RESEARCH ARTICLE

Association Between EGF, TGF-β1 and TNF-α Gene Polymorphisms and Hepatocellular Carcinoma

Hai-Zhou Shi[1], Peng Ren[1], Qing-Jun Lu[1], Marco Niedrgethmnn[2], Guo-Yang Wu[3]*

Abstract

Introduction: Up to present, EGF 61*A/G, TGF-β1 -509*T/C and TNF-α-308*A/G gene polymorphisms have been analysed in other cancer entities than hepatocellular carcinoma (HCC). We here investigated the frequency of these gene polymorphisms among HCC patients. Materials and Methods: A total of 73 HCC patients and 117 cancer-free healthy people were recruited at the Surgical Department of Zhongshan Hospital. Genomic DNA was isolated from peripheral blood and gene polymorphisms were analyzed by PCR-RFLP. Results: The distribution of EGF 61*G/G homozygotes among HCC patients was more frequent than that in the control group (24.7% vs 11.1%, OR=2.618, 95%CI=1.195-5.738). In parallel, the frequency of the "G" allele in the HCC 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 could be found for the TGF-β1-509 and TNF-α-308 genotypes. Conclusion: EGF 61*G/G genotype and G allele are significantly increased among patients with HCC. TGF-β1-509*T/C and TNF-α-308*A/G gene polymorphisms are not related to this cancer entity.

Keywords: Hepatocellular carcinoma - polymorphism - growth factor - EGF

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Introduction

Hepatocellular carcinoma is a disease with a limited prognosis, which is characterized by its propensity to infiltrate adjacent tissues and to metastasize at early stages (Yang et al., 2010). Absence of specific symptoms, aggressive tumor growth, and resistance to conventional chemotherapy and radiotherapy regimens conspire to culminate in a median overall survival of < 6 months and thus annual mortality figures virtually equaling incidence numbers (Jemal et al., 2005).

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 tumorigenesis (Balkwill et al., 2002; Ikeguchi et al., 2005; Luca et al., 2008). It has been hypothesized that certain polymorphisms for these factors result in functional changes in expression which may influence susceptibility to hepatocellular carcinoma. Up to present, EGF 61*A/G, TGF-β1 -509*T/C and TNF-α-308*A/G gene polymorphisms haven't been reported in hepatocellular carcinoma. We investigated the frequency of these gene polymorphisms among hepatocellular carcinoma patients by means of a case-control study.

Materials and Methods

Patients

Between June 2000 and Septemper 2010, a total of 73 hepatocellular carcinoma patients were recruited at the Surgical Department of the Zhongshan Hospital. 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 (28 females and 45 males). The diagnosis of hepatocellular carcinoma was confirmed histologically in the Pathological Department of Zhongshan Hospital. The control group comprised of 117 cancer-free healthy people who received a control sonograhy as preventive measure. The age range was 61-67 years (43 females and 74 males).

Genotyping

For genetic analyses, genomic DNA was isolated from peripheral EDTA-blood of hepatocellular carcinoma patients and healthy controls using QIAampTM DNA Mini and QIAampTM DNA Blood Mini Kits from QIAGENTM Company according to the manufacturer's instructions. DNA concentrations were determined by A280 using a UV spectrophotometer.

*[1]Department of General Surgery, Binzhou Central Hospital, Binzhou medical College, Bin Zhou, [3]Department of General Surgery, Zhongshan Hospital, Xiamen University, Xiamen, China, [2]Surgical Clinic, University Hospital Mannheim, D-68267 Mannheim, Germany *For correspondence: lucy00234@gmail.com*

Gene polymorphisms were determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP). Primers, lengths of the amplified PCR fragments are given in Table 1. PCR conditions are summarized in Table 2.

The EGF (61*A/G) PCR product was digested with restriction endonuclease Alu I (sequence of restriction site: AG▼CT) for 2 hours. TGF-β1 (-509*T/C) PCR products were digested with the restriction endonuclease Bsu36 I (sequence of restriction site: CC▼TNAGG) for 2 hours. TNF-α (-308*A/G) PCR products were digested with restriction endonuclease NcoI (sequence of restriction site: C▼CATGG) for 2 hours. DNA-fragments were analyzed on 2-3% agarose gels stained with ethidium bromide(Schulte et al., 2001; Sakao et al., 2002; Shahbazi et al., 2002).

Statistics

p-value were calculated with Pearson's chi-square-test or Fisher's exact test. Threshold for significance was $p<0.05$. Statistical analysis was performed by using standard software (V10, SPSS for MS Windows)

Results

*TGF-β1-509*T/C gene polymorphism in hepatocellular carcinoma patients and healthy controls*

The PCR fragments of TGF-β1 –509* C/C genotype were digested into 2 fragments of 273 and 257bp. 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 257bp.

When hepatocellular carcinoma patients were compared with the healthy controls, there were no statistically significant differences ($p > 0.05$, Chi-square; Table 3).

TNF-α-308A/G gene polymorphisms in hepatocellular carcinoma and healthy controls

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

When hepatocellular carcinoma patients were compared with the healthy controls, there were no statistically significant differences ($p > 0.05$, Chi-square; Table 3).

