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Association Between EGF, TGF-β1, VEGF Gene Polymorphism and Colorectal Cancer

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Abstract
Introduction Up to the present, EGF 61 A/G, TGF-β1 −509 T/C, and VEGF 936 T/C gene polymorphisms have been analyzed in other cancer entities than colorectal cancer. We have now investigated the frequency of these gene polymorphisms among colorectal cancer patients.
Material and methods A total of 157 colorectal cancer patients and 117 cancer-free healthy people were recruited at the Surgical Department of the Universitätsklinikum Mannheim. All patients and healthy people are Caucasians. Genomic DNA was isolated from peripheral blood, and gene polymorphisms were analyzed by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP).
Results The distribution of EGF 61 G/G homozygotes among colorectal cancer patients was more frequent than that in the control group (33.1% versus 11.1%; Odds Ratio [OR] = 3.962; 95% Confidence Interval [CI] = 2.036–7.708). The frequency of the "G" allele in the colorectal cancer patient group was also higher than that in the control group (51.3% versus 33.3%; OR = 2.105; 95% CI = 1.482–2.988). No difference could be found for the TGF-β1 and VEGF genotypes among colorectal cancer patients and healthy controls.
Conclusions The EGF 61 G/G genotype and the G allele are significantly related to colorectal cancer. The TGF-β1 −509 T/C and VEGF 936 T/C gene polymorphisms are not related to colorectal cancer.

Introduction

Colorectal cancer is the second-leading cause of cancer-related deaths in Europe and the United States [1]. Although the primary therapy is surgical, the elucidation of different novel prognostic markers that could also serve as therapeutic targets is necessary to better understand this cancer entity and to improve outcome. Single nucleotide polymorphisms (SNPs) are the most common sources of human genetic variation, and they may contribute to an individual's susceptibility to cancer. The occurrence of growth factor gene polymorphisms has also been illustrated in colorectal cancer, although no association in tumor susceptibility was found for the −308 G > A polymorphism of the TNF-α gene when comparing colorectal cancer patients and healthy controls [2]. For the −238 G > A site of the TNF-α gene, Jang et al. [3] reported that the A allele decreases the risk of developing colorectal cancer. Up to now, however, most studies have focused more on other cancer entities, such as melanoma and breast cancer, than on colorectal cancer.

Thus far, EGF 61 A/G, TGF-β1 −509 T/C, and VEGF 936 T/C gene polymorphisms have been analyzed in other cancer entities than colorectal cancer [4–6]. Although the functional effects of these polymorphisms have not yet been elucidated, we hypothesized that they may play a role

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in modulating susceptibility to colorectal cancer. To test this hypothesis, we performed a case-control study to investigate the association between these gene polymorphisms and the risk of colorectal cancer.

Materials and methods

Patients

Between October 2000 and March 2003, a total of 157 colorectal cancer patients were recruited at the Surgical Department of the Universitätsklinikum Mannheim. Blood samples were collected after informed patient consent was given and the study was approved by the local ethics committee. The age range was 33–91 years (57 women and 100 men). All patients were histologically diagnosed as having colorectal cancer in the Pathological Department of Universitätsklinikum Mannheim. The control group comprised 117 colorectal cancer-free healthy people. They are volunteers undergoing colonoscopy as preventive measure and who had a normal colonoscopy. The age range was 61–67 years (43 women and 74 men). All patients and healthy controls were Caucasians. Tumor pathology stages were classified according to the tumor-node-metastasis (TNM) classification of the Union Internationale Contra le Cancer (UICC). Pathology grades were determined according to the criteria of World Health Organization (WHO).

Genotyping

For genetic analyses, genomic DNA was isolated from peripheral blood of colorectal cancer patients and healthy controls by standard methods. Gene polymorphisms were analyzed by polymerase chain reaction–restriction fragment length polymorphism analysis (PCR–RFLP). For the purification of genomic DNA, QIAamp^{TM} DNA Mini and QIAamp^{TM} DNA Blood Mini Kits from the QIAGEN Company were used according to the manufacturer's instructions. DNA concentrations were determined by A_{280} with a UV spectrophotometer.

Polymerase chain reactions were performed in a total volume of 50 µl primer; lengths of the amplified PCR fragments are given in Table 1, and PCR conditions are summarized in Table 2 [4–6].

The EGF (61 A/G) PCR product was digested with restriction endonuclease Alu I (sequence of restriction site: AG▼CT) for 2 h. Fragments were analyzed on 3% agarose electrophoresis gels stained with ethidium bromide.

