Association of epidermal growth factor genotype with angiogenesis in Egyptian hepatocellular carcinoma and cirrhotic patients

Iman Helwa[a], Haiam A. Raouf[a], Naglaa Kholoussi[a], Nora N. Esmaiel[b], Rania F. M. Abdelkawy[a]

Departments of [a]Immunogenetics and [b]Molecular Genetics and Enzymology, Human Genetics and Genome Research Division, Center of Excellence for Human Genetics, National Research Centre, Cairo, Egypt

Correspondence to Iman Helwa, MD, Immunogenetics Department, Human Genetics and Genome Research Division, Center of Excellence for Human Genetics, National Research Centre, Cairo 12622, Egypt
Tel: +20 100 157 7973;
Fax: +23 762 1028;
e-mail: helwa1971@yahoo.com

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Background
Worldwide, hepatocellular carcinoma (HCC) is considered a common cancer ranking number six. It is considered number four in Egypt, and is strongly related to hepatitis B and C viruses. In liver cirrhosis, epidermal growth factor (EGF) gene polymorphism genotype is linked to developing HCC. Nevertheless "vascular endothelial growth factor" (VEGF) and "angiopoietin-2" (Ang-2) play a major role as well. Among angiogenic proteins; VEGF is effective and has an effective role in neovascularization. Facts suggest its role in tumor progression and hepatocarcinogenesis.

Objective
The objective of this study was to evaluate the relationship among human EGF genotype and HCC through monitoring of the potent angiogenic proteins (VEGF and Ang-2).

Methods
A total of 81 adults were prospectively enrolled and stratified into three groups: apparently healthy participants ($n = 15$), patients suffering from liver cirrhosis ($n = 29$), and HCC ($n = 37$). Genotyping of EGF single-nucleotide polymorphism was carried out in whole blood of the study participants by sequencing directly using ABI3730XL sequencer. In addition, serum VEGF and Ang-2 levels were determined in all participants using enzyme-linked immunosorbent assay technique.

Results
The study results revealed that six participants of the 61GA heterozygote genotype group (four cirrhosis and two HCC) had high VEGF levels compared with three participants of the 61GG wild-type homozygote group (one cirrhosis and two HCC) and nine participants of the 61AA homozygote genotype group (three controls, one cirrhosis, and five HCC). Moreover, Ang2 was 1.5-fold upregulated in four HCC patients with homozygote genotype compared with three participants of the heterozygote group (one cirrhosis and two HCC). Considerable upregulation was noted in VEGF levels in HCC and cirrhotic patients compared with controls. Also, Ang-2 levels increased in both the liver cirrhosis and HCC groups.

Conclusion
EGF polymorphism genotype (whether heterozygote or homozygote) is associated with increased levels of serum VEGF, an angiogenic protein related to risk for development of HCC. EGF genotype is related to risk for development of HCC in liver cirrhosis through its effect on VEGF and Ang-2 levels.

Keywords:
Ang-2, angiogenesis, cirrhosis, EGF gene, HCC, VEGF

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Introduction

One of the major health problems worldwide is hepatocellular carcinoma (HCC), which accounts for nearly 620,000 or more new cases annually. The observed increase in the occurrence of HCC is chiefly attributable to infection with hepatitis C virus (HCV; Balogh *et al.*, 2016).

Host genetic factors strongly affect the pathogenesis of HCC and so do environmental factors. Moreover, hepatocyte malignant transformation is implicated by changes in molecular signaling pathways and leads to tumor progression (Duan *et al.*, 2019). This process is also linked to disease pathogenesis. Hepatitis B virus (HBV)- and/or HCV-associated cirrhosis as well as alcohol are very well recognized environmental risk factors for HCC worldwide. Despite of the fact that only a fraction of patients with cirrhosis and HCV develop HCC, yet still cirrhosis is considered a precancerous stage (Rawla *et al.*, 2018).

Unfortunately, when cirrhosis occurs, there is a high possibility for HCC to develop. Moreover, in follow-up to diagnose HCC, hepatologists use ultrasound in their regular practice to screen patients with cirrhotic liver, after which if suspicions continue, they may move to computed tomography or magnetic resonance imaging in addition to serum α1-fetoprotein. Despite using present imaging techniques, it is not easy or in fact it may be impossible to diagnose small cancerous lesions (Inarrairaegui and Sangro, 2007).

