

# Serum levels of $\beta$ -catenin as a potential marker for genotype 4/ hepatitis C-associated hepatocellular carcinoma

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**Abstract.** The global rising incidence of hepatocellular carcinoma (HCC), which parallels the increase of hepatitis C virus (HCV) prevalence, has sparked a renewed interest in discovering additional HCC serum markers. In this study, we investigated the clinical use of serum E-cadherin, ICAM, MMP-2, VEGF, OPN and  $\beta$ -catenin as potential diagnostic makers for HCV/genotype 4-associated HCC. Twenty cases of healthy subjects, 11 cases with asymptomatic HCV/genotype 4 carriers (ASC), 28 chronic hepatitis (CH) cases and 32 patients with HCC were enrolled in this study. Serum levels of proteins were measured by a sandwich-enzyme-linked (ELISA) assay. The diagnostic accuracy of each candidate marker was evaluated using receiver-operating characteristic (ROC) curve analysis, reporting the area under the curve (AUC) and its 95% confidence interval (CI). We demonstrated that serum  $\beta$ -catenin levels were significantly elevated in patients with HCC compared to those with CH, ASC and healthy controls. Among the six studied markers,  $\beta$ -catenin was also found to be the only marker that can significantly discriminate between patients with HCC and those with CH; therefore,  $\beta$ -catenin could be considered

as a potential marker for early diagnosis of HCV-associated HCC in patients infected with HCV genotype 4.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and one of the leading causes of cancer death in the world (1). It has heterogeneous geographical distribution, with its greatest incidence in Asia and sub-Saharan Africa, where hepatitis B infection is endemic (2). Its incidence has also been increasing steadily in the United States and Western Europe due to the high prevalence of hepatitis C (3,4). Egypt has the highest prevalence of hepatitis C virus (HCV) infection, with 14% of the population infected and seven million with chronic liver hepatitis (5,6). Little is known about the molecular mechanisms by which HCV initiates HCC. Molecular studies directed at mapping the etiology of HCV disease progression to HCC are expected to provide new insights on the management of this increasing problem and hence are of great global health interest. However, the lack of molecular makers that can characterize the stage of HCC, regardless of the etiologic factor, and tumor progression precludes the effective diagnosis and prognosis of HCC. Currently, HCC diagnosis relies on the radiology imaging systems, and elevated serum  $\alpha$ -fetoprotein (AFP). Serum AFP is not always elevated to a diagnostic level in all patients in small HCC, thus, considerable numbers of patients with more advanced stages would be missed unless other diagnostic tools are used (7,8). Moreover, the level of AFP may be elevated in non-malignant chronic liver diseases, including chronic hepatitis and cirrhosis (9,10). Several biomarkers, such as des- $\gamma$ -carboxyprothrombin/prothrombin induced by vitamin K absence-II, lens cularis agglutinin-reactive (AFP-L3), and glypican-3, have been examined for their ability to diagnose early HCC (11). However, there is a need for additional serum biomarkers to improve the detection of HCC at its early stage. Protein expression profiling studies have detected many proteins that are associated with hepatocarcinogenesis. For example, serum level of soluble E-cadherin was shown to be elevated in patients with HCC, and is associated with early recurrence or extrahepatic metastasis (12). The

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*Abbreviations:* HCC, hepatocellular carcinoma; sE-cadherin, soluble human epithelial cadherin; ICAM-1, intercellular adhesion molecule; total MMP-2, active and pro-matrix metalloproteinase 2; VEGF, vascular endothelial growth factor; OPN, human osteopontin; CH, chronic hepatitis; ASC, asymptomatic HCV carriers; HCV, hepatitis C virus; AFP,  $\alpha$ -fetoprotein; NO, nitric oxide; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of MMPs; RGD, arginine-glycine-aspartic acid; ROC, receiver-operating characteristic curve

*Key words:* hepatocellular carcinoma, chronic hepatitis C, hepatitis C virus genotype 4, serum markers,  $\beta$ -catenin, E-cadherin

Table I. Clinical characteristics of study subjects.

