Evaluation of serum endoglin as noninvasive marker in hepatocellular carcinoma

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Introduction
Hepatitis C viral (HCV) infection is a major risk factor for liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC). A number of laboratory-based methods has been developed for the noninvasive diagnostic evaluation of HCC. Endoglin (CD105) is a homodimeric membrane glycoprotein expressed on endothelial cells that can bind to transforming growth factor-b1 and transforming growth factor-b3.

Aim of the study
The aim of this study was to evaluate the diagnostic value of endoglin and a-fetoprotein (AFP) in patients with chronic HCV infection with and without HCC.

Patients and methods
A total of 50 HCV patients were chosen and divided into two patients groups, group I (26 cirrhotic patients) and group II (24 HCC patients), and compared with group III (10 healthy volunteers) as controls. For all participants, thorough clinical examination, blood picture, liver function tests, HCV antibody, AFP, and serum endoglin were performed. Abdominal ultrasound, abdominal triphasic computed tomographic scan, and liver biopsy for those diagnosed HCC by triphasic computed tomography were performed.

Results
We found highly significant increase in serum endoglin in HCV patients with HCC (group III) compared with HCV patients with liver cirrhosis (group I) and controls (group III). There was significant positive correlation between serum endoglin and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, and AFP. In addition, there was significant negative correlation between serum endoglin and hemoglobin, albumin, and prothrombin concentration. The cutoff value for serum AFP for which HCC is suspected was greater than 2.17 ng/ml with sensitivity 98% and specificity 80%, whereas the cutoff value for serum endoglin was greater than 6.05 ng/ml with sensitivity 98% and specificity 90%.

Conclusion
Serum endoglin surpassed serum AFP due to the higher degree of diagnostic specificity, as well as sensitivity. Serum endoglin showed a better diagnostic performance and proved to be more reliable as a tumor marker for HCC. Serum endoglin may be used with serum AFP as complementary biomarker as noninvasive technique to aid diagnosis of HCC.

Keywords:
hepatitis C virus, hepatocellular carcinoma, serum a-fetoprotein, serum endoglin

Introduction
Hepatitis C virus (HCV) is recognized as a major cause of chronic liver disease [1]. The prevalence of HCV infection is 2.2% of the world's population. An estimated 27% of cirrhosis and 25% of hepatocellular carcinoma (HCC) worldwide occur in HCV-infected people [2]. HCV is responsible for infecting over 20 million in Arab countries (3). The prevalence of that virus is reported to be higher in Egypt than in any other Arab country; the prevalence of HCV among the general populations is variable from 13 to 22% [4]. It is found to be 14.5% among blood donors, 7.7% among healthcare workers, and 47.2% in chronic liver disease patients [5].

The predominant HCV genotype among Egyptians was found to be genotype 4, particularly subtype 4a. However, other genotypes and subtypes such as 1a, 1b, and 2a are also present indicating that HCV genotypes are variable [6]. HCC is one of the most common and aggressive human malignancies worldwide (7). In 2012, more than half a million people worldwide were diagnosed as having HCC (8).

Most of patients with HCC are diagnosed at a late stage. Screening strategies including imaging with ultrasound, triphasic computed tomographic (CT) scan, or gadolinium-enhanced MRI [9]. Although a-fetoprotein (AFP) is the most widely used biomarker of HCC, AFP is insufficiently sensitive or specific for use as a diagnostic assay. AFP can be elevated in

patients with liver diseases other than HCC, including viral hepatitis, intrahepatic cholangiocarcinoma, and in some metastases from colon cancer. In addition, only 10–20% of early-stage HCC patients have abnormal AFP serum levels; thus, AFP cannot be used as the sole tool to screen for HCC [10].

Endoglin is expressed as a 180-kDa homodimer formed by disulfide-linked monomers. It is an accessory receptor for transforming growth factor-β (TGF-b) and its expression is upregulated in actively proliferating endothelial cells [11]. Endoglin has been suggested as an appropriate marker for tumor-related angiogenesis and neovascularization. Several studies demonstrate the role of endoglin in tumor diagnosis, prognosis, and therapy [12]. HCC is mainly angiogenesis dependent; hence, serum endoglin may be a useful marker for diagnosis and follow-up (13).

The aim of the study was to evaluate the role of serum endoglin as noninvasive biomarkers in diagnosis of HCC associated with HCV infection and to compare it with its level in patients with HCV infection with cirrhosis. The diagnostic performance of endoglin as a possible tumor marker is compared with that of serum AFP.

