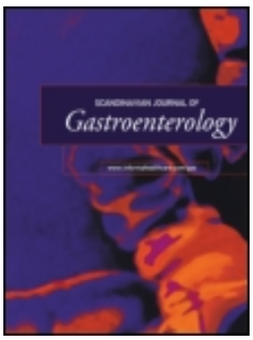

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ORIGINAL ARTICLE

Combined use of AFP, PIVKA-II, and AFP-L3 as tumor markers enhances diagnostic accuracy for hepatocellular carcinoma in cirrhotic patients

TAE SEOP LIM[1], DO YOUNG KIM[1], KWANG-HYUB HAN[1], HYON-SUK KIM[2], SEUNG HWAN SHIN[1], KYU SIK JUNG[1], BEOM KYUNG KIM[1], SEUNG UP KIM[1], JUN YONG PARK[1] & SANG HOON AHN[1]

[1]Department of Internal Medicine and [2]Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Republic of Korea

ABSTRACT

Objective: As data on the effectiveness of tumor markers in detecting hepatocellular carcinoma (HCC) in cirrhotic patients are limited, we investigated the diagnostic accuracy of alpha-fetoprotein (AFP), protein induced by vitamin K absence or antagonist-II (PIVKA-II), and *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3). *Material and methods*: This retrospective study enrolled 361 cirrhotic patients with HCC, and 276 cirrhotic patients without HCC occurrence. *Results*: Most patients were men ($n = 431$, 67.7%); the median age was 57.0 years. The main etiology of chronic liver disease was chronic hepatitis B ($n = 467$, 73.3%). The sensitivity and specificity of combined three biomarkers was 87.0 and 60.1% in overall HCC, and 75.7 and 60.1% in early HCC, respectively (cutoff: 20 ng/mL for AFP, 40 mAU/mL for PIVKA-II, and 5% for AFP-L3). The area under the receiver operating characteristic curve (AUROC) for HCC diagnosis was 0.765 (95% confidence interval [CI], 0.728–0.801) for AFP; 0.823 (95% CI, 0.791–0.854) for PIVKA-II; and 0.755 (95% CI, 0.718–0.792) for AFP-L3. The AUROC for early HCC diagnosis was 0.754 (95% CI, 0.691–0.816) for AFP, 0.701 (95% CI, 0.630–0.771) for PIVKA-II, and 0.670 (95% CI, 0.596–0.744) for AFP-L3. Combining the three tumor markers increased the AUROC to 0.877 (95% CI, 0.851–0.903) for HCC diagnosis, and 0.773 (95% CI, 0.704–0.841) for early HCC diagnosis. *Conclusion*: Diagnostic accuracy improved upon combining the AFP, PIVKA-II, and AFP-L3 tumor markers compared to each marker alone in detecting HCC and early HCC in cirrhotic patients.

KEY WORDS
AFP, AFP-L3, biomarker, hepatocellular carcinoma, PIVKA-II

HISTORY
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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer, and the third most frequent cause of cancer-related mortality worldwide [1]. As HCC patients often have a poor prognosis when it is diagnosed in the advanced stages, early diagnosis of HCC is of paramount importance to improve the survival of HCC patients. Patients with chronic liver disease who are at risk of HCC undergo surveillance for HCC, leading to detection of malignancies in the early stages and improved patient survival; however, the surveillance tools and the actual yield of surveillance are still debated. At present, the guidelines established by the American Association for the Study of Liver Disease (AASLD) and the European Association for Study of the Liver (EASL) recommend surveillance using ultrasonography (US), repeated every 6 months [2,3]. Although US is a non-invasive and accurate method to detect HCC, its accuracy may be unsatisfactory in cirrhotic patients, who are at the highest risk of developing HCC [4]. Indeed, a recent meta-analysis reported that the sensitivity of US was only 63% in cirrhotic patients, suggesting that the detection of HCC might be influenced by the coarse echogenic pattern of cirrhosis [5]. Therefore, the performance of the surveillance program for cirrhotic patients needs to be improved; tumor markers that can be easily obtained by a blood sample may be good candidates in providing acceptable diagnostic accuracy and cost-effectiveness.

