

Direct comparison of five serum biomarkers in early diagnosis of hepatocellular carcinoma

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Background: Although a number of serum biomarkers for detection of hepatocellular carcinoma (HCC) have been explored, their exact diagnostic value remains unclear. We aimed to conduct a direct comparison of five representative serum biomarkers for detecting HCC and to derive multi-marker prediction algorithms.

Patients and methods: In total, 846 patients were recruited from three hospitals in China, including 202 HCC patients, 226 liver cirrhosis patients, 215 chronic hepatitis B virus-infected patients, and 203 healthy volunteers. Serum levels of alpha-fetoprotein (AFP), lens culinaris agglutinin-reactive AFP (AFP-L3), des-gamma-carboxyprothrombin (DCP), squamous cell carcinoma antigen, and centromere protein F autoantibody were measured by ELISA. The diagnostic performances of individual biomarkers and multi-marker combinations were evaluated by receiver operating characteristics analysis. The bootstrapping method was adopted to adjust for potential overfitting of all diagnostic indicators.

Results: DCP exhibited the best diagnostic performance, with areas under the curve (AUC) for detecting HCC of 0.82 (95% CI 0.64–0.80) and sensitivity of 65.2% (95% CI 63.3–82.1%) at 90% specificity. Of note, DCP showed similar diagnostic efficacy for detecting AFP-positive and AFP-negative HCC. After a comprehensive search for multi-marker combinations, a two-marker prediction algorithm including AFP and DCP was constructed and yielded an AUC of 0.87 (95% CI 0.68–0.84) for detecting HCC. In addition, the combination showed good ability in discriminating early-stage HCC and decompensated liver cirrhosis, with an AUC of 0.81 (95% CI 0.75–0.86).

Conclusion: DCP could be a complementary biomarker in the early diagnosis of HCC. The constructed multi-marker prediction algorithms could contribute toward distinguishing HCC from non-malignant chronic liver diseases.

Keywords: early detection, liver cirrhosis, prediction model

Introduction

Liver cancer is the sixth most commonly diagnosed cancer worldwide and the second leading cause of cancer-related death.^{1,2} Hepatocellular carcinoma (HCC) accounts for 70–90% of all new liver cancer cases, largely in association with chronic hepatitis B virus (HBV) infection.^{1–4} Stage at diagnosis is the most important prognostic factor, with a 5-year overall survival rate of 50–70% at early stages and less than 5% at advanced stages. Therefore, diagnosis of HCC at a curative stage and surveillance of non-malignant chronic liver diseases carrying a risk of HCC are key to improving the poor prognosis of HCC.

Abdominal ultrasonography is the main recommended tool in HCC diagnosis and surveillance.^{3,5} However, abdominal ultrasonography presents rather limited sensitivity

in detecting HCC and is further hampered by inter- and intra-observer variability.⁶ Alpha-fetoprotein (AFP) is currently the best established serum biomarker for the diagnosis of HCC.^{7,8} However, the diagnostic performance of AFP in detecting HCC is suboptimal, with sensitivities of 41–65% and specificities of 80–90% at a commonly used cutoff value of 20 ng/mL.^{9,10} In particular, much lower sensitivity was observed in detecting early-stage HCC and smaller tumors.⁹ Therefore, the identification of effective and reliable non-invasive biomarkers for diagnosis and surveillance of HCC is urgently needed and is of high clinical and public health relevance.

To date, a number of serum biomarkers carrying diagnostic potential for detecting HCC have been identified,^{11,12} such as lens culinaris agglutinin-reactive AFP (AFP-L3),^{13,14} des-gamma carboxyprothrombin (DCP),^{15,16} squamous cell carcinoma antigen (SCCA),^{17,18} and centromere protein F autoantibody (anti-CENPF).¹⁹ Although these biomarkers have been evaluated in several studies, the diagnostic performance varied greatly across studies, and evidence of a direct comparison of the diagnostic performance of multiple biomarkers in the same population is sparse, especially for Chinese populations.^{7,8} In addition, promising results were commonly reported in some studies which tried to combine different biomarkers and constructed multi-marker prediction models.^{7,8,14} However, some results should be interpreted with caution because of a lack of validation (lacking either the use of external independent validation populations or adoption of appropriate internal validation methods), which would lead to significant overfitting regarding the diagnostic performance.

In the present study, we therefore selected five representative biomarkers (AFP, AFP-L3, DCP, SCCA, and anti-CENPF) with diagnostic potential for detecting HCC. Serum levels of the five biomarkers were simultaneously measured in a large sample set (N=846) comprising patients with HCC, liver cirrhosis, chronic HBV infection, and healthy controls, recruited in three hospitals in China. We aimed to conduct a direct comparison of the diagnostic performance of the five biomarkers for detecting HCC and to derive and validate multi-marker algorithms carrying good diagnostic potential in distinguishing HCC from non-malignant chronic liver diseases.

Patients and methods

Study design and population

This study was conducted based on a cross-sectional design. Eight hundred eighty-four eligible participants were consecutively recruited from three hospitals in China (Cancer Hospital of Chinese Academy of Medical Science, Beijing Youan Hospital, and Beijing Friendship Hospital) from November

2013 to December 2014, including 202 HCC patients, 226 patients with liver cirrhosis, 215 patients with chronic HBV infection, and 203 healthy volunteers.

HCC was diagnosed according to the Chinese guidelines of diagnosis and treatment for HCC (2012 version, China).²⁰ Liver cirrhosis was diagnosed according to the guidelines of prevention and treatment for chronic hepatitis jointly proposed by the Chinese Society of Hepatology and the Chinese Society of Infectious Diseases (2015, China),²¹ and the following diagnostic criteria were adopted: 1) histological or clinical evidence of liver cirrhosis, including definite chronic hepatitis based on clinical manifestation of portal hypertension, diagnosis of liver cirrhosis indicated by ultrasound, and/or computed tomography (CT); 2) evidence of HBV infection; and 3) for patients without histological evidence, at least two of the following criteria should be met: i) presentation of liver cirrhosis changes indicated by imaging techniques such as ultrasound and CT; ii) platelets $<100 \times 10^9/L$; iii) serum albumin <35 g/L or prothrombin time >1.3 second; iv) gastroesophageal varices diagnosed by gastroscopy; and v) fibroscan >12.4 kPa (alanine transaminase $<5 \times$ upper limit of normal). Chronic HBV infection was diagnosed according to the guidelines of the prevention and treatment for chronic hepatitis (2015, China).²¹ Healthy volunteers were outpatients with normal liver biochemistry, no history of liver disease, and no malignant disease. Patients with heart, lung, or kidney diseases were excluded from the study.

