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A simple diagnostic index comprising epithelial membrane antigen and fibronectin for hepatocellular carcinoma

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ABSTRACT

Background and rationale for the study. Continuing search for suitable tumor-markers is of clinical value in managing patients with various malignancies. These markers may be presented as intracellular substances in tissues or may be released into the circulation and appear in serum. Therefore, this work is concerned with identification and quantitative determination of epithelial membrane antigen (EMA) and fibronectin and estimating their performances as surrogate markers for identifying hepatocellular carcinoma (HCC). **Results.** A total of 627 individuals constituted this study [fibrosis (F1-F3) = 217; cirrhosis = 191; HCC = 219]. Western-blot was used for identifying EMA and fibronectin in sera. As a result, a single immunoreactive band was shown at 130-kDa and 90-kDa corresponding to EMA and fibronectin, respectively. They were quantified using ELISA providing values in HCC higher than fibrosis or cirrhosis with a significant difference ($P < 0.0001$). For identifying HCC, EMA showed 0.82 area under receiver-operating characteristic curve (AUC) with sensitivity = 70% and specificity = 78% while fibronectin yielded AUC = 0.70 with sensitivity = 67% and specificity = 82%. FEBA-Test comprising fibronectin and EMA together with total-bilirubin and AFP was constructed yielding AUC = 0.92 for identifying HCC from cirrhosis with sensitivity = 89% and specificity = 85%. FEBA-Test was then tested for differentiating HCC from fibrosis showing AUC = 0.97 with sensitivity = 90% and specificity = 89%. FEBA-Test enabled the correct identification of HCC patients with CLIP 0-1 and size ≤ 3 cm with AUC = 0.80 and AUC = 0.84, respectively, indicating its ability in identifying early HCC. **Conclusions.** A four-marker index may improve the early detection of HCC with a high degree of accuracy.

Key words. Liver fibrosis. Cirrhosis. HCC. Markers. FEBA-Test.

INTRODUCTION

Hepatitis C virus (HCV) is considered as a major cause of liver associated diseases throughout the world. People with HCV have 2% annual risk and 7 to 14% five-year risk for hepatocellular carcinoma (HCC).[1-3] HCC is the most common primary malignancy of the liver, being the fifth most frequent cancer worldwide and the third most frequent cause of mortality among oncological patients.[4] For these reasons, it is mandatory to establish a definite strategy for diagnosing HCC at a stage where curative treatments can be performed. Many physicians screen high-risk populations with various strategies including serum α-fetoprotein (AFP) and ultrasonography.[5] However, despite the large number of published studies in recent years, efficacy and cost/effectiveness of screening and surveillance of cirrhotics for diagnosing HCC is still debated.[6] The use of AFP, a tumor marker variably secreted by hepatocellular carcinomas, to detect these tumors has been widely debated.[7-9] AFP had 39-65% sensitivity and 76-94% specificity for detecting HCC in previously published studies.[10] Several investigators concluded that AFP is not a useful diagnostic test,[7-9,11] but AFP continues to be commonly used.[5] Additionally, it is almost impossible to evaluate the actual sensitivity of ultrasonography and other imaging techniques from the published studies on screening

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and surveillance, since the gold standard remains undefined. However the analysis of these studies by Gebo, *et al.* graded the evidence for the use of ultrasonography in this setting as weak evidence.[6] This work is concerned with determining the levels of epithelial marker such as epithelial membrane antigen (EMA) together with fibronectin and then estimating their performance as surrogate markers for detecting HCC. Moreover, we aimed to create a simple diagnostic discriminant index utilizing EMA, fibronectin together with other markers for HCC diagnosis.

MATERIAL AND METHODS

Blood samples

A total of 373 consecutive Egyptian individuals [133 with liver fibrosis (F1-F3), 115 with liver cirrhosis (F4) and 125 with HCC] constituted the estimation group. In addition, a total of 254 patients [84 with F1-F3, 76 with F4 and 94 with HCC] constituted the validation group. Histopathological classification of liver fibrosis and cirrhosis was performed according to the METAVIR score.[12] Liver fibrosis was defined as a Metavir score of ≤ 3 (F1-F3) whereas cirrhosis was defined as a Metavir score of 4 (F4). The diagnosis of HCC was carried out according to the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines.[13] The diagnosis of HCC was based on AFP levels above 400 U/L, presence of hepatic focal lesion (s) detected by liver ultrasound (US), and was confirmed by computed tomography (CT) and/or magnetic resonance imaging (MRI) techniques. The final diagnosis was confirmed by histopathologic analysis on US-assisted fine-needle biopsy, when indicated. Patients with other causes of liver diseases, or other suspected malignancies were excluded from the present study. None of the HCC patients had received transarterial embolisation or chemotherapy or underwent radiofrequency ablation or surgical interference. Staging of HCC was conducted as follows: Cancer of the Liver Italian Program (CLIP) score, based on four items and with a score ranging from 0 to 6. These four items included:

- I. Child-Pugh stage (A = 0, B = 1, and C = 2).[14]
- II. Tumor morphology (uninodular < 50% = 0; multinodular > 50% = 1; and massive or > 50% = 2).
- III. AFP level (< 400 U/L = 0; > 400 U/L = 1); and
- IV. Presence of portal vein thrombosis (no, 0; yes, 1).[15]

Liver function tests [albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP)] were all measured using fresh serum in automated biochemistry analyzer (Roche/Hitachi 917, Mannheim, Germany). AFP level was estimated by chemiluminescence, with Immulite (1000) AFP kit (Diagnostic Products Corporation; Los Angeles, CA, USA).

Western-blot and gel electroelution

First of all, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 0.75 mm-thick, 12% vertical slab gels according to the method of Laemmli.[16] This technique facilitated the separation of a mixture of proteins according to their molecular weights. Following electrophoretic separation, Western electroblotting is used for transferring the separated protein bands onto a nitrocellulose membrane (0.45 mm pore size, Sigma) in a protein transfer unit according to Towbin, *et al.*[17] Then, they were immunostained using their specific monoclonal antibodies corresponding to EMA and fibronectin separately. Finally, both EMA and fibronectin bands were cut and electroeluted separately from preparative polyacrylamide gels at 200 V for 3 h in a dialysis bag (Sigma). The protein content of the purified bands was determined[18] and the remainder was stored at -20°C. Western-blot is primarily a research tool which is useful and highly sensitive and specific, but not routinely used for diagnosis for large numbers of samples. Therefore, low cost ELISA test was used for quantifying these biomarkers.