In early or borderline cases, HCC diagnosis may encounter some difficulties, especially if tumor is well differentiated or when tissue sample is small. In cirrhosis, macronodules are large regenerative nodules from which HCC may develop. Based on their morphological characteristics alone, it is not easy to predict the biological performance of these early precancerous macronodules that developed on top of cirrhosis, inspite of major efforts done to standardize and classify them (Jain 2014).

By studying the expression of known genes that are deregulated in HCC using worldwide genomic analysis, important hints for diagnosis of these difficult cases have been declared. This needs defining HCC tissue versus non-HCC, whether totally normal or cirrhotic through exclusive gene markers and genome-sequencing studies, which has exposed much about the genomic landscape of HCC. Moreover, systematic analysis was done previously that contained precised commonly mutated genes, which included tumor suppressor genes, chromatin remodeling genes, antioxidant defense genes, and others, which were accordingly applied as sequencing panels for HCC (Zucman-Rossi *et al.*, 2015).

Regarding Egypt, HCC developed in over roughly 4.0% of patients with chronic liver disease. It is well known that malignancy occurs as a complication of cirrhosis that occurs as a complication of HCV. Moreover, over 70% of liver malignancies are HCC. Unfortunately, in Egypt, the incidence of HCV is very high, around 6 million having viremia represent 7.3% (Doss *et al.*, 2015; Holah *et al.*, 2015.).

“Epidermal growth factor” (EGF) is a polypeptide growth factor that has chief effects in survival, migration in addition to cell proliferation through EGFR receptor binding. It is secreted by inflammatory as well as tumor cells in the microenvironment (Hanahan and Weinberg Robert, 2011). Furthermore, growth as well as differentiation of malignant cells are stimulated by EGF. This is also the case with normal epithelial cells (Bernardes *et al.*, 2010). Moreover, EGF plays a significant role in hepatocyte morphology. EGF overexpression could be a chief step to liver cancer development and is assumed to have a specific role in spontaneous tumor development (Lindsey and Langhans 2015).

Biological functions of EGF include stimulation of epidermal and epithelial tissues, as well as proliferation, differentiation, and tumorigenesis (Limaye *et al.*, 2008). Multiple human cancers have been linked to single-nucleotide polymorphism relating the A-to-G mutation at position 61 of the 5' untranslated region of the EGF gene (61*A/G, rs4444903) with the possibility of developing tumor (Xu *et al.*, 2010). This was hypothesized because tissue-specific EGF gene expression is modulated by this polymorphism. Although several studies studied the association between EGF + 61A/G polymorphism and HCC susceptibility, their outcomes remain uncertain and controversial (Tanabe *et al.*, 2008; Qi *et al.*, 2009; Suenaga *et al.*, 2013; Wu *et al.*, 2013; Yuan *et al.*, 2013).

Single-nucleotide polymorphism (SNP) as a germline genomic DNA variant increases susceptibility to develop HCC and is among the significant host genetic factors. Elevated risk to develop HCC was linked to several SNPs and other polymorphisms. These were identified through surveys done by Genome-wide association study (GWAS) and single-gene-based or biological hypothesis-based studies (Nahon and Zucman-Rossi, 2012; Fujiwara *et al.*, 2018).

In Shahbazi *et al.* (2002) studied the EGF gene, particularly, the region from position –1350 to 164. They noted a G-to-A substitution at position 61 in the 5' untranslated region. The 61-A-allele variant was found to cause decreased EGF production *in vitro* on demonstration in peripheral blood mononuclear cells. Consequently, the risk of gastric cancer was linked to this promoter variant. Six years later, Tanabe *et al.* (2008) related developing HCC in liver cirrhosis to EGF gene polymorphism genotype.

The process of formation of new microvessels is called angiogenesis. As this permits oxygen and nutrient transport, it is essential for growth and progression of different human solid tumors. Different factors promote angiogenesis, as angiopoietins and vascular endothelial growth factor (VEGF) that is a well known angiogenic factor secreted by various tumor cells (Sherbet 2011).