All participants were recruited before receiving any radiotherapy, chemotherapy, or operative treatment and had no other malignant diseases. The tumor stages of HCC patients were defined according to the Union for International Cancer Control (UICC) TNM classification (7th version). TNM stage I was defined as early-stage HCC in our analyses.

The study was approved by the ethics committees of the respective hospitals (Ethics Committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, the Clinical Research Ethics Committee of Beijing Friendship Hospital, Capital Medical University, and the Ethics Committee of Beijing Youan Hospital of Capital Medical University). Written informed consent was obtained from each participant.

Laboratory analysis

Preparation of serum samples

Blood samples were collected from each participant at the hospital visit. After completion of blood clotting, blood samples were immediately centrifuged at 2000–2500 g for 10 min and stored at -80°C until analyses.

AFP

A commercial ELISA kit was used (CanAg, Fujirebio Diagnostics, Göteborg, Sweden). The test was conducted as per the instructions of the kit. In brief, 100 μ L antibody solution and 25 μ L AFP calibrators were added to each well. Serum samples were then added into the strip wells. The wells were incubated for 1 h at room temperature, and then, each strip was washed six times, followed by the addition of 100 μ L tetramethylbenzidine (TMB) horseradish peroxidase (HRP) substrate for 30 min at room temperature. The absorbance at 620 nm was read on the microplate reader SpectraMax M3 (Molecular Devices LLC, Sunnyvale, CA, USA).

SCCA

Serum SCCA levels were determined using the SCCA detection kit (Xeptagen, Marghera, Italy). The test was conducted as per the instructions of the kit. In brief, 100 μ L was dispensed per well of standard solution starting from 8 ng/mL and performing in-plate twofold serial dilutions to a final concentration of 0.5 ng/mL. Then, 100 μ L of 1:8 diluted samples was added in duplicate with 100 μ L dilution buffer as a blank. Samples were incubated for 1 h at room temperature and washed six times. Following this, 100 μ L of diluted enzyme-conjugated streptavidin solution was added and incubated for 1 h at room temperature, then washed six times. Then, 100 μ L chromogen solution per well was applied. The color was allowed to develop for 10–15 min at room temperature in the dark and then stopped by adding 100 μ L of stop solution. The optical density values of each well were measured at 450 nm on the microplate reader SpectraMax M3 (Molecular Devices LLC).

AFP-L3

Serum AFP-L3 levels were determined using the AFP-L3 detection kit (Beijing Re-jing Biotechnology Company, Beijing, China). In brief, 400 μ L of serum sample was pipetted and mixed with 600 μ L washing buffer. A column was inserted in a collection tube and centrifuged to equilibrate the column. After this, 600 μ L of diluted serum sample was transferred to the column and left to stand at room temperature for 10 min. Then, 1200 μ L of washing buffer was added to the column and centrifuged at 3000 rpm for 20 s at room temperature. The flowthrough was discarded and 600 μ L elution buffer was added to the column and centrifuged at 3000 rpm for 20 s. Serum AFP-L3 was detected in the collection tube and the ratio of AFP-L3 was calculated using the predefined formula.

DCP

A commercial Lumipulse protein induced by vitamin K absence/antagonist II (PIVKA-II) detection kit (Fujirebio, Tokyo, Japan) was used to determine DCP levels. The test was conducted as per the instructions of the kit. Briefly, the magnetic particle in each test tube was combined with PIVKA-II monoclonal antibody and antiprothrombin polyclone antibody labeled by alkaline phosphatase. The Lumipulse G PIVKA-II calibration solution and washing buffer were prepared. The LUMIPULSE G1200 immunochemistry system was used for detection of DCP using a 100 μ L serum sample (Fujirebio, Japan).

CENPF autoantibody

Serum autoantibody to CENPF was determined using an in-house CENPF ELISA kit. In brief, 96-well microplates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) were coated with 100 μ L CENPF antigen (8 μ g/mL) and incubated at 4°C overnight. The reaction was blocked with 10% newborn bovine sera (Life Technologies & Invitrogen Corporation, Burlington, Canada). Serum diluted with 10% newborn bovine sera was incubated for 1 h at 37°C. The samples were washed five times, followed by the addition of 100 μ L 1:8000 dilution of anti-human immunoglobulin G-peroxidase antibody produced in rabbit (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C. Then, 100 μ L of TMB HRP-Substrate (Solarbio Science & Technology, Beijing, China) was added for 10 min at 37°C, and the reaction was stopped by adding 50 μ L of stop solution (Solarbio Science & Technology, Beijing, China). The absorbance was read immediately at 450 nm or 620 nm on the microplate reader SpectraMax M3 (Molecular Devices).

Statistical analysis

Study population characteristics were first described and compared between different study groups. The differences in serum levels of the five biomarkers between different groups were examined by chi-square tests. For evaluation of the diagnostic performance of biomarkers, the following diagnosis-related indicators were used: sensitivity (true-positive rate), specificity (true-negative rate), and the area under the receiver operating characteristics (ROC) curve (AUC). For each individual biomarker, a logistic regression model using the original test values was adopted to construct the prediction model. Based on the predicted probabilities derived from the prediction models, AUCs and 95% CIs (calculated based on 2000 bootstrap samples) were calculated and reported,

and the differences in AUCs of individual biomarkers were examined by the bootstrap method (2000 bootstrap samples). Moreover, sensitivities of each individual biomarker at cut-offs yielding 90% specificity were calculated. In addition to the apparent estimates of indicators, the .632+ bootstrap method (1000 bootstrap samples with replacement) was applied to adjust for potential overestimation of diagnostic performance.²²

Multi-marker algorithms were further explored using logistic regression models based on different biomarker combinations. ROC analyses were further conducted using the approach described above. All the diagnostic-related indicators (e.g., sensitivity, specificity, and AUC) were further adjusted for potential overestimation using the .632+ bootstrap method. The differences in AUCs for different multi-marker algorithms were examined by the bootstrap method (2000 bootstrap samples).

