Serum Vascular Endothelial Growth Factor AND ANGIOSTATIN as Potential Markers in Patients with Hepatocellular Carcinoma

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Abstract: Objective: to evaluate the prognostic role of serum VEGF and angiostatin levels in patients with HCC. **Patients and methods:** Between April 2010 and April 2012, 40 patients diagnosed with HCC, presented to the Departments of Gastroenterology and clinical oncology, Assiut Univ. Hospital were recruited in this study. The control group consisted of 40 healthy individuals and another group of 40 cirrhotic patients with no evidence of HCC attending the Gastroenterology clinic of our hospital were included. Serum samples were prospectively collected from all groups for estimation of α-FP, VEGF, and angiostatin levels using ELISA technique. Patients with HCC were managed according to the BCLC strategy. All patients were reviewed in the Gastroenterology and oncology clinics at least every 1 to 2 months. **Results:** The mean serum VEGF concentrations (632.3±5.1 pg/mL) were significantly higher in patients with HCC than in liver cirrhosis patients and healthy controls (mean value148.0±23.32 pg/mL, and 45.0±6.4 pg/mL, respectively) ($P < 0.05$). In addition, HCC patients showed increased serum VEGF concentrations with increased BCLC score (Odd's Ratio1.05 - 95% confidence interval 1.11–3.9). On multivariate analysis, serum VEGF level was an independent prognostic factor (hazard ratio 1.86 (95 per cent confidence interval 1.10 to 3.92); $P = 0.032$). We also found that angiostatin levels were significantly lower in HCC patients compared with patients with liver cirrhosis and control subjects ($P <0.05$). Furthermore, there was no significant correlation between serum angiostatin levels and VEGF levels. We did not find any correlation between angiostatin serum levels and overall survival. **Conclusion:** this study demonstrated that serum VEGF level is a prognostic marker for HCC that can help guidance in clinical decision-making regarding therapy and outcome. Our study also showed that angiostatin is potential diagnostic marker that may aid in early detection of HCC. However, further studies should be performed.

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1. Introduction:

The incidence of HCC is predicted to increase over the next several decades as survival in patients with predisposing diseases, such as cirrhosis, is expected to increase over time (1). Because of this, there has been great interest in evaluating factors that influence prognosis in HCC. The most widely studied prognostic factors are related to pathological characteristics of the neoplasm, including tumor size, grade, stage and vascular invasion (2-5). A variety of other potential serum prognostic markers, however, remain to be further characterized (2).

Angiogenesis, defined as the formation of new blood vessels from existing vasculature, is an important process regulating the growth and development of malignancies including HCC. The extensive hypervascularity associated with HCC is thought to be driven in part by the pro-angiogenic factor known as vascular endothelial growth factor (VEGF) (6,7). Furthermore, the invasiveness of certain HCC lesions has recently been linked to high levels of VEGF, leading several authors to conclude that an important relationship between VEGF and prognosis exists for HCC (8, 9). The three most commonly used methods of measuring VEGF are serum-based VEGF quantitation using enzyme-linked immunosorbent assay, tissue-based semi-quantitative VEGF immunohistochemistry and tissue-based mRNA measurement (6). However, immunohistochemistry has a limitation because it requires a tumor specimen. On the other hand, the measurement of VEGF in blood does not require a tumor specimen thus it is applicable to every cancer patient (1).

There is a natural balance between promotors of angiogenesis such as VEGF and inhibitors of angiogenesis such as angiostatin (10). Today it is widely accepted that angiostatin is produced by stromal cells of the primary tumor. However, the origin of circulating angiostatin in cancer patients and its prognostic significance are not yet clear (11).

The aim of this study was to evaluate the prognostic role of serum VEGF and angiostatin levels in patients with HCC.

2. Patients and Methods

In this study, 40 patients were diagnosed with HCC were included and presented to the Departments of Gastroenterology and clinical oncology, Assiut Univ. Hospital in period Between April 2010 and April 2012, . The study protocol was approved by the Ethics Committee of our institution. All patients provided a written informed consent.