Quantitation of epithelial membrane antigen and fibronectin using ELISA

To quantitate EMA, serum samples were diluted (1:40) in coating buffer (50 mM arbonate/bicarbonate buffer, pH 9.6) and were added (50 μL/ well) to 96-well microtiter plates at 4°C overnight. After blocking with 0.5% BSA in coating buffer (200 μL/ well), the specific mouse monoclonal antibodies at dilution 1:100 in PBS was added (50 μL/well) and was incubated at 37°C for 2 h. Then, goat anti-mouse antibody conjugated with alkaline phosphatase (Sigma) diluted (1:300) in 0.2% BSA in PBS-T20 was incubated at 37°C for 1 h. The plate was washed with PBS + 0.5% Tween 20 after every step. The substrate was 1 mg/ml p-nitrophenyl phosphate and the intensity of the signal was determined by measuring the absorbance at 450 nm after 10 min using a microtiter plate reader (Σ960, Metretech Inc, Germany). Fibronectin was quantified using ELISA and fibronectin specific monoclonal antibody. Serial concentrations of the purified EMA (1.2-37.5 μg/mL) and fibronectin (200-1,600 mg/L) were tested in parallel to establish a dose-response curve as a function of the concentration in serum samples.

Statistical analysis

All statistical analyses were performed by SPSS software version 15.0 (SPSS Inc., Chicago, IL) and GraphPad Prism package; version 5.0 (GraphPad Software, San Diego, CA). Continuous variables were expressed as mean ± standard deviation. The correlation was evaluated by Spearman's rank correlation coefficient. A value of $p < 0.05$ was considered statistically significant. The deviation of AFP was successfully corrected by log transformation of the data. The main endpoint was the identification of HCC patients *vs.* cirrhotic patients using a simple predictive score. For formulating the predictive score, univariate analysis based on Student's *t* test was performed to identify variables that were significantly different between HCC patients *vs.* those with cirrhosis. All variables with a high AUC and a high significance on univariate analysis were entered in stepwise linear regression analysis to develop a model for identifying HCC. Based on the receiver-operating characteristic (ROC) analysis, the best cutoff points were selected and then common indicators of score performance (sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV)) were derived from such a 2 x 2 contingency table.

RESULTS

Patient characteristics

Laboratory characteristics of all patients were summarized in table 1. The mean age of the patients included in this study was 51.5 ± 10.2 years, 253 (67.8%) were men while 120 (32.2%) were women. Overall, 35.7% had liver fibrosis (F1-F3), 30.8% had liver cirrhosis (F4) and 33.5% had HCC. As anticipated, patients that developed F1-F3 were young with a mean (± SD) of 42.7 (± 7.2) years as compared to those who developed F4 or HCC. Patients with HCC produced a range of AFP values from normal to more than 49,268 U/L. The main endpoint of the current study was concerned with discriminating HCC patients from cirrhotic patients. To determine which factors could differentiate HCC from cirrhosis, the laboratory data of these two groups were analyzed. As expected, the data showed that the distribution of all evaluated laboratory blood markers (AST, ALT, ALP, albumin, total bilirubin and AFP) differed significantly ($P < 0.05$) between

Table 1. Patients (n = 627) characteristics in estimation and validation study.

Marker	Estimation study (n = 373)			P *	Validation study (n = 254)			P *
	F1-F3 (n = 133)	F4 (n = 115)	HCC (n = 125)		F1-F3 (n = 84)	F4 (n = 76)	HCC (n = 94)	
Age (years)	42.7 ± 7.2	55 ± 8.3	57.6 ± 9.4	< 0.05	44.9 ± 5.6	52.3 ± 4.4	59.0 ± 8.4	< 0.05
AST (U/L)[a]	54 ± 30	70 ± 35	94 ± 76	< 0.05	52 ± 33	76 ± 38	99 ± 85	< 0.05
ALT (U/L)[a]	66 ± 39	48 ± 24	64 ± 59	< 0.05	64 ± 41	50 ± 23	66 ± 49	> 0.05
AST/ALT ratio	0.9 ± 0.3	1.6 ± 0.6	1.9 ± 1.3	> 0.05	0.7 ± 0.3	1.7 ± 0.5	2.0 ± 0.8	< 0.05
ALP (U/L)[a]	72 ± 37	151 ± 80	223 ± 184	< 0.05	78 ± 41	149 ± 72	242 ± 197	< 0.05
Albumin (g/dL)[a]	4.3 ± 0.4	3.3 ± 0.4	3.1 ± 0.5	< 0.05	4.5 ± 0.4	4.0 ± 0.5	3.0 ± 0.6	< 0.05
Total bilirubin (mg/dL)[a]	0.8 ± 0.4	1.8 ± 1.5	2.7 ± 1.8	< 0.05	0.7 ± 0.4	1.9 ± 1.6	2.8 ± 2.0	< 0.05
AFP (U/L)[a]	5.9 ± 1.0	18.3 ± 3.7	8873 ± 3848	< 0.05	6.3 ± 1.5	18.0 ± 612	10873 ± 5038	< 0.05
Log (AFP (U/L)[a]	0.5 ± 0.4	0.9 ± 0.6	2.3 ± 1.2	< 0.05	0.5 ± 0.1	0.9 ± 0.1	2.3 ± 0.2	< 0.05
EMA (μg/mL)[b]	2.6 ± 1.8	3.3 ± 1.8	8.0 ± 1	< 0.05	2.9 ± 1.7	3.5 ± 1.8	7.4 ± 1.0	< 0.05
Fibronectin (mg/L)	447 ± 157	496 ± 137	661 ± 332	< 0.05	440 ± 141	490 ± 150	647 ± 291	< 0.05

[a] Reference values: aspartate aminotransferase (AST) up to 40 U/L; alanine aminotransferase (ALT) up to 45 U/L; albumin 3.8-5.4 g/dL; total bilirubin up to 1 mg/dL; alkaline phosphatase (ALP) 22-92 U/L; α-fetoprotein (AFP) up to 10 U/L. [b] EMA: epithelial membrane antigen. *P value for F4 *vs.* HCC (P > 0.05 is considered non significant; P < 0.05 is considered significant).