Variables	HCC (n=32)	CH (n=28)	ASC (n=11)	Control (n=20)	P-value
Age mean, years (range)	54 <sup>a</sup> (40-76)	50.5 <sup>ab</sup> (31-63)	44 <sup>bc</sup> (22-56)	32 <sup>c</sup> (27-53)	<0.001
Gender (male/female)	29/3	21/7	9/2	16/4	
AFP	855 <sup>a</sup> (175-1390)	13.5 <sup>b</sup> (0.4-975.8)	1.4 <sup>c</sup> (0.53-7.35)	0.69 <sup>c</sup> (0.43-1.8)	<0.001
D-Bilirubin	0.92 <sup>a</sup> (0.14-20.4)	0.64 <sup>a</sup> (0.2-9.24)	0.78 <sup>a</sup> (0.05-0.93)	0.05 <sup>b</sup> (0.02-0.09)	<0.001
I-Bilirubin	1.05 <sup>a</sup> (0.55-9.4)	0.86 <sup>a</sup> (0.5-6.38)	0.14 <sup>b</sup> (0.04-0.59)	0.11 (0.03-0.45)	<0.001
AST	58 <sup>a</sup> (23-138)	68.5 <sup>a</sup> (25-138)	29 <sup>b</sup> (15-39)	9 <sup>b</sup> (5-13)	<0.001
ALT	46 <sup>a</sup> (26-100)	55 <sup>a</sup> (12-89)	35 <sup>b</sup> (14-51)	6 <sup>c</sup> (3-11)	<0.001
Alk-phosphatase	111 <sup>a</sup> (63-389)	117 <sup>a</sup> (37-304)	122 <sup>a</sup> (60-194)	36 <sup>b</sup> (23-56)	<0.001
Albumin	2.65 <sup>b</sup> (1.3-3.2)	4 <sup>a</sup> (1.9-5.4)	2.8 <sup>b</sup> (2-3.2)	2.7 <sup>b</sup> (2.3-3.7)	<0.001
Urea	37 (12-129)	28 (18-150)	30 (16-58)	44 (9.6-112)	0.285
Creatinine	1 (0.5-6.39)	0.95 (0.5-2.7)	0.9 (0.6-1.4)	1.11 (0.52-1.9)	0.348
HB	10.95 <sup>b</sup> (6.2-12.8)	11.5 <sup>b</sup> (7.9-13.3)	14.1 <sup>a</sup> (12.3-17)	13.3 <sup>a</sup> (11.9-14.5)	<0.001
TLC	4 <sup>b</sup> (0.53-11.4)	6.45 <sup>ab</sup> (2.7-12.5)	5.3 <sup>ab</sup> (3.6-9)	7.5 <sup>a</sup> (4.45-10)	<0.001
PLT	105.5 <sup>c</sup> (62-650)	105.5 <sup>bc</sup> (7.6-293)	190 <sup>ab</sup> (20-400)	195 <sup>a</sup> (159-315)	<0.001
PT	16.05 (14-24)	15.8 (13-20.7)	14.4 (13.0-15.1)	13.0 (13- 14.5)	0.303
PC	50.5 <sup>b</sup> (33-84)	52 <sup>b</sup> (20-90)	85 <sup>a</sup> (68-100)	100 <sup>a</sup> (100-100)	<0.001
FBS	97.5 (60-254)	95 (70-199)	98 (67-162)	112.5 (67-145)	0.285

Groups indicated by different superscripted initials are significantly different from each other. HCC, hepatocellular carcinoma; CH, chronic hepatitis; ASC, asymptomatic HCV carriers; AFP,  $\alpha$ -fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

level of other serum proteins, such as serum intercellular adhesion molecule-1 (sICAM-1) (13), serum matrix metalloproteinase-2 (MMP-2) (14), vascular endothelial growth factor (VEGF) (15), plasma osteopontin (OPN) (16), and  $\beta$ -catenin (17) has been shown to be elevated in patients with HCC and hence could be used as prognostic markers for early detection of HCC. However, limited information is available about the extent to which these proteins could be used as prognostic markers for viral-associated HCC.