They met the following inclusion Criteria:

1) Diagnosis of localized or metastatic hepatocellular carcinoma (HCC) by biopsy and/or imaging studies.
2) Age: 18 and over.
3) Performance status: ECOG 0-2.
4) Severity of liver disease was assessed by Child-Pugh classification [13]Table (A).
5) The stage and management were defined according to the Barcelona-Clinic Liver Cancer Group diagnostic and treatment strategy (BCLC) [14]. Sorafenib was not given due to financial reasons, instead, best supportive care was prescribed Table (B).
6) Patients with other types of malignancy, advanced organ failure, active infection and advanced medical co-morbidity were excluded from the study.

Table (A): Child-Pugh scoring system to assess severity of liver disease:

	Points		
	1	2	3
Encephalopathy (grade)	none	1-2	3-4
Ascites	Absent	Slight or Controlled By diuretics	At least Moderate Despite Diuretic Treatment
Bilirubin (mg/di)	<2	2-3	>3
Albumin (g/di)	>3.5	2.8-3.5	<2.8
Prothrombin time (seconds) prolonged	<4	4-6	>6
Or/INR	<1.7	1.7-2.3	>2.3
For primary biliary Clcirrhosis, Primary Sclerosing Cholangitis or other Cholestatic liver Bilirubin (mg/dl)	<4	4-10	>10

Table (B): Barcelona clinic liver cancer (BCLC)-classification

Tumor Stage	General State of Heath	Tumour-Characteristics	Child-Stage
0 Very early	Good	Single nodule<2cm	A & B
A Early	Good	Single nodule <5 cm, 3 nodules< 3 cm	A & B
B Intermediate	Good	Large, multiple nodules	A & B
C Advanced	Reduced	Vascular invasion, extrahepatic secondaries	A & B
D Terminal	Severely	Any form	C

Control group

The control group consisted of 40 healthy individuals with no apparent evidence of active disease or medical disorders.

As most HCC patients in this study were expected to have underlying liver cirrhosis, another group of 40 cirrhotic patients with no evidence of HCC attending the Gastroenterology clinic of our hospital was included.

Work up: all patients had undergone

1) Detailed history and full clinical examination.
2) Routine laboratory investigations: complete blood count, liver function tests, prothrombin time and kidney function tests using standard methodologies.
3) Viral markers: HBs antigen by ELISA (Monolisa, Biorad, USA) and HCV antibody by 4th generation ELISA, antigen antibody (Biorad, USA) and confirmed by detection of HCV RNA by PCR
4) **HCV RT-PCR: RNA extraction** was performed by the kit supplied by Qiagen (Viral RNA Mini Kit Lot No. 11233766). **HCVRNA amplification** (RT-nested PCR amplification) was done by the reagent supplied by (Promega) **first amplification mix** containing (10 ul 10x-buffer, 2 ul MgSO4, 2 ul primer I, 2 ul primer 2, 1 ul dNTPs mix 10 umol, 1 ul RT, 1 ul Taq DNA polymerase and 24 ul RNAse free water). Amplification cycles profile were (48 °C 45 min. and 95 °C 5 min one cycle), (95 °C 5 min., 60 °C 45 sec. &72°C 2 min ,5cycles), (95 °C 5 min.,60 °C 45 sec. & 72°C 2 min, 30 cycles) and (72 °C 2min., 5 cycles), **second amplification mix** containing (5ul 5x-buffer, 2 ul Mgcl 6mmol, 2 ul primer 3, 2ul primer 4, 1ul dNTPs mix, 10 umol, 0.5ul Taq DNA polymerase & 32.3 ul RNAse free water). Amplification cycles profile were (95°C 5min one cycle), (95°C 5 min., 60°C 45 sec & 72°C 2 min, 5 cycles), (95°C 5 min., 60°C 45 sec.& 72°C 2 min, 30 cycles) and (72°C 2 min , 5 cycles). **Detection** was done by 2% agarose gel electropheresis in TAE buffer, positive bandswere detected at 150 bp in comparison to PCR ladder.
5) Estimation of serum α-fetoprotein level (α-FP), using commercially available ELISA kits (Quantikine Human α-FP Immunoassay; R & D Systems, Minneapolis, MN).
6) Sonographic examination and triphasic study of the liver by CT scan.
7) X-ray of the chest and bone scan to detect metastasis.
8) Liver biopsy from HCC patients. Ultrasound-guided core needle biopsies were obtained from the hepatic tumors and immediately fixed in 10%

formalin and sent to the pathologist for histopathological examination. The tumor was graded as well differentiated HCC (grade I), moderately differentiated (grade II) and poorly differentiated (grade III-IV) according to Edmondson and Steiner (15).