The TGF-β1 (−509 T/C) PCR product was digested with the restriction endonuclease Bsu36 I (sequence of restriction site: CC▼TNAGG) for 2 h. Fragments were analyzed on 2% agarose electrophoresis gels stained with ethidium bromide [5]. The VEGF (936 T/C) PCR product was digested with restriction endonuclease NIa III (sequence of restriction site: CATG▼) for 2 h. Fragments were analyzed on 3% agarose gels stained with ethidium bromide.

Statistics

The p values were calculated with Pearson's chi-square test or Fisher's exact test. The threshold for significance was $p < 0.05$. Statistical analysis was performed with standard SPSS software (v 10 of SPSS for MS Windows)

Results

EGF 61 A/G gene polymorphisms in colorectal cancer patients and healthy controls

The PCR fragments of the A/A genotype were digested into three fragments of 193, 34, 15 bp, respectively, while digestion of the EGF 61 G/G genotype yielded 4 fragments of 102, 91, 34, and 15 bp each. The EGF 61 A/G genotype PCR-products were digested into 5 fragments of 193, 102, 91, 34, and 15 bp.

The distribution of polymorphisms in the healthy controls was as follows: G/G homozygote in 11.1%, A/G heterozygote in 44.4%, and A/A homozygote in 44.4%. The frequency of G/G homozygotes among colorectal cancer patients was higher than that in the control group (33.1% versus 11.1%). The odds ratio for carriers of the 61 G/G

Table 1 Primer sequence and resulting fragment length for growth factors gene polymerase chain reaction (PCR)

Gene	Primer direction	Primer sequence	Resulting fragment bp
EGF	For	5′-TGTCACTAAAGGAAAGGAGGT-3′	242
	Rev	5′-TTCACAGAGTTTAACAGCCC-3′	
TGF-β1	For	5′-CGGACACCCAGTGATGGG-3′	530
	Rev	5′-CCTCCTGGCGGCCAAGCGC-3′	
VEGF	For	5′-AAGGAAGAGGAGACTCTGCGC-3′	198
	Rev	5′-TATGTGGGTGGGTGTGTCTACAGG-3′	

Table 2 Technical data for growth factors gene polymorphism detection methods

PCR reaction condition	EGF		TGF-β1		VEGF	
	Temperature	Cycles	Temperature	Cycles	Temperature	Cycles
	94°C 5 min	1	94°C 1 min	1	94°C 1 min	1
	94°C 1 min	35	94°C 1 min	30	94°C 1 min	30
	57°C 1 min		60°C 1 min		60°C 1 min	
	72°C 1 min		72°C 1.5 min		72°C 1 min	
	72°C 10 min	1	72°C 10 min	1	72°C 10 min	1
Mastermix (μl)						
10× PCR buffer	5		5		5	
dNTP (10 mM)	2		1		1	
Primer-forward (10 μM)	3		2		1	
Primer-reverse (10 μM)	3		2		1	
$MgCl_2$ (50 mM)	3		1.5		1.5	
Taq polymerase (5 U/μl)	0.4		0.4		0.4	
Restriction enzyme	AluI		Bsu36 I		NIa III	
Restriction time (h)	2		2		2	
Restriction pattern length (bp)	A: 102 + 91 + 34 + 15		C: 273 + 257		T: 112 + 86	
	G: 193 + 34 + 15		T: 530		C: 198	
Agarose gel concentration	3%		2%		3%	
Reference	Shahbazi et al. 2002 [4]		Schulte et al. 2001 [5]		Krippl et al. 2003 [6]	

genotype for colorectal cancer was 3.962 (95% CI 2.036–7.708). The frequency of the "G" allele in the colorectal cancer patient group (51.3%) was also greater than that in the control group (33.3%). The odds ratio for carriers of the 61 G allele for colorectal cancer was 2.105 (95% CI = 1.482–2.988). These differences in the distribution of the EGF 61 G/G genotype and G allele frequency between colorectal cancer patients and healthy controls were significantly different as determined by a chi-square test.

Table 3 shows the distribution of EGF genotypes according to tumor stage and tumor grading. The genotypes were not statistically significantly associated with the stage or grading of colorectal cancer.

TGF-β1 −509 T/C gene polymorphism in colorectal cancer patients and healthy controls

The PCR fragments of the TGF-β1 –509 C/C genotype were digested into 2 fragments of 273 and 257 bp. The T/T genotype PCR-products could not be digested. The heterozygote T/C genotype PCR-products were digested into 3 fragments of 530, 273, and 257 bp.