Patients and methods

In a cross-sectional study, 50 chronic infected HCV Egyptian patients were recruited from the Internal Medicine Department, AL Zahraa Hospital after informed consent. An approval of the ethical committee was also obtained before the start of this study. Clinical and biochemical profiles were assessed. Patients were excluded if they have hepatitis B virus, HIV, inflammatory diseases, hematological malignancy, and cancer of any organ other than the liver.

Ten healthy volunteers for validation of our findings were included.

Patients were divided as follows

Group I included 26 patients with liver cirrhosis due to HCV. Cirrhotic patients were diagnosed by clinical examination and biochemical and ultrasonographic criteria.

Group II included 24 HCV patients with HCC along with cirrhotic liver. HCC patients were diagnosed by serum AFP level, abdominal ultrasound, and abdominal triphasic CT scan and were confirmed by liver biopsy.

Group III included 10 apparently healthy individuals, age and sex matched, having no acute or chronic illness, with normal liver functions as controls.

All individuals were subjected to the following:

Full history taking, complete clinical examination, abdominal ultrasound, CT scan, and laboratory investigations including blood picture, liver function tests [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, total bilirubin, prothrombin time, and concentration], HCV antibody, serum AFP, creatinine, and serum endoglin.

Sampling

Five milliliters of fasting venous blood samples were taken and divided into parts; 1.8 ml of venous blood was added to tube containing 0.2% citrate for prothrombin time determination using tissue thromboplastin method on automated blood coagulation analyzer (Siemens AG, Erlangen, Germany). A volume of 1.2 ml of venous blood was added to a tube containing EDTA for complete blood count determination on Coulter counter (Coulter LH 750 analyzer; Berlin, Germany). The rest of blood was left to clot, then the serum was separated by centrifugation at 1000g for 15 min and stored at -20°C for determination of liver function tests (albumin and total bilirubin, prothrombin concentration) and liver enzymes (AST, ALT, ALP), AFP, and endoglin.

Liver function tests were performed on Hitachi autoanalyzer 912 (Hitachi, Roche, Japan) using colorimetric techniques, and serum AFP was determined using quantitative sandwich immunoassay [14] and the kit was supplied from Anogen (Mississauga, Ontario, Canada). HCV antibody was detected by enzyme immunoassay.

Serum endoglin was measured by quantitative sandwich enzyme immunoassay technique supplied from R&D Systems Inc. (Minneapolis, Minnesota, USA) [15].

Statistical analysis

Results were collected, tabulated, and statistically analyzed by personal computer and statistical package SPSS version 10 (Chikago, USA). Two types of statistics were performed: Descriptive statistics – for example, mean (X) and SD – and analytic statistics. The Student t-test is a test of significance used for comparison of the means between two groups having quantitative variables, and the Mann–Whitney U-test (nonparametric test) is a test of significance used for comparison between two groups not normally

distributed having quantitative variables. In addition, Spearman correlation coefficient (*r*) (nonparametric test) is a test used to measure the association between two quantitative variables. The level of significance was set as *P* value less than 0.05.

Results

Table 1 shows highly significant decrease in hemoglobin, platelets, serum albumin, and prothrombin concentration in both HCV and HCC groups compared with the control group (*P* < 0.01). In addition, there was highly significant increase in serum AST, serum ALT, serum ALP, and total bilirubin in both patient groups compared with the control group (*P* < 0.01).

Table 2 reveals highly significant increase in serum endoglin and serum AFP in both patient groups compared with the control group (*P* < 0.01). There was highly significant increase in both parameters in HCC patients compared with HCV patients (*P* < 0.01).

Table 3 shows significant negative correlation between serum endoglin and hemoglobin, serum albumin, and prothrombin concentration. In addition, there was significant positive correlation between serum endoglin and serum AST, serum ALT, serum ALP, and serum AFP.

Table 4 reveals the cutoff value for both serum endoglin and serum AFP above which HCC is suspected. For serum endoglin the cutoff value was greater than 6.05 ng/ml with sensitivity 98%, specificity 90%, and accuracy 99%. For serum AFP, the cutoff value was greater than 2.17 ng/ml with sensitivity 98%, specificity 80%, and accuracy 99%.

Discussion

HCV is a serious growing problem in Arab countries; the prevalence of HCV in Egypt is expected to be continuing at a rate of ≈6.9/1000 persons per year, indicative of possibly ongoing hyperepidemic transmission in this region [16].

One of the major complications of HCV infection is the development of hepatic fibrosis and progression to cirrhosis and HCC with an estimated 5-year risk of 5–20% [17].

