Association Between EGF, TGF-β1, TNF-α Gene Polymorphisms and Cancer of the Pancreatic Head

GUO-YANG WU[1], QINGJUN LU[1], TILL HASENBERG[2], MARCO NIEDERGETHMANN[2], STEFAN POST[2], JÖRG W. STURM[2] and MICHAEL KEESE[2]

[1]*Department of General Surgery, Zhongshan Hospital, Xiamen University, Xiamen, P.R. China;*
[2]*Surgical Clinic, University Hospital Mannheim, D-68167 Mannheim, Germany*

Abstract. *Background: To date, EGF 61*A/G, TGF-β1 -509*T/C and TNF-α -308*A/G gene polymorphisms have been not been analysed in pancreatic carcinoma. This study investigated the frequency of these gene polymorphisms among patients with cancer of the pancreatic head. Patients and Methods: A total of 73 pancreatic head cancer patients and 117 cancer-free healthy people were recruited at the Surgical Department of the University Hospital Mannheim. Genomic DNA was isolated from peripheral blood and gene polymorphisms were analysed by PCR-RFLP. Results: The distribution of EGF 61*G/G homozygotes among pancreatic head cancer patients was more frequent than that in the control group (24.7% vs 11.1%, odds ratio (OR)=2.618, 95% confidence interval (CI)=1.195-5.738). In addition, the frequency of the G allele in the pancreatic head cancer patient group was also higher than that in the control group (45.9% vs. 33.3%, OR=1.696, 95% CI=1.110-2.592). No difference was found for the TGF-β1 -509 and TNF-α -308 genotypes among pancreatic head cancer patients and healthy controls. Conclusion: The frequencies of the EGF 61*G/G genotype and G allele are significantly increased among patients with pancreatic head cancer. TGF-β1-509*T/C and TNF-α -308*A/G gene polymorphisms are not related to this cancer entity.*

Carcinoma of the pancreatic head is a disease with limited prognosis, characterized by its propensity to infiltrate adjacent tissues and to metastasise in its early stages (1). Absence of specific symptoms, lack of early detection markers, aggressive tumour growth and resistance to conventional chemotherapy and radiotherapy regimens conspire to culminate in a median overall survival smaller than nine months and, thus, annual mortality figures virtually equalling incidence numbers (2). In addition to differences in the expression of certain oncogenes and tumour suppressor genes, amplified autocrine and paracrine growth factor signalling loops, such as the epidermal growth factor pathway, evidently contribute to the aggressive growth pattern of pancreatic cancer (3).

The growth factors TGF-β1, TNF-α and EGF have been shown to be involved in growth, differentiation and epithelial transformation in the multistep processes of tumourigenesis (4-6). It has been hypothesised that certain polymorphisms for these factors result in functional changes in expression which may influence susceptibility to pancreatic head cancer. Until now, no *EGF* 61*A/G, *TGF-β1* -509*T/C and *TNF-α* -308*A/G gene polymorphisms have been reported in pancreatic head cancer. The present case–control study investigated the frequency of these gene polymorphisms among pancreatic head cancer patients.

Patients and Methods

Patients. Between June 2000 and September 2004, a total of 73 pancreatic head cancer patients (28 females and 45 males) were recruited at the Surgical Department of the University Hospital Mannheim, Germany. Blood samples were collected with informed patient consent and the study was approved by the local Ethics Committee. The age range was 30-82 years. The diagnosis of adenocarcinoma of the pancreatic head was confirmed histologically in the Pathological Department of the University Hospital Mannheim. The control group comprised of 117 cancer-free healthy people (43 females and 74 males) who received a control sonography as a preventive measure. The age range was 61-67 years.

Genotyping. For genetic analyses, genomic DNA was isolated from peripheral EDTA-blood of pancreatic head cancer patients and healthy controls using QIAamp DNA Mini and QIAamp DNA Blood Mini Kits (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were determined by A_{280} using an ultraviolet spectrophotometer.

