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HCC-Check: A Novel Diagnostic Tool for Early Detection of Hepatocellular Carcinoma Based on Cytokeratin-1 and Epithelial Membrane Antigen: A Cross-Sectional Study

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Abstract

Background: Hepatocellular carcinoma is frequently diagnosed in advanced stages, leading to a poorer prognosis. Therefore, early diagnosis and identification of biomarkers may significantly improve outcomes. **Methods:** This cross-sectional study enrolled 486 participants distributed among 3 groups: F1 to F3 = 184, F4 = 183, and hepatocellular carcinoma = 119. Liver fibrosis staging was performed using FibroScan, while imaging features were used for hepatocellular carcinoma detection. Epithelial membrane antigen and cytokeratin-1 levels in serum were quantified through Western blot and ELISA, respectively. **Results:** Patients diagnosed with hepatocellular carcinoma exhibited significantly elevated levels of epithelial membrane antigen and cytokeratin-1 compared to non-hepatocellular carcinoma patients, with a highly significant statistical difference ($P < .0001$). Epithelial membrane antigen demonstrated diagnostic performance with an area under the curve of 0.75, a sensitivity of 69.0%, and a specificity of 68.5%. Cytokeratin-1 for the identification of hepatocellular carcinoma showed a sensitivity of 79.0% and a specificity of 81.4%, resulting in an area under the curve of 0.87. The developed HCC-Check, which incorporates epithelial membrane antigen, cytokeratin-1, albumin, and alpha-fetoprotein, displayed a higher area under the curve of 0.95 to identify hepatocellular carcinoma, with a sensitivity of 89.8% and a specificity of 83.9%. Notably, HCC-Check values exceeding 2.57 substantially increased the likelihood of hepatocellular carcinoma, with an estimated odds ratio of 50.65, indicating a higher susceptibility to hepatocellular carcinoma development than those with lower values. The HCC-Check diagnostic test exhibited high precision in identifying patients with hepatocellular carcinoma, particularly those with small tumor sizes (<5 cm) and a single nodule, as reflected in area under the curve values of 0.92 and 0.85, respectively. HCC-Check was then applied to the validation study to test its accuracy and reproducibility, showing superior area under the curves for identifying different stages of hepatocellular carcinoma. These outcomes underscore the effectiveness of the test in the early detection of hepatocellular carcinoma. **Conclusion:** The HCC-Check test presents a highly accurate diagnostic method for detecting hepatocellular carcinoma in its early stages.

Keywords

cytokeratin-1, diagnosis, epithelial membrane antigen, HCC-Check, liver

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Abbreviations

AFP, alpha-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; CK-1, cytokeratin-1; ELISA, enzyme-linked immunosorbent assay; EMA, epithelial membrane antigen; HCC, hepatocellular carcinoma; PT-INR, prothrombin time, international normalized ratio; PVT, portal vein thrombosis; ROC, receiver operating characteristics; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; US, ultrasound.

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Introduction

Liver cancer, specifically hepatocellular carcinoma (HCC), is a predominant cause of cancer-related deaths worldwide, comprising 80% of all liver cancers.[1] Despite advances in understanding the epidemiology and risk factors of the disease, the global incidence and mortality rates of HCC persistently escalate. Unfortunately, a substantial proportion of HCC cases are diagnosed at advanced stages. Incidence and mortality rates exhibit variability globally, influenced by environmental and communication risk factors, the accessibility of healthcare resources, and the effectiveness of early-stage detection and treatment. The rapid progression of HCC often leads to untimely fatalities, exacerbated by its limited responsiveness to radiation and chemotherapy. Hence, the imperative for early detection of precancerous HCC nodules is to enhance the 5-year survival rate and potentially mitigate incidence and mortality. However, the main treatments for HCC are liver resection and transplantation[2]; however, therapeutic alternatives are limited by low sensitivity to radiation and chemotherapy, coupled with high rates of relapse and metastases, resulting in grim prognoses.[3] The screening program's main objective is to identify nodules before malignancy or symptoms manifest, facilitating early stage treatment. Comprehensive evaluation, which includes clinical evaluation, imaging, laboratory tests, and histopathological examination, is essential for accurate diagnosis and effective HCC treatment. Imaging plays an essential role in diagnosis, with outcomes significantly influenced by techniques, examiner expertise, and imaging device quality. Alpha-fetoproteins (AFP) lack utility as early-stage HCC screening tests due to their low sensitivity, sometimes as low as 35% in cases of cirrhosis. Therefore, it is imperative to improve early diagnosis and identify effective treatments.[4] Epithelial membrane antigen (EMA) is a protein associated with epithelial cell abnormalities, found to exhibit abnormal mucin expression in human carcinomas of various digestive organs. On the contrary, cytokeratin (CK) serves as a differentiation-dependent tissue-specific epithelial cell marker, present in numerous malignant epithelial cells.[5] Hepatocellular carcinoma manifests several CK subtypes. Cytokeratin is the largest member of the intermediate filament superfamily and exists in many malignant epithelial cells.[6,7] Hepatocellular carcinoma manifests several CK subtypes.[8] Studies indicate that intact and partially degraded CK fragments are released into the bloodstream after necrosis induced by liver inflammation, detectable through serological tests.[9–11] Previously, cytokeratin-1 (CK-1) was identified as a valuable clinical marker for the prediction of HCC.[12] Therefore, we hypothesized that incorporating a new mathematical combination consisting of EMA, CK-1, and the prominent liver function test parameter, albumin, would improve predictive efficacy in the identification of early HCC.

Materials and Methods

Patients

After reviewing HCC prevalence, we aimed to include 513 patients ($\alpha = 5\%$, power = 80%). From the initial pool of 513 consecutive participants in this cross-sectional study, 27 individuals were excluded due to reasons such as mortality, lactation, pregnancy, and inadequate samples, as illustrated in Figure 1. Enrolling 486 from Mansoura University hospitals, Mansoura, Egypt, from February 2021 to June 2022 within a 95% CI enhanced early effect detection. From those cohorts, a total of 225 patients [75 with liver fibrosis (F1-F3), 75 with liver cirrhosis (F4), and 75 with HCC] constituting the validation group were randomly enrolled. The expected sensitivity and specificity were based on preliminary data. The entire patient record was anonymized to protect privacy. The selected sample size, denoted 486, ensures robust statistical power to identify diagnostic accuracy differences and improve the reliability of the study.

The patients were categorized into 3 groups based on the severity of their liver disease. The first group consisted of 184 patients with F1 to F3, comprising 130 men and 54 women with an average age of 42.9 (±8.5) years. The second group included 183 patients with F4. The average age of this group, which included 117 men and 66 women, was 52.5 (±9.0) years. F1 to F3 and F4 were histopathologically classified according to FibroScan (Echosens).[10] The third group consisted of 119 patients diagnosed with HCC. In this group, there were 95 males and 24 females, with an average age of 57.4 (±9.2) years. Strengthening the reporting of observational studies in epidemiology (STROBE) principles are strictly followed in this manuscript, which demonstrates our dedication to thorough and transparent reporting in observational research. We strengthen our study's credibility and importance in the scientific community by adhering to these recommendations.[13] The diagnosis of HCC followed the guidelines set by the American Association for the Study of Liver Disease Practice.[11] Hepatocellular carcinoma was established for patients with AFP levels of 400 U/L or higher through the detection of focal liver lesions by ultrasound (US).