Statistical analyses were performed with the statistical software R version 3.3.2.²³ R package “pROC” was employed to perform the ROC analysis. R package “Daim” was used to conduct the .632+ bootstrap analyses. All tests were two sided and *p*-values of 0.05 or less were considered to be statistically significant.

Results

Overall, 846 participants were recruited in this study, including 202 HCC patients, 226 patients with liver cirrhosis, 215 patients with chronic HBV infection, and 203 healthy volunteers. The characteristics of the study population are shown in Table 1 and Table S1. Overall, more men and older patients were included in the HCC, liver cirrhosis, and chronic HBV infection groups compared to the healthy control group. It was found that 17.3% and 6.9% of the patients in the HCC group and 18.6% and 8.9% of the patients in the liver cirrhosis group also carried HBV infection and hepatitis C virus (HCV) infection, respectively. For the HCC group, 46.5% of the patients were diagnosed at TNM stage I, and 93.6% of the patients had no metastasis. For the liver cirrhosis group, 40.2% (n=91), 29.2% (n=66), and 16.8% (n=38) of the patients had a Child–Pugh score of A, B, and C, respectively. Moreover, 77.9% (n=176) and 22.1% (n=50) of the liver cirrhosis patients were at the decompensated and compensated stage, respectively.

Table S2 presents the associations between the clinicopathological factors and the serum levels of five individual markers in HCC patients. Apart from SCCA, the other four markers, AFP, AFP-L3, DCP, and CENPF, showed

Table 1 Study population characteristics

Characteristics	HCC (n=202)	CHB (n=215)	LC (n=226)	HC (n=203)
Age (years), mean±SD	57.2±11.5	39.2±11.8	50.5±10.4	48.2±11.0
Gender, n (%)				
Male	170 (84.2)	134 (62.3)	161 (71.2)	97 (47.8)
Female	32 (15.8)	80 (37.2)	65 (28.8)	106 (52.2)
Missing	–	1 (0.5)	–	–
HBV infection, n (%)				
HBV+	132 (65.3)	215 (100.0)	184 (81.4)	0 (0)
HBV–	27 (13.3)	0 (0.0)	6 (2.7)	203 (100.0)
Missing	43 (21.3)	0 (0.0)	36 (15.9)	0 (0)
HCV infection, n (%)				
HCV+	14 (6.9)	0 (0)	2 (8.9)	0 (0)
HCV–	133 (65.8)	31 (14.4)	100 (44.2)	203 (100.0)
Missing	55 (27.2)	184 (85.6)	124 (54.9)	0 (0)
TNM tumor stage, n (%)				
I	94 (46.5)	–	–	–
>I	97 (48.0)	–	–	–
Missing	11 (5.4)	–	–	–
Child–Pugh, n (%)				
A	–	–	91 (40.2)	–
B	–	–	66 (29.2)	–
C	–	–	38 (16.8)	–
Missing	–	–	31 (13.7)	–
Metastasis, n (%)				
Yes	13 (6.4)	–	–	–
No	189 (93.6)	–	–	–

Note: “–” = not applicable.

Abbreviations: CHB, chronic hepatitis B virus infection; HBV, hepatitis B virus; HC, healthy control; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, liver cirrhosis; TNM, tumor-node-metastasis.

significantly higher serum levels in advanced-stage HCCs (TNM stage >I) than in early-stage HCCs (TNM stage I). Other factors, including age, gender, HBV antigens (HBsAg and HBeAg), and HCV infection, were found to have no association with serum levels of the five biomarkers (all p -values < 0.05).

We conducted a direct comparison of the diagnostic performance of the five individual biomarkers in detecting HCC. ROC analyses were conducted based on the logistic regression model and the AUC values of each biomarker in predicting the presence of HCC were calculated. To minimize the potential overestimation, all the diagnostic indicators were further adjusted using the .632+ bootstrap method. (Comparisons between the apparent and .632+ adjusted ROC curves for the five individual biomarkers in discriminating HCC with controls are shown in Figures S2–S6.) The AUCs decreased slightly after adjusting the potential overfitting by the .632+ bootstrap method. For instance, the apparent and .632+ adjusted AUCs (95% CIs) of AFP for discriminating early-stage HCC with liver cirrhosis were 0.74 (0.67–0.79) and 0.62 (0.60–0.77), respectively. Detailed results regarding the comparison of the diagnostic performance of the five biomarkers for detecting HCC are shown in Table 2, Figure 1 and [Figure S1](#). Overall, four of the biomarkers (AFP, AFP-L3,

DCP, and CENPF; but not SCCA) presented diagnostic potential in detecting HCC. DCP presented the highest diagnostic value for discriminating HCC versus controls, with a .632+ adjusted AUC of 0.82 (95% CI 0.64–0.80), which is statistically significantly higher than the traditional biomarker AFP (AUC=0.72, 95% CI 0.70–0.82) ($p=0.045$). When defining cutoffs to yield 90% specificity, the sensitivities of DCP, AFP, AFP-L3, and CENPF in detecting HCC were 65.2% (95% CI 63.3–82.1%), 43.7% (95% CI 36.4–62.3%), 42.6% (95% CI 33.3–53.8%), and 20.4% (95% CI 10.8–42.6%), respectively. When restricting the outcome to early-stage HCC only, the diagnostic performance of all five biomarkers in terms of AUC decreased slightly, but DCP still carried the best diagnostic value, with an AUC of 0.74 (95% CI 0.68–0.84) and sensitivity of 51.0% (95% CI 40.0–75.0%) at 90% specificity. Notably, DCP also presented diagnostic potential in discriminating early-stage HCC versus liver cirrhosis, with an AUC of 0.73 (95% CI 0.70–0.85).