Based on animal studies, the hypothesis of VEGF-driven splanchnic angiogenesis was put forward (Fernández *et al.*, 2009). There are several factors present in patients with cirrhosis, that is, tissue hypoxia, cytokine imbalance, and shear stress are

known to promote VEGF expression (Alanio *et al*., 2015; Hengst *et al*., 2016).

Therefore, in this study, we aimed at evaluating the association of EGF promoter G61A SNP with the angiogenesis process in HCC and cirrhotic patients by carrying out genotyping analyses for EGF G61A SNP in cirrhotic and HCC cases and evaluation of angiogenic factors such as VEGF and angiopoietin-2 (Ang-2) in these cases.

Patients and methods

Twelve hours of fasting venous blood samples (10 ml) were collected from 81 participants from Ain Shams University hospitals. These participants were categorized into three groups according to different clinical characteristics: group I included 15 apparently healthy participants with normal liver functions, group II included 29 patients suffering from liver cirrhosis but without liver tumor, and group III included 37 HCC patients. Written consent forms were signed and documented by all of the patients who participated in this study, and the study was approved by the Ethical Committee of the National Research Centre (Ethics no. 11010172).

Quantification of angiogenic proteins by enzyme-linked immunosorbent assay

Serum levels of VEGF and Ang-2 were assayed using a standard sandwich enzyme-linked immunosorbent assay (ELISA) commercial kits (Human VEGF ELISA Kit, RayBio, Norcross, Georgia, USA) for VEGF and (Human Ang-2 ELISA Kit Invitrogen, ThermoFisher, Germany) for Ang-2. VEGF and Ang-2 were performed in duplicate according to the manufacturer's instructions.

DNA extraction and genotyping of epidermal growth factor gene

DNA was extracted from whole blood of 37 randomly selected samples (11 controls, 13 cirrhosis, and 13 HCC). Genotyping of EGF SNP was sequenced directly by ABI3730XL sequencer in Macrogen sequencing service, Korea. Genomic DNA was subjected to polymerase chain reaction (PCR) amplification, with initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 51°C for 30 s, and 72°C for 30 s, with the final extension step of 72°C for 7 min using the following primers: forward—TGTCACTAAAGGAAAGGAGGT and reverse—TTCACAGAGTTTAACAGCCC. Then amplified fragments were verified by agarose gel electrophoresis containing ethidium bromide. The verified amplicons were purified and sequenced.

Statistical methods

Data were statistically analyzed using the SPSS version 19.0 software (SPSS Inc., Chicago, Illinois, USA). Nonparametric Mann–Whitney *U* test was used to compare VEGF and Ang-2 levels between groups, and Spearman's rank correlation to test the association of EGF genotyping with VEGF and Ang-2 levels. Data were presented as median (min–max). *P* value of less than 0.05 was considered statistically significant. Receiver-operating characteristic (ROC) curve was constructed for VEGF and Ang-2.

Results

Angiogenesis is critical for the progression of HCC, and EGF gene polymorphism genotype may be connected to the angiogenesis processes and leads to hepatocellular carcinoma progression. We studied whether EGF gene polymorphism genotype correlates with angiogenic factor levels in cirrhotic and HCC patients or not.

The results of quantitation of VEGF, as analyzed by ELISA technique, are summarized in (Table 1). These results indicated nonsignificant upregulation in VEGF levels in cirrhotic patients and a significant increase in HCC patients.

Our group found a highly significant increase in circulating Ang-2 levels in HCC patients and a significant upregulation in cirrhotic patients compared with controls ($P < 0.001$ and 0.009, respectively; Table 2). Furthermore, Ang-2 levels were upregulated but not significant in HCC patients in comparison with cirrhotic patients. While results of VEGF indicated a highly significant increase in HCC patients versus cirrhotic patients (*P* 0.001) (Table 3).

Among 37 participants (11 controls, 13 cirrhotic patients, and 13 HCC patients), the genotyping was successful for EGF polymorphism in 27 participants (Table 4), resulting in an overall success rate of 72.97%.