*EGF61*A/G gene polymorphisms in hepatocellular carcinoma patients and healthy controls*

The PCR fragments of A/A genotype were digested into 3 fragments of 193, 34, 15bp respectively while digestion of the EGF 61*G/G genotype yielded 4 fragments of 102, 91, 34, 15bp each. The EGF 61*A/G genotype PCR-products were digested into 5 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 hepatocellular carcinoma patients was higher than that in the control group (24.7% versus 11.1%). The odds ratio for carriers of the 61*G/G genotypes for hepatocellular carcinoma was 2.618 (95% confidence interval 1.195-5.738). The frequency of the "G" allele in the hepatocellular carcinoma patient group (45.9%) was also greater than that in the control group (33.3%). The odds ratio for carriers of 61*G allele for hepatocellular carcinoma 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 hepatocellular carcinoma patients and healthy controls were significantly different as determined by a chi-square test.

Table 1. Primer Sequence and Resulting Fragment Length for Growth Factors Gene PCR

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

Table 2. Technical Data for Growth Factors Gene Polymorphism Detection Methods

	TGF-β1		TNF-α		EGF	
PCR reaction condition	Temperature	Cycles	Temperature	Cycles	Temperature	Cycles
	94ºC 1min	1	94ºC 5min	1	94ºC 5min	1
	94ºC 1min	30	94ºC 1min	30	94ºC 1min	35
	60ºC 1min		60ºC 1min		57ºC 1min	
	72ºC 1.5min		72ºC 1min		72ºC 1min	
	72ºC 10min	1	72ºC 5min	1	72ºC 10min	1
Mastermix (μl)						
10*PCR buffer	5		5		5	
dNTP (10mM)	1		1		2	
Primer-for (10μM)	2		1		3	
Primer-rev (10μM)	2		1		3	
$MgCl_2$ (50mM)	1.5		1.5		3	
Taq polymerase (5u/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. 2001		Sakao et al. 2002		Shahbazi et al. 2002	

Table 3. EGF, TGF-1 and VEGF Genotypes and Allele Frequencies in Patients with Hepatocellular Carcinoma and in Healthy Controls

	Healthy controls in literature	HCC patients (n=73)	Healthy controls (n=117)	x^2	P	OR	95%CI
TGF-β1 -509 genotype	Grainger 1999						
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-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-308 allele							
A		24(16.4)	34(14.7)	0.219	0.64	1.146	0.648-2.024
G		122(83.6)	198(85.3)				
EGF61 genoyype	Amend et al. 2004						
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)				
EGF61 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)				

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 tumors producing their own growth factors (e.g. and prevention of apoptosis (Balkwill et al., 2002; Ikeguchi et al., 2005; Korc et al., 2007; Bussink et al., 2008; Luca et al., 2008). This also appears to protect cancer cells from the toxic actions of chemotherapy and radiotherapy, rendering these treatment modalities less effective. Many epithelial tumor 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 (Stuelten et al., 2005; Jakowlew et al., 2006; Van et al., 2006; Bussink et al., 2008; Wu et al., 2009). 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 tumor stages (Friess et al., 1999; Ghaneh et al., 2002).

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 promotes apoptosis as well (Ikeguchi et al., 2005). 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 (Derynck et al., 2001; Shin et al., 2005). Here, increased levels of TGF-β1 frequently detected in human tumors may contribute either to tumor 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 (Kim et al., 2003). Grainger reported that individuals homozygous for –509T/T had higher plasma concentrations of TGF-β1 than heterozygous C/T or homozygous C/C individuals (Grainger et al., 1999). The genotype distribution and allele frequencies among the healthy controls in our study were parallel to the literature. Our Result show that –509 T allele does not influence the risk to develop hepatocellular carcinoma.

More and more research indicate that TNF may promote cancer development and dissemination in a range of animal experiments (Balkwill et al., 2002). Although we did not measure circulating TNF-α levels, TNF-α-308*A allele has already been shown to increase the constitutive and inducible expression of TNF-α protein, possibly caused by the differential binding of a nuclear protein to the TNF-α-308*A allele (Kroeger et al., 1997; Wilson et al., 1997). It has been reported that some malignant tumors such as prostate cancer, non-Hodgkin's lymphoma and breast carcinoma are related to TNF-α-308*A/G gene polymorphism (Chouchane et al., 1997; Oh et al., 2000; Jeng et al., 2007). Our result shows that TNF-α-308*A/G gene polymorphism is not related to hepatocellular carcinoma. This finding parallels other research, which could prove no association between the TNF-α-308 polymorphism and gastric cancer, uterine cervical cancer, colorectal cancer, or renal cell carcinoma (Jang et al., 2001).

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 (Luca et al., 2008). A clear impact of EGF polymorphisms on skin cancer has been described. Shahbazi reported that the 61*G/G genotype was significantly associated with Breslow thickness and the risk to develop 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 (Shahbazi et al., 2002). It was also demonstrated that the EGF 61* gene polymorphism played a role for the progression of malignant melanoma (McCarron et al., 2003). Recently, it has been reported that gastric cancer and glioma were related to EGF 61* gene polymorphism (Hamai et al., 2005; Costa et al., 2007). Therefore, we hypothesized that the EGF 61* gene polymorphism might be correlated to hepatocellular carcinoma.

We could confirm that the EGF 61*G/G genotype and G allele are significantly related to hepatocellular carcinoma. Hepatocellular carcinoma 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, we may propose that a higher EGF production is associated with an increased risk of hepatocellular carcinoma. 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 might itself be 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.

The more frequent occurrence of the G allele in EGF 61* gene polymorphism among hepatocellular carcinoma 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 to develop hepatocellular carcinoma which could then be subjected to a more careful or earlier routine screening for hepatocellular carcinoma.

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The authors declare that there are no conflicts of interest.

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