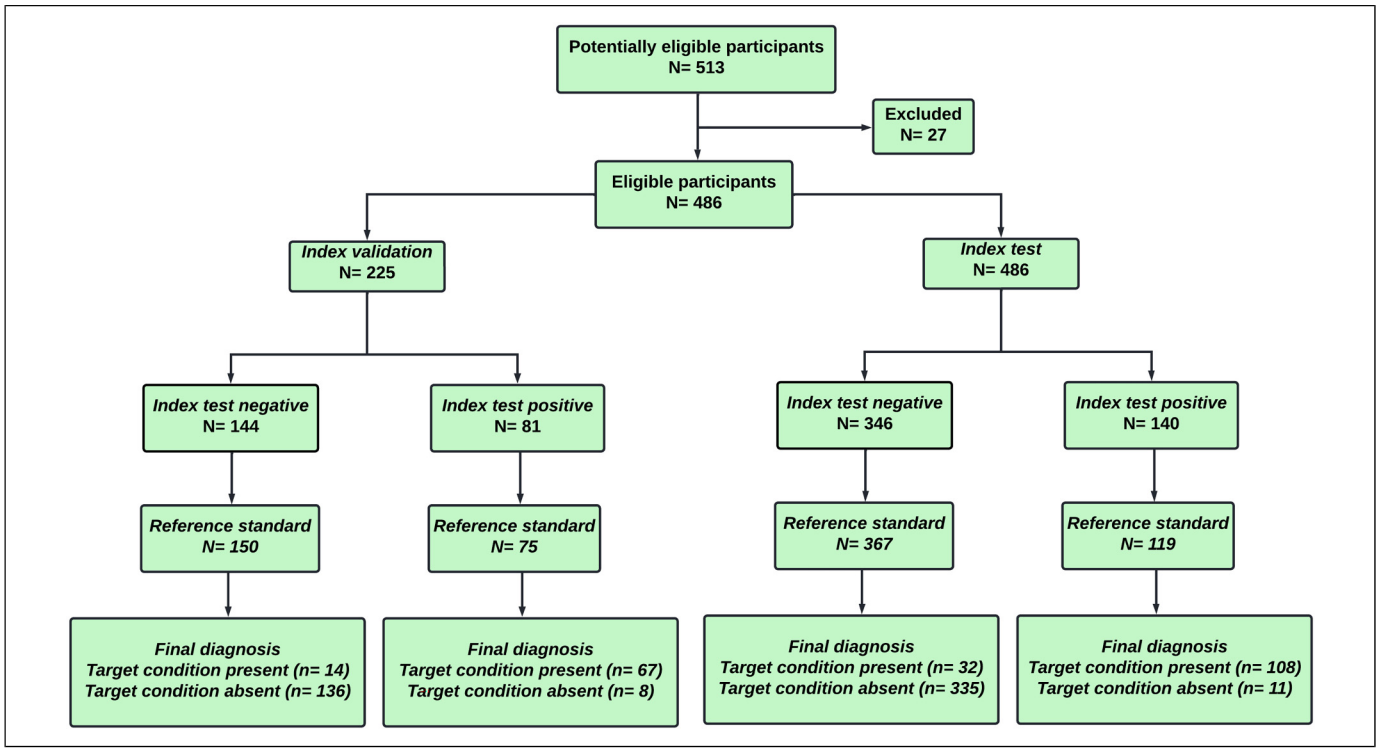

Figure 1. Flowchart showing the participants included in the study.

Subsequently, the diagnosis was confirmed by computed tomography and/or magnetic resonance imaging.

Patients with additional liver disorders or suspected malignancies were excluded to focus specifically on HCC cases. Furthermore, the study cohort consisted of individuals who had not undergone specific treatments such as radiofrequency ablation, chemotherapy, or surgical intervention, aiming to minimize confounding factors associated with these therapies. All participants provided their informed consent in writing prior to enrollment, allowing the use of their serum samples for the evaluation of 2 biomarkers, EMA and CK-1. Furthermore, participants were extensively informed about the study's diagnostic techniques and were educated about the characteristics and implications of the disease, ensuring an informed decision regarding their voluntary participation. Additionally, as part of our dedication to protecting patient privacy, we have carefully removed any personal information using a deidentification procedure. All study participants' privacy and confidentiality are strengthened by this all-inclusive strategy, which guarantees complete anonymization of patient data. Our devotion to following this stringent protocol demonstrates our steadfast support for the moral conduct of research.

Blood samples were collected within 2 days after FibroScan and computed tomography. Fresh serum samples were analyzed for liver function tests, including AST, ALT, ALP, total bilirubin, and albumin, using an automated biochemical analyzer (A15, Biosystem). Platelet count was determined using the KX-21 Sysmex automated hematology analyzer (Sysmex Corporation) on treated blood EDTA-K3. The PT-INR ratio was calculated using a citrate solution in another fraction of the sample, while the AFP levels were measured using the chemiluminescence method and the immulite of the AFP kit (1000) (Diagnostic Products Corporation).

Identification and Quantification of EMA and CK-1

Electrophoresis of sodium dodecyl sulfate-polyacrylamide gel was carried out on a 0.75-mm thick vertical slab gel of 0.75-mm thickness, 12%, using the Laemmli method.[9] The separated bands were then transferred to a nitrocellulose membrane (0.45 mm diameter, Sigma) by Western Blot, as described by Towbin *et al*.[14] The bands were immunostained with corresponding antibodies (ABC Diagnostics) and matched separately with EMA and CK-1.

In the study, it was observed that when the antigens were treated with monoclonal antibodies CK-1 and EMA separately, a single immunoreactive band was detected at 67 and 130 kDa, respectively. This finding aligns with previous reports by Attallah *et al*.[12,15] Subsequently, the samples were quantified using the ELISA technique with monospecific antibodies (ABC Diagnostics). The intensity of the color was directly proportional to the number of conjugates bound, depending on the concentration of the estimated biomarkers in serum samples. As per the manufacturer's instructions, positive results, regardless of visibility, should always be considered positive. In cases of indeterminate results (weak positives), it is recommended to collect another sample 2 weeks later. An indeterminate result, indicating a mild positive reaction, may be caused by an

Table 1. Laboratory Characteristics of All Patients Included in This Study (N = 486).