In Table 5, the results showed that patients with 61GA heterozygote genotype had 1.45-fold upregulation in VEGF levels compared with the 61GG wild-type homozygote and the 61AA homozygote patients,

Table 1 Level of serum VEGF (pg/ml) between the studied groups

	n	VEGF (pg/ml)	*P*
Control	15	1050 (490-1890)	
Cirrhosis	29	2190 (290-6000)	0.051*
HCC	37	1690 (510-6000)	0.026*

Results expressed as median (min-max). HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor. *Significant vs controls (by Mann-Whitney U test).

where the latter showed 1.32-fold increase compared with wild-type homozygote. Nevertheless, Ang-2 was 1.5-fold upregulated in patients with 61AA homozygote genotype compared with 61GA heterozygote group. Unexpectedly, Ang-2 was decreased in 61GA heterozygote genotype and 61AA homozygote genotype patients in comparison with 61GG wild-type homozygote patients.

As shown in Table 6, no significant difference was noticed in the VEGF level comparing participants with mutant genotype compared with those with wild-type homozygote. Moreover, Ang-2 level was lower but not significantly in mutant genotype compared with wild homozygote state.

Association analysis showed that although different EGF genotype states were not associated with VEGF and Ang-2 levels, VEGF nor Ang-2 had a significant correlation with any of the three genotyping states (Table 7 and Fig. 1).

The figure illustrates ROC curves that presented a higher classification power of VEGF compared with Ang-2. VEGF estimation is able to precisely differentiate participants with HCC from healthy controls with 93% sensitivity and a specificity of 72% and a cutoff point of 1018.0 pg/ml (Fig. 2), whereas Ang-2 sensitivity was 71.4%, specificity was 71.1%, and the cutoff point was 2018.0 pg/ml.

Table 2 Ang-2 (pg/ml) level in the studied groups

	n	Ang-2 (pg/ml)	*P*
Control	15	728 (40-1349)	
Cirrhosis	29	1282 (233-6000)	0.009*
HCC	37	3117 (330-6000)	<0.001*

Results expressed as median (min-max). Ang-2, angiopoietin-2; HCC, hepatocellular carcinoma. *Significant vs controls (by Mann-Whitney *U* test).

Table 3 Levels of serum angiogenic factors in HCC patients compared with cirrhotic patients

	Cirrhosis	HCC	*P*
VEGF (pg/ml)	2190 (290-6000)	1690 (510-6000)	0.001*
Ang-2 (pg/ml)	1282 (233-6000)	3117 (330-6000)	0.852

Results expressed as median (min-max). Ang-2, angiopoietin-2; HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor. *Significant cirrhosis versus HCC (by Mann-Whitney *U* test).

Table 4 EGF genotyping results

	Control group (*n*=7)	Cirrhotic group (*n*=9)	HCC group (*n*=11)
G/G N (genotype %)	2 (28.5%)	2 (28.5%)	3 (42.8%)
A/G N (genotype %)	2 (25%)	4 (50%)	2 (25%)
A/A N (genotype %)	3 (25%)	3 (25%)	6 (50%)

EGF, epidermal growth factor; HCC, hepatocellular carcinoma.

Table 5 Comparison between angiogenic protein (VEGF and Ang-2) levels in all the study participants regarding their EGF genotype

	Genotype	*n*	Mean
VEGF (pg/ml)	G/G genotype	7	2155.71
	A/G genotype	8	3132.50
	A/A genotype	12	2848.33
Ang-2 (pg/ml)	G/G genotype	7	2537.29
	A/G genotype	8	1393.88
	A/A genotype	12	2133.58

Ang-2, angiopoietin-2; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor.

Table 6 Comparison of VEGF and Ang-2 in groups of patients with wild genotype vs patients with mutant EGF genotype

Wild/mutant	*n*	Mean (pg/ml)
VEGF wild	7	2155.71
Mutant	20	2962.00
Ang-2 wild	7	2537.29
Mutant	20	1837.70

Ang-2, angiopoietin-2; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor.

