RESEARCH ARTICLE

Increased Serum Endoglin and Transforming Growth Factor ß1 mRNA Expression and Risk of Hepatocellular Carcinoma in Cirrhotic Egyptian Patients

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Abstract

Transforming growth factor-B1 (TGF-ß1)and its coreceptor endoglin (ENG) have been shown to contribute to hepatocellular tumor development and malignant progression. Our aim was to evaluate the serum expression levels of ENG/ TGF-ß1 mRNAs and risk of hepatocellular carcinoma in cirrhotic Egyptian patients. Our study included 77 subjects. Real time polymerase chain reaction was used to evaluate the expression level of ENG and TGF-β1mRNAs. The relative expression ratio of ENG mRNA was 0.82 (0.1 -3.2), 0.66 (0.15-5.3), 0.38(0.007-2.8) and 0.12 (0.00-0.22) and the relative expression ratio of TGF-β1mRNA was 1.4 (0.19 -6.2), 1.2 (0.22-4.3), 1.0 (0.15-4.4) and 0.6 (0.00-2.2) for cirrhotic HCC cirrhotic, HCC only and healthy control groups respectively. Increased ENG and TGF-β1 mRNA gene expression was correlated with TNM clinical stage. The expression ratio in TNM stage III–IV 1.1 (0.07-3.2), 1.55 (0.15-6.2) was statistically significantly higher than that in stage I-II 0.47 (0.007-2.8), 1.0 (0.31-4.4) (P<0.05). Our data suggested that increased ENG and TGF-β1 gene expression may participate in hepatocarcinogenesis and increased risk of HCC in individuals with cirrhosis. Early screening for evidence of cirrhosis and consideration of ENG and TGF-β1 as targets for therapy and treatment strategies are warranted.

Keywords: Endoglin - TGFß1 - mRNA - quantitative PCR - hepatocellular carcinoma

Asian Pac J Cancer Prev, **17 (5)**, 2429-2434

Introduction

CHepatocellular carcinoma (HCC) is an aggressive dismal tumor known by its high rate of morbidity and mortality. Worldwide, it is the third leading cause of cancer deaths, with over 500.000 people affected (Thomas et al., 2010). Cirrhosis of any etiology is the major risk factor for HCC. Cirrhosis which is a progressive disease, developing slowly over many years, until eventually it can stop liver function (liver failure) or HCC. Established cirrhosis has a 10-year mortality of 34-66%, largely dependent on the cause of the cirrhosis (Altekruse et al., 2009). In Egypt, HCC annually affects 5-7 cases per 100.000 populations with a nearly equivalent mortality rate (6 per 100.000) reflecting its high disease fatality (El-Zanaty and Way, 2014).

The human transforming growth factor beta (TGF-β) is an important multifunctional family of cytokines. This family includes more than 40 structurally related factors such as the TGF-β isoforms (TGF- β 1~5), activins as well as bone morphogenetic proteins (BMPs) (Gordon et al., 2008, Bertolino et al., 2008; Lebrin et al., 2005). TGFβ-1 is one of TGF-β isoforms and its gene is precisely located on the long (q) arm of chromosome 19 at position 13.1 from base pair 41,836,811 to base pair 41,859,830. In normal liver tissues; TGFβ-1 is generally produced by nonparenchymal cells (Kupffer's and endothelial cells) (Bissell et al., 2001and Dong et al., 2008).

TGFβ-1 can functionally arrest the cell cycle in the G1 phase. This will inhibit cell proliferation and will trigger apoptosis. In tumor development, TGF-ß 1plays a paradoxical role: it functions as a tumor suppressor in the early stages of epithelial malignant transformation due to its pro-apoptotic, anti-proliferative and tumor growth inhibiting actions. Surprisingly, it can subsequently act as a tumor promoter factor through stimulation of the epithelial-to-mesenchymal transition (EMT) as well as through invasiveness of cancer cells and inhibition of immune surveillance (Ferrara et al., 2004). Importantly, TGF-ß 1 has an additional important role in angiogenesis enrolled by promoting proliferation and migration of the endothelial cells at low concentrations,this ends by vessel maturation at high concentrations (Guerrero-Esteo et al., 2002; Elliott et al., 2005). Hepatic TGF-β1 is over-

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expressed in HCC tissues and correlated with tumor formation, progression and prognosis of HCC (Flisiak et al., 2000). This increased expression of TGF-β1 can be a useful marker to early detect HCC due to the higher sensitivity and specificity than alpha fetoprotein (AFP) in the diagnosis of small HCC lesions (Jakowlew, 2006).