In view of the possible diagnostic role of these serum proteins in the development of HCC initiated by the HCV/genotype 4, the serum levels of the above mentioned proteins were measured in 91 Egyptian subjects using a sandwich ELISA method in four groups. We have analyzed the correlation and predictive value of these markers in screening patients at risk of HCC.

## Materials and methods

**Patients and sample collection.** Serum samples for the assessment of biomarkers were obtained from 91 consecutive individuals. Samples were obtained from patients presented before treatment to the specialized liver clinic of the National Cancer Institute, Cairo University during the period of January to December 2009. Informed consent was obtained from each patient and the study protocols conformed to the ethics guidelines of the Institutional Review Board. Subjects were divided into four groups. Group 1 (HCC; n=32) included patients with histological proven HCC. Group 2 (CH; n=28) included patients with chronic hepatitis, with or without cirrhosis. Group 3 (ASC; n=11) included patients infected

with HCV with histological confirmed non-cirrhotic chronic hepatitis (asymptomatic carriers). All patients were HCV positive as detected by hepatitis C antibody and HCV RT-PCR (18), and HBV negative as detected by hepatitis B antigen and HBV DNA/PCR (19). Group 4 (Control; n=20) included normal healthy subjects with no history of liver disease that were negative for HCV and HBV. The clinicopathological data of the subjects in this study at initial diagnosis were collected, which included gender, age, liver function tests, kidney function tests, and AFP levels are presented in Table I.

Sera collected from 10 ml of coagulated blood by centrifugation were immediately separated and frozen at -80°C until assayed.

**Measurement of serum biomarkers.** Serum levels of soluble human epithelial cadherin (sE-cadherin), sICAM-1, active and proMMP-2 (total MMP-2), VEGF, human OPN and  $\beta$ -catenin were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Each sample was examined in duplicate and the average value (mean) was used for data analysis. The cut-off was considered as mean  $\pm$  2SD of the negative controls.

**Statistical analysis.** Data are expressed as the mean  $\pm$  SD. Comparisons between groups were analyzed by the  $\chi^2$  test or Fisher's exact test for categorical variables, and by the Mann-Whitney test or Student's t-test when appropriate for quantitative variables. Receiver-operating characteristics curves (ROC) were constructed to evaluate the diagnostic performance of the serum makers in discriminating HCC from other groups.