Discussion

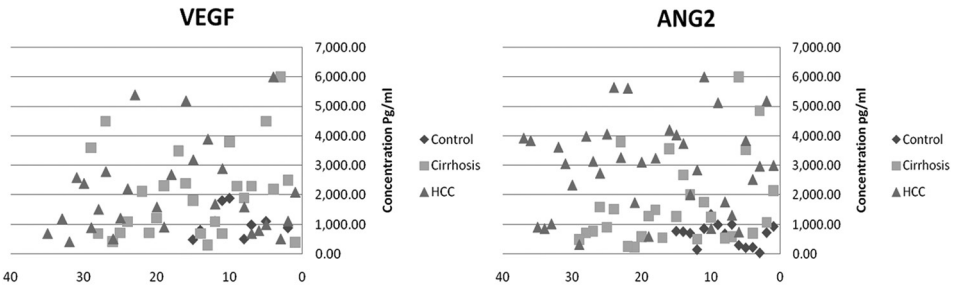
HCC is a progressive cancer with a high mortality rate in the fifties affecting black women and Hispanic men in the United States (Torre *et al.*, 2015; Petrick *et al.*, 2016; Bertuccio *et al.*, 2017; Yang *et al.*, 2017). Egypt ranks third in Africa and 15th worldwide (Rashed *et al.*, 2020).

Several guidelines are available for screening high-risk populations. In 2018, a national screening campaign was started by the Ministry of Health (MOH) in Egypt, to fight high HCV predominance by 2020 (Esmat *et al.*, 2018). Advanced liver fibrosis or cirrhosis is considered a significant predisposing factor for liver cancer, namely HCC.

As HCC is a hypervascular tumor, its development and progression is promoted by angiogenesis (Asayama *et al.*, 2008; Kaseb *et al.*, 2009). Several angiogenic factors regulate this complicated process as vascular endothelial growth factor (VEGF) (Yamaguchi *et al.*, 2006).VEGF is one of the most important angiogenesis regulators and has been suggested as a useful biological marker of tumor invasiveness and prognosis in HCC (Zhang *et al.*, 2006; Abdel-Haleem *et al.*, 2007).

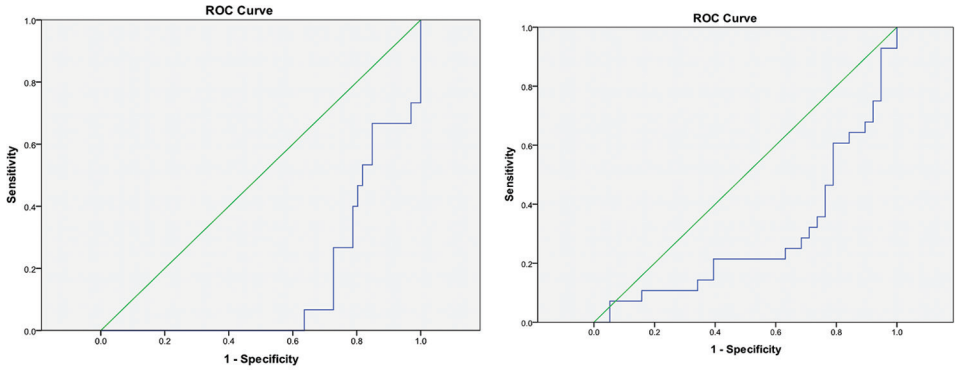
In advanced cirrhosis, conditions of slow flow, and the influence of VEGF induces Ang-2 expression (Goettsch *et al.*, 2008). Our results indicated nonsignificant upregulation in VEGF levels in cirrhotic patients and a significant increase in HCC patients compared with controls (*P* 0.051 and 0.026, respectively; Table 1). Moreover, these results indicated a highly significant increase in VEGF levels of HCC patients versus cirrhotic patients (*P* 0.001; Table 3).

Figure 1



Scatterplot of vascular ehdothelial growth factor and angiopoietin-2 quantities in different study groups (pg/ml).

Figure 2



Receiver-operating characteristic analysis of significant mean difference of vascular ehdothelial growth factor and angiopoietin-2 between the designed groups [control, cirrhosis, and hepatocellular carcinoma].

Table 7 Spearman's rho correlation between VEGF and Ang-2 and genotyping states

Spearman's rho	VEGF	Ang-2	Genotyping
VEGF			
Correlation coefficient	1.000	0.057	0.124
P (2-tailed)	.	0.618	0.536
n	81	81	27
Ang-2			
Correlation coefficient	0.057	1.000	−0.098
P (2-tailed)	0.618	.	0.626
n	81	81	27
Genotyping			
Correlation coefficient	0.124	−0.098	1.000
P (2-tailed)	0.536	0.626	.
n	27	27	27

Ang-2, angiopoietin-2; VEGF, vascular endothelial growth factor.