Variables	Fibrosis (n = 184)	Cirrhosis (n = 183)	HCC (n = 119)	Tukey's post hoc test[a]		
				Fibrosis versus cirrhosis	Fibrosis versus HCC	Cirrhosis versus HCC
Male/female	130/54	117/66	95/24			
Age (years)	42.9 ± 8.5	52.5 ± 9.0	57.4 ± 9.2	<0.0001	<0.0001	<0.0001
ALT (U/L)	68 ± 38	50 ± 33	55 ± 38	<0.0001	0.015	0.631
AST (U/L)	55 ± 31	67 ± 41	85 ± 58	0.031	<0.0001	0.003
ALP (U/L)	77 ± 39	124 ± 43	156 ± 131	<0.0001	<0.0001	0.023
T. bilirubin (mg/dL)	0.8 ± 0.4	1.6 ± 2.5	2.7 ± 2.5	<0.0001	<0.0001	<0.0001
Albumin (g/dL)	4.3 ± 0.3	3.6 ± 0.6	2.9 ± 0.5	<0.0001	<0.0001	<0.0001
Platelets ($\times 10^9$/L)	191.3 ± 54.0	135.5 ± 74.6	127.9 ± 90.7	<0.0001	<0.0001	0.717
AFP (ng/mL)	5.3 ± 10.8	9.7 ± 13.5	615.4 ± 2641.4	0.999	<0.0001	<0.0001
Log AFP (ng/mL)	0.47 ± 0.41	0.83 ± 0.42	1.89 ± 0.97	<0.0001	<0.0001	<0.0001
EMA (μg/mL)	2.2 ± 0.4	2.3 ± 0.7	5.3 ± 7.3	0.935	<0.0001	<0.0001
CK-1 (μg/mL)	2.2 ± 3.7	3.2 ± 4.9	33.8 ± 45.6	0.917	<0.0001	<0.0001

Abbreviations: HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; AFP, alpha-fetoprotein; CK-1, cytokerain-1; EMA, epithelial membrane antigen.
The data were presented as mean ± standard deviation. Reference values: HCC and ALT (up to 45 U/L), AST (up to 40 U/L), ALP (22–92 U/L), total bilirubin (up to 1 mg/dL), albumin (3.8-5.4 g/dL), platelet counts [(150-400 ($\times 10^9$/L)], AFP (up to 10 U/L).
[a]$P > 0.05$ is considered non-significant; $P < 0.05$ is considered significant.

unidentified antibody cross-reaction, contamination with a different person's sample, or some other technical error.

Statistical Analysis

For statistical analysis, the GraphPad Prism package (version 5.0) from GraphPad Software in San Diego, CA, and SPSS software (version 22.0) from SPSS Inc. in Chicago, IL, were utilized. The patient's characteristics were depicted by the mean and standard deviation (SD). A P value less than 0.05 was considered significant, while P values greater than 0.05 were considered insignificant. The Spearman rank correlation coefficient was used to assess the correlation. The AFP divergence was reversed by successfully transforming the data log. The primary goal of this study was to detect patients with clinically active HCC as soon as possible. ROC curves were used to assess the diagnostic potential of different factors. An HCC identification index was established through backward stepwise logistic regression analysis. To determine the best cutoff points, the ROC analysis was utilized, and sensitivity and specificity were derived from a 2×2 contingency table.

Results

Characteristics of the Patient

The present study comprised 486 patients, with the primary goal being the discrimination of patients with early HCC from cirrhotic patients. Table 1 summarizes the characteristics of all the patients included in this study. The participants had a mean age of 49.7 (±10.6) years; 342 were men and 144 were women. In general, 24.5% of the patients had HCC, 37.7% had F4, and 37.9% had F1 to F3. As expected, patients with F1 to F3 were younger than those with F4 or HCC, with a mean age (±SD) of 42.9 (±8.5) years, as provided in Table 1. The liver biochemical profiles of the studied groups revealed a significant difference between HCC patients and cirrhosis in mean values of AST, ALP, albumin, total bilirubin, and AFP. Albumin in the HCC group was significantly lower than in the other 2 groups ($P < .0001$). On the other hand, there was no significant difference between HCC and cirrhosis in terms of mean values of ALT and platelet count.

Prediction of HCC

This study compared CK-1 and EMA levels in patients with HCC, F1 to F3, and F4. The box plots in Figure 2A to D show the results of this comparison. Hepatocellular carcinoma patients exhibited significantly higher levels of CK-1 and EMA compared to those with F1 to F3 and F4, with a highly significant difference ($P < .0001$). The median values for CK-1 and EMA were higher in patients with HCC (12.00 μg/mL and 3.00 μg/mL, respectively) compared to those who developed F1 to F3 (0.67 μg/mL for CK-1 and 2.23 μg/mL for EMA) or F4 (1.25 μg/mL for CK-1 and 2.42 μg/mL for EMA). Interestingly, the EMA concentration increased in HCC patients over those with F1 to F3 and F4 by 2.38 and 2.25 times, respectively, as shown in Figure 2E and F. Unlike individuals who developed F1 to F3 or F4, HCC patients showed a 15.49-fold and a 10.79-fold increase in CK-1 levels, respectively (Figure 2G and H).

Diagnostic Performance of EMA and CK-1 in HCC

ROC analysis was used to assess the diagnostic abilities of EMA and CK-1 compared to AFP, and the results are presented in Table 2. According to the findings, the use of EMA per se