We further examined the differences in diagnostic value of the four biomarkers in detecting AFP-positive or AFP-negative patients with HCC, and the results are shown in Table 3. At the commonly used positivity threshold of 20 ng/mL, 99 of the 202 HCC patients (49.0%) were defined as AFP positive, including 38 early-HCC cases (18.8%).

Table 2 Direct comparison of the diagnostic performance of five markers in detecting HCC

AFP		AFP-L3		DCP		SCCA		CENPF	
AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b
(a) HCC vs LC, CHB, and HC									
0.72 (0.70–0.82)	43.7 (36.4–62.3)	0.67 (0.64–0.75)	42.6 (33.3–53.8)	0.82 (0.64–0.80)	65.2 (63.3–82.1)	0.48 (0.40–0.59)	7.5 (0–24.2)	0.65 (0.62–0.77)	20.4 (10.8–42.6)
(b) HCC vs LC and CHB									
0.68 (0.67–0.79)	38.8 (29.4–50.0)	0.65 (0.62–0.73)	39.1 (28.3–52.8)	0.80 (0.62–0.80)	64.1 (59.7–80.3)	0.47 (0.35–0.60)	8.8 (0–31.0)	0.53 (0.44–0.68)	14.0 (0–29.0)
(c) HCC vs LC									
0.67 (0.68–0.80)	40.1 (31.8–57.1)	0.65 (0.62–0.74)	40.5 (30.0–53.3)	0.78 (0.64–0.81)	57.2 (48.6–75.9)	0.47 (0.35–0.60)	8.8 (0–31.0)	0.52 (0.33–0.73)	11.8 (0–28.6)
(d) Early-stage HCC vs LC, CHB, and HC									
0.64 (0.60–0.78)	36.5 (24.2–57.6)	0.56 (0.51–0.67)	23.4 (7.7–39.2)	0.74 (0.68–0.84)	51.0 (40.0–75.0)	0.48 (0.36–0.61)	8.0 (0–24.1)	0.45 (0.34–0.59)	6.8 (0–18.2)
(e) Early-stage HCC vs LC and CHB									
0.61 (0.59–0.75)	30.2 (18.8–43.8)	0.54 (0.50–0.66)	22.1 (9.5–38.1)	0.72 (0.65–0.83)	45.5 (38.9–72.4)	0.47 (0.37–0.61)	7.0 (0–30.8)	0.52 (0.33–0.73)	11.8 (0–28.6)
(f) Early-stage HCC vs LC									
0.62 (0.60–0.77)	30.6 (21.6–53.1)	0.55 (0.49–0.67)	22.8 (8.3–40.0)	0.73 (0.70–0.85)	48.3 (41.9–76.5)	0.48 (0.36–0.61)	8.0 (0–24.1)	0.45 (0.34–0.59)	6.8 (0–18.2)

Notes: ^aAUC was adjusted for potential overfitting by the .632+ bootstrap method. ^b.632+ bootstrap adjusted sensitivity at cutoffs yielding 90% specificity.

Abbreviations: AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive AFP; AUC, area under the curve; CENPF, centromere protein F autoantibody; CHB, chronic hepatitis B virus infection; DCP, des-gamma-carboxyprothrombin; HC, healthy control; HCC, hepatocellular carcinoma; LC, liver cirrhosis; SCCA, squamous cell carcinoma antigen; SEN, sensitivity; SPE, specificity.