The combination of ultrasound with AFP is not recommended for HCC surveillance because the small 6–8% gain in the detection rate does not balance the increase in false-positive results and the cost of early-stage HCC diagnosis. Furthermore, ultrasound has suboptimal sensitivity for detecting early-stage HCC. Thus, it is warranted for serological tests to identify patients with early-stage HCC (18).

Table 1 Comparison between the patient and control groups regarding serum biochemical markers (mean ± SD)

Variables	Groups			*P*
	HCV patients with cirrhosis	HCV patients with HCC	Controls	
WBC ($10^3/cm^2$)	5.98 ± 3.08	6.20 ± 3.83	6.58 ± 1.55	P_1>0.05P_2>0.05P_3>0.05
Hemoglobin (g/dl)	11.32 ± 1.61	10.65 ± 1.36	12.64 ± 1.33	P_1<0.05*P_2<0.01*P_3>0.05
Platelets ($10^3/cm^2$)	154.12 ± 70.20	150.13 ± 42.10	291.00 ± 78.84	P_1<0.01**P_2<0.01**P_3>0.05
AST (U/l)	61.10 ± 19.80	99.33 ± 64.11	35.10 ± 8.54	P_1<0.01**P_2<0.01**P_3<0.05*
ALT (U/l)	50.77 ± 18.12	92.21 ± 86.53	30.00 ± 6.36	P_1<0.01**P_2<0.01**P_3>0.05
ALP (U/l)	39.25 ± 13.87	143 ± 21.96	30.7 ± 9.7	P_1<0.01**P_2<0.01**P_3<0.05*
Albumin (g/dl)	2.85 ± 0.68	2.78 ± 0.58	3.97 ± 0.09	P_1<0.01**P_2<0.01**P_3>0.05
Total bilirubin (mg/dl)	2.74 ± 1.38	3.62 ± 2.94	0.64 ± 0.30	P_1<0.01**P_2<0.01**P_3>0.05
Prothrombin concentration %	63.38 ± 12.58	56.29 ± 2.51	87.00 ± 3.01	P_1<0.01**P_2<0.01**P_3>0.05

P_1 is the difference between HCV patients with the cirrhosis group and the control group, P_2 is the difference between HCV patients with the HCC group and the control group, and P_3 is the difference between HCV patients with the cirrhosis group and HCV patients with the HCC group; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; WBC, white blood cell; **P < 0.05 (significant), *P < 0.001 (highly significant), P > 0.05 (nonsignificant).

Table 2 Comparison between the patient group and the control group regarding serum endoglin and serum a-fetoprotein levels

Variables	Groups			*P*
	HCV patients with cirrhosis	HCV patients with HCC	Controls	
Endoglin (ng/ml)	9.10 ± 1.74	13.61 ± 3.47	5.00 ± 1.03	P_1<0.01*P_2<0.01*P_3<0.01*
AFP (ng/ml)	110.80 ± 166.89	1988.56 ± 7040.42	1.48 ± 0.80	P_1<0.01*P_2<0.01*P_3<0.01*

P_1 is the difference between HCV patients with the cirrhosis group and the control group, P_2 is the difference between HCV patients with the HCC group and the control group, P_3 is the difference between HCV patients with the cirrhosis group and HCV patients with the HCC group; AFP, a-fetoprotein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; *P < 0.05 (significant), *P < 0.01 (highly significant), P > 0.05 (nonsignificant).

Table 3 Correlation coefficient of serum endoglin with different laboratory parameters

Variables	Correlation with endoglin	
	Correlation coefficient	
	R	P
WBC	−0.015	>0.05
Hemoglobin	−0.389	<0.01*
Platelets	−0.135	>0.05
AST	0.528	<0.01*
ALT	0.507	<0.01*
ALP	0.525	<0.01*
Albumin	−0.370	<0.05*
Total bilirubin	0.541	<0.01*
Prothrombin concentration	−0.694	<0.01*
AFP	0.678	<0.01*

AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; WBC, white blood cell; *P < 0.05 (significant), *P < 0.01 (highly significant), P > 0.05 (nonsignificant).

Table 4 Sensitivity and specificity of α-fetoprotein and endoglin in the hepatitis C virus, hepatocellular carcinoma, and control groups

Cutoff value	Sensitivity (%)	Specificity (%)	Accuracy (%)
Serum endoglin >6.05 (ng/ml)	98	90	99
Serum AFP at >2.17 (ng/ml)	98	80	99

AFP, α-fetoprotein.