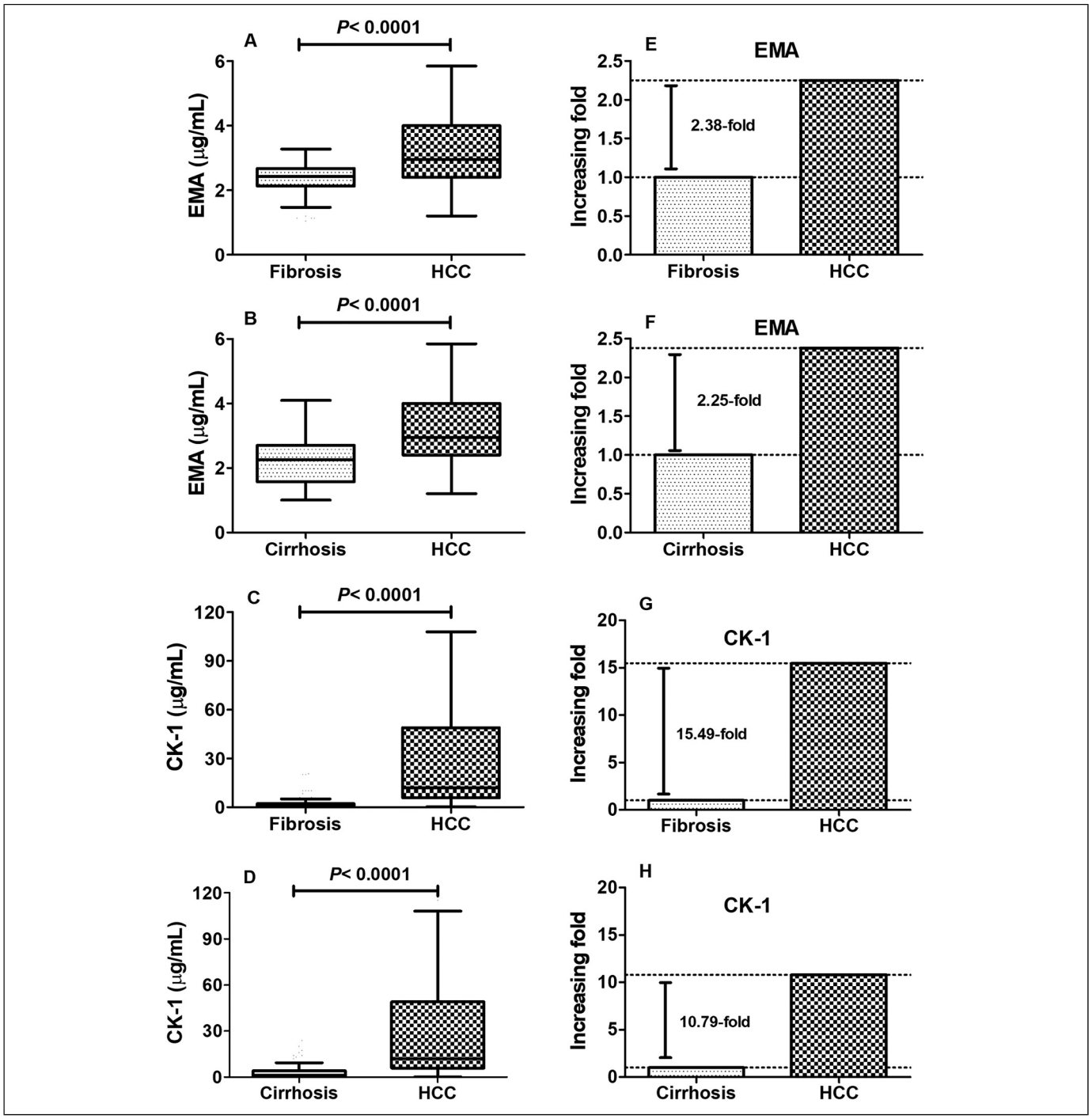

Figure 2. Distribution of epithelial membrane antigen (EMA) and cytokeratin-1 (CK-1) values and their increasing folds in different groups of patients. Whiskers indicate the highest and lowest values, and the line across the box indicates the median value. (A) EMA level in the fibrotic group compared to the HCC group; (B) EMA level in the cirrhotic group compared to the HCC group; (C) CK-1 level in the fibrotic group compared to the HCC group; (D) CK-1 level in the cirrhotic group compared to the HCC group; (E) Increasing fold of EMA in the HCC group versus the fibrotic ones; (F) Increasing fold of EMA in the HCC group versus the cirrhotic ones; (G) Increasing fold of CK-1 in the HCC group versus the fibrotic ones; and (H) Increasing fold of CK-1 in the HCC group versus the cirrhotic ones.

distinguishes between HCC patients and those who developed F1 to F3 and F4 with area under the curves (AUCs) of 0.76 and 0.75, respectively. However, CK-1 per se produced AUCs of 0.90 and 0.87, which were higher than those generated by EMA and AFP to identify HCC patients from those who developed liver F1 to F3 or F4, respectively.

To enhance the diagnostic precision of these biomarkers in detecting HCC, CK-1, EMA, and AFP were combined into a

Table 2. Diagnostic Performances of Different Candidate Markers Compared to AFP and HCC-Check for Predicting HCC.

Variables	Cutoff	AUC (95% CI)	Diagnostic performance (%)		
			Sensitivity (95% CI)	Specificity (95% CI)	χ^2; *P* value [a]
Fibrosis versus HCC					
AFP (ng/mL)	≥400	0.89 (0.85-0.93)	26.9 (19.2-35.8)	99.5 (97.0-100.0)	51.69; <.0001
CK-1 (μg/mL)	>4.55	0.90 (0.86-0.93)	79.0 (70.6-85.9)	84.8 (78.8-89.6)	122.20; <.0001
EMA (μg/mL)	>2.59	0.75 (0.68-0.81)	69.0 (59.8-77.1)	66.7 (59.3-73.5)	36.61; <.0001
HCC-Check[b]	>2.57	0.99 (0.98-1.00)	90.7 (82.9-94.6)	98.4 (95.3-99.7)	246.182; <.0001
Cirrhosis versus HCC					
AFP (ng/mL)	≥400	0.80 (0.75-0.85)	26.9 (19.2-35.8)	99.5 (97.0-100.0)	51.42; <.0001
CK-1 (μg/mL)	>4.55	0.87 (0.82-0.91)	79.0 (70.6-85.9)	81.4 (75.0-86.8)	107.77; <.0001
EMA (μg/mL)	>2.59	0.76 (0.71-0.82)	69.0 (59.8-77.1)	68.5 (61.2-75.1)	40.64; <.0001
HCC-Check[b]	>2.57	0.95 (0.93-0.97)	90.7 (82.9-94.6)	83.9 (77.7-88.9)	159.738; <.0001
Non-HCC (Fibrosis & Cirrhosis) versus HCC					
AFP (ng/mL)	≥400	0.85 (0.80-0.89)	26.9 (19.2-35.8)	99.7 (98.5-100)	95.86; <.0001
CK-1 (μg/mL)	>4.55	0.88 (0.85-0.92)	79.0 (70.6-85.9)	83.1 (78.9-86.8)	158.99; <.0001
EMA (μg/mL)	>2.59	0.75 (0.70-0.81)	69.0 (59.8-77.1)	67.6 (62.5-72.3)	49.32; <.0001
HCC-Check[b]	>2.57	0.97 (0.95-0.98)	90.7 (82.9-94.6)	91.2 (87.8-93.9)	291.171; <.0001

Abbreviations: AUC, area under the curve, AFP, alpha-fetoprotein; CK-1, cytokerain-1; EMA, epithelial membrane antigen; HCC, hepatocellular carcinoma; CI, confidence interval; χ^2, qui square.
[a]$P > 0.05$ is considered non-significant; $P < 0.05$ is considered significant.
[b]HCC-Check: [2.879 + 0.009 × EMA (μg/mL) − 0.236 × Albumin (g/dL) + 0.03 × CK-1 (μg/mL) + 0.299 × Log AFP (ng/mL)].

Table 3. Variables Associated With HCC Existence.

Variables	Univariate analysis		Multivariate analysis	
	P value[a]	AUC (95% CI)	*P* value[a]	Odds ratio (95% CI)
AFP (ng/mL)	<.0001	0.80 (0.75-0.85)	<.0001	4.417 (2.609-7.476)
CK-1 (μg/mL)	<.0001	0.87 (0.82-0.91)	<.0001	1.110 (1.047-1.175)
EMA (μg/mL)	<.0001	0.76 (0.71-0.82)	.021	1.892 (1.103-3.246)
Albumin (g/dL)	<.0001	0.95 (0.93-0.97)	<.0001	0.217 (0.111-0.424)

Abbreviations: AUC, area under the curve; AFP, alpha-fetoprotein; CK-1, cytokerain-1; EMA, epithelial membrane antigen; CI, confidence interval.
[a]$P > 0.05$ is considered non-significant; $P < 0.05$ is considered significant.

single predictive function. Notably, AST, ALP, total bilirubin, and albumin, which were significantly associated with HCC ($P < .05$), were included in the analysis. Albumin emerged as the most efficient marker, yielding an AUC of 0.80. The best formula for predicting HCC, determined through stepwise logistic regression analysis (Table 3), included albumin, AFP, EMA, and CK-1, resulting in the creation of the HCC-Check index: HCC-Check = 2.879 + 0.009 × EMA (μg/mL) − 0.236 × Albumin (g/dL) + 0.03 × CK-1 (μg/mL) + 0.299 × Log AFP (ng/mL). The diagnostic performance of HCC-Check based on the ROC analysis is presented in Table 2. The best cutoff point for HCC-Check and its candidate markers was determined.

The box plots in Figure 3A and B show the comparison of HCC-check levels in HCC patients and those with F1 to F3 or F4. As expected, HCC patients had significantly higher HCC-Check levels ($P < .0001$). The mean (±SD) value of HCC-Check was 3.8 (±1.5) in HCC patients, compared to 2.1 (±0.2) and 2.3 (±0.3) for F1 to F3 and F4, respectively. A strong correlation (Spearman rank correlation coefficient = 0.60, $P < .0001$) was found between HCC-Check and the progression of liver disease. HCC-Check accurately identified HCC, with superior AUCs of 0.99 and 0.95 in patients with F1 to F3 and F4, respectively (Figure 3C and D), surpassing the performance of each marker alone. Notably, HCC-Check values greater than 2.57 increased the probability of the existence of HCC, with an estimated odds ratio (95% CI) of 50.65 (24.24-105.83), indicating a higher susceptibility to developing HCC than those with lower values.