Endoglin (ENG) is a TGF-β binding receptor. It is expressed at low levels in resting endothelial cells while its expression promptly increases in active vascular endothelial cells with tumor angiogenesis (Lebrin et al., 2005). The ENG gene is 40 kb long that is located on the long (q) arm of chromosome 9 at position 9q34 from base pair 130,577,290 to base pair 130,617,051(Balza et al., 2001and Conley et al., 2004). Its protein is a 180 kDa homodimeric transmembrane glycoprotein that forms a complex with growth factors and different proteins included in vascular angiogenesis and remodeling (Balza et al., 2001). Endoglin is assigned the "cluster of differentiation number 105" at the Fifth International Workshop on Human Leukocyte Differentiation Antigens, so it is subsequently known as CD105 (Kopczyńska and Makarewicz 2012). It has two different isoforms long and short (L and S) that have a capacity to bind TGF. Both modulate TGF functions through interaction with TGF receptors (TGF-ßRI and TGF-ßRII) (Barbara et al., 1999). Lebrin et al. (2005) previously suggested that changes of endoglin expression in tumor cells can contribute to the deregulation of TGF- β (dependent and independent) signaling pathways and tumor progression . Quantitative analysis of ENG expression ratio in HCC patients has scarcely been performed.

In our study, we aimed to evaluate the expression levels of ENG/ TGF-ß1 mRNAs and risk of hepatocellular carcinoma in cirrhotic Egyptian patients.

Materials and Methods

Subjects

Seventy seven subjects were included in our study and were categorized to three groups:

Group I: It included 48 HCC patients 32 males and 16 females, Their ages were between 42-67 with a mean age value ±SD of (58.3±9.7); include; 38 cirrhotic patients with HCC (n=38), 10 patients with HCC only (n=10).

2- Group II: It included 14 cirrhotic patients without HCC, 11 males and 3 females. Their ages were between 40-66 years with a mean value ±SD of (59.4±7.9).

3- Group III: It included 15 apparently healthy volunteers, 9 males and 6 females, matched for age with a mean age value ±SD of (57.2±12.6).

HCC was confirmed are according to international guidelines. The clinical diagnostic, staging, surgical and pathological data were collected in a standardized manner. HCC was staged according to the TNM staging system , the studied liver cancer patients were 8 patients (16.7%) stage I, 19 (39.6%) stage II, 16 (33.3%) stage III and 5 (10.4%) stage IV. Baseline characteristics of HCC patients are demonstrated in (Table 1).

Cirrhotic patients were classified according to the Child Pugh's clinical classification. In group I, 8 patients (21%) were Child class A, 17 patients (44.7 %) Child B and 13 patients (34.3%) Child C. In group II, 3 patients (21.4%) were Child class A, 4 patients (28.6%) Child B and 7 patients (50%) Child C.

Exclusion criteria: We primarily excluded HCC patients who previously performed any specific therapeutic or palliative interventions for their lesions to avoid bias from such procedures on interpreted results.

Ethical Statement: Informed written consent is obtained from all participants according to human ethics committee approval. The study protocol was reviewed and approved by the Ethics Committee of the National Cancer Institute

All Subjects included in the study were subjected to the following: Full history taking and complete clinical examination.

Routine laboratory investigations including: complete blood count, liver biochemical profile, viral markers.

Specific laboratory investigations including: Serum α-fetoprotein level by electro-chemiluminescence assay using cobase411 auto analyzer (Roche diagnostics).

Relative expression ratio of ENG mRNA and TGFß1 mRNA was determined using Real-Time PCR System 7500

Radiological investigations include: Abdominal ultrasonography, triphasic CT abdomen and/or dynamic MRI abdomen with MRI diffusion.

Sampling and RNA Extraction

Peripheral blood samples (10 mL in EDTA), were collected. Nucleated cells were isolated by the osmotic red blood cell lysis method and the resulting cell pellets were stored at -80°C until RNA extraction.

Extraction of total RNA from nuclear cells

Total RNA of nuclear cells was extracted using RNA extraction kit QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, and USA) according to the manufacturer instruction. All RNA preparation and handling steps took place in a laminar flow hood, under RNAase free conditions. The RNA concentration was assessed by absorbance reading at 260 nm with (Nano-Drop ND-100). All the procedure followed the manufacture instruction.