AFP is the most widely investigated biomarker for HCC diagnosis. Persistent elevation of AFP has been shown to be a risk factor for developing HCC and is used to define at-risk populations. However, AFP has suboptimal diagnostic performance for HCC surveillance. In patients with liver cirrhosis, fluctuating levels of AFP may reflect flare-ups of viral hepatitis, exacerbation of underlying liver disease, or HCC development. In addition, only 10–20% of early-stage HCC patients have abnormal AFP serum levels (19).

Several biological studies investigated the clinical usefulness of endoglin, and they found that it may facilitate the screening and diagnosis of HCC and assess HCC progression. A possible role for endoglin is suggested as a novel complementary biomarker to detect the risk of development of HCC in cirrhotic patients [20].

This study tried to evaluate the role of serum endoglin as noninvasive biomarkers in diagnosis of HCC. The present study showed statistical significant difference between group I (HCV with cirrhosis), II (HCV complicated by HCC), and III (control) with respect to platelet, ALT, AST, bilirubin, and prothrombin time and concentration. These results were in agreement with those of Schuppan and Afdhal [21] who revealed that, in advanced liver disease, thrombocytopenia is seen, along with impaired hepatic biosynthesis and impairment of the detoxifying function of the liver. High transaminase levels are usually a sign of clinically relevant liver disease and are associated with significantly increased mortality from liver disease [22].

In addition, our study showed statistical significant difference between groups I, II, and III with respect to hemoglobin, which is in agreement with the study by McHutchison et al. [23] who stated that chronic liver diseases are frequently associated with hematological abnormalities. Anemia of diverse etiology occurs in about 75% of patients with chronic liver disease. A major cause of anemia associated with chronic liver disease is hemorrhage, especially into the gastrointestinal tract. Patients with severe hepatocellular disease develop defects of blood coagulation as a consequence of endothelial dysfunction, thrombocytopenia, deficiencies of coagulation factors, and various associated disorders. Other mechanisms of anemia include side effects of treatment of hepatitis with interferon and ribavirin [24].

This study also showed significant increase in ALP level in HCV patients with HCC, which is in agreement with the study by Yu et al. [25] who found that preoperative level of ALP was one of the most important independent predictors of recurrence, even more important than AFP as noticed that elevation of ALP above 82 U/l predicted poor prognosis in patients where AFP level was less than 66 ng/ml.

In our study, the serum endoglin levels were highly significantly elevated in the HCC group than in both the cirrhotic group and the control group ($P < 0.01$). These findings were in accordance with those of Preativatanyou et al. (26), who reported increased levels of soluble endoglin in the circulation during liver fibrosis. TGF-β is one of the most potent profibrotic cytokines known, and excessive TGF-β signaling has been implicated in a number of fibrotic conditions [27]. TGF-β contributes to all stages of liver disease progression ranging from the early events in injury and inflammation to the later processes of fibrosis/cirrhosis and HCC [28]. A study by Meurer et al. [29] showed that endoglin expression is increased in transdifferentiating hepatic stellate cells, which are the most fibrogenic cell type in the liver.

The elevation of endoglin level in HCC patients than in cirrhotic patients may be due to neoangiogenesis accompanied to hypoxia associated with neoplastic tissue [30] or due to the development of an angiogenic response, which may depend on the balance between TGF-β and endoglin expression as TGF-β level was elevated in HCC. In addition, on the molecular basis,

hypoxia and TGF-β1 have been described to have a direct regulatory role in endoglin gene expression in endothelial cells [31].

In the present study, there were positive significant correlations between serum endoglin and AST, ALT, ALP, total bilirubin, and serum AFP, whereas negative significant correlations were found between serum endoglin and hemoglobin, albumin, and prothrombin concentration.

With respect to serum AFP, the present work demonstrated highly significant elevation in serum AFP in the HCC group compared with the other two groups (*P* < 0.001 for both). This in concordance with the study by Ezzat *et al.* [32] who reported the same finding in the HCC and cirrhotic groups compared with the control group. However, the sensitivity of this marker is limited (41–65%). Given the high heterogeneity of HCC, it is currently thought that an optimal serological test for HCC will be based on the simultaneous measurement of two or three highly specific serological markers [33].

In our study, we found a positive significant correlation between serum endoglin and serum AFP for all individuals, which is in accordance with the study by Yagmur *et al.* [34] who found that serum endoglin levels were moderately positively correlated with AFP in patients with liver cirrhosis and higher positive correlation was found in patients with liver cirrhosis and HCC.