All patients were reviewed in the Gastroenterology and oncology clinics at least every 1 to 2 months.

Measurement of Serum VEGF and Angiostatin Levels

Serum samples were prospectively collected from all groups. Venous blood samples were drawn into a serum separator tube and centrifuged at 3,000 rpm for 10 minutes, then stored at −80°C until VEGF and Angiostatin levels were determined.

Serum levels of angiostatin were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) using Duo-Set ELISA kit. Serum VEGF levels were quantitatively measured by an enzyme-linked immunosorbent assay kit designed to measure human VEGF concentration in serum (Quantikine Human VEGF Immunoassay; R & D Systems, Minneapolis, MN). This assay has been shown to be reliable and reproducible in previous studies (16). Briefly, 100 µL recombinant human VEGF standard and serum sample was serially diluted and pipetted into a microtiter plate coated with murine monoclonal antibody specific for human VEGF and incubated for 2 hours at room temperature. Any VEGF present was bound by the immobilized antibody. After washing away any unbound substances, a horseradish peroxidase-linked polyclonal antibody specific for VEGF was added to each well to sandwich the VEGF. After further washings to remove any unbound antibody–enzyme reagent, tetramethylbenzidine was added. The intensity of color developed, which was in proportion to the amount of VEGF bound, was measured by reading absorbance at 450 nm. Each measurement was made in duplicate, and the VEGF level was determined from a standard curve generated for each set of samples assayed. The sensitivity of the assay was 9 pg/mL, and the coefficients of variation of intraassay and interassay determinations were in the range given by the manufacturer (4.5–6.7% and 6.2–8.8%, respectively).

Statistical Methods

The nonparametric 2-sided Wilcoxon rank-sum test (Mann–Whitney *U* test) for paired group comparisons was applied for statistical analysis. Univariate overall survival analyses were performed using Kaplan-Meier and univariate Cox analysis. For multivariate analysis, the Cox proportional hazards regression model was used. In all tests, a *P* value of at least .05 was considered statistically significant. All statistical analyses were done with the SPSS software package, version 18.0 (SPSS, Inc., Chicago, IL).

3. Results

Patients' characteristics were summarized in Table 1. The liver functions of patients were summarized in Table 2. There was a significant increase in serum concentration of α-fetoprotein in HCC patients as compared to patients with liver cirrhosis and the control group (Table 3).

Similarly, a significant increase in serum VEGF was found in HCC patients as compared with patients with liver cirrhosis and control subjects (632.3±5.1, 148.0±23.32, 45.0±6.4 respectively), (Table 3). In addition, HCC patients showed increased serum VEGF concentration with increased BCLC score (Tables 4,5). Moreover, serum VEGF level was positively correlated with serum α-fetoprotein (Table 6). Significant positive correlations between serum VEGF and serum activities of ALT and AST were found in HCC patients, (Table 6).

In this study, we used the median level of serum VEGF as a cut-off value. On correlation with survival data of patients with HCC, it was found that high level of serum VEGF was correlated with poor overall survival. On multivariate analysis, serum VEGF level was an independent prognostic factor (hazard ratio 1.86 (95 per cent confidence interval 1.10 to 3.92); $P = 0.032$).