The cross-tabulation of the results of the HCC-Check diagnostic test with the results of the reference standard is presented in Table 4. The most balanced cutoff point was chosen based on ROC analysis, where the sensitivity and specificity are optimized.

Hepatocellular carcinoma patients were grouped according to tumor morphology and the presence of portal vein thrombosis (PVT). Tumor morphology included 4 groups: HCC patients with a single nodule (56.52%), those with multiple

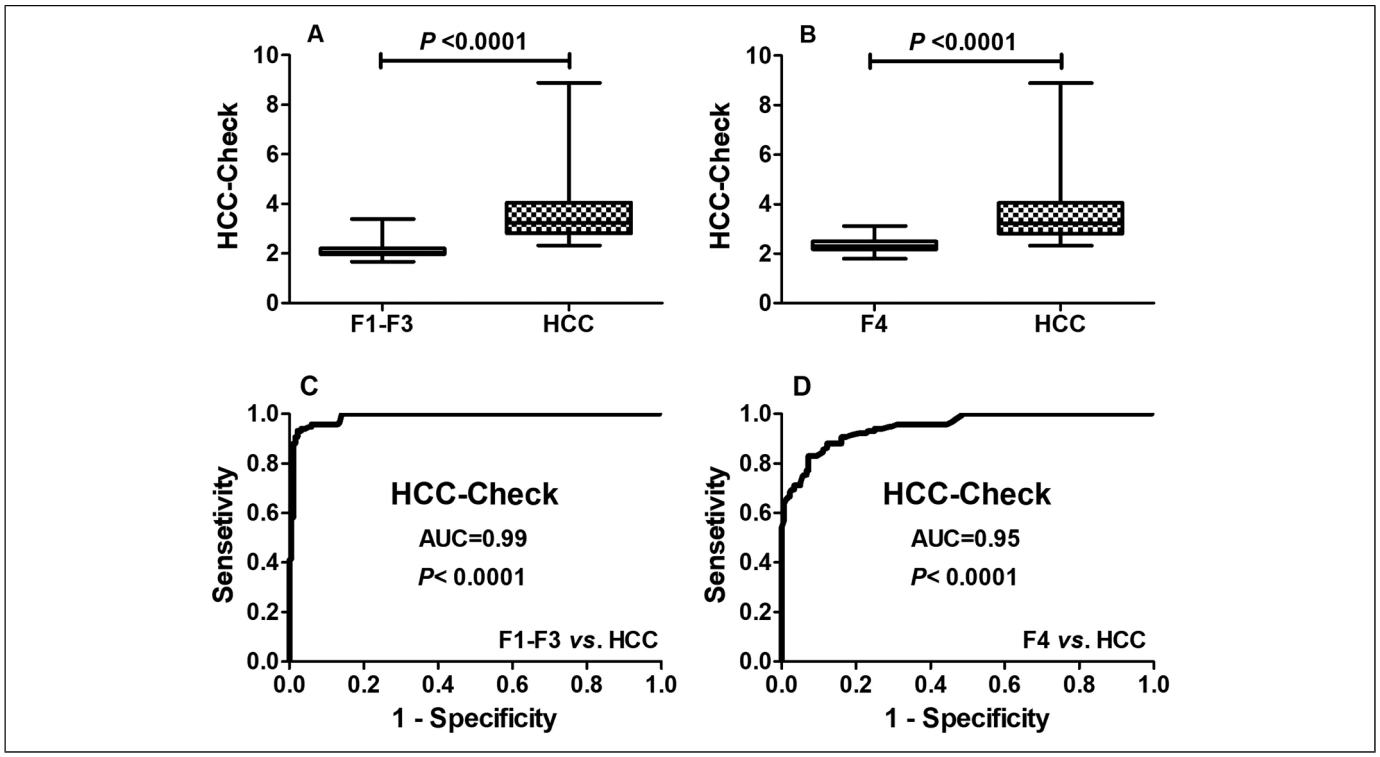

Figure 3. Distribution and diagnostic precision of HCC-check in different groups of patients (A) HCC-check level in the fibrotic group (F1-F3) compared to the HCC group, (B) HCC-check level in the cirrhotic group (F4) compared to the HCC group; area under the curve to discriminate (C) HCC from F1 to F3 and (D) HCC from F4. An AUC of 1.0 is characteristic of an ideal test, whereas an AUC of 0.5 or less indicates a test with no diagnostic value. HCC-Check = [2.879 + 0.009 × EMA (μg/mL) – 0.236 × Albumin (g/dL) + 0.03 × CK-1 (μg/mL) + 0.299 × Log AFP (ng/mL)].

Table 4. Contingency Table From the Study Evaluating the Accuracy HCC-Check for the Diagnosis of Patients With HCC.

	HCC based on computed tomography		
HCC-Check test[a]	Positive HCC	Negative HCC	Total
Positive	108	32	140
Negative	11	335	346
Total	119	367	486

[a]HCC-Check: [2.879 + 0.009 × EMA (μg/mL) – 0.236 × Albumin (g/dL) + 0.03 × CK-1 (μg/mL) + 0.299 × Log AFP (ng/mL)].

nodules (43.48%), those with a nodule size under 5 cm (57.41%), and those with a nodule size of 5 cm or larger (42.59%). Portal vein thrombosis classification included 2 groups: HCC patients with PVT (62.71%) and HCC patients without PVT (37.28%). The diagnostic performance of HCC-Check was compared to AFP for recognizing different types of HCC, demonstrating strong diagnostic utility with higher AUCs in Table 5.

Overall, our findings demonstrated that HCC-Check allowed correct identification of HCC with a single nodule and size <5 cm, producing higher AUCs of 0.92 and 0.85, respectively. Furthermore, HCC-Check yielded a higher AUC of 0.93 for the prediction of patients with HCC without PVT (Figure 4). AFP, with limited diagnostic accuracy for early HCC in clinical practice, exhibited a comparable subpar performance lower than that obtained by HCC-Check, making the diagnosis more reliable (Table 5). The HCC-Check was then applied to a validation cohort comprising 225 patients to test its accuracy and reproducibility (Table 6). The HCC-Check was significantly correlated with liver fibrosis stages ($r = 0.80$, $P < .0001$). The diagnostic power of HCC-Check was assessed in the validation group by the ROC curve showing promising AUCs to identify different stages of HCC, as illustrated in Table 7. It is evident that our findings were reproduced in the validation study with no significant difference.

Discussion

It's worth noting that primary liver cancer, also referred to as HCC, is the most common type of liver cancer in adults.[16] Additionally, it's the primary cause of mortality among individuals with cirrhosis. Risk factors for HCC include chronic liver disease, which can result in cirrhosis and eventually HCC. Unfortunately, HCC is often diagnosed at advanced stages, resulting in limited treatment options and a poor prognosis.[17] Yearly estimates suggest that liver cancer will cause the deaths of over 1 million individuals, with only an 18% 5-year survival rate projected for 2030.[18] As a result, regular examinations are recommended for patients with cirrhosis to detect possible early HCC.[18] Establishing a clear strategy for early diagnosis

Table 5. Diagnostic Performances of HCC-Check Compared to AFP for Discriminating HCC From Cirrhosis Based on Tumor Morphology and Portal Vein Thrombosis.