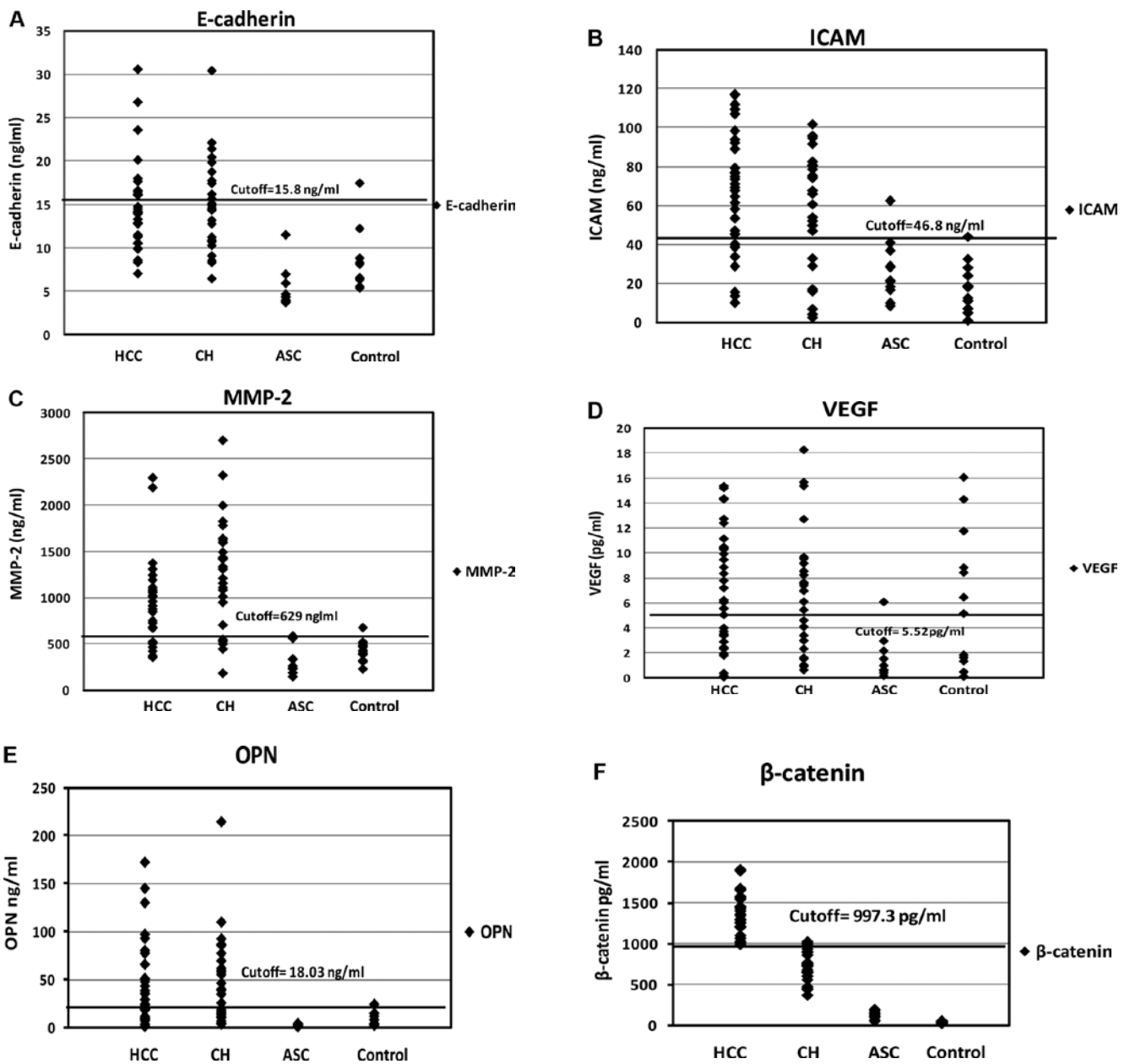


Figure 1. Scatter plots for (A) E-cadherin, (B) sICAM, (C) MMP-2, (D) VEGF, (E) OPN, (F)  $\beta$ -catenin. Levels are presented as ng/ml in hepatocellular carcinoma. HCC; chronic hepatitis, CH; asymptomatic carriers (ASC) and healthy controls. The cut-off value is indicated.

Sensitivity, specificity, positive and negative predictor values and diagnostic accuracy were calculated in accordance with standard methods.  $P < 0.05$  for a two-tailed test was considered statistically significant. All statistical analyses were performed using the SPSS software version 15.0 (SPSS, Chicago, IL).

### Results and Discussion

**Patient profiles.** A total of 91 subjects, 75 men and 16 women were included in the study. Detailed demographic data are shown in Table I. The age of patients with HCC, CH and ASC were significantly older than healthy controls ( $P < 0.001$ ). Likewise, the mean age of patients with CH was significantly higher than that of ASC patients or healthy controls ( $P < 0.001$ ). The level of serum AFP in patients with HCC was significantly higher than those of healthy controls, CH and ASC ( $P < 0.001$ ).

The serum AFP levels in the CH patients were also significantly higher than those in ASC patients and healthy controls ( $P < 0.001$ ).