Table 3 Tumor stage-specific and grade-specific distribution of the EGF, TGF-β1, and VEGF polymorphism genotype among colorectal cancer patients

	Stage I	Stage II	Stage III	Stage IV	*p* Value	Grade I	Grade II	Grade III	*p* Value
EGF 61 genotype									
A/A	17 (34.0%)	13 (36.1%)	8 (21.6%)	7 (28.0%)	>0.05	1 (50.0%)	39 (30.5%)	4 (30.8%)	>0.05
A/G	16 (32.0%)	13 (36.1%)	16 (43.2%)	9 (36.0%)		1 (50.0%)	46 (35.9%)	5 (38.3%)	
G/G	17 (34.0%)	10 (27.8%)	13 (35.1%)	9 (36.0%)		0 (0.0%)	43 (33.6%)	4 (30.8%)	
TGF-β1 −509 genotype									
T/T	6 (12.0%)	6 (16.7%)	3 (8.1%)	1 (4.0%)	>0.05	0 (0.0%)	14 (10.9%)	1 (7.7%)	>0.05
C/C	29 (58.0%)	15 (41.7%)	19 (51.4%)	13 (52.0%)		2 (100.0%)	64 (50.0%)	6 (46.2%)	
T/C	15 (30.0%)	15 (41.7%)	15 (40.5%)	11 (44.0%)		0 (0.0%)	50 (39.1%)	6 (46.2%)	
VEGF 936 genotype									
T/T	0 (0.0%)	1 (2.8%)	1 (2.7%)	1 (4.0%)	>0.05	0 (0.0%)	3 (2.3%)	0 (0.0%)	>0.05
C/C	39 (78.0%)	26 (72.2%)	31 (83.8%)	20 (80.0%)		1 (50.0%)	99 (77.3%)	12 (92.3%)	
T/C	11 (22.0%)	9 (25.0%)	5 (13.5%)	4 (16.0%)		1 (50.0%)	27 (21.1%)	1 (7.7%)	

Table 4 EGF, TGF-β1, and VEGF genotypes and allele frequencies in patients with colorectal cancer and in healthy controls

	Healthy controls in literature	CRC patients (n = 157)	Healthy controls (n = 117)	χ^2	p Value	OR	95% CI
EGF 61 genotype	Amend et al. 2004 [7]						
G/G	30(12.9)	52 (33.1)	13 (11.1)	17.948	0.000	3.962	2.036–7.708
A/A + A/G	84 + 118 (36.2 + 50.9)	48 + 57 (30.6 + 36.3)	52 + 52 (44.4 + 44.4)				
EGF 61 allele							
G		161 (51.3)	78 (33.3)	17.549	0.000	2.105	1.482–2.988
A		153 (48.7)	156 (66.7)				
TGF-β1 −509 genotype	Grainger et al. 1999 [8]						
T/T	24 (7.5)	16 (10.2)	9 (7.7)	0.981	0.612		
C/C	146 (45.0)	78 (49.7)	55 (47.0)				
T/C	152 (47.0)	63 (40.1)	53 (45.3)				
TGF-β1 −509 allele							
T		95 (30.3)	71 (30.3)	0.000	0.982	0.996	0.689–1.439
C		219 (69.7)	163 (69.7)				
VEGF 936 genotype	Krippl et al. 2003 [6]						
T/T	10 (2.0)	3 (1.9)	1 (0.9)	1.143	0.565		
C/C	353 (70.6)	123 (78.3)	88 (75.2)				
T/C	137 (27.4)	31 (19.7)	28 (23.9)				
VEGF 936 allele							
C		277 (88.2)	204 (87.2)	0.134	0.714	1.101	0.658–1.841
T		37 (11.8)	30 (12.8)				

Statistical results are for comparison of genotypes or allele frequencies between colorectal cancer patients and healthy controls

CRC colorectal cancer; *OR* odds ratio; *CI* confidence interval

When colorectal cancer patients were compared with the healthy controls, there was no statistically significant difference (Table 4).

Table 3 shows the tumor-stage or tumor-grade specific distribution of TGF-β1 genotypes among colorectal cancer patients. The genotypes were not statistically significantly associated with the tumor stage or grading of colorectal cancer.

VEGF 936 T/C gene polymorphisms in colorectal cancers and healthy controls

The PCR fragments of the T/T genotypes at VEGF 936 were digested into 2 fragments of 112 and 86 bp. The C/C genotype PCR-products could not be digested. The T/C genotype PCR-products were digested into 3 fragments of 198, 112, and 86 bp.

When colorectal cancer patients were compared with the healthy controls, there was no statistically significant difference ($p > 0.05$, chi-square; Table 4).

Table 3 shows the stage- and grade-specific distribution of VEGF genotypes among colorectal cancer patients. The genotype was again not statistically significantly associated with tumor stage or grading in colorectal cancer.