HCC patients and cirrhotic patients. However, AST/ALT ratio did not differ between the two groups (P > 0.05) as shown in table 1.

Identification and quantitation of both EMA and fibronectin

The target EMA and fibronectin were identified based on SDS-PAGE procedure followed by Western-blot. A single immunoreactive band was shown at 130-kDa and 90-kDa molecular weight corresponding to epithelial membrane antigen and fibronectin, respectively, in sera of patients with cirrhosis (F4) and HCC (Figure 1). Then, these bands were purified from sera using the electroelution technique and were quantified using dose-response curves of their serial concentrations. As a result, the concentration of EMA and fibronectin were higher in HCC

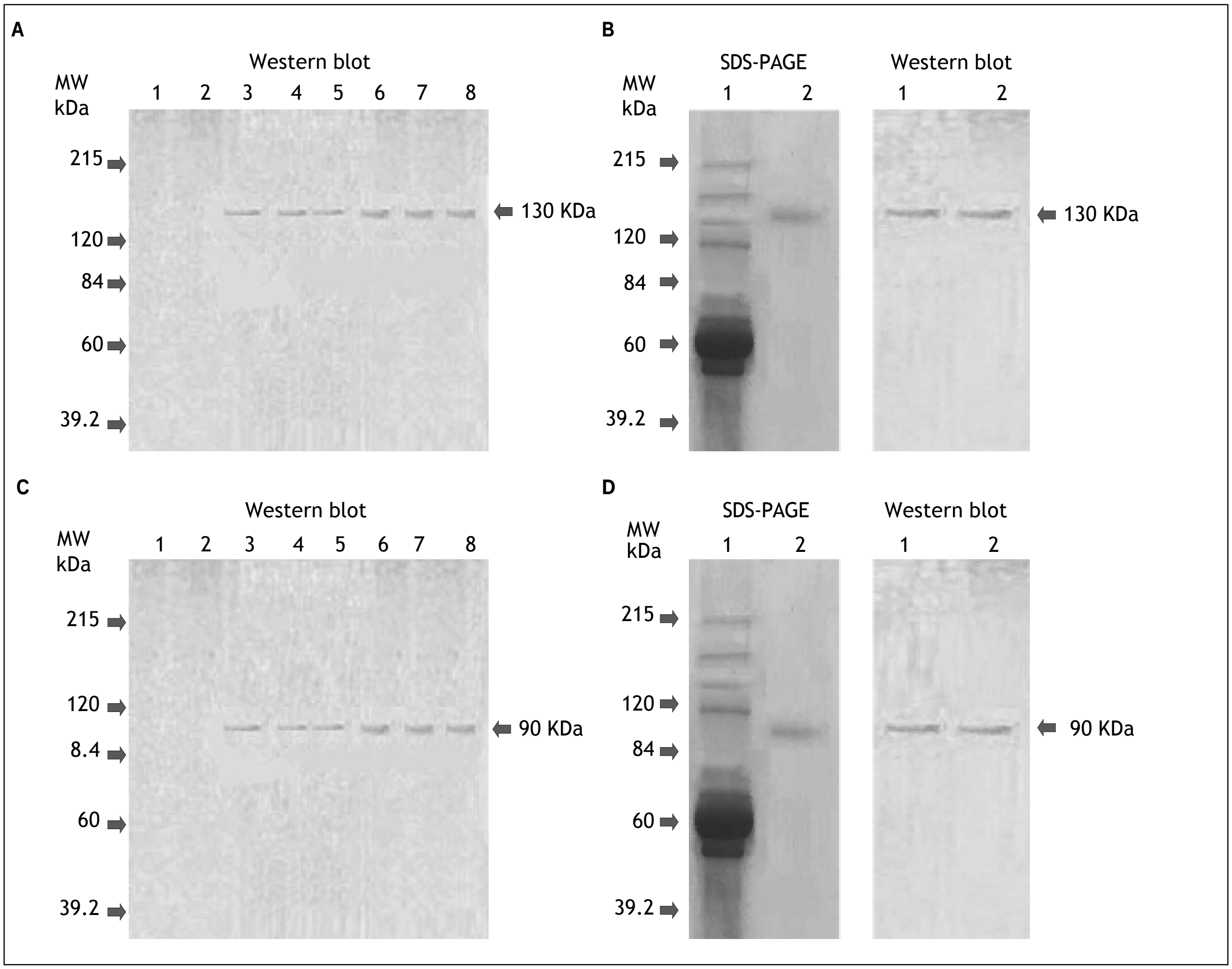

Figure 1. Identification and purification of epithelial membrane antigen (EMA) and fibronectin. A. Western-blot of selected serum samples using mouse monoclonal antibody specific to EMA; lanes 1-2: sera of healthy individuals, lanes 3-5: sera of patients with liver cirrhosis, lanes 6-8: sera of HCC patients. B. SDS-PAGE and Western-blot of HCC serum sample and purified EMA antigen. lane 1: serum of patients with HCC, lane 2: purified EMA antigen from patients with HCC. C. Western-blot of selected serum samples using mouse monoclonal antibody specific to fibronectin; lanes 1-2: sera of healthy individuals, lanes 3-5: sera of patients with liver cirrhosis, lanes 6-8: sera of HCC patients. D. SDS-PAGE and Western-blot of HCC serum sample and purified fibronectin antigen. Lane 1: serum of HCC patients with, lane 2: purified fibronectin antigen from HCC patients. Molecular weight markers were myosin (215 kDa), phosphorylase B (120 kDa), bovine serum albumin (84 kDa), ovalbumin (60 kDa) and carbonic anhydrase (39.2 kDa).