**Serum levels of E-cadherin, ICAM-1, MMP-2, VEGF, OPN, and  $\beta$ -catenin as diagnostic markers.** The results of the present study showed that the serum levels of four out of the six studied markers ( $\beta$ -catenin, E-cadherin, sICAM and OPN) were significantly higher in patients with HCC and CH compared to ASC patients and healthy controls (Fig. 1). The high serum levels of those cell adhesion markers could be related to the process of fibrosis and cirrhosis in HCV-infected livers, which usually precedes the development of carcinoma, especially that no significant difference was detected between HCC and CH patients. Among those four markers, the serum levels of  $\beta$ -catenin were significantly higher in patients with HCC

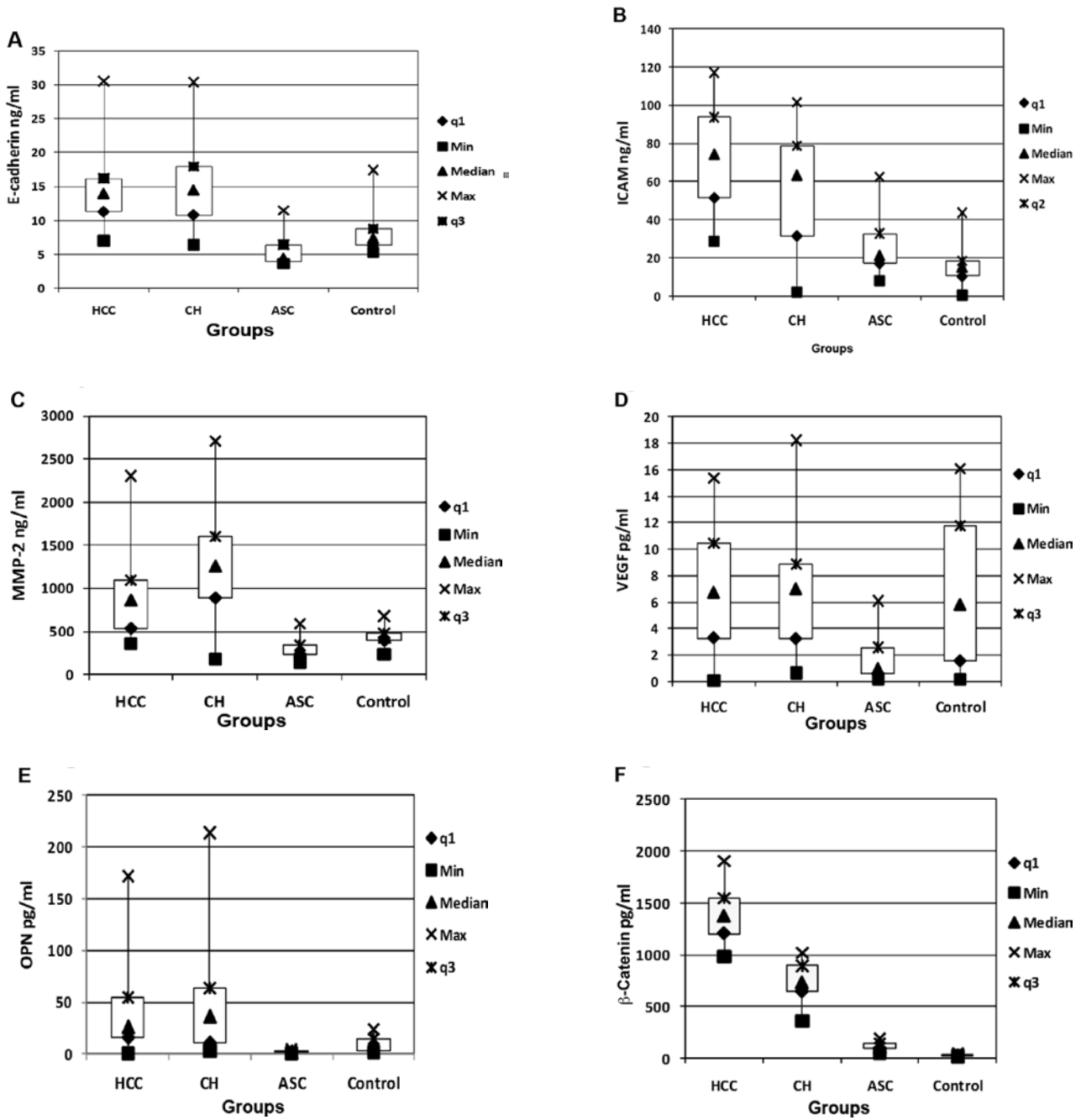


Figure 2. Serum levels of (A) E-cadherin, (B) sICAM, (C) MMP-2, (D) VEGF, (E) OPN, (F)  $\beta$ -catenin in hepatocellular carcinoma (HCC); chronic hepatitis (CH); asymptomatic HCV carriers (ASC); and healthy controls. The box plots indicate the median values (triangles), the minimum and maximum values (squares and crosses, respectively). The box defines the boundaries of the first and third quartiles of data.

compared to CH patients (cut-off value of 1379.5 pg/ml for HCC patients vs. 737 pg/ml for CH patients) (Fig. 1). Similarly, the serum levels of  $\beta$ -catenin were also higher in patients with CH than in ASC patients and healthy controls. These data are in agreement with previous reports by Sun *et al* (20), where higher serum level of  $\beta$ -catenin were measured in patients with HCC compared