Correspondence to: Michael Keese, Surgical Clinic, University Hospital Mannheim, D-68167 Mannheim, Germany. E-mail: michael.keese@umm.de

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Table I. *Primer sequences and resulting fragment lengths for growth factor gene PCR.*

Gene	Primer direction	Primer sequence	Resulting fragment bp
TGF-β1	Forward	5'-CGGACACCCAGTGATGGG-3'	530
	Reverse	5'-CCTCCTGGCGGCCAAGCGC-3'	
TNF-α	Forward	5'-AGGCAATAGGTTTTGAGGGCCAT -3'	345
	Reverse	5'-GAGCGTCTGCTGGCTGGGTG -3'	
EGF	Forward	5'-TGTCACTAAAGGAAAGGAGGT-3'	242
	Reverse	5'-TTCACAGAGTTTAACAGCCC-3'	

Gene polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Primers and lengths of the amplified PCR fragments are given in Table I. PCR conditions are summarised in Table II.

The *EGF* (61*A/G) PCR product was digested with restriction endonuclease Alu I (sequence of restriction site: AG▼CT) for two hours. *TGF-β1* (-509*T/C) PCR products were digested with the restriction endonuclease Bsu36 I (sequence of restriction site: CC▼TNAGG) for two hours. *TNF-α* (-308*A/G) PCR products were digested with restriction endonuclease NcoI (sequence of restriction site: C▼CATGG) for two hours. DNA fragments were analysed on 2-3% agarose gels stained with ethidium bromide (7-9).

Statistics. *P*-values were calculated with the Pearson's chi-square test or Fisher's exact test. Threshold for significance was $p<0.05$. Statistical analysis was performed using SPSS for MS Windows, v. 10 (SPSS, Inc., Chicago, IL, USA).

Results

*TGF-β1 -509*T/C gene polymorphism in pancreatic head cancer patients and healthy controls*. The PCR fragments of *TGF-β1* -509* C/C genotype were digested into two fragments of 273 and 257bp. T/T genotype PCR products were not digested. The heterozygote T/C genotype PCR products were digested into three fragments of 530, 273 and 257 bp. When pancreatic head cancer patients were compared with the healthy controls, there were no statistically significant differences ($p>0.05$, chi-square; Table III).

TNF-α-308A/G gene polymorphisms in pancreatic head cancer and healthy controls. The PCR fragments of the T/T genotypes at VEGF 936 were digested into two fragments of 112 and 86 bp. C/C genotype PCR products were not digested. The T/C genotype PCR products were digested into three fragments of 198, 112 and 86 bp, respectively. When pancreatic head cancer patients were compared with the healthy controls, there were no statistically significant differences ($p>0.05$, chi-square; Table III).

*EGF61*A/G gene polymorphisms in pancreatic head cancer patients and healthy controls*. The PCR fragments of A/A genotype were digested into three fragments of 193, 34 and 15 bp, while digestion of the *EGF* 61*G/G genotype yielded four fragments of 102, 91, 34 and 15 bp. The *EGF* 61*A/G genotype PCR products were digested into five fragments of 193, 102, 91, 34, 15bp.

The distribution of polymorphisms in the healthy controls was: G/G homozygotes in 11.1%, A/G heterozygotes in 44.4%, and A/A homozygotes in 44.4%. The frequency of G/G homozygotes among pancreatic head cancer patients was higher than that in the control group (24.7% *versus* 11.1%). The odds ratio (OR) for carriers of the 61*G/G genotypes for pancreatic head cancer was 2.618 (95% confidence interval (CI)=1.195-5.738). The frequency of the G allele in the pancreatic head cancer patient group (45.9%) was also greater than that in the control group (33.3%). OR for carriers of 61*G allele for pancreatic head cancer was 1.696 (95% CI=1.110-2.592). These differences in the distribution of the *EGF* 61*G/G genotype and G allele frequency between pancreatic head cancer patients and healthy controls were significantly different as determined by the chi-square test.