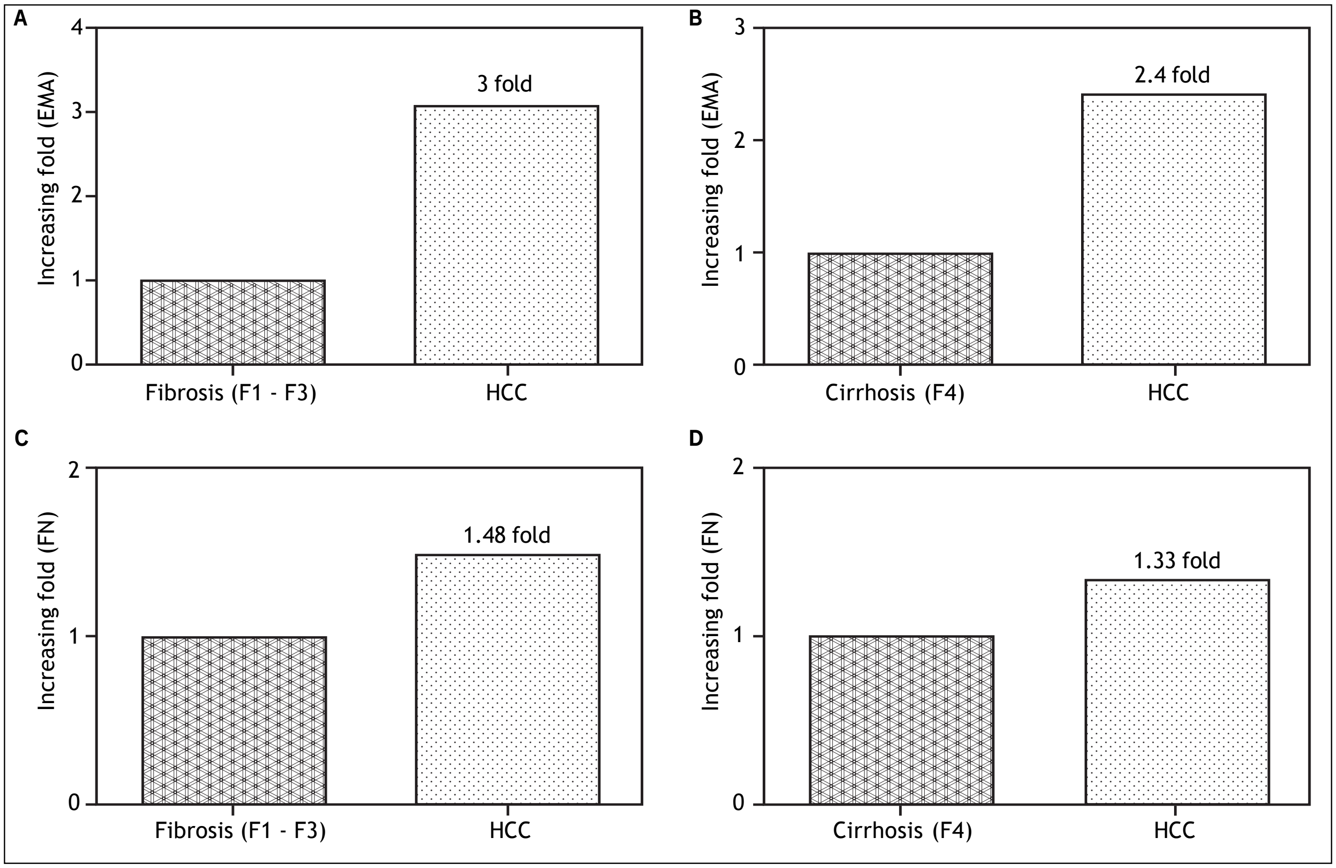

Figure 2. The distribution of observed fold changes for epithelial membrane antigen (EMA) and fibronectin (FN) between HCC patients versus those with liver fibrosis (F1-F3) or cirrhosis (F4). A. EMA (F1-F3 vs. *HCC). B. EMA (F4* vs. *HCC). C. FN (F1-F3* vs. *HCC). D. FN (F4* vs. *HCC).*

patients than patients with fibrosis (F1-F3) or cirrhosis (F4) with a significant difference ($P < 0.0001$) as shown in table 1. HCC patients had a 3-fold and 2.4-fold increase in EMA concentration over F1-F3 and F4 patients, respectively (Figures 2A-2B). On the other hand, patients with HCC displayed a 1.48-fold and 1.33-fold increase in fibronectin concentration over those who developed F1-F3 and F4, respectively (Figure 2C-2D).

Predictors of HCC

Univariate analysis of all variables tested in this study revealed that age, AST, ALT, ALP, albumin, total bilirubin, log AFP, fibronectin and EMA were all significant ($P < 0.05$) and were identified as predictors of HCC. Bivariate Spearman's rank correlation coefficient was then calculated to measure the relationship between individual serum markers to the progression of liver pathology (Figure 3A). In order to estimate and compare the diagnostic accuracies of these individual markers, ROC curves were used (Figure 3B). Using this method, the following biomarkers EMA (AUC = 0.81) followed by AFP, fibronectin and total bilirubin (AUC = 0.70) had the best diagnostic accuracies for identifying HCC and showing strong correlations with histological disease progression. Next, based on ROC analysis, optimal cutoff points for these four candidate markers were determined and their diagnostic performances for identifying HCC were measured and presented in Figure 3C. Then, AFP, EMA, fibronectin and total bilirubin were combined in a single predictive function to improve their diagnostic accuracies for predicting HCC.

Development of FEBA-Test

Although baseline levels of age, AST, ALT, ALP, albumin, total bilirubin, AFP, fibronectin and EMA were all significant on univariate analysis and were identified as predictors of HCC, only AFP, EMA,