VEGF expression levels were found to increase due to gene polymorphisms and this was linked to higher risk of HCC (Yvamoto *et al.*, 2015). Moreover, its level could be used as a prognostic factor in HCC, as suggested by some studies (Llovet *et al.*, 2012; Mukozu *et al.*, 2013).

Our group found a highly significant increase in circulating Ang-2 levels in HCC patients and a significant upregulation in cirrhotic patients with respect to controls ($P < 0.001$ and 0.009, respectively; Table 2). Furthermore, Ang-2 levels were upregulated but not significant in HCC patients in comparison with cirrhotic patients (*P* 0.852; Table 3).

That came in agreement with Goettsch *et al.* (2008) and Li *et al.* (2014), who proved that Ang-2 is a vital shear-stress-regulated gene. In investigational circumstances that simulate slow blood flow that occurs in case of portal hypertension rising through development of prolonged liver damage, Ang-2 mRNA, protein expression, and release was found to be upregulated after 24 h of this shear-stress application influenced by such circumstances.

The genotyping was successful for EGF polymorphism in 27 participants (Table 4), resulting in an overall success rate of 72.97%. As shown in Table 5, patients with 61GA heterozygote genotype had 1.45-fold upregulation in VEGF levels compared with the 61GG wild-type homozygote and the 61AA homozygote patients showed 1.32-fold increase in respect to wild-type homozygote. On the other hand, Ang-2 was 1.5-fold upregulated in the patients with 61AA homozygote genotype compared with the 61GA heterozygote group. Nevertheless, Ang-2 was decreased in 61GA heterozygote genotype and 61AA homozygote genotype patients compared with 61GG wild-type homozygote patients.

These results are in agreement with Ferrara *et al*. (2003) who discovered that activation of EGFR pathway causes upregulation of the VEGF ligand and its receptor (VEGFR2) on endothelial cells, hence motivating angiogenesis and vascular permeability.

Likely results were found by Tanabe *et al*. (2008), Abu Dayyeh *et al*. (2011), and Jiang *et al*. (2015) when the EGF 61AG variant was declared as a risk allele for HCC in Eastern and Western participants with severe liver fibrosis mostly affected with viral hepatitis. Moreover Fuchs *et al*. (2014) and Lanaya *et al*. (2014) in their experimental studies found that hepatocarcinogenesis was linked to *EGF* overexpression in hepatic stellate cells and macrophages.

Suenaga *et al*. (2013) experienced similar results in hospital-based studies when they found that EGF + 61A/G polymorphism was considerably associated with HCC risk.

Similarly, Tanabe *et al*. (2008) studied the relation between EGF polymorphism and HCC risk. They stated that the risk of developing HCC was linked to EGF gene polymorphism 61*A/G.

Nevertheless, the connection between the EGF gene polymorphism 61*A/G and risk of HCC was evaluated in various ethnicities in epidemiological studies (Abu Dayyeh *et al*., 2011). However, their results were conflicting. In the same context, various studies have indicated that higher vulnerability to HCC is present in patients having G/G genotypes (Abu Dayyeh *et al*., 2011), yet others have not found any link (Li *et al*., 2010; Chen *et al*., 2011). Moreover, many meta-analyses have shown that some gene polymorphisms significantly correlate with HCC susceptibility (Minmin *et al*., 2011; Wei *et al*., 2011).

On the other hand, Zhong *et al*. (2012) suggested that having the EGF 61*G/G genotype increases HCC risk on one side, while on the other, the A/A genotype has a protective effect. They also defined the EGF 61*A/G polymorphism as an HCC genetic susceptibility element that provided chronic HBV infection and/or cirrhosis. As a matter of fact, the number of deaths related to HCC nearly matches the number of diagnosed annual cases (Michielsen *et al*., 2005).

Therefore, in order to improve prevention and treatment strategies, recognition of molecular markers associated with high HCC risk would better define high-risk populations of HCC.

Conclusion

Serum VEGF and Ang-2 levels are frequently elevated in HCC patients more than in patients with cirrhotic liver.

EGF polymorphism genotype (whether heterozygote or homozygote) associated with increased levels of serum VEGF was recorded particularly in the HCC patients' group although statistically insignificant, yet may be further elucidated in larger studies.

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Conflicts of interest

There are no conflicts of interest.

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