Variables	Cutoff	Diagnostic performance (%)		
		Sensitivity (95% CI)	Specificity (95% CI)	χ^2; P value [a]
Liver cirrhosis vs HCC (Single nodule)				
AFP (ng/mL)	≥400	14.1 (6.6-25.0)	99.5 (97.0-100.0)	212.843; .024
HCC-Check	>2.57	84.4 (73.1-92.2)	83.9 (77.7-88.9)	98.021; <.0001
Liver cirrhosis vs HCC (Multiple nodules)				
AFP (ng/mL)	≥400	44.9 (30.7-59.8)	99.5 (97.0-100.0)	211.992; .003
HCC-Check	>2.57	97.9 (88.9-100.0)	83.9 (77.7-88.9)	114.119; <.0001
Liver cirrhosis vs HCC (Nodule size <5 cm)				
AFP (ng/mL)	≥400	16.1 (8.1-27.7)	99.5 (97.0-100.0)	26.223; <.0001
HCC-Check	>2.57	71.0 (58.1-81.8)	83.9 (77.7-88.9)	95.085; <.0001
Liver cirrhosis vs HCC (Nodule size ≥5 cm)				
AFP (ng/mL)	≥400	47.8 (32.9-63.1)	99.5 (97.0-100.0)	90.949; <.0001
HCC-Check	>2.57	100 (92.3-100)	83.9 (77.7-88.9)	109.545; <.0001
Liver cirrhosis vs HCC (without PVT)				
AFP (ng/mL)	≥400	20.3 (11.8-31.2)	99.5 (97.0-100.0)	173.874; .021
HCC-Check	>2.57	86.3 (76.3-93.2)	83.9 (77.7-88.9)	137.686; <.0001
Liver cirrhosis vs HCC (with PVT)				
AFP (ng/mL)	≥400	20.3 (11.8-31.2)	99.5 (97.0-100.0)	213.104; .026
HCC-Check[b]	>2.57	95.5 (84.5-99.4)	83.9 (77.7-88.9)	114.408; <.0001
Liver cirrhosis vs HCC (Single nodule & Size <5 cm & without PVT)				
AFP (ng/mL)	≥400	21.8 (13.7-32.0)	99.5 (97.0-100.0)	237.076; .013
HCC-Check[b]	>2.57	89.7 (81.3-95.2)	83.9 (77.7-88.9)	267.000; .437
Liver cirrhosis vs HCC (Multiple nodules & Size ≥5 cm & with PVT)				
AFP (ng/mL)	≥400	28.4 (19.6-38.6)	99.5 (97.0-100.0)	237.102; .013
HCC-Check	>2.57	90.4 (82.6-95.5)	83.9 (77.7-88.9)	274.000; .438

Abbreviations: AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; PVT, portal vein thrombosis; CI, confidence interval.
[a] $P > 0.05$ is considered non-significant; $P < 0.05$ is considered significant; χ^2, qui square.
[b] HCC-Check: $[2.879 + 0.009 \times \text{EMA (μg/mL)} - 0.236 \times \text{Albumin (g/dL)} + 0.03 \times \text{CK-1 (μg/mL)} + 0.299 \times \text{Log AFP (ng/mL)}]$.

is crucial, as curative treatments have higher success rates at this stage. Current research has sparked a discussion on the economic viability of implementing screening procedures for cirrhosis and facilitating early identification of HCC.

It is important to consider that the diagnostic tools used in screening must be simple, affordable, noninvasive, well received by the target population, and have high sensitivity and specificity. The most common screening method currently used in clinical practice is US, although it has a sensitivity of just around 50%, particularly in obese patients and those with severe steatosis, as well as its operator-dependent technique, which can result in variability in image quality and interpretation. The sensitivity to finding an early stage HCC is still around 70%, even when paired with AFP.[19,20] AFP is an additional clinically accessible indicator that shows comparable subpar performance.[21] Research has been conducted on the effectiveness of using AFP as a tumor marker to detect HCC. It was discovered that a serum AFP level of 400 ng/mL marked a significant shift in the biological processes of the tumor and the surrounding noncancerous tissues. Wei *et al.* revealed 4 druggable targets, primarily related to metabolism and immunology, that might be used as therapeutic targets and prognostic indicators.[22] It has been established that a risk factor for HCC is a persistently elevated AFP level, which has been linked to an aggressive histological morphology. According to reports, 30% of patients with HCC continued to be AFP-negative (<20 ng/mL), and several organizations no longer advise using AFP for HCC surveillance. For these reasons, AFP has been combined with other variables in some previous research.[23–25]

When the AFP threshold is set at 400 ng/mL, it has a sensitivity of 32% and a specificity of 99%.[3] However, using AFP as a diagnostic test with a cutoff value of 20 ng/mL has good sensitivity but low specificity. If a higher cutoff value is used, such as 200 ng/mL or more, there is high specificity but low sensitivity.[26] Our study also confirms these findings, as an AFP value of 400 U/L as the cutoff value resulted in a specificity of 99.5%, but the sensitivity decreased to 26.9%. It is worth noting that only about 27% of patients with HCC have elevated serum AFP. This cutoff value was selected due to its frequent association with the diagnosis of HCC. Unfortunately, the results mentioned above indicated the number of patients with HCC who had negative AFP, which subsequently explained why the diagnostic importance of AFP has been questioned and debated.

Patients with HCC can greatly benefit from the identification of new biomarkers that aid in early diagnosis. However, when searching for a new index to detect HCC, a major question arises: how sensitive is it to detect HCC in those who already have it, and how specific is it to exclude HCC when it is not present?