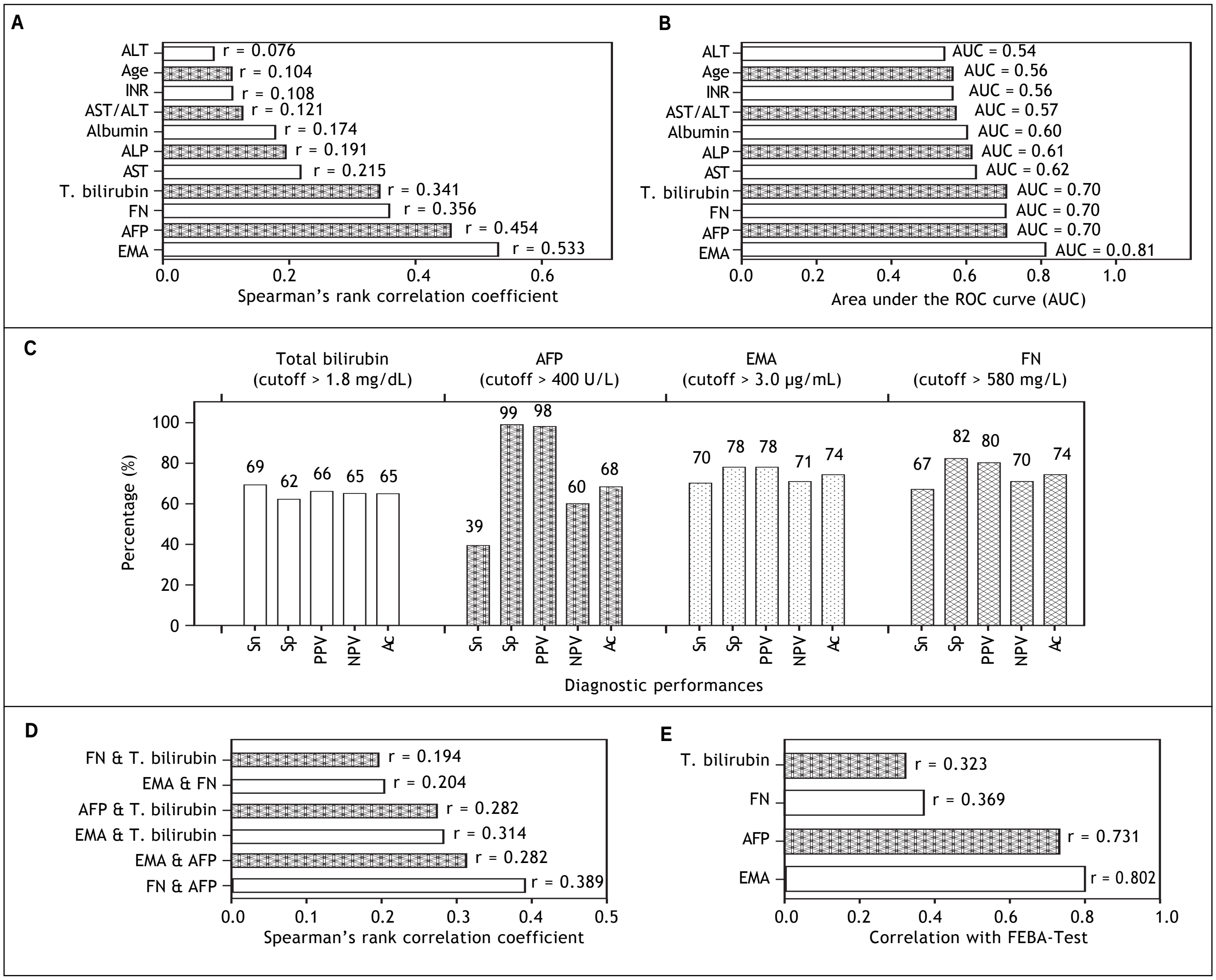

Figure 3. Development of FEBA-Test in the estimation group (n = 373). A. Spearman's rank correlation coefficient of individual serum markers in relation to the progression of liver pathology. B. Area under curve of individual serum markers to discriminate patients with HCC from those with liver cirrhosis. C. diagnostic performances of candidate markers incorporated in the FEBA-Test for identifying HCC, D. Spearman's rank correlation coefficient of candidate markers in relation to each other. E. Spearman's rank correlation coefficient between the constructed FEBA-Test and its individual components. FEBA-Test = [0.001 x fibronectin (mg/L) + 0.13 x EMA (µg/mL) + 0.036 x total bilirubin (mg/dL) + 0.83 x log AFP (U/L) - 0.063].

fibronectin and total bilirubin retained significance when combined with each other and the following function called FEBA-Test was generated that could improve the prediction of HCC.

FEBA-Test = [0.001 x fibronectin (mg/L) + 0.13 x EMA (μg/mL) + 0.036 x total bilirubin (mg/dL) + 0.83 x log AFP (U/L) - 0.063].

There is no correlation between theses candidate markers incorporated in the FEBA-Test as shown in figure 3D. They were not related with no redundancy. In addition, Spearman's rank correlation coefficient between the FEBA-Test and its candidate markers were measured for the impact of each marker on the predictive criteria (Figure 3E).

Diagnostic ability of FEBA-Test

The areas under the ROC curve was used to estimate the diagnostic value of FEBA-Test derived from our data set that showed an AUC = 0.92 for differentiating HCC from F4 (Figure 4A) which was evident to be more efficient than that produced by AFP