with those in CH patients. The authors of this study suggested that serum level of  $\beta$ -catenin could be used clinically to complement the current AFP diagnostic test for more accurate detection of early HCC. Therefore, the ability to measure  $\beta$ -catenin in the peripheral blood of HCC patients would offer a valuable, non-invasive diagnostic and prognostic marker for HCC. The expression of  $\beta$ -catenin in HCC has also

been detected immunohistochemically by several groups. In these studies, mutated nuclear  $\beta$ -catenin overexpression has been associated with increased cell proliferation, poorer cellular differentiation and reduced survival rate (21,22). These mutations of  $\beta$ -catenin lead to a nuclear accumulation of aberrant  $\beta$ -catenin proteins that stimulate the activity of other transcription factors, such as cyclin D1 and c-myc (23,24). In dysplastic nodules, a cytoplasmic expression of  $\beta$ -catenin has been observed (25). Axin, an important regulator of  $\beta$ -catenin, is mutated in about 10% of HCC cases, leading to an activation of the Wnt pathway (26). However, mutations in the *axin* gene have been identified only in HCC that lack mutations in the *\beta*-catenin gene (27,28).

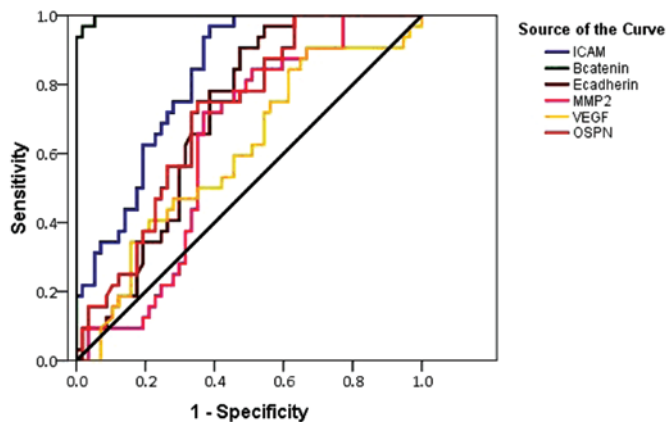


Figure 3. Receiver-operating characteristic (ROC) curve analyses of the candidate bio-markers. The diagnostic accuracy of each candidate biomarker, in terms of sensitivity and specificity, are presented after ROC curve analysis. The AUC values for  $\beta$ -catenin compared to sICAM are shown in Table II.