Reverse transcription and real time PCR assays:

Reverse transcription reaction was carried out in 20 µL reaction mixture by using first strand cDNA synthesis kit (Promega; USA) according to manufacture instruction. For quantitative real-time PCR, cDNA was amplified in an Real-Time PCR System 7500 (Applied Biosystems) using SYBR Green Master Mix Reagent (Applied Biosystems). The forward (F) and reverse (R) primers used were:

TGF-β1: F: CCCAGCATCTGCAAAGCTC; R: GTCAATGTACAGCTGCCGCA; Endoglin: F: CATCCTTGAAGTCCATGTCCTCTT, R: GCCAGGTGCCATTTTGCTT; GADPH: F: TGCACCACCAACTGCTTAGC; R: GGCATGGACTGTGGTCATGAG.

Each primer was used at a concentration of 0.3 µM in each reaction. Cycling conditions were as follows: step 1, 10 min at 95°C; step 2, 15 sec at 95°C; step 3, 30 sec at 55°C; step 4, 30 sec at 60°C, repeating from step 2 to

step 4 40 times. Data from the reaction were collected and analyzed by the complementary computer software (Sequence Detection Software, Applied Biosystems, and Version 1.3). Melting curves were run to confirm specificity of the signal.

Quantitative analysis

Relative quantification of gene expression was calculated using comparative ΔΔ Ct and normalized to GADPH for the assessment of quantitative differences in the cDNA target between samples in each sample using Real-Time PCR software, the mathematical model of Pfaffl (2001) was applied.

Statistical analysis

All the statistics were performed with the software Statistical Package for the Social Sciences (SPSS) (Version 17 SPSS Inc, Chicago) and the p value was considered significant when it was less than 0.05. Statistical data were expressed as mean ± standard deviation. Median used for nonparametric data. Statistical analysis was done using t test. ANOVA and linear regression were applied when data were normally distributed. The Pearson χ^2 test was used to compare the results of two or more subgroups. Mann Whitney test was used to compare median values with clinicopathological variables.

Results

Non statistically significant difference as regards age or gender between three studied groups (p>0.05). Mean age was 58.3±9.7, 59.4±7.9, 57.2±12.6 in HCC patients, cirrhotic patients and healthy controls

Biochemical laboratory data

Our study showed statistically significant difference between group I, II and III regarding the laboratory parameters as ALT, AST, ALP, ALB, total bilirubin and serum AFP (p<0.001). Pairwise comparison showed non statistical significant difference between groups I and II regarding the laboratory parameters as ALT, AST, ALP, ALB, total bilirubin (p>0.05), with statistical significant difference between groups I ,II and III p<0.05). Statistical significant difference between serum AFP and both control groups (p<0.05) (Table 2).

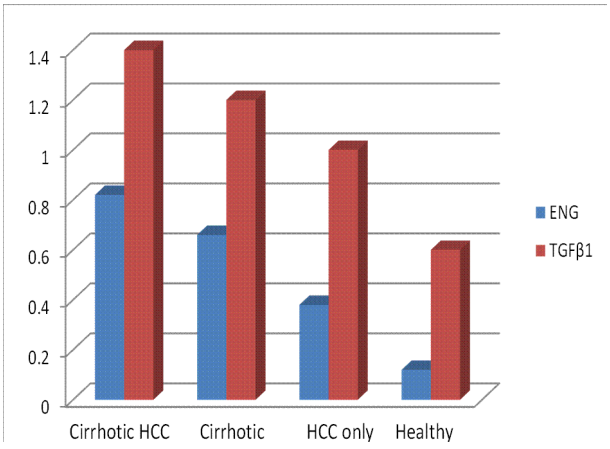

Figure 1. Relative Expression Ratio of ENG/ TGF-β1mRNAs among the Three Studied Groups. The relative expression ratio of ENG mRNA was 0.82 (0.1 -3.2), 0.66 (0.15-5.3), 0.38(0.007-2.8) and 0.12 (0.00-0.22) and the relative expression ratio of TGF-β1mRNA was 1.4 (0.19 -6.2), 1.2 (0.22-4.3), 1.0 (0.15-4.4) and 0.6 (0.00-2.2) for cirrhotic HCC, cirrhotic, HCC only and healthy control groups respectively

mRNA Expression

Quantitative PCR analysis was applied to examine ENG, TGFβ1 genes at the mRNA level in cancerous and control group. Expression of ENG and TGFβ1 mRNAs were observed in all studied patients samples.