Discussion

Up-regulation and overexpression of growth factors and growth factor receptors has been correlated to many processes related to cancer, including uncontrolled cellular proliferation, autocrine stimulation of tumours producing their own growth factors and prevention of apoptosis (4-6, 10, 11). This also appears to protect cancer cells from the toxic actions of chemotherapy and radiotherapy, rendering these treatment modalities less effective. Many epithelial tumour entities, including gastric and cervical cancer, as well as cancers of head, neck, breast and lung, express high levels of EGF, TGF-β1 and TNF-α, which are associated with advanced disease and poor clinical prognosis (11-14). Extensive expression studies have also been performed in pancreatic carcinoma *in vivo* and *in vitro* (10, 15). Higher level expression of EGF, TGF-β1 and TNF-α have been inversely correlated to survival in these patients and high expression levels have been found in advanced tumour stages (16, 17).

TGF-β1 regulates growth, differentiation, and epithelial transformation in the multistep processes of tumourigenesis,

Table II. *Technical data for growth factor gene polymorphism detection methods.*

	TGF-β1		*TNF-α*		*EGF*	
PCR reaction condition	Temperature	Cycles	Temperature	Cycles	Temperature	Cycles
	94˚C 1 min	1	94˚C 5 min	1	94˚C 5 min	1
	94˚C 1 min	30	94˚C 1 min	30	94˚C 1 min	35
	60˚C 1 min		60˚C 1 min		57˚C 1 min	
	72˚C 1.5 min		72˚C 1 min		72˚C 1 min	
	72˚C 10 min	1	72˚C 5 min	1	72˚C 10 min	1
Mastermix (µl)						
10*PCR buffer	5		5		5	
dNTP (10 mM)	1		1		2	
Primer, forward (10 µM)	2		1		3	
Primer, reverse (10 µM)	2		1		3	
$MgCl_2$ (50 mM)	1.5		1.5		3	
Taq polymerase (5 u/µl)	0.4		0.4		0.4	
Restriction enzyme	Bsu36 I		NcoI		AluI	
Restriction time (hours)	2		2		2	
Restriction pattern length (bp)	C: 273+257		G: 325+20		A: 102+91+34+15	
	T: 530		A: 345		G: 193+34+15	
Agarose gel concentration	2%	3%	3%			
Reference	Schulte *et al*. (7)		Sakao *et al*. (8)		Shahbazi *et al*. (9)	

Table III. *EGF, TGF-β1 and VEGF genotypes and allelic frequencies in patients with pancreatic head cancer and healthy controls. Statistical results are for comparison of genotypes and allele frequencies between pancreatic head cancer patients and healthy controls. Percentage rates are shown in parentheses.*

	Healthy controls in literature	PHC patients (n=73)	Healthy controls (n=117)	Chi-square	*p*-Value	OR	95% CI
TGF-β1 -509 genotype	Grainger *et al*. (21)						
T/T	24 (7.5)	8 (11.1)	9 (7.7)	3.526	0.172		
C/C	146 (45.0)	24 (33.3)	55 (47.0)				
T/C	152 (47.0)	40 (55.6)	53 (45.3)				
TGF-β1 -509 allele							
T		56 (38.9)	71 (30.3)	2.919	0.088	1.461	0.945-2.259
C		88 (61.1)	163 (69.7)				
TNF-α -308 genotype							
A/A		2 (2.7)	2 (1.7)	0.299	0.861		
A/G		20 (27.4)	30 (25.9)				
G/G		51 (69.9)	84 (72.4)				
TNF-α -308 allele							
A		24 (16.4)	34 (14.7)	0.219	0.640	1.146	0.648-2.024
G		122 (83.6)	198 (85.3)				
EGF 61 genotype	Amend *et al*. (22)						
G/G	30 (12.9)	18 (24.7)	13 (11.1)	6.042	0.014	2.618	1.195-5.738
A/A+A/G	84+118 (36.2+50.9)	24+31 (32.9+42.5)	52+52 (44.4+44.4)				
EGF 61 allele							
G		67 (45.9)	78 (33.3)	6.007	0.014	1.696	1.110-2.592
A		79 (54.1)	156 (66.7)				