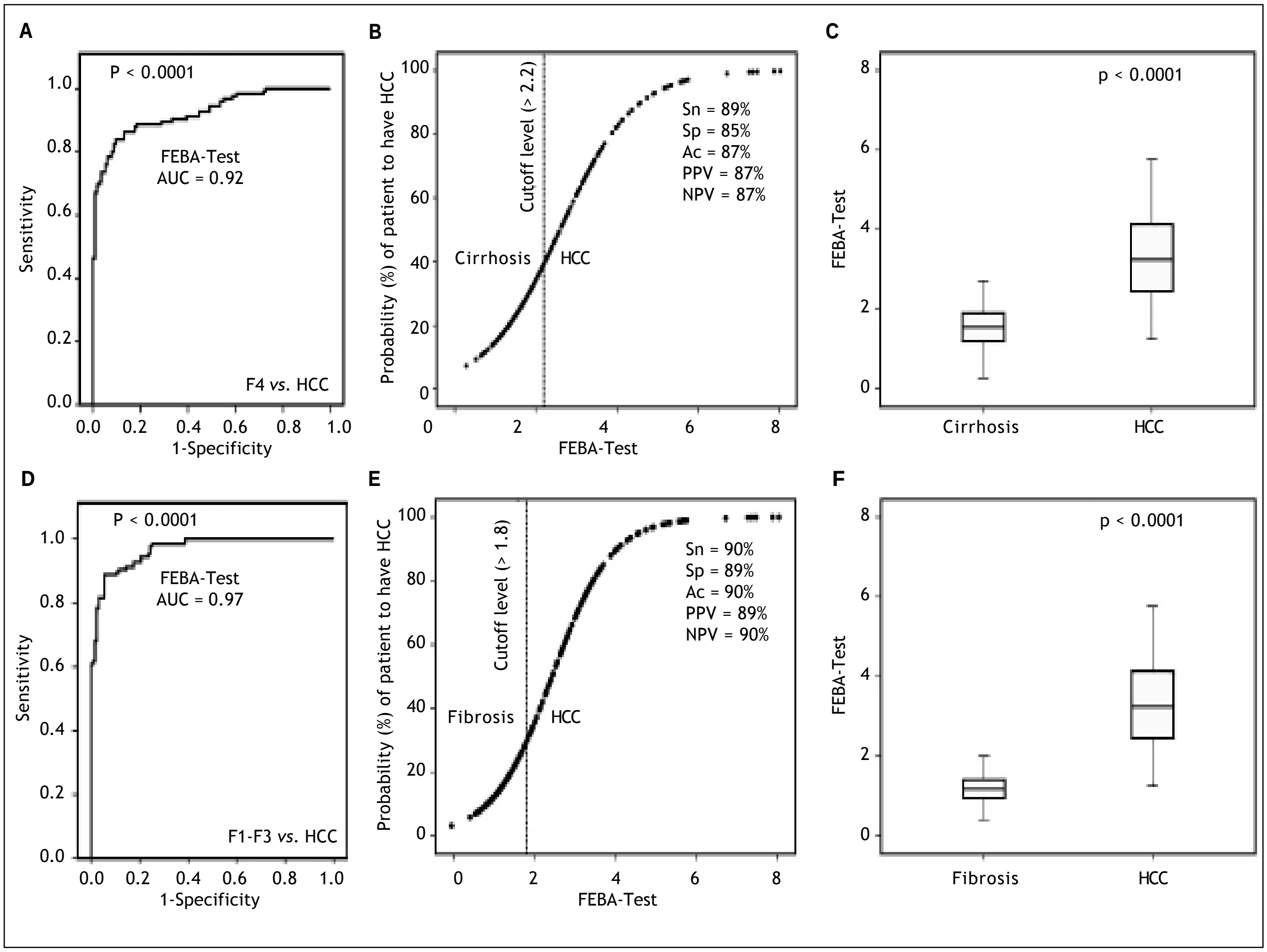

Figure 4. Diagnostic performances and distribution of FEBA-Test. A-C. For separating patients with HCC from those with liver cirrhosis (F4). D-F. For separating patients with HCC from those with liver fibrosis (F1-F3). AUC: area under the ROC curve. Sn: sensitivity. Sp: specificity. Ac: accuracy. PPV: positive predictive value. NPV: negative predictive value.

(AUC = 0.70). Next, an optimal cutoff point > 2.2 was selected and the diagnostic performances were shown in figure 4B. For FEBA-Test > 2.2, the probability of patient to have HCC increases. On contrary, for FEBA-Test ≤ 2.2, the probability of patient to have HCC decreases as shown in figure 4B. The distribution of FEBA-Test levels in HCC patients in relation to F4 patients were compared and presented as box plots (Figure 4C). As seen in figure 4C, the values of FEBA-Test in HCC patients were higher than those in cirrhotic patients. The median value in F4 was 1.6 while that of HCC was 3.6 with an extremely significant difference ($P < 0.0001$). Moreover, FEBA-Test significantly correlated with histological disease progression with a Spearman's rank correlation coefficient of 0.72 ($P < 0.0001$). On the other hand, the effectiveness of FEBA-Test for discriminating HCC patients from those who developed F1-F3 was then estimated using ROC curve that showed a superior AUC = 0.97 (Figure 4D). Likewise, an optimal cutoff point > 1.8 was then selected to separate HCC patients from F1-F3 patients and the diagnostic performances were clarified in figure 4E. At this point, 112 of 126 (PPV = 89%) would have HCC and only 14 of 322 without HCC would be classified incorrectly. In addition, 118 out of 131 (NPV = 90%) would have F1-F3 and only 13 out of 125 without fibrosis would be classified falsely. The distribution of FEBA-Test levels in patients with HCC in relation to those who developed F1-F3 were compared and were presented as box plots in figure 4F. The median value in F1-F3 was 1.2 while that of HCC was 3.6 with an extremely significant difference ($P < 0.0001$).