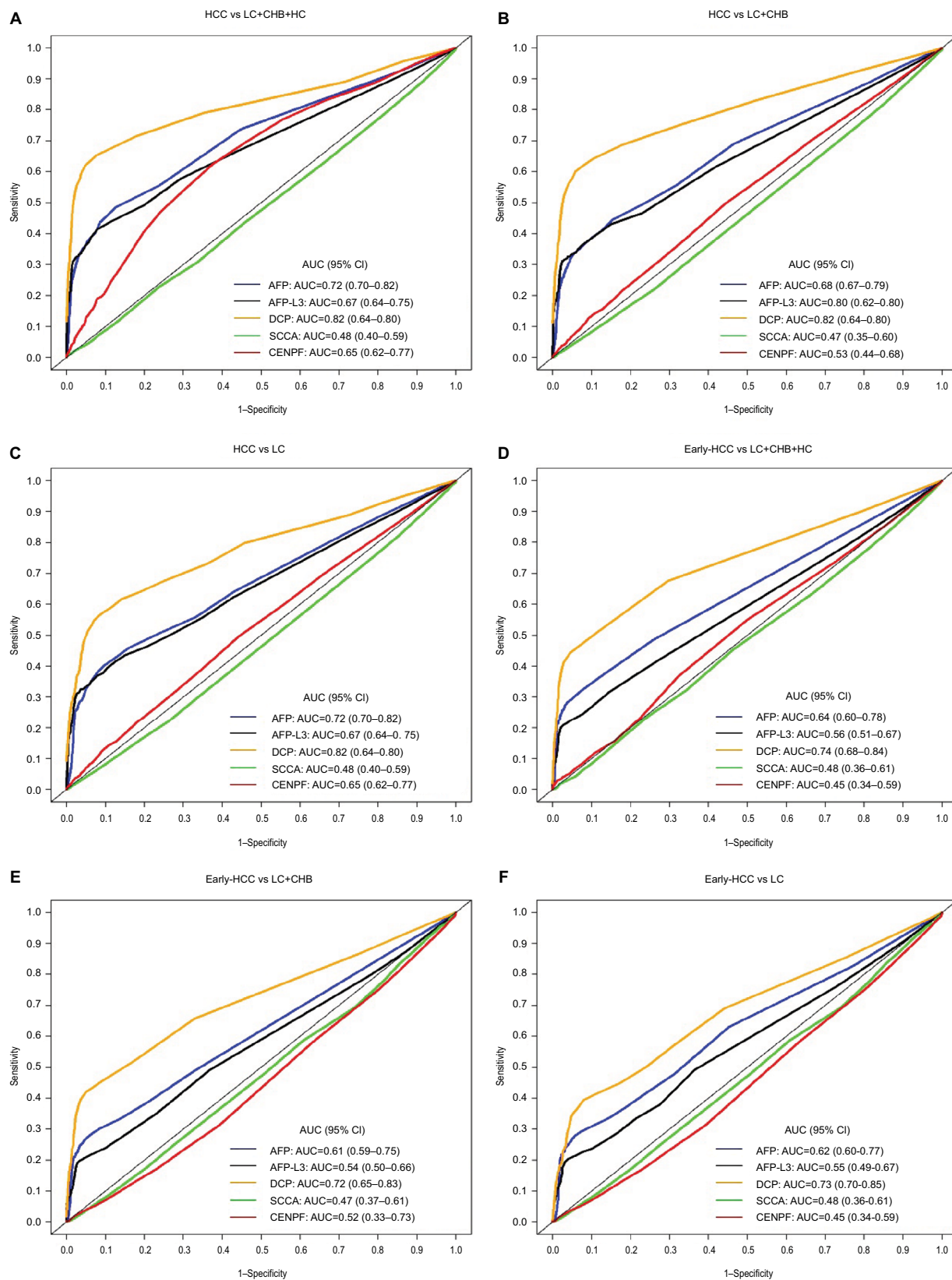


Figure 1 Comparison of .632+ adjusted receiver operating characteristics curves of AFP, AFP-L3, DCP, SCCA, and CENPF for discriminating: **(A)** HCC vs CHB+LC+HC; **(B)** HCC vs CHB+LC; **(C)** HCC vs CHB; **(D)** early-stage HCC vs CHB+LC+HC; **(E)** early-stage HCC vs CHB+LC; and **(F)** early-stage HCC vs LC.

Abbreviations: AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive AFP; AUC, area under the curve; CENPF, centromere protein F autoantibody; CHB, chronic hepatitis B virus infection; DCP, des-gamma-carboxyprothrombin; HC, healthy control; HCC, hepatocellular carcinoma; LC, liver cirrhosis; SCCA, squamous cell carcinoma antigen.

Table 3 (a) Diagnostic performance of four markers in detecting AFP-positive or AFP-negative patients with HCC

Marker	HCC vs LC+CHB+HC		HCC vs LC+CHB		HCC vs LC	
	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b
AFP-positive^c						
AFP-L3	0.95 (0.89–0.99)	89.4 (82.6–100.0)	0.93 (0.90–0.98)	85.1 (70.0–100.0)	0.93 (0.91–0.99)	87.9 (71.4–100.0)
DCP	0.84 (0.63–0.85)	65.4 (61.4–88.9)	0.82 (0.62–0.84)	64.5 (59.5–87.9)	0.78 (0.63–0.84)	59.3 (50.0–83.3)
SCCA	0.46 (0.36–0.61)	7.8 (0–21.4)	0.45 (0.31–0.62)	8.4 (0–29.4)	0.45 (0.31–0.62)	8.4 (0–29.4)
CENPF	0.65 (0.62–0.81)	21.7 (5.9–47.6)	0.53 (0.40–0.71)	11.7 (0–29.2)	0.53 (0.40–0.71)	11.7 (0–29.2)
AFP-negative^c						
AFP-L3	0.48 (0.46–0.53)	6.7 (0–11.5)	0.46 (0.44–0.52)	7.2 (0–10.3)	0.46 (0.44–0.52)	7.0 (0–10.7)
DCP	0.78 (0.62–0.81)	56.3 (48.4–77.8)	0.76 (0.61–0.82)	56.6 (48.1–75.8)	0.73 (0.61–0.81)	52.1 (41.4–72.5)
SCCA	0.47 (0.36–0.59)	6.8 (0–27.3)	0.47 (0.34–0.63)	7.8 (0–38.1)	0.47 (0.34–0.63)	7.8 (0–38.1)
CENPF	0.60 (0.55–0.77)	20.0 (6.7–41.2)	0.50 (0.38–0.67)	12.5 (0–31.6)	0.50 (0.38–0.67)	12.5 (0–31.6)

(b) Diagnostic performance of four markers in detecting AFP-negative or AFP-positive patients with early-stage HCC

Marker	Early-HCC vs LC+CHB+HC		Early-HCC vs LC+CHB		Early-HCC vs LC	
	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b
AFP-positive^c						
AFP-L3	0.85 (0.61–100.0)	75.4 (25.0–100.0)	0.83 (0.62–1)	74.0 (33.3–100.0)	0.83 (0.63–1)	73.7 (33.3–100.0)
DCP	0.72 (0.63–0.90)	41.4 (26.7–83.4)	0.70 (0.62–0.88)	42.6 (28.6–83.3)	0.68 (0.62–0.87)	37.1 (25.0–76.5)
SCCA	0.45 (0.33–0.60)	7.9 (0–22.2)	0.47 (0.33–0.64)	7.0 (0–33.3)	0.47 (0.33–0.64)	7.0 (0–33.3)
CENPF	0.54 (0.40–0.84)	12.2 (0–50.0)	0.44 (0.21–0.73)	4.9 (0–25.0)	0.44 (0.21–0.73)	4.9 (0–25.0)
AFP-negative^c						
AFP-L3	0.47 (0.44–0.54)	9.2 (0–14.3)	0.46 (0.43–0.53)	5.4 (0–13.3)	0.46 (0.43–0.53)	9.4 (0–13.3)
DCP	0.69 (0.61–0.82)	48.0 (28.6–70.0)	0.69 (0.59–0.82)	39.0 (29.4–68.8)	0.66 (0.60–0.81)	36.7 (23.8–64.7)
SCCA	0.49 (0.35–0.62)	10.8 (0–31.3)	0.46 (0.35–0.60)	7.2 (0–33.3)	0.46 (0.35–0.60)	7.2 (0–33.3)
CENPF	0.45 (0.30–0.67)	9.0 (0–22.2)	0.44 (0.32–0.60)	6.3 (0–30.0)	0.44 (0.32–0.60)	6.3 (0–30.0)

Note: ^aAUC was adjusted for potential overfitting by the .632+ bootstrap method. ^b.632+ bootstrap adjusted sensitivity at cutoffs yielding 90% specificity. ^cThe threshold is 20 ng/mL to define the positivity of AFP.