Soluble E-cadherin was found to be circulating in the biological fluids of healthy individuals, but elevated in cancer patients (29) as well as in individuals with systemic inflammatory response syndrome (30). The median concentration of serum E-cadherin reported in our study was significantly higher in patients with CH and HCC compared to those with ASC and healthy controls (Fig. 1). In contrast to  $\beta$ -catenin, no significant difference was observed in the median concentration of serum E-cadherin between CH and HCC patients (Fig. 2A). Earlier studies showed that the median serum E-cadherin levels were significantly elevated in HCC patients before surgery compared to healthy subjects (10.759 ng/ml vs. 5.798 ng/ml,  $P < 0.05$ ) (15). Moreover, high serum E-cadherin ( $\geq 8.000$  ng/ml) was significantly associated with early recurrence and extra-hepatic metastasis. Therefore, serum E-cadherin could be considered as a potential prognostic marker for patients with HCC (31), albeit with limited prognostic value in the early detection of HCC.

There have been few reports investigating the relationship between serum ICAM-1 concentrations, hepatitis and hepatocarcinogenesis. These reports showed that ICAM-1 is produced and secreted by tumor cells and that serum ICAM-1 could be used as a marker for disease progression and prognosis in patients with HCC (32). In the present study, the median level of sICAM was significantly higher in patients with HCC and CH compared to those of ASC and healthy controls (Fig. 1). However, there was no significant difference in the level of sICAM between HCC and CH (Fig. 2B). Our findings thus confirmed previous reports by Shimizu *et al* (32) who reported that high serum concentrations of ICAM-1 or an increasing level of sICAM-1 over time are significant risk factors for the occurrence of HCC in patients with HCV-associated CH or liver cirrhosis (LC). Thus, regular measurements of sICAM-1 concentrations would be of clinical significance and could be used not only as a prognostic marker for early detection of HCC in patients with CH or LC, but also as a marker of disease progression after HCC treatment.

The median concentrations of serum MMP-2 reported in the present study were significantly higher in patients with CH

compared to those with HCC (Fig. 2C). These results demonstrate the important role of MMP-2 in the development of HCV-induced fibrosis as well as in disease progression to cirrhosis. Our data are in agreement with those of Kuyvenhoven *et al* (33) who demonstrated that serum levels of MMP-2 as detected by ELISA, were significantly higher in HCC patients than those of healthy controls but comparable to patients with chronic liver disease. Additional studies showed that circulating serum MMP-2 levels were increased in patients with liver cirrhosis and reported a wide overlap in patients with CH and healthy controls (34). Serum levels of proMMP-2 were determined to be highly elevated in chronic liver diseases than in normal controls and were strongly correlated to type IV collagen in sera of CH patients. Therefore, it is possible to consider the serum level of proMMP-2 as a follow-up marker in patients with CH (35).

The present study showed no significant increase in the serum level of OPN in patients with CH compared to those with HCC (Fig. 2C). However, this level was significantly higher in patients with CH and HCC than in ASCs and healthy controls (Fig. 1). Huang *et al* (36) have demonstrated that serum OPN level correlates well with liver fibrosis and inflammation. They reported that a significant difference in the mean plasma OPN levels between HCV patients with severe fibrosis and those with mild fibrosis ( $4.29 \pm 1.01$  vs.  $2.15 \pm 0.63$  ng/ml, respectively  $P < 0.001$ ) and suggested that plasma OPN could be used as a marker to evaluate the severity of liver damage in HCV-infected persons. In contrast to our data, Kim *et al* (37) reported that plasma OPN levels in HCC patients (median 954 ng/ml, range 168-5,742) were significantly higher than in those with chronic liver diseases (381 ng/ml, 29-1,688) or healthy controls (155 ng/ml, 10-766) ( $P < 0.001$ ) with a diagnostic sensitivity and specificity of 87 and 82%, respectively, suggesting a superior diagnostic accuracy for serum OPN. This discrepancy in the results between both studies could be attributed to the fact that all patients included in our study were positive for HCV with a preponderance of genotype 4.