Endoglin mRNA

The relative expression ratio of ENG mRNA was 0.82 (0.1 -3.2), 0.66 (0.15-5.3), 0.38(0.007-2.8) and 0.12 (0.00-0.22) in cirrhotic HCC, cirrhotic, HCC only and healthy control group respectively. Statistically significant difference was evident when comparing the levels of ENG mRNA expression of cirrhotic HCC and cirrhotic patients and healthy volunteers (p<0.05). Increased expression was observed in HCC only cases compared to healthy volunteer with no statistically significant difference (p>0.05). The expression of ENG mRNA was much higher in cirrhotic HCC patients and cirrhotic patients than HCC only cases while no significant difference was observed (p>0.05) (Table 3) (Figure 1).

TGFβ1 mRNA

The relative expression ratio of TGF-β1mRNA was 1.4 (0.19 -6.2), 1.2 (0.22-4.3), 1.0 (0.15-4.4) and 0.6 (0.00-2.2) for cirrhotic HCC, cirrhotic, HCC only and healthy control groups respectively. Statistically significant difference was

Table 1. Patient and HCC Characteristics

Criteria	Frequency (%)
Presenting symptoms	
Pain	31(64.6)
Abdominal distention	21(43.8)
Jaundice	4 (8.3)
Encephalopathy	1 (2.1)
Comorbidity	
Diabetes	8 (16.7)
Other comorbidity with diabetes	6 (12.6)
PV thrombosis	9 (18.8)
HCV	21 (43.5)
Cirrhosis	38 (79.2)
Lesion	
Multiple hepatic focal lesions	24 (50)
Single hepatic focal lesion <5cm	14 (44.7)
Single hepatic focal lesion >5cm	10 (26.3)
Child pugh score	
A	8 (21)
B	17 (44.7)
C	13 (34.3)
Lesion	
Multiple hepatic focal lesions	24 (50)
Single hepatic focal lesion <5cm	14 (29.2)
Single hepatic focal lesion >5cm	10 (20.8)
Stage	
I	8(16.7)
II	19 (39.6)
III	16 (33.3)
IV	5 (10.4)

Table 2. Comparison of Laboratory Parameters among Studied Group

	HCC (group I) Mean ±SD	Cirrhosis(group II) Mean ±SD	Healthycontrol (group III) Mean ±SD	P value
TLC($X10^3$) Median (range)	5.1(2.4-26.4)	5.2(3-26.4)	6.9(6.9-7.2)	0.05
Hb (g/dl)	10.7±2.6 a	10.5±3 a	12.7±1	0.001
Platelets($X10^3$) Median (range)	144(2.2-674) a	150(85-401) a	280(150-450)	0.001
T.Bil. (mg/dl)	1.8±1.1 a	1.1±1.2 a	0.2±0.03	0.001
ALT(U/L)	68±47.5 a	62.1±35.8 a	17.3±4.4	0.001
AST (U/L)	75.4±27.4 a	99.1±54.2 a	19.6±5.2	0.001
ALP(U/L)	181.7±77.7 a	198.1±78.1 a	80.8±8.4	0.001
Albumin (g/dl)	2.5±0.6 a	2.4±0.6 a	0.6±0.3	<0.001
AFP<400 (ng/ml) Median (range)	75(15-380) a	46(15-134) a	5(1.2-12.4)	<0.001
AFP>400 (ng/ml) Median (range)	220(84-1103) a			<0.001

a= significant when compared with healthy control; T.Bil. Total bilirubin ALT; alanine aminotransferase, AST; aspartate aminotransferase, ALP; alkaline phosphatase, AFP; α-fetoprotein

Table 3. Expression of ENG/ TGFß1 mRNAs in Different Groups

	HCC (group I)	Cirrhosis (group II)	Healthy control (group III)	P*
Endoglin				
Median	0.71[a]	0.66 a	0.12	<0.05
Range	0.007-3.2	0.15-5.3	0.00-0.22	
Cirrhotic HCC	0.82 a			
	0.1 -3.2			
HCC only	0.38			
	0.007-2.8			
TGFB1				
Median	1.35 a	1.2 a	0.6	<0.05
Range	0.15-6.2	0.22-4.3	0.00- 2.2	
Cirrhotic HCC	1.4a			
	0.19 -6.2			
HCC only	1			
	0.15-4.4			

a= significant when compared with healthy control

Table 4. Correlation between Endoglin and TGFß1 Expression and Clinico-pathological Parameter in Hepatocellular Carcinoma Patients