wound healing and embryogenesis. It has been shown that TGF-β1 acts as a potent inhibitor of proliferation and migration, and promotes apoptosis as well (4). A model was proposed in which TGF-β1 inhibits the development of early, benign lesions but promotes invasion and metastasis when its tumour suppressor activity is overridden by oncogenic mutations in other pathways (18, 19). Increased levels of TGF-β1 frequently detected in human tumours may contribute either to tumour suppression or progression. Previous studies have shown that the -509 T allele (T/T or

C/T genotype) is associated with a decreased risk for the occurrence of hepatocellular carcinoma in patients with chronic hepatitis B virus infection in the Korean population (20). Grainger *et al*. (21) reported that individuals homozygous for -509T/T had higher plasma concentrations of TGF-β1 than heterozygous C/T or homozygous C/C individuals. The genotype distribution and allele frequencies among the healthy controls in the present study were in agreement with those quoted in the literature (21). These results showed that -509 T allele does not influence the risk of developing pancreatic head cancer.

An increasingly growing body of research involving a range of animal experiments indicates that TNF may promote cancer development and dissemination (23). Although circulating TNF-α levels were not measured in the present study, the *TNF-α* -308*A allele has already been shown to increase the constitutive and inducible expression of TNF-α protein, possibly by the differential binding of a nuclear protein to the *TNF-α* -308*A allele (23, 24). It has been reported that some malignant tumours such as hepatocellular carcinoma, prostate cancer, non-Hodgkin's lymphoma and breast carcinoma are related to *TNF-α* -308*A/G gene polymorphism (24-26). The results of the present study showed that *TNF-α* -308*A/G gene polymorphism is not related to pancreatic head cancer. This finding is in agreement with those reported in the literature, which demonstrated no association between the *TNF-α* -308 polymorphism and gastric cancer, uterine cervical cancer, colorectal cancer, or renal cell carcinoma (29).

EGF exerts effects on cell proliferation and differentiation by binding to the tyrosine kinase EGF receptor. The EGF receptor system is an important mediator within the tumour microenvironment of autocrine and paracrine circuits that result in enhanced tumour growth (6). A clear impact of *EGF* polymorphisms on skin cancer has already been described. Shahbazi *et al*. (9) reported that the 61*G/G genotype was significantly associated with Breslow thickness and the risk of developing a malignant melanoma, and melanocytes cultured from individuals homozygous for the 61*A allele produced significantly less EGF than cells derived from 61*G homozygous or heterozygous A/G individuals. It was also demonstrated that the *EGF* 61* gene polymorphism plays a role in the progression of malignant melanoma (30). Recently, it has been reported that gastric cancer and glioma are related to the *EGF* 61* gene polymorphism (31, 32). Therefore, the present study hypothesised that the *EGF* 61* gene polymorphism may be correlated to pancreatic head cancer.

The present study confirmed that the *EGF* 61*G/G genotype and G allele are significantly related to pancreatic head cancer. Pancreatic head cancer patients were found to have a higher distribution of G/G genotypes and G alleles. Since the G/G genotype leads to a higher production of EGF (9), it is proposed that a higher EGF production is associated with an increased risk of pancreatic head cancer. The mechanism by which the *EGF* 61*G/G genotype increases EGF production remains to be determined. Possible reasons may be: (i) the polymorphism may itself be functional; (ii) the G to A substitution may affect the DNA folding or processing of the mRNA transcript and (iii) the allelic variation at position 61 may be closely linked to a functional polymorphism elsewhere in the gene.

The more frequent occurrence of the G allele in *EGF* 61* gene polymorphism among pancreatic head cancer patient needs now to be confirmed by an independent second study, since it may be a useful marker to detect patients with an increased risk of developing pancreatic head cancer, allowing them to be subjected to a more careful or earlier routine screening for pancreatic head cancer.

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