Previous reports by Jinno *et al* (38) indicated that serum VEGF levels increased gradually along with disease progression in patients with hepatitis, cirrhosis and HCC. There was a significant difference in the level of serum VEGF between HCC and other patient groups, but not among hepatitis patients, cirrhotic patients and normal controls. Zhao *et al* (39) reported that serum VEGF detected by ELISA was significantly higher in HCC patients than in those with benign liver lesions and healthy controls; however, no significant difference was found between patients with benign hepatic diseases and healthy controls or between patients with benign hepatic diseases and those with cirrhosis. In the present study, the median VEGF level showed no statistically significant difference between patients with CH, HCC and healthy controls (Fig. 2D). The data are consistent with that reported by Niu *et al* (40) who reported that the mean serum levels of VEGF in normal healthy controls, cirrhotic patients, patients with benign liver tumor, and those with HCC were  $158.46 \pm 41.84$ ,  $90 \pm 22.42$ ,  $156.34 \pm 41.32$  and  $164.42 \pm 76.07$  ng/ml; respectively. As a result, serum levels of VEGF were significantly higher in HCC than in cirrhotic patients, whereas no significant differences between patients with HCC and healthy controls were noted. These data highlight the important role of VEGF

Table II. ROC curve values of studied markers between HCC and CH patients.

ROC values	ICAM $\geq 55.9$ ng/ml (%)	$\beta$ -catenin $\geq 997.3$ pg/ml (%)
Sensitivity	71.9	96.9
Specificity	47	92.6
AUC	0.642	0.995
PPV	60.53	93.93
NPV	59	96.15

ROC, receiver-operating characteristic; AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value; CH, chronic active hepatitis; HCC, hepatocellular carcinoma.

in disease progression from liver cirrhosis to HCC, and its limited prognostic value in differentiating between benign liver tumors and HCC (15).

*Cut-off values for measured serum markers in different groups.* The ROC curves for measured markers were plotted on the same graph to identify a cut-off value for each marker that would best distinguish HCC from the other groups investigated (Fig. 3). Based on the ROC analysis, the optimal cut-off values for sICAM and  $\beta$ -catenin for HCC and CH patients were  $\geq 55.9$  ng/ml, and  $\geq 997.3$  pg/ml, respectively. There were no satisfactory cut-off values for the other studied markers when comparing these two groups. For sICAM, we found that 23/32 (71.8%) HCC patients had an optimal cut-off level of  $\geq 55.9$  ng/ml, compared to 15/28 (53.6%) cases with CH. In contrast, 31/32 (96.9%) of HCC patients had  $\beta$ -catenin levels  $\geq 997.3$  pg/ml compared to 2/28 (7.1%) in patients with CH. The area under the curves (AUC) for E-cadherin, sICAM, MMP-2, VEGF, OPN and  $\beta$ -catenin were 0.708, 0.821, 0.630, 0.603, 0.712 and 0.998, respectively. Therefore, the serum level of  $\beta$ -catenin could possibly be used as a valuable marker to discriminate patients with hepatocellular carcinoma from those with chronic active hepatitis. As shown in Table II, the sensitivity and specificity values for  $\beta$ -catenin, at a cut-off value of  $\geq 997.3$  pg/ml, were 96.9 and 92.6%, respectively. For sICAM, these values at a cut-off value  $\geq 55.9$  ng/ml were 71.9 and 47%, respectively. This suggests that the optimal value for  $\beta$ -catenin showed a higher sensitivity and specificity in discriminating between HCC and CH than sICAM.

In conclusion, our study showed that serum  $\beta$ -catenin levels were significantly elevated in patients with HCC as compared to those with CH, ASC and healthy controls. Among the six studied markers,  $\beta$ -catenin was found to be the only one that can significantly discriminate between patients with HCC and those with CH; therefore,  $\beta$ -catenin could be considered as a potential marker for early diagnosis in selecting high-risk patients for the HCC surveillance program. Further large scale studies are worthwhile to confirm these observations and to elucidate the clinical significance of serum  $\beta$ -catenin in patients with HCC.

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