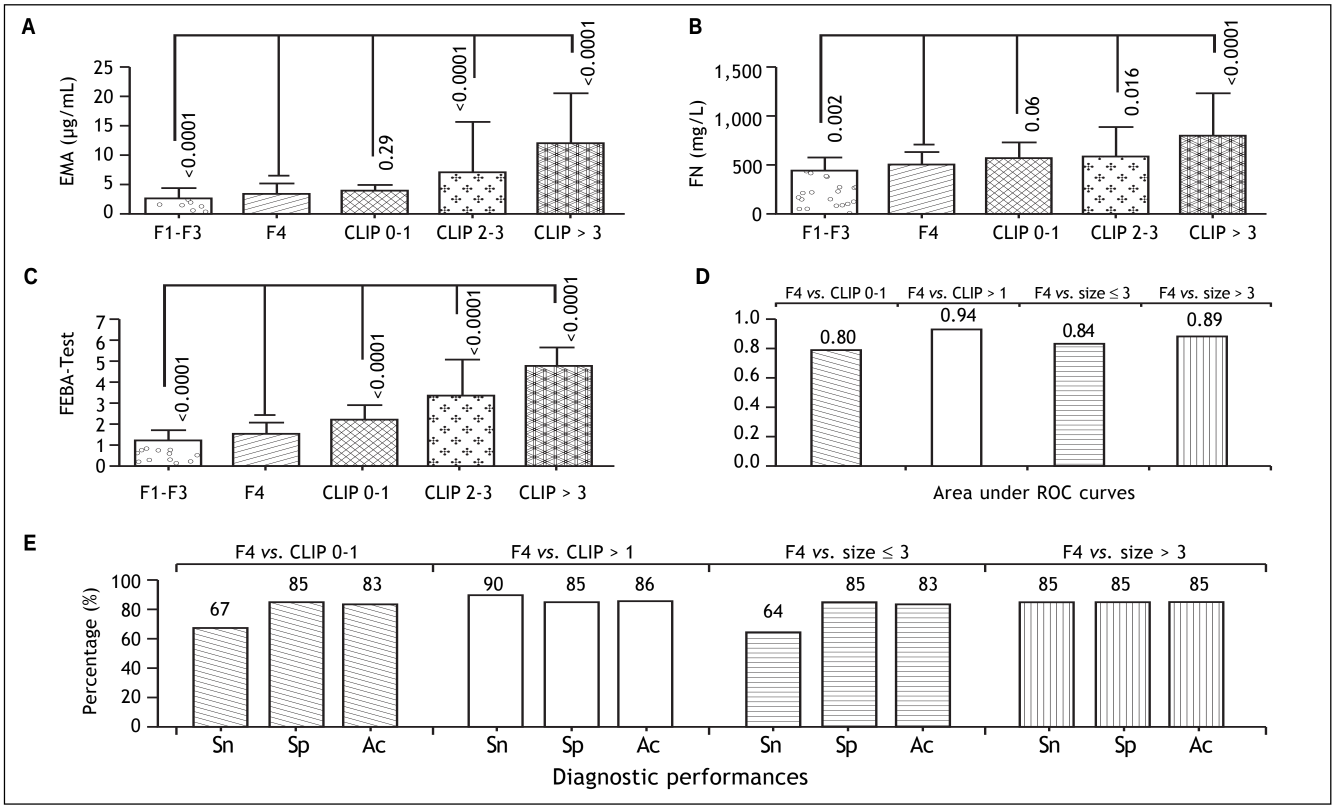

Figure 5. Distribution of epithelial membrane antigen (EMA), fibronectin (FN) and FEBA-Test levels among different groups of patients with liver pathology in addition to diagnostic performances of FEBA-Test. A. Distribution of EMA. B. Distribution of FN. C. Distribution of FEBA-Test. D. Area under curves for FEBA-Test for separating patients with F4 from patients with different stages of HCC. E. Calculated sensitivity (Sn), specificity (Sp) and accuracy (Ac) for FEBA-Test for discriminating patients with F4 from patients with different stages of HCC.

FEBA-Test and CLIP scoring system

Also, we aimed to estimate the diagnostic ability of FEBA-Test in identifying the percent of cases at different stages of HCC that were categorized according to CLIP scoring system (CLIP 0, 9.3%; CLIP 1, 18.6%; CLIP 2, 34.9%; CLIP 3, 23.3%; CLIP 4, 14%). Patients with HCC who had CLIP 5-6 were missing in this study. We used CLIP score (0-1) to define early stages of HCC. To stage patients according to CLIP scoring system, multiple clinical indexes, such as Child-Pugh score, tumour morphology, AFP level and presence of portal vein thrombosis, were taken into account. The distribution of FEBA-Test levels in addition to EMA and fibronectin in different liver pathological groups is depicted in figure 5A-5C. The differences in FEBA-Test values were statistically extremely significant ($P < 0.0001$) between patients who developed cirrhosis *vs.* those who had liver fibrosis, HCC (CLIP 0-1), HCC (CLIP 2-3) and HCC (CLIP > 3). Our results showed that the use of FEBA-Test could discriminate patients with HCC who had CLIP 0-1 and size ≤ 3 cm from those who developed F4 with AUCs of 0.80 and 0.84, respectively, indicating its potential ability in identifying early HCC. In addition, FEBA-Test could discriminate patients with HCC who had CLIP > 1 and size > 3 cm from those who developed F4 with AUCs of 0.94 and 0.89, respectively (Figure 5D). Additionally, the diagnostic performances for FEBA-Test for identifying these different groups were calculated and presented in figure 5E.

Validation study

We evaluated whether the predictive criteria identified in the estimation study were able to reproduce their predictive ability in a subsequent different, but related group of patients. The characteristics of the validation group were similar to that of estimation group with no significant differences in any of the assessed variables as previously depicted in table 1. It is evident that the aforementioned results were reproduced in the validation study with no significant difference. At cutoff level > 2.2, FEBA-Test yielded sensitivity = 83% with PPV = 90% and accuracy = 91%, specificity = 88% with NPV = 81% for discriminating HCC from F4. As well, at cutoff level > 1.8, FEBA-Test generated sensitivity = 88% with PPV = 89% and accuracy = 96%, specificity = 88% with NPV = 87% for discriminating HCC from F1-F3.