Discussion

EGF 61 A/G gene polymorphism in colorectal cancer

EGF exerts effects on cell proliferation and differentiation by binding to the tyrosine kinase EGF receptor (EGFR). The EGFR system is an important mediator within the tumor microenvironment of autocrine and paracrine circuits that result in enhanced tumor growth [9]. Both EGF and EGFR expression have been described to be significantly increased in neoplastic tissues from patients with colorectal adenocarcinoma compared with that in the adjacent normal mucosa [10]. An impact of EGF polymorphisms on cancer has been described. Shahbazi et al. [4] reported that 61 G/G was significantly associated with Breslow thickness and the risk of developing a malignant melanoma; also, cells from individuals homozygous for the 61 A allele produced significantly less EGF than cells from 61 G homozygous or heterozygous A/G individuals. It was also demonstrated that the EGF +61 polymorphism played a role for the progression of malignant melanoma [11]. Recently, it has been shown that EGF 61 gene polymorphism has a functional influence on EGF gene expression in normal colon in colorectal cancer patients [12]. We

hypothesized that the EGF 61 gene polymorphism might be correlated to colorectal cancer.

From our results we can confirm that the EGF 61 G/G genotype and G allele are significantly related to colorectal cancer. Colorectal cancer patients were found to have a higher distribution of G/G genotypes and G alleles. Because the G/G genotype leads to a higher production of EGF [4], we could propose that a higher EGF production is associated with an increased risk of colorectal cancer. The mechanism by which the EGF 61 G/G genotype increases the EGF production remains to be defined. Possible reasons could be: (1) the polymorphism itself is functional; (2) the G to A substitution might affect the DNA folding or processing of the mRNA transcript; (3) the allelic variation at position 61 could be closely linked to a functional polymorphism elsewhere in the gene.

Our results do not show a correlation of the EGF +61 polymorphism to the tumor stages or to the tumor grading in colorectal cancer. However, a further study involving a larger patient collective is required.

The more frequent occurrence of the G allele in EGF +61 gene polymorphism among colorectal cancer patient needs to be confirmed by a second study, because it may be a useful marker for detecting patients with an increased risk of colorectal cancer, who could then be subjected to a more careful or earlier routine screening for colorectal cancer.

TGF-β1−509 T/C gene polymorphism in colorectal cancer

TGF-β1 regulates growth, differentiation, and epithelial transformation in the multistep processes of tumorigenesis, wound healing, and embryogenesis. It has been shown that TGF-β1 acts as a potent inhibitor of proliferation and migration, and it promotes apoptosis [13]. A model was proposed in which TGF-β1 inhibits the development of early, benign lesions but promotes invasion and metastasis when its tumor-suppressor activity is overridden by oncogenic mutations in other pathways [14, 15]. Here, increased levels of TGF-β1 frequently detected in human tumors may contribute either to tumor suppression or to tumor progression. Previous studies have shown that the −509 T allele (T/T or C/T genotype) is associated with a decreased risk of the occurrence of hepatocellular carcinoma in patients with chronic hepatitis B virus infection in the Korean population [16].

The genotype distribution and allele frequencies among the healthy controls in our study were parallel to those reported in the literature [8]. Our results show that the −509 T allele does not influence the risk of colorectal cancer. Here, differences in ethnic background and the different cancer entities (hepatocellular carcinoma versus colorectal cancer) may be the reasons for the different results. The TGF-β1 polymorphism −509C/T does not correlate with the stage or the grading of colorectal cancer. This is in concordance with the literature because, so far, no report shows that TGF-β1 polymorphism −509C/T is related to tumor progression.

VEGF 936 T/C gene polymorphism in colorectal cancer

Evidence from preclinical and clinical studies shows that vascular endothelial growth factor (VEGF) is a predominant angiogenic factor in human colorectal cancer that is associated with formation of metastases and poor prognosis [17]. Several VEGF gene polymorphisms have been reported in some cancer entities. The VEGF −1154 A/A genotype has been related to a decreased risk of developing prostate cancer and to a reduction in the invasive potential of malignant melanoma [18, 19]. Hofmann et al. concluded that VEGF 936C/T, −2578C/A, −634G/C gene polymorphisms are not associated with individual susceptibility to colorectal cancer [20].

In our study, the VEGF 936 genotype distribution and allele frequencies among the healthy controls were parallel to the findings reported in the literature [6]. It has also been reported that carriers of the VEGF 936T allele are at a decreased risk for breast cancer [6]. However, the VEGF 936 C/T gene polymorphism cannot be associated with colorectal cancer in our study. Also no correlation between the VEGF 936 gene polymorphism and the tumor stage or tumor grading could be shown.

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