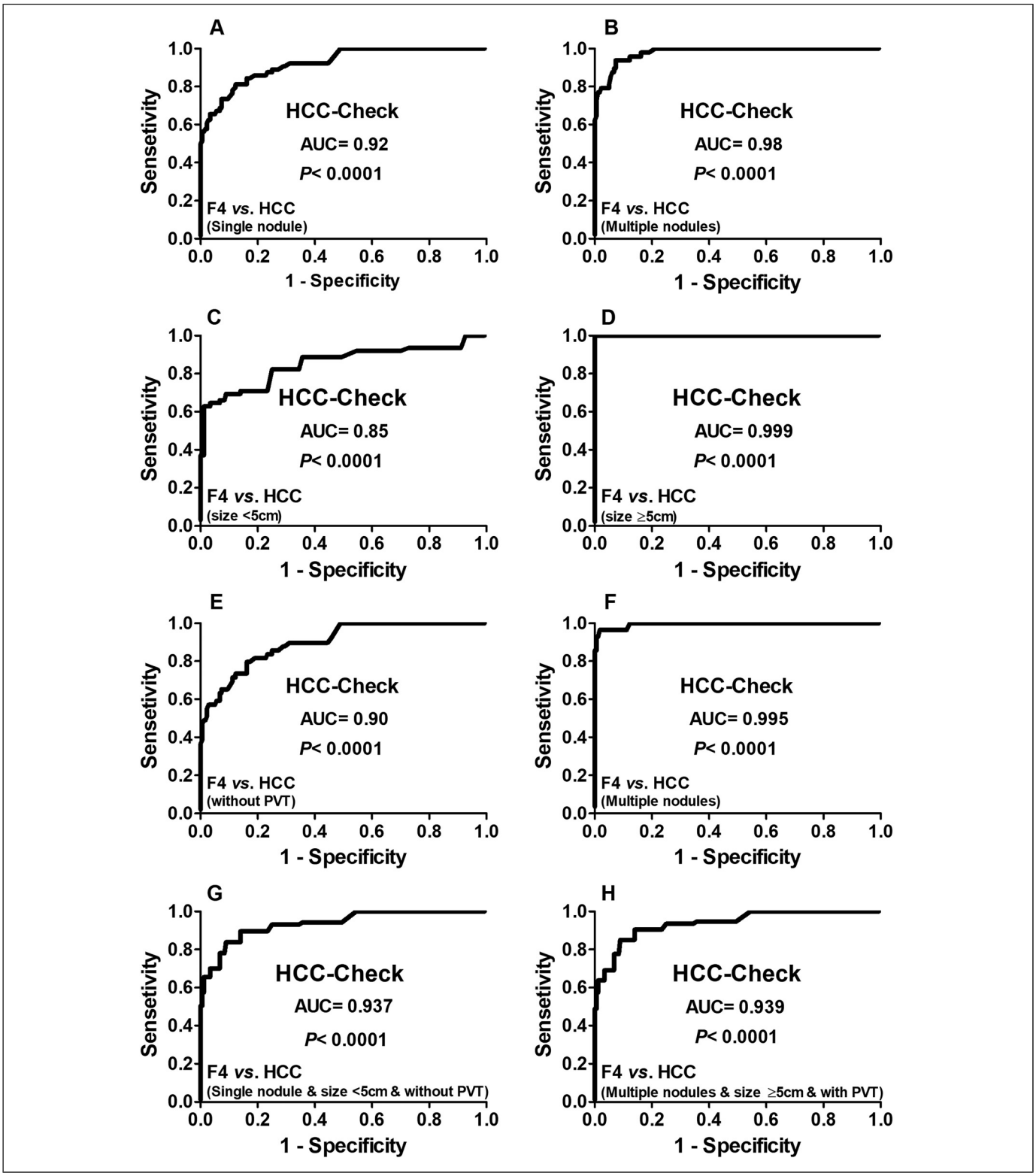

Figure 4. Area under the curve (AUC) showing the diagnostic precision of HCC-check to discriminate HCC from cirrhosis based on tumor morphology and portal vein thrombosis (PVT). An AUC of 1.0 is characteristic of an ideal test, whereas an AUC of 0.5 or less indicates a test with no diagnostic value. (A) HCC with a single nodule versus F4, (B) HCC with multiple nodules versus F4, (C) HCC with a nodule size <5 cm versus F4, (D) HCC with a nodule size ≥5 cm versus F4. (E) HCC without PVT versus F4, (F) HCC with PVT versus F4, (G) HCC with a single nodule, size <5 cm, and without PVT versus F4. (H) HCC with multiple nodules, size ≥5 cm; and with PVT versus F4. HCC-Check = [2.879 + 0.009 × EMA (μg/mL) − 0.236 × Albumin (g/dL) + 0.03 × CK-1 (μg/mL) + 0.299 × Log AFP (ng/mL)].

Table 6. Laboratory Characteristics of All Patients Included in the Validation Study (N = 225).

Variables	Fibrosis (n = 75)	Cirrhosis (n = 75)	HCC (n = 75)	Tukey's post hoc test [a]		
				Fibrosis versus cirrhosis	Fibrosis versus HCC	Cirrhosis versus HCC
Male/female	50/25	52/23	61/14			
Age (years)	43.4 ± 7.5	52.6 ± 10.2	57.9 ± 9.4	<0.0001	<0.0001	0.003
ALT (U/L)	62 ± 32	49 ± 36	53 ± 38	0.091	0.318	0.788
AST (U/L)	53 ± 28	64 ± 41	91 ± 66	0.403	<0.0001	0.003
ALP (U/L)	83 ± 35	118 ± 33	168 ± 154	0.376	0.001	0.084
T. bilirubin (mg/dL)	0.7 ± 0.3	1.4 ± 0.9	2.3 ± 1.5	0.002	<0.0001	<0.0001
Albumin (g/dL)	4.1 ± 0.2	3.3 ± 0.4	3.0 ± 0.5	<0.0001	<0.0001	<0.0001
Platelets ($\times 10^9$/L)	195.3 ± 56.3	121.8 ± 69.7	131.7 ± 97.2	<0.0001	<0.0001	0.770
AFP (ng/mL)	10.8 ± 47.3	7.7 ± 9.8	909.2 ± 3297.5	0.999	0.012	0.011
Log AFP (ng/mL)	0.48 ± 0.50	0.63 ± 0.46	1.98 ± 1.07	0.408	<0.0001	<0.0001
EMA (μg/mL)	2.4 ± 0.4	2.2 ± 0.7	5.3 ± 7.5	0.977	<0.0001	<0.0001
CK-1 (μg/mL)	2.4 ± 3.7	2.7 ± 4.7	37.0 ± 48.9	0.997	<0.0001	<0.0001

Abbreviations: HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; AFP, alpha-fetoprotein; CK-1, cytokerain-1; EMA, epithelial membrane antigen.
The data were presented as the mean ± standard deviation. Reference values: HCC and ALT (up to 45 U/L), AST (up to 40 U/L), ALP (22-92 U/L), total bilirubin (up to 1 mg/dL), albumin (3.8-5.4 g/dL), platelet counts [(150-400 ($\times 10^9$/L)], AFP, alpha-fetoprotein (up to 10 U/L).
[a] $P > 0.05$ is considered non-significant; $P < 0.05$ is considered significant.

Table 7. Validation of HCC-Check for Identifying Different Categories of HCC.

Variables	AUC (95% CI), *P* value [a]	Diagnostic performance (%)	
		Sensitivity (95% CI)	Specificity (95% CI)
Fibrosis versus HCC			
HCC-Check[b]	0.97 (0.95-1.00), <.0001	89.3 (80.1-95.3)	96.0 (88.8-99.2)
Cirrhosis versus HCC			
HCC-Check[b]	0.93 (0.89-0.97), <.0001	89.3 (80.1-95.3)	85.3 (75.3-92.4)
Non-HCC (Fibrosis & Cirrhosis) versus HCC			
HCC-Check[b]	0.95 (0.92-0.98), <.0001	89.3 (80.1-95.3)	90.7 (84.8-94.8)
Liver cirrhosis vs HCC (Single nodule)			
HCC-Check[b]	0.89 (0.82-0.96), <.0001	81.82 (67.3-91.8)	85.3 (75.3-92.4)
Liver cirrhosis vs HCC (Multiple nodules)			
HCC-Check[b]	0.99 (0.98-1.00), <.0001	100.0 (88.8-100.0)	85.3 (75.3-92.4)
Liver cirrhosis vs HCC (Nodule size <5 cm)			
HCC-Check[b]	0.89 (0.82-0.96), <.0001	81.4 (66.6-91.6)	85.3 (75.3-92.4)
Liver cirrhosis vs HCC (Nodule size ≥5 cm)			
HCC-Check[b]	0.99 (0.98-1.00), <.0001	100.0 (88.8-100.0)	85.3 (75.3-92.4)
Liver cirrhosis vs HCC (without PVT)			
HCC-Check[b]	0.89 (0.83-0.96), <.0001	83.0 (69.2-92.4)	85.3 (75.3-92.4)
Liver cirrhosis vs HCC (with PVT)			
HCC-Check[b]	0.99 (0.99-1.00), <.0001	100.0 (87.7-100.0)	85.3 (75.3-92.4)
Liver cirrhosis vs HCC (Single nodule & Size <5 cm & without PVT)			
HCC-Check[b]	0.92 (0.87-0.97), <.0001	87.5 (76.9-94.5)	85.3 (75.3-92.4)
Liver cirrhosis vs HCC (Multiple nodules & Size ≥5 cm & with PVT)			
HCC-Check[b]	0.92 (0.87-0.97), <.0001	87.1 (76.2-94.3)	85.3 (75.3-92.4)