Abbreviations: AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive AFP; AUC, area under the curve; CENPF, centromere protein F autoantibody; CHB, chronic hepatitis B virus infection; DCP, des-gamma-carboxyprothrombin; HC, healthy control; HCC, hepatocellular carcinoma; LC, liver cirrhosis; SCCA, squamous cell carcinoma antigen; SEN, sensitivity; SPE, specificity.

Excellent diagnostic performance was observed for AFP-L3 in detecting AFP-positive HCCs, with AUCs higher than 0.90. However, in contrast, AFP-L3 presented no diagnostic value in detecting AFP-negative HCCs. For DCP and CENPF, similar diagnostic values were observed in detecting AFP-negative and AFP-positive HCCs, and the differences were not statistically significant (all $p > 0.05$). For instance, the .632+ adjusted AUCs of DCP in detecting AFP-positive and AFP-negative HCCs versus patients with liver cirrhosis were 0.68 (95% CI 0.62–0.87) and 0.66 (95% CI 0.60–0.81), respectively ($p = 0.743$).

To further enhance the diagnostic performance, a comprehensive search for different biomarker combinations was conducted, and multi-marker prediction algorithms were constructed using logistic regression models. To avoid potential overfitting, all diagnosis-related indicators were further adjusted using the .632+ bootstrap method. Comparisons of

the diagnostic performance of the multi-marker algorithms are shown in Table 4 and [Table S3](#) and comparison of the ROC curves of different prediction algorithms in detecting HCC is shown in Figure 2. Overall, for two-marker combinations, a prediction algorithm including AFP and DCP showed the best diagnostic value. The apparent AUC (without correction) of the two-marker algorithm in detecting early-stage HCC was 0.86 (95% CI 0.81–0.90). After correction for potential overfitting, the .632+ adjusted AUC was 0.79 (95% CI 0.73–0.88) and the .632+ adjusted sensitivity was 59.8% (95% CI 46.4–77.4%) at 90% specificity. Further combining AFP-L3 with AFP and DCP did not improve the overall diagnostic performance, but yielded reductions in terms of AUC and sensitivity (Table 4). We further examined the diagnostic performance of the biomarker combinations for discriminating early-stage HCC versus decompensated liver cirrhosis and compensated liver cirrhosis, as shown

Table 4(a) Diagnostic performance of marker combinations in detecting HCC

Marker	HCC vs LC+CHB+HC		HCC vs LC+CHB		HCC vs LC	
	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b
AFP+AFP-L3	0.69 (0.65–0.78)	46.5 (35.2–57.7)	0.66 (0.63–0.76)	40.9 (30.8–53.7)	0.66 (0.63–0.77)	43.4 (30.8–55.3)
AFP+DCP	0.87 (0.68–0.84)	73.8 (63.6–84.2)	0.84 (0.67–0.83)	68.2 (59.4–78.5)	0.83 (0.68–0.84)	64.2 (53.9–76.6)
AFP-L3+DCP	0.84 (0.64–0.83)	71.0 (60.4–83.3)	0.82 (0.62–0.82)	66.3 (54.5–77.8)	0.81 (0.63–0.83)	63.7 (52.8–77.8)
AFP+AFP-L3+DCP	0.85 (0.64–0.84)	73.7 (60.8–85.1)	0.83 (0.63–0.83)	65.7 (54.7–78.6)	0.81 (0.64–0.84)	64.5 (52.8–78.7)

(b) Diagnostic performance of marker combinations in detecting early-stage HCC

Marker	Early-stage HCC vs LC+CHB+HC		Early-stage HCC vs LC+CHB		Early-stage HCC vs LC	
	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b	AUC (95% CI) ^a	SEN at 90% SPE (95% CI) ^b
AFP+AFP-L3	0.58 (0.49–0.69)	26.8 (10.0–42.9)	0.56 (0.46–0.68)	25.0 (9.5–41.7)	0.56 (0.45–0.68)	25.7 (7.7–42.1)
AFP+DCP	0.79 (0.73–0.88)	59.8 (46.4–77.4)	0.77 (0.71–0.86)	56.0 (43.2–70.6)	0.75 (0.71–0.87)	52.6 (37.0–68.6)
AFP-L3+DCP	0.72 (0.64–0.84)	47.9 (33.3–72.2)	0.69 (0.61–0.83)	42.9 (25.0–64.3)	0.67 (0.61–0.83)	42.5 (22.2–63.7)
AFP+AFP-L3+DCP	0.72 (0.61–0.85)	49.7 (34.8–73.3)	0.70 (0.58–0.83)	45.3 (25.0–64.3)	0.68 (0.59–0.83)	42.7 (23.1–64.7)

Note: ^aAUC was adjusted for potential overfitting by the .632+ bootstrap method. ^b.632+ bootstrap adjusted sensitivity at cutoffs yielding 90% specificity.

Abbreviations: AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive AFP; AUC, area under the curve; CHB, chronic hepatitis B virus infection; DCP, des-gamma-carboxyprothrombin; HC, healthy control; HCC, hepatocellular carcinoma; LC, liver cirrhosis; SEN, sensitivity; SPE, specificity.

in [Table S4](#). The combination of AFP and DCP also exhibited good ability in discriminating early-stage HCC versus decompensated liver cirrhosis or compensated liver cirrhosis, with apparent AUCs of 0.81 (95% CI 0.75–0.86) and 0.84 (95% CI 0.78–0.90), respectively, and the difference was not statistically significant ($p=0.422$).

When further combining age and gender with AFP and DCP, the new prediction algorithm showed better diagnostic performance compared to the previous algorithm including AFP and DCP, with a .632+ AUC of 0.88 (95% CI 0.80–0.93) and a sensitivity of 65.4% at 90% specificity in detecting early-stage HCC ([Table S5](#)). The comparison of the ROC curves for the two prediction algorithms is shown in [Figure S7](#), and detailed descriptions of the regression equations and optimal probabilities for the two algorithms are shown in [Tables S6](#) and [S7](#).