When comparing the diagnostic performance of serum endoglin as a possible tumor marker for HCC with serum AFP, receiver operating characteristic curves were performed, and the best generated cutoff value above which HCC diagnosis is suspected was for AFP greater than 2.17 ng/ml with sensitivity 98% and specificity 80%. However, the cutoff value of endoglin was greater than 6.05 ng/ml with sensitivity 70% and specificity 81% (Fig. 1).
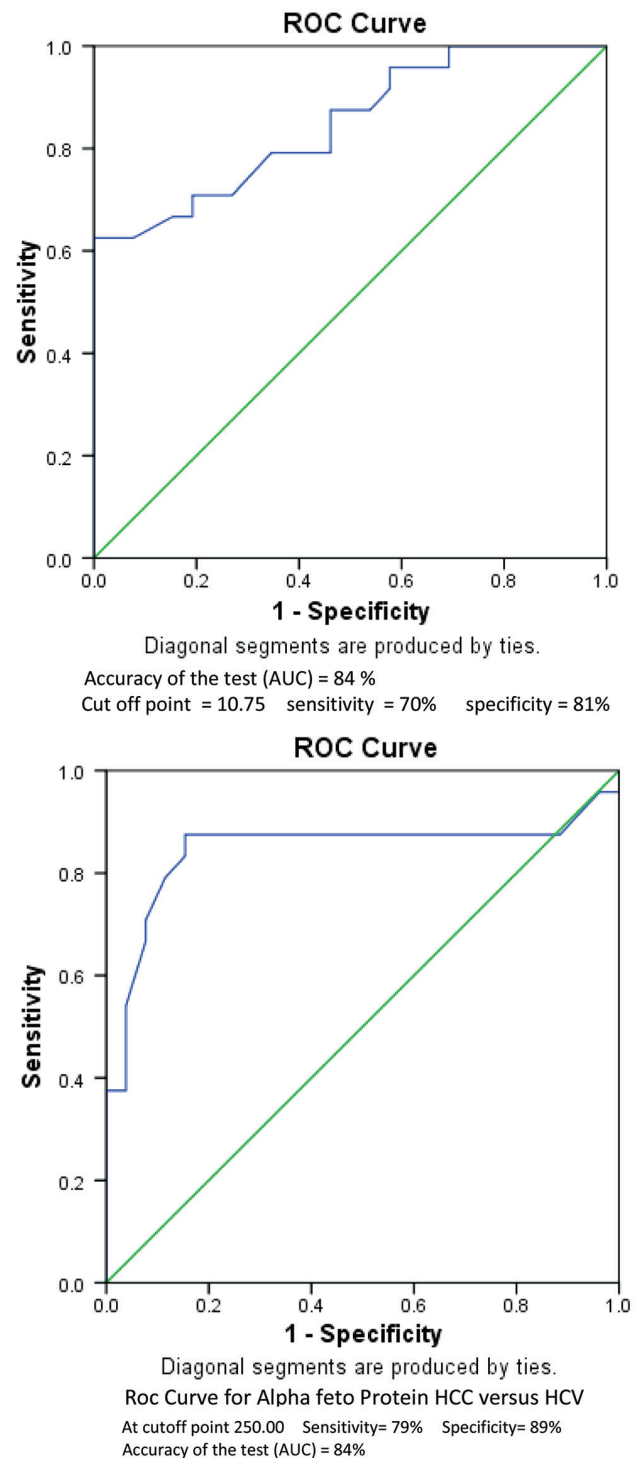
Conclusion

Serum endoglin showed a better diagnostic performance and proved to be more reliable as a tumor marker for HCC. Serum endoglin may be used with serum AFP as complementary biomarker as noninvasive technique to aid diagnosis of HCC.

Figure 1

Accuracy of the test (AUC) = 84 %
Cut off point = 10.75   sensitivity = 70%   specificity = 81%

Roc Curve for Alpha feto Protein HCC versus HCV
At cutoff point 250.00   Sensitivity= 79%   Specificity= 89%
Accuracy of the test (AUC) = 84%

Endoglin HCC versus HCV, accuracy of the test (AUC) = 84%, cutoff point, sensitivity, and specificity=10.75, 70%, and 81%. ROC curve for a-fetoprotein HCC versus HCV, at cutoff point 250.00, sensitivity = 79%, specificity = 89%, accuracy of the test (AUC) = 84%. AUC, area under the curve; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ROC, receiver operating characteristic.

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