As shown in Table 3, angiostatin levels were significantly lower in HCC patients compared with patients with liver cirrhosis and control subjects ($P < 0.05$). In contrast to VEGF, we did not find any significant correlation between serum angiostatin levels, BCLC score and serum α-fetoprotein levels, (Table 4, 5). Furthermore, there was no significant correlation between serum angiostatin levels and VEGF levels. We did not find any correlation between angiostatin serum levels and overall survival

Tab. 1 Patients characteristics

	HCC (n=40)	Cirrhosis (n=40)
Age: (mean ±SD)	52.36 ± 13.7	50.83 ± 17.9
Sex: Male/female	40/0	40/0
Hepatitis serology: positive for		
HBV	19 (47.5%)	7(17.5%)
HCV	14(35%)	20(50%)
Both	7(17.5%)	13(32.5%)
Child-Pugh score		
A	5(12.5%)	8(16.7%)
B	25(62.5%)	20(50%)
C	10(25%)	12(33.3%)
BCLC		
A	10(25%)	
B	20(50%)	
C	6(15%)	
D	4(10%)	

Treatment		
Surgical resection	3(7.5%)	
TACE	25(62.5%)	
Radiofrequency	5(12.5%)	
Supportive care	7(17.5%)	

[n] number of patients; BCLC…Barcelona-Clinic Liver Cancer Group diagnostic and treatment strategy.

Tab. 2: Liver function tests of patients with cirrhosis and HCC as compared with the control subjects (mean±SE).

	Control group (n=40)	Cirrhotic group (n=40)	HCC group (n=40)
ALT (U/ml)	10.2±1.7	54.4±13.3[*]	61.5±5.3[*#]
AST (U/ml)	9.5±1.5	77.7±9.13[*]	94.06±11.4[*#]
Total bilirubin (mg/dl)	0.56±.01	4.4±0.4[*]	7.6±0.06[#]
Albumin (g/dl)	4.70±0.47	2.20±0.40[*]	2.80±1.3[*]
ALP (U/l)	27.3±0.85	75.0±6.6[*]	182.4±10.5[*#]
γGT (U/l)	16.5±1.0	42.6±8.9[*]	194.9±26.8[*#]

[n] number of patients;
[*]significant difference as compared with the control group at $p<0.05$;
[#]significant difference as compared with the cirrhotic group at $p<0.05$.

Tab. 3: Serum concentration of α-fetoprotein, VEGF and Angiostatin in cirrhotic and HCC patients as compared with the control group (mean±SE).

	Control group (n=40)	Cirrhotic group (n=40)	HCC group (n=40)
α-fetoprotein (ng/ml)	2.7±0.4	20.4±5.3[*]	346.09±15.8[*#]
VEGF (pg/ml)	45.0±6.4	148.0±23.32[*]	632.3±5.1[*#]
Angiostatin	171.4±19.0	153.5±17.4	19.2±8.4[*#]

[n] number of patients;
[*]significant difference as compared with the control group at $p<0.05$;
[#]significant difference as compared with the cirrhotic group at $p<0.05$.

Tab. 4: Serum concentration of VEGF in HCC patients in relation to BCLC staging (mean±SE).

	A (n=10)	B (n=20)	C & D (n=10)
VEGF (pg/ml)	501.23±1.5	586.68±6.07[*]	635.23±7.25[#]

[n] number of patients;
[*]significant difference as compared with BCLC A group at $p<0.05$;
[#]significant difference as compared with BCLC B group at $p<0.05$.

Tab. 5: Multivariate analysis of serum concentration of α-fetoprotein, VEGF and Angiostatinin HCC patients in relation to BCLC staging.

Variable	B	Odd's Ratio	95% CI
VEGF (pg/ml)	3.1	1.05[*]	1.11–3.9
A-fetoprotein (ng/ml)	2.8	0.59	0.72–5.3
Angiostatin	2.7	0.034	0.84-4.3

[*]significant at $p<0.05$.

Tab. 6: Correlation between serum VEGF with the measured parameters in cirrhotic and HCC patients.

	VEGF	
Parameters	**Cirrhotic patients**	**HCC patients**
ALT	−0.07	0.47[*]
AST	0.33[*]	0.42[*]
Total bilirubin	0.27[*]	0.10
Albumin	0.05	−0.31[*]
Alkaline phosphatase	0.16	−0.09
Γ-glutamyl transferase	0.33	−0.4
α-fetoprotein	0.12[*]	0.59[*]

[*]significant difference at $p<0.05$.