DISCUSSION

It is a worthy of noting that liver fibrosis eventually leads to end-stage cirrhosis and/or HCC in a significant number of patients.[19] HCC develops in a cirrhotic liver in 80% of cases, and this pre-neoplastic condition is the strongest predisposing factor.[20] Consequently, proper definition of early HCC has critical implications as one third of dysplastic lesions will develop a malignant phenotype.[20,21] Thus, there is an urgent need to identify better tools to characterize these lesions. Several tumor markers have been proposed for HCC diagnosis. Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3)[22] and Des-gamma carboxyprothrombin (DCP)[23] have been approved by the Food and Drug Administration as plasma markers for HCC. DCP is a more specific HCC marker than AFP because other liver diseases don't cause an increase of DCP serum levels. DCP measurement for HCC has 48-62% sensitivity and 81-98% specificity. However, it has been reported that AFP-L3[24] and DCP[24,25] are less sensitive than AFP for the diagnosis of early stage HCC. On the other hand, Attallah, *et al.* reported that cytokeratin-1 may be clinically valuable as a surrogate marker for identifying HCC.[26] Herein, this work was concerned with the identification and quantitative determination of EMA and fibronectin and then estimating their performances for HCC diagnosis. With respect to EMA, it is a type of mucins which are a group of high molecular weight glycoproteins found on epithelial surfaces. In general, mucins are produced by secretory epithelial cells for the lubrication and protection of ducts and lumen.[27] The loss of cell architecture and polarity associated with malignant disease means that EMA, normally confined to luminal surfaces, is shed into the bloodstream and thus has potential as a tumor marker. The abnormal EMA molecules reveal new protein epitopes or carbohydrate antigens, and may be recognized by the immune system as notable tumor associated antigens.[28] It was reported that EMA is expressed by adenocarcinomas of the breast, ovary and colon and has been suggested as a circulating tumour marker.[29] However, the expression levels of EMA in liver cancer and cirrhotic liver tissues and their correlation with carcinogenesis still remain to be elucidated. EMA could detect small deposits of malignant cells in organs such as liver.[30] There are discrepant results for EMA expression in HCC. Sasaki and Nakanuma[31] reported that EMA core protein is expressed in intrahepatic bile duct carcinoma, but not in HCC. However, Cao, *et al.*[32] demonstrated that EMA is remarkably expressed in HCC cells and can be considered as an indicator of HCC prognosis. In this study, EMA was identified using Western-blot at 130-kDa. Several previous authors identified EMA in different body fluids as a high-molecular-weight glycoprotein (35-1500 kDa).[33-35] Our results showed that patients with HCC have a significantly elevated EMA compared to patients who developed F1-F3 or F4. Additionally, EMA significantly was correlated with histological disease progression with a Spearman's rank correlation coefficient of 0.533 and enabled the correct identification of patients who have HCC with 0.81 AUC. On the other hand, fibronectin is a major component of the extracellular matrix that is considered to have an important role in chronic inflammatory periodontal disease.[36] Fibronectin also plays important roles in the development and pathogenesis of many disorders, including cancer.[37-39] Fibronectin plays an important role in signal transduction and cell adhesion and is partially regulated by TGF-β,[40] a cytokine that plays a central role in controlling the growth of normal hepatocytes. Abnormal fibronectin protein expression in HCC may be a manifestation of dysregulation of this important cell cycle control point. Abnormal fibronectin expression may also cause abnormal cell-to-cell or cell-to-extracellular matrix adhesion, contributing to tumor development. In fact, fibronectin has been implicated in the development of multiple types of human cancer and it has been associated with cell migration and invasion in several metastatic models.[41] Studies of fibronectin in breast carcinomas have shown stronger expression than in normal breast parenchyma, and a different distribution.[42] In addition, it was reported that fibronectin levels increase with the progression of colorectal cancer and this expression is a useful marker of the degree of disease advancement.[43] Apparently, fibronectin plays an important role in cancer progression, and thus is hypothesized to be highly associated with HCC progression.[44] It was stated that the expression of fibronectin is increased in liver tumor growth and this elevation has been linked to resistance to therapy.[45] Furthermore, fibronectin either regulates, or is regulated by, a number of important cell cycle proteins thought to play a role in HCC pathogenesis including cyclin D1, β-catenin[46] and p53.[47] Previous authors have suggested that four main fragments were produced at 150, 120, 90 and 80 kDa after fibronectin proteolysis.[48] In this work, Western-blot analysis revealed that monoclonal antibody reacted against fibronectin at an apparent molecular weight of 90-kDa in sera. Our results showed that patients with HCC have a significantly elevated fibronectin compared to patients with F1-F3 or F4. Fibronectin enabled the correct identification of patients who have HCC with 0.70 AUC. These finding suggest that these molecules, EMA and fibronectin, could be potential markers for HCC diagnosis in patients with chronic liver disease. In order to improve the diagnostic accuracies of EMA and fibronectin, it is necessary for them to be combined with other markers for HCC diagnosis in clinical setting. On univariate and multivariate analyses, only total bilirubin and AFP together with EMA and fibronectin retained significance when combined with each other. Consequently, AFP and total bilirubin were also identified as diagnostic markers for HCC. It is well known that persistently elevated AFP levels are related to the presence of HCC and its determination can be helpful for a better definition of at-risk patients (i.e., patients with cirrhosis);[49] hence, AFP is the most widely tested biomarker in HCC. When used as a diagnostic test, AFP value of 20 ng/mL shows good sensitivity but low specificity, whilst the higher cutoff value such 200 ng/mL presents a high specificity but low sensitivity.[50] Consistent to these findings, our results showed that AFP value at a cutoff of 400 U/L presents a superior specificity of 99% but the sensitivity dropped to 35%. Our results were almost similar to that obtained by Marrero, *et al.*[51] who investigated the diagnostic performance of AFP in differentiating patients with HCC from cirrhosis yielding sensitivity = 34% and specificity = 100%. This cutoff value was chosen because this value is frequently reported to be specific for the diagnosis of hepatocellular carcinoma. With regard to bilirubin, it is well known that increased level of bilirubin in the blood, hyperbilirubinemia, cause Jaundice. It occurs in 5-44% of patients with HCC. It is an important clinical presentation as the different aetiological causes of jaundice in HCC determine the therapeutic approach and the prognosis.[52] Under these aforementioned analysis, we introduced a predictive function called FEBA-Test combining four markers (fibronectin, EMA, total bilirubin and AFP) yielding AUC = 0.92 for identifying HCC. At a cutoff value > 2.2, FEBA-Test has sensitivity = 89% and specificity = 85% for predicting HCC. At this point, HCC could be confirmed with a PPV = 87% and could be excluded with NPV = 87%. The sensitivity achieved by FEBA-Test was almost similar to that produced by Ishida, *et al.*[53] based on AFP, AST, LD, hemoglobin, prothrombin time and male ratio (82 *vs.* 85%) whilst the specificity was higher for FEBA-Test (74 *vs.* 90%). Next, the effectiveness of FEBA-Test for discriminating HCC from F1-F3 was performed using ROC curve that showed a superior diagnostic accuracy of AUC = 0.97. These results were reproduced in the validation study with no significant difference. In an additional part of this study the ROC curve analysis was used to evaluate the diagnostic accuracy of FEBA-Test in identifying different stages of HCC that were categorized according to CLIP scoring system. We used CLIP 0-1 to define early HCC. To stage patients in the CLIP score, multiple clinical indexes, such as Child-Pugh score, tumour morphology, AFP level, and presence of portal vein thrombosis were taken into account. FEBA-Test gave AUCs 0.80 and 0.84 for identifying patients with HCC who had CLIP 0-1 and size ≤ 3 cm, respectively. This result may indicate the ability of FEBA-Test in identifying early stages of HCC. In addition, FEBA-Test enabled the correct identification of patients with HCC who had CLIP > 1 and size > 3 cm with AUCs 0.94 and 0.89, respectively. In summary, we showed that FEBA-Test may improve the detection of HCC with a high degree of accuracy. Further prospective multicenter studies involving a greater number of patients are warranted to validate the usefulness of the produced score in clinical practice.

ABBREVIATIONS

- **AAR:** AST/ALT ratio.
- **AFP:** alpha fetoprotein.
- **ALP:** alkaline phosphatase.
- **ALT:** alanine aminotransferase.
- **AST:** aspartate aminotransferase.
- **EMA:** epithelial membrane antigen.
- **HCC:** hepatocellular carcinoma.
- **HCV:** hepatitis C virus.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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