Discussion

In the present study, serum levels of five biomarkers were simultaneously measured in a large set of samples (N=846). After direct comparison of the diagnostic performance of the five biomarkers, DCP was identified as the best performing biomarker in distinguishing HCC from non-malignant chronic liver diseases, with a .632+ adjusted AUC of 0.82 (95% CI 0.64–0.80) and a .632+ adjusted sensitivity of 65.2% (95% CI 63.3–82.1%) at 90% specificity. We further

constructed a prediction algorithm combining AFP and DCP, which presented enhanced diagnostic performance compared to individual biomarkers. The .632+ adjusted AUC of the two-marker algorithm in distinguishing HCC from non-malignant chronic liver diseases was 0.87 (95% CI 0.68–0.84), and the adjusted sensitivity was 73.8% at a specificity of 90%. Notably, the algorithm also showed good ability in discriminating HCC versus liver cirrhosis, with an adjusted AUC of 0.83 (95% CI 0.68–0.84). Furthermore, additionally combining sociodemographic factors of age and gender slightly improved the diagnostic efficacy for detecting HCC.

The definition of the cutoff values directly affects the sensitivity and specificity of the respective biomarkers. In our study, the cutoffs were defined in such a way as to yield the same specificity in order to make a fair comparison of sensitivities for the five biomarkers. The sensitivities were further corrected for potential overestimation using the state-of-the-art bootstrap method. Therefore, the diagnostic performance of the respective biomarkers may be underestimated compared to other published results. Comparisons between the apparent and the .632+ adjusted ROC curves for all five biomarkers are also provided in [Figures S2–S6](#).

DCP, also known as PIVKA-II, has been proposed as a potential serological biomarker for HCC detection in a variety of studies.^{15,16,24,25} In a meta-analysis summarizing 12

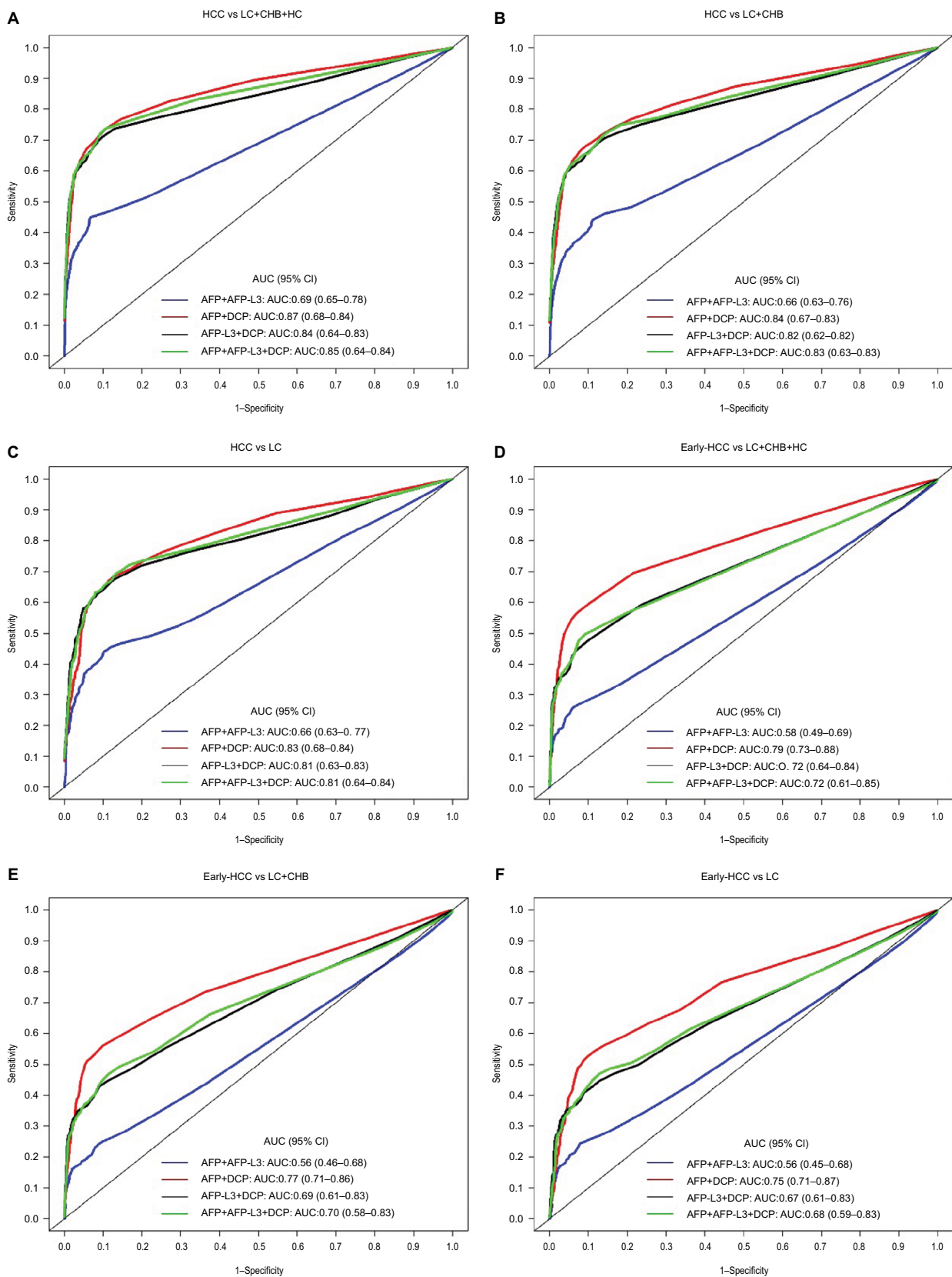


Figure 2 Comparison of .632+ adjusted receiver operating characteristics curves of four different multi-marker combinations for discriminating: **(A)** HCC vs CHB+LC+HC; **(B)** HCC vs CHB+LC; **(C)** HCC vs CHB; **(D)** early-stage HCC vs CHB+LC+HC; **(E)** early-stage HCC vs CHB+LC; and **(F)** early-stage HCC vs LC.