Site	n	ENG	p*	TGFβ-1	p*
Bilobar	20	0.77 (0.07-2.8)	0.14	1.2 (0.15-3.4)	0.05
Left lobe	8	0.30 (0.1-2)		1.1 (0.25-3.8)	
Right lobe	20	1.30 (0.07-3.2)		1.4(0.33-4.4)	
Portal vein thrombosis					
Yes	9	0.42(0.007-1.3)	0.12	1.4 (0.31-4.4)	0.55
No	39	0.80 (0.07-3.2)		1.3 (0.15-6.2)	
Cirrhosis					
Yes	38	0.82(0.1-3.2)	0.14	1.4 (0.19-6.2)	0.33
No	10	0.38 (0.007-2.8)		1.0 (0.15-4.4)	
Lesions					
Multiple hepatic lesions	24	0.78 (0.07-3.2)	0.48	1.40 (0.15-4.4)	0.87
Single hepatic lesion <5cm <<5cm	14	0.55(0.07-1.7)		1.0 (0.19-6.2)	
Single hepatic lesion>5cm	10	0.87 (0.007-2.8)		1.1 (0.31-3.8)	
Child pugh score**					
A	8	0.44(0.07-3.)	0.66	1.45(5.3-2.2)	0.72
B	17	1.0(0.1-2.8)		1.45(5.3-2.2)	
C	13	0.8(0.16-2.8)	1,4(0.31-3.8)	1.4(0.25-3.1)	
Stage		0.8(0.16-2.8)			
Stage					
Stage I	8	0.38 (0.007- 1.4)	0.18	0.74 (0.44-3.6)	0.66
Stage II	19	0.57 (0.07 -2.8)		1.3 (0.15-6.2)	
Stage III	16	0.92 (0.1- 3.2)		1.4 (0.31-3.8)	
Stage IV	5	1.3 (0.07-2.5)		1.7 (0.73-4.4)	

* p<0.05 is significant/ p value <0.05 comparison between stage I & II and stage III & IV ; ** Child Pugh score was performed for 38 cases

evident when we comparing the levels of TGF-β1mRNA expression of cirrhotic HCC, cirrhotic patients and healthy control ($p<0.05$). Increased expression was observed in HCC only cases compared to healthy volunteer with no statistically significant difference ($p>0.05$). The expression of TGF-β1mRNA was higher in cirrhotic HCC patients and cirrhotic patients than HCC only cases while no significant difference was discovered ($p>0.05$) (Table 3) (Figure 1).

Endoglin/TGFß1 mRNAs expression levels and the pathological findings

The expression ratio of ENG and TGFß1 mRNAs in TNM stage III-IV 1.1 (0.07-3.2), 1.55 (0.15-6.2) were significantly higher than that in stage I- II 0.47 (0.007-2.4), 1.0 (0.31-4.4) ($P<0.05$). No significant difference was detected between the relative expression ratio of ENG and TGFß1 mRNAs as regard the site, size or portal vein thrombosis ($p>0.05$). The detection of ENG mRNA in peripheral blood correlated with that of TGFß1 mRNA (Table 4).

Discussion

Hepatocarcinogenesis is a complex process requiring multiple factors and multiple steps. We present the results of ENG/TGF-β1 mRNAs expression assessment in hepatocellular cancer patients using real time quantitative PCR technique and their risk in HCC development and progression in cirrhotic patients. Real time quantitative PCR allows the measurement with a great level of precision avoiding the sources of error in many immunological techniques (Gómez-esquer et al., 2004).