Abbreviations: AUC, area under the curve; HCC, hepatocellular carcinoma; PVT, portal vein thrombosis; CI: confidence intervals.
[a] $P > 0.05$ is considered non-significant; $P < 0.05$ is considered significant. Diagnostic performances of HCC-Check were assessed at a cutoff greater than 2.57.
[b] HCC-Check: [2.879 + 0.009 × EMA (μg/mL) − 0.236 × Albumin (g/dL) + 0.03 × CK-1 (μg/mL) + 0.299 × Log AFP (ng/mL)].

In some cancer studies, EMA and CK-1 were used as serum tumor markers to keep track of the progression of the disease in patients.[27,28] EMA is a glycoprotein that is present in the membranes of mucin and is widely expressed in epithelial cells. The expression of EMA increases in carcinomas.[19] Therefore, EMA, along with keratins, can be an effective diagnostic marker for carcinoma. Furthermore, EMA can sometimes be considered in the differential diagnosis of soft tissue tumors.[29] In a study in which hepatoma cells and activated liver stellate cells were cultured together, it was discovered

that EMA was increased. Additionally, clinical studies have shown that there is a correlation between EMA expression and a negative prognosis in several types of cancer, including HCC.[30] Cytokeratins are the main filament proteins in the liver, where damage to the integrity of the hepatocyte membrane causes their release into the blood circulation. The main function of these filament proteins is to enable cells to withstand mechanical stress. Cytokeratins have been reported to be particularly useful tools for cancer diagnostics, as they reflect tumor cell activity.[27] Therefore, by following patients with the CK marker in combination with other markers that reflect tumor burden, the oncologist can obtain critical details about tumor growth activity, especially if the tumor has already been clinically confirmed. This was the aim of this work: to combine CK-1 and EMA with one of the oncofetal proteins to increase the diagnostic rate for the early detection of HCC.

This study aimed to identify and measure the presence of CK-1 and EMA in the blood of patients. Those with HCC had higher levels of these biomarkers compared to those without HCC (including those with F1-F3 and F4). The use of a single biomarker for early detection of HCC had varied results in terms of sensitivity and specificity, indicating that it was not sufficient for an accurate diagnosis. However, when combined with routine AFP and albumin tests, CK-1 and EMA showed increased early diagnostic rates, which led to the creation of the HCC-Check index. Patients with HCC had significantly higher levels of HCC-Check compared to those with F1 to F3, or F4.

We evaluated the effectiveness of the HCC-Check in diagnosing patients with HCC through ROC curve analysis. Our index was successful in accurately identifying patients with HCC as opposed to those with F1 to F3 and F4, yielding AUCs of 0.99 and 0.95, respectively. Other notable HCC biomarkers, such as PIVKA-II and GPC3, have shown promise in previous studies and have demonstrated good diagnostic efficacy.[18] Interestingly, our findings yielded AUCs higher than those produced by either glypican-3 per se (AUC = 0.779) or its combination with AFP (AUC = 0.936) for identifying HCC or PIVKII which yielded lower AUC of 0.90 for discriminating HCC from F4.[31,32]

At a cutoff value higher than 2.57, the HCC-Check had a sensitivity of 90.7% for diagnosing HCC. Additionally, the HCC-Check demonstrated 98.4% specificity in distinguishing HCC from fibrotic patients and 83.9% specificity in distinguishing HCC from cirrhotic patients. At this cutoff, the HCC-Check had a higher negative predictive value of 93.2%, meaning only 6.79% of patients with an HCC-Check lower than 2.57 would be misdiagnosed. Compared to AFP alone, which had a sensitivity of 26.9%, the HCC-Check had a sensitivity of 90.7% in identifying patients with HCC. The AUC achieved in this study was comparable to the one generated by the GALAD score, which takes into account gender, age, AFP-L3, AFP, and descarboxy prothrombin.[28] However, it was relatively higher than the AUC produced by the HMC-CU score, a recently published method that considers age, hemoglobin, INR, albumin, AFP, and gender.[33]

It is a known fact that early diagnosis and immediate treatment can positively impact long-term outcomes for patients. Unfortunately, most patients with HCC are diagnosed at advanced stages of the disease, leading to limited treatment options. Like any cancer, the treatment and prognosis of HCC depend on various factors, such as tumor histology, size, spread, liver function, and detection time. As a result, this study also used ROC curve analysis to assess HCC-Check's diagnostic ability in identifying different clinical statuses of HCC based on PVT and tumor morphology to detect early HCC. HCC-Check was found to have better AUCs of 0.92 and 0.98 in discriminating between HCC patients who had a single nodule or multiple nodules among cirrhotic patients. Interestingly, HCC-Check produced AUCs of 0.85 and 0.996 to identify HCC patients who had nodules smaller than 5 cm and nodules ≥5 cm, respectively.

Surprisingly, our findings showed that HCC-Check allowed the correct identification of HCC with a single nodule with a size of less than 5 cm, producing an AUC of 0.92. All these findings may indicate the ability of our developed index to identify not only HCC in general but also the early stages of this type of cancer. Moreover, these results were reproduced in the validation study with no significant difference.

Generally, one of the limitations of cross-sectional studies is the use of convenience samples that may not be entirely representative of the population. Consequently, the results of this study might not be extrapolated to the general population. Researchers must employ suitable sampling techniques and guarantee that the study sample is representative of the population of interest if they are to generalize about their results. Another limitation is the relatively small number of patients included in this work. The financial burden of reagents, diagnostic procedures, radiological CT, MRI, and pathological examinations contributes to the issue at hand. To validate the usefulness of the index in clinical practice and reduce potential bias, it is necessary to conduct more prospective multicenter studies with a larger number of patients. This will increase the precision of the results.

Conclusions

In terms of improving patient outcomes and individualized treatment plans, the HCC-Check diagnostic test may exhibit promise in the field of diagnostic medicine. Our study is hampered by its observational design and perhaps inadequate statistical analysis, even though it shows positive results in precision and early illness diagnosis. To validate these early findings, further research with larger sample sizes is necessary.

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Authors' Note

The 1975 Helsinki Declaration's ethical principles were followed by the study protocol. The study protocol and informed consent were both approved by the research ethics committee, Faculty of Pharmacy, Cairo University, Cairo, Egypt (BC 2912) on 25/01/2021.

Authors' Contribution

All authors have made a substantial, direct, and intellectual contribution to the work. This work is a part of KAA's PhD thesis, Faculty of Pharmacy, Cairo University. NF, KF, and SMR were involved in the supervision of the study. MSA was an internal supervisor at the Biotechnology Research Center. All authors contributed to the final manuscript.

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