Abbreviations: AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive AFP; AUC, area under the curve; CENPF, centromere protein F autoantibody; CHB, chronic hepatitis B virus infection; DCP, des-gamma-carboxyprothrombin; HC, healthy control; HCC, hepatocellular carcinoma; LC, liver cirrhosis; SCCA, squamous cell carcinoma antigen.

studies, a pooled sensitivity of 71% (95% CI 68.0–73.0%) and a pooled specificity of 84.0% (95% CI 83.0–86.0%) were reported,¹⁵ which are similar to the results of our study. Of note, our analyses additionally showed that DCP had a similar diagnostic efficacy in detecting AFP-negative or AFP-positive patients with HCC, indicating that DCP could be potentially used as a complement biomarker of AFP in the diagnosis of HCC. With regard to the laboratory methods of detecting DCP, techniques such as ELISA have been developed and widely used in previous studies.^{8,26,27} Therefore, the combination of DCP and AFP could be potentially implemented in a cost-efficient manner when translated into clinical application.

Our results of the low sensitivity of AFP-L3 in detecting AFP-negative HCC cases were in line with previous studies.^{13,28} These findings indicated that AFP-L3 may have limited utilization as an independent diagnostic biomarker for HCC, given the relatively high proportion of HCC patients who could be missed by AFP. Evidence on whether combining AFP-L3 and AFP could yield better diagnostic performance than single biomarkers is still conflicting. Although enhanced diagnostic performance for the combination of AFP-L3 and AFP was reported in some studies,^{29,30} this was not observed in our study. Therefore, the role of AFP-L3 in the diagnosis of HCC should be further evaluated in future studies.

In our study, SCCA was found to show no diagnostic value in the early detection of HCC, which was in line with a previous study conducted by Soyemi and colleagues.¹⁷ In a meta-analysis including 12 studies, the pooled sensitivity and specificity of SCCA in detecting HCC were 59% and 76%, respectively.¹⁸ However, strong heterogeneity existed owing to the different sample sizes, the different study designs, and the various cutoffs adopted in different studies. Further large prospective studies are required to rigorously evaluate the diagnostic accuracy of SCCA in detecting HCC.

In our previous study, CENPF autoantibody showed potential diagnostic value for early HCC and the majority of early HCC cases with negative AFP were positive for autoantibody to CENPF. Furthermore, the combination of autoantibody to CENPF with AFP improved the ability to diagnose HCC at an early stage, with an AUC of 0.882, or with a sensitivity of 86.7% and a specificity of 68.8%.¹⁹ However, in the present study, the performance of CENPF autoantibody showed a lower AUC value in distinguishing HCC from controls, probably as a result of the different controls used and autoimmune diseases which may exist in the cases with liver cirrhosis and hepatitis.

Some biomarkers examined in our study were reported to have a prognostic value in previous studies.^{26,31,32} For instance, the levels of AFP, AFP-L3, and DCP usually increase as HCC progresses, i.e., with the increase in the size and number of lesions and progression to portal vein invasion.^{33,34} Moreover, elevated levels of biomarkers were associated with a higher rate of recurrence and poorer survival rate, as reported in previous studies.³² Owing to the limitation of the cross-sectional design, the prognostic value of the individual biomarkers and multi-marker combinations cannot be assessed in our study. However, the exact prognostic value of the multi-marker combinations for predicting progression and survival of HCC patients deserves to be further studied in future investigations.

It should be noted that the diagnostic performance of all five individual biomarkers still does not meet the requirement for HCC screening, given the suboptimal sensitivity for early-stage HCC. Exploration of novel effective biomarkers and the construction of multi-marker combinations are possible ways to further enhance the diagnostic performance in the future. Although promising results regarding multi-marker prediction models were frequently reported in many studies, few of them were independently validated or used rigorous statistical methods to adjust for potential overestimation of diagnostic indicators, as done in our study.^{24,29,30} For example, in a study by Ertle and colleagues,²⁴ the AUC for the prediction model including AFP and DCP in detecting HCC was reported to be 0.91 using a sample set of 164 HCC and 422 controls, but no external validation or interval validation was adopted.

Patients with liver cirrhosis carry a great risk for the development of HCC, particularly patients at the decompensated stage.^{35–37} The identification and surveillance of patients with liver cirrhosis at high risk of HCC are highly clinically relevant. Current guidelines from the American Association for the Study of Liver Disease (AASLD) and the European Association for the Study of the Liver recommend surveillance of cirrhotic patients with ultrasound with or without AFP every 6–12 months. However, evidence suggested that ultrasound was suboptimal for detecting early-stage HCC and AFP provided no additional benefit to ultrasound.^{38–41} Our results showed that combining DCP with AFP improved diagnostic efficacy in distinguishing early-stage HCC with liver cirrhosis, indicating that the combination could be considered as a complementary tool for liver cirrhosis surveillance. However, important issues such as surveillance interval and cost-effectiveness of surveillance strategies need to be further explored in future studies.

To our knowledge, our study is the first to conduct a direct comparison of the diagnostic performance of five recently recognized biomarkers in the diagnosis of HCC using a large sample set from China. Specific strengths and limitations deserve careful consideration when interpreting our study. Strengths include that the five biomarkers were simultaneously measured in a large sample set. We also adopted state-of-the-art methods to adjust for potential overestimation of the diagnostic indicator for the single and multi-marker prediction models. The limitations include that the diagnostic performance of biomarkers in detecting HCC with different tumor sizes or tumor differentiation status was not evaluated in our current analysis, and this should be addressed in further studies. Moreover, since the diagnostic performance of the constructed prediction algorithms is still not optimal regarding early diagnosis of HCC, further improvement by combination with other promising biomarkers or established risk prediction models (such as the Child–Pugh–Turcotte score and the model for end-stage liver disease score) would be highly recommended.

In summary, DCP could be used as a complementary biomarker in the diagnosis of HCC and to improve the identification of patients with AFP-negative HCC. The constructed multi-marker prediction algorithms would contribute toward distinguishing HCC from non-malignant chronic liver diseases and may be useful for the surveillance of cirrhosis for HCC.

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Disclosure

The authors report no potential conflicts of interest in this work.

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