This study revealed that the expression of TGF β 1 was significantly greater in cirrhotic HCC patients and cirrhotic patients than healthy control group. Increased expression was observed in HCC only cases compared to healthy volunteer with no statistically significant difference ($p>0.05$). The relative expression ratio of TGF- β 1 was 1.4 (0.19-6.2) in cirrhotic HCC patients, 1.2 (0.22-4.3) in cirrhotic group were higher than HCC only cases 1.0 (0.15-4.4). Our finding is in agreement with El Demerdash and Abdalla, (2012) who reported increased expression of TGF β 1 mRNA in both cirrhotic and cancer patients; denoting a possible important role for development of HCC in cirrhotic patients . The possible explanation of their increased expression in cirrhotic group was mainly due to enhancement of intracellular signals that are related to inflammation and fibrotic activity in liver. Hepatocytes are recognized as important cellular sources of latent TGFβ1release. This initial step in the up-regulation of TGFβ1 (being related to oxidative stress associated with hepatic injury and damage to hepatocytes) is a signal for macrophages and platelet activation that result in release of TGFβ1 and over-expression of genes responsible for morphological and functional changes in liver cells. Sheble et al. (2013) reported that TGF-β1 mRNA is a more reliable marker for diagnosis of HCC and that amplification of TGF-β1 mRNA by means of PCR is a sensitive method for detection of HCC in peripheral blood.

ENG is able to be shed into the circulation, with high levels detected in patients with different types of tumors as well as cancer metastases (Markowitz and Roberts 1996, Kumar et al., 1999). This suggests its direct involvement either by modulating the response of tumor cells to TGF-β or by an unidentified mechanism that is TGF-β-independent. Some studies suggested that ENG expression increases phosphorylation of TGFß1 with a consequent increase of phosphorylation of Smad 2 protein that can interact with transcription factors, coactivators, and suppressors. Such action can be explained as integrators of multiple signals to modulate the gene transcription (Kim et al., 2002).

Although some studies used serum tests to detect endoglin, our study could be the first to test ENG mRNA. Studies of ENG at the mRNA level in cancer are lacking, most probably due to the practical ease with serum endoglin detection. However, studies at mRNA are interesting as many tumor markers are known to be subjected to posttranscriptional regulation, and mRNA expression directly correlates with the level of translation and therefore the ultimate level of protein expression. Many studies have reported the expression of ENG in malignant cells of different primary tumors with diagnostic and prognostic significance, such as breast cancer (Takahashi et al., 2001) , squamous cell carcinoma of oral cavity (Schimming et al., 2002) , prostatic cancer (Wikstrom et al., 2002) and renal cell carcinoma (Yagasaki et al., 2003).

In our study, ENG mRNA relative expression ratio significantly increased in cirrhotic HCC and cirrhotic patients compared to healthy control group. Increased expression was observed in HCC only cases compared to healthy volunteer with no statistically significant difference ($p>0.05$). The highest relative expression ratio of ENG mRNA was 0.82 (0.1-3.2) in cirrhotic HCC patients and 0.66 (0.15-5.3) in cirrhotic group much higher than HCC only cases was 0.38 (0.007-2.8).

However, the increased expression of ENG/ TGF-β1 mRNAs in cirrhotic HCC and cirrhotic patients than HCC only cases without cirrhosis and our limited sample size (38 cirrhotic HCC cases , 14 cirrhotic ,10 HCC only and 15 healthy controls) and heterogeneity in cancer with wide variability in level of expression may help to explain why no statistically significant difference were found but increased their expression in cirrhotic and cancer cases compared to healthy control and increased expression in cirrhotic than cancer only indicate that they may contribute to hepatocarcinogenesis and increased risk of hepatocellular carcinoma in cirrhotic patients.

Our results stated that ENG/ TGF-β1 mRNAs correlated with advanced stage of disease. No significant difference was detected as regard their expression in relation to site, size of tumor or associated cirrhosis or portal vein thrombosis.

As cancer develops, cancer cells become more resistant to the growth inhibitory properties of TGF β1and both the cancer cells and the stromal cells often increase the production of TGF β 1 which stimulates angiogenesis and cell motility. Also, it suppresses immune response with the extracellular matrix and increases the interaction of tumor cell leading to greater invasiveness and metastatic

potential of the cancer (Perez et al., 2010) acting as a promoter of malignancy during tumor progression (Shariat et al., 2001). This is in agreement with previous results (Derynck et al., 2001; Giannelli et al., 2002; Lebrun, 2012).

In conclusion, Increased expression ENG/ TGF β 1 in cirrhotic and cancer may contribute to hepatocarcinogenesis and increased risk of hepatocellular carcinoma in cirrhotic patients. Early screening for evidence of cirrhosis and consider ENG and TGF-β1 as ideal targets for therapy and treatment strategies.

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