4. Discussion

In most solid malignancies, tumor stage at presentation determines prognosis and plan of management. However, most patients with HCC have two diseases, liver cirrhosis or HCC, and complex interactions between the two have major implications for prognosis and treatment choice (17). Therefore, there has been great interest in identifying prognostic markers for patients with HCC as these markers can help guide clinical decision-making regarding therapy and outcomes. Various studies have evaluated the prognostic value of VEGF levels in HCC. Its overall test performance remains unclear. Conflicting data, however, have emerged regarding the ability of VEGF to predict disease progression and overall survival (OS) in HCC. This may be related to differences in the methods of measuring and reporting quantitative VEGF measurements (18).

In their metanalysis, Schoenleber *et al.* (18) reported that serum-based studies tended to be of slightly higher methodological quality than tissue-based studies although this was not statistically significant. In addition, data from the serum VEGF studies appear to be generalisable to all patients with HCC, as the included populations were treated using a variety of curative therapies. Although tissue studies only included surgically treated patients, serum studies included patients treated with surgical or medical management, chemoembolisation, or radiofrequency ablation. When VEGF levels for both surgically and non-surgically treated groups were examined, no

difference was found between groups. This suggests that choice of therapy was not potentially associated with serum VEGF levels (18).

In this study, we evaluated a possible prognostic value of serum VEGF in patients with HCC and the correlation of its high levels with α-fetoprotein and OS. We found a significant increase in serum concentration of α-fetoprotein in HCC patients as compared to patients with liver cirrhosis and the control group. Similar results were obtained by many studies [19-22]. Some reports have indicated that α-fetoprotein has limited utility of differentiating HCC from benign hepatic disorders for its high false-positive and false-negative rates, and patients with acute exacerbation of viral hepatitis but no HCC may also have markedly increased α-fetoprotein levels [23].

Therefore, we aimed to measure the serum concentration VEGF in patients with liver cirrhosis and HCC to evaluate its activity as tumor marker for liver malignancies.

We found a significant increase in serum VEGF in HCC patients as compared with patients with liver cirrhosis and control subjects. These results coincided with those recorded by other studies [24-28]. Moreover, serum VEGF level was positively correlated with serum α-fetoprotein. In their study, Corradini *et al.* (29) found that serum AFP concentration correlated positively ($r=0.755$; $P< 0.01$) with serum VEGF-A in the HCC patients with serum AFP above 20 ng/ml, but not in those with serum AFP below 20 ng/ml (27). In another study by Gadelhak *et al.* (30), there was no association between either p53 Abs or VEGF and AFP concentrations in the HCC patients. However, a greater incidence of VEGF and accumulation of p53 Abs expression was detected in positive cases for AFP where VEGF was detected in 85.3% and p53 Abs was detected in 83.3% of positive cases for AFP concentration divided by the platelet count (30).

In addition, significant positive correlations between serum VEGF and serum activities of ALT and AST in HCC patients were found. Moreover, serum VEGF increased significantly with increasing stage of BCLC and correlated with poor OS, coinciding with similar studies (24-28, 31, 32).

Our results revealed significantly higher angiostatin concentration in samples of healthy controls and patients with liver cirrhosis as compared with HCC patients. This is in agreement with that reported by Szarvas *et al.* (11), although their study was on bladder cancer patients. One possible explanation is that tumor cells produce substances that inhibit angiostatin production. This change in the balance of proangiogenic and antiangiogenic factors may contribute to an "angiogenic switch," which may provide a systemic proangiogenic milieu supporting tumor-induced angiogenesis. However, the process leading to downregulation of circulating angiostatin levels is uncertain, (11).

In conclusion, this study demonstrated that serum VEGF level is a prognostic marker for HCC that can help guide clinical decision-making regarding therapy and outcomes. Our study also showed that angiostatin is potential diagnostic marker that may aid in early detection of HCC. However, further studies should be performed.

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