrene metabolites and PhIP were markedly altered (table 2). For *UGT1A7**3 that was identified as a main allelic association and risk factor in CRC, catalytic analysis indicated loss of activity for all tested substrates. The *UGT1A7**4 allele exhibited substantially reduced phenolic UGT activities and undetectable activity towards carcinogen metabolites such as 8- and 9-hydroxybenzo(α)pyrene and the human precarcinogen PhIP. Expression of *UGT1A7**2 demonstrated substantially reduced overall UGT activity with preservation of carcinogen metabolite glucuronidation (table 2). Preservation of this activity may explain the high incidence of N129K/R131K in the normal population represented in the *UGT1A7**2/*1 (21%) and *UGT1A7**2/*2 (16%) alleles. The lowest glucuronidation activity was found with the *UGT1A7**3 allele, which confirms previous data.²⁵ In an earlier study, *UGT1A7* activities were analysed using stably transfected HEK cell clones in addition to a higher concentration of UDP-glucuronic acid in the UGT enzymatic assays. In our assay, *UGT1A7**3 was characterised by undetectable UGT activity with the substrates shown in table 2, which was confirmed by several individual transfection experiments. Although Guillemette *et al* showed some remaining UGT activity of *UGT1A7**3 in their stably expressing clones, both studies arrived at the conclusion that *UGT1A7**3 represented the allele with the least benzo(α)pyrene metabolite glucuronidation activity. The catalytic analysis supports the finding that the *UGT1A7**3 allele is significantly associated with the cancer group, identifying it as the main risk allele of CRC.

The magnitude of the altered catalytic activities encoded by the polymorphic alleles is an unexpected finding based on structural and biological considerations. The *UGT1A7**3 allele has three amino acid changes, all of which were conserved with either *UGT1A8* or *UGT1A9* (fig 2). The high degree of homology between *UGT1A7*, *UGT1A8*, *UGT1A9*, and *UGT1A10* has led to the assumption of great similarity of substrate specificity, as evidenced by glucuronidation of many phenolic compounds.^{3,4} However, examined in the absence of homology to other UGTs, these changes could be perceived to

Table 2 Alteration in catalytic activity: *UGT1A7* polymorphisms (activities in pmol/min/mg)

Substrate	UGT1A7*1 wild-type	UGT1A7*4 W208R	UGT1A7*2 N129K/R131K	UGT1A7*3 N129K/R131K/W208R
1-Nitrophenol	1773 (615)	32 (23)	13 (2)	nd
1-Naphthol	592 (100)	36 (16)	nd	nd
4-Methylumbelliferone	12110 (361)	191 (136)	13 (2)	nd
4-tert-Butylphenol	121 (2)	nd	12 (0.2)	nd
7-Hydroxybenzo(α)pyrene	1263 (20)	51 (10)	14 (0.5)	nd
8-Hydroxybenzo(α)pyrene	98 (6)	nd	12 (1)	nd
7-8-Hydroxybenzo(α)pyrene	79 (7)	nd	15 (1)	nd
9-Hydroxybenzo(α)pyrene	198 (15)	nd	16 (3)	nd
PhIP	48 (4)	nd	17 (5)	nd

Mean catalytic activities of the missense mutations identified in exon 1 of *UGT1A7*. The wild-type *UGT1A7**1 exhibited high activities with phenolic compounds as well as with benzo(α)pyrene metabolites, as previously characterised.³ The analysis demonstrates reduced glucuronidation activities of the polymorphic *UGT1A7* proteins. The major risk allele identified in the patient analysis, *UGT1A7**3, exhibited the lowest carcinogen detoxification activity. PhIP, amino-1-methyl-6-phenylimidazo-[4,5- β]-pyridine (PhIP); nd, not detected. Values are mean (SD).

impact on function as the N129K and W208R differences represent non-conservative substitutions.

The majority of CRC patients with the UGT1A7*3 (129K/131K/208R) genotype were heterozygous (table 1). Similar findings have been reported for the mismatch repair genes in familial CRC.¹³⁻¹⁶ In the *UGT1A* genes, the presence of heterozygous mutant alleles with a reduced activity towards carcinogens can be influenced by additional occurrence of polymorphisms in exons 2–5 which are common to all UGT1A gene products. This combination would introduce an added risk factor capable of modifying the catalytic activity of all UGT1A proteins and would exert an increased influence on the already altered activity of polymorphic *UGT1A7* alleles described in this study. In view of the previous identification of 20 individual polymorphisms located within the common UGT1A exon 2–5 sequences (reviewed by Tukey and Strassburg³), additional combinations with the *UGT1A7* polymorphisms described here are likely.³²

Our findings implicate the combination of genetic predisposition and environmental factors via the cellular detoxifying enzyme UGT1A7 in human CRC. The UGT1A7*3 allele exhibited a highly significant association with CRC. It was present in only 27% of the normal population studied (allelic frequency 0.17) but a tumour free state at the time of blood sampling does not preclude the development of CRC in normal controls at a later time. This suggests that screening for the presence of UGT1A7*3 would have a negative predictive value of 73%. Screening for 129K/131K/208R of the human *UGT1A7* gene in selected patients may represent an attractive diagnostic tool to identify interindividual CRC risk profiles. Based on these findings, sporadic CRC may have a higher degree of individual genetic predisposition than previously thought. As CRC is a potentially curable disease when diagnosed at an early stage, the clinical implementation of our findings has the potential to improve the primary prevention of CRC in risk groups and to affect the decision process for endoscopic evaluation. In addition, *UGT1A7* may serve as an attractive candidate gene for gene therapeutic strategies.

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