Although the current guidelines of the AASLD and EASL do not include any tumor markers in the surveillance program, the tumor markers alpha-fetoprotein (AFP) or protein induced by vitamin K absence or antagonist-II (PIVKA-II) are widely used in clinical practice. Recently, a novel blood parameter, the *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), an AFP-isoform that reflects changes in the carbohydrate chain, has been considered an effective tumor marker for HCC [6]; AFP-L3 proved beneficial in early diagnosis

Correspondence: Do Young Kim, MD, Department of Internal Medicine, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodeamun-gu, Seoul 120-752, Korea. Tel: +82-2-2228-1992. Fax: +82-393-6884. dyk1025@yuhs.ac

of HCC [6,7], and in predicting prognosis after treatment [8–11]. Furthermore, a highly sensitive AFP-L3 assay is now available, with an advanced micro-total analysis system (μTAS), and AFP-L3 can be estimated accurately even in patients with very low AFP levels, suggesting that it may be effective in diagnosing HCC and predicting the prognosis [12,13].

Although AFP, PIVKA-II, and AFP-L3 might be good candidates for HCC surveillance in cirrhotic patients, their diagnostic accuracy, especially for the highly sensitive AFP-L3, has rarely been studied in patients with cirrhosis. A previous study in the US reported the superiority of PIVKA-II compared to AFP and AFP-L3 in patients with chronic liver disease and HCC. However, the study suggested that combining tumor markers is not more accurate than PIVKA-II alone [14]. Thus, we investigated the diagnostic accuracy of AFP, PIVKA-II, and AFP-L3, either alone, or in combination, in detecting the overall and early HCC among patients with cirrhosis.

Patients and methods

Patients

This retrospective study firstly enrolled 686 consecutive patients with cirrhosis who underwent HCC surveillance between September 2009 and February 2013. Of these, 277 non-cirrhotic patients and 18 with non-evaluated biomarkers were excluded. Out of the remaining 391 cirrhotic patients, 97 developed HCC (annual incidence, 4.9%). Among 294 cirrhotic patients without HCC, 18 were again excluded because HCC was diagnosed within 12 months after evaluation of biomarkers in those patients (see the exclusion criteria below). Thus, finally 276 cirrhotic patients were enrolled as control. During the study period, 335 HCC patients with cirrhosis who were referred from other hospitals were also enrolled in addition to the 97 HCC patients who were diagnosed by surveillance in our institution. Among these 432 HCC patients, 71 were excluded by various reasons (Figure 1). Thus, finally 361 HCC patients with cirrhosis were enrolled. The inclusion criteria were as follows: (1) age $>$ 20 years, (2) patients with cirrhosis, (3) available AFP, PIVKA-II, and AFP-L3 levels. The exclusion criteria were as follows: (1) previously diagnosed HCC, (2) previous history of liver transplantation or liver resection for a reason other than HCC, (3) any cancer other than HCC. Furthermore, in cirrhosis without HCC, patients who had been diagnosed with HCC within 12 months from the tumor marker measurement (AFP, PIVKA-II, and AFP-L3 levels) were also excluded. In this study, cirrhosis was defined by histological information or clinical criteria. When the histological information was not available, cirrhosis was defined as follows: (1) platelet count $<$ 100,000/μL and ultrasonographic findings suggestive of cirrhosis, including a blunted, nodular liver edge accompanied by splenomegaly ($>$12 cm); (2) esophageal or gastric varices; or (3) overt complications of liver cirrhosis, such as ascites, variceal bleeding, and hepatic encephalopathy [15]. This study protocol was approved by the institutional ethics review board and was in compliance with the Declaration of Helsinki.

Diagnosis of HCC

The diagnosis of HCC was made either histologically or non-invasively, and based on the guidelines of AASLD or EASL [2,3]. Briefly, HCC was diagnosed when the typical hallmark of HCC (hypervascular in the arterial phase with washout in the portal venous or delayed phases) was observed on one of the following imaging techniques: 4-phase multidetector computed tomography or dynamic contrast-enhanced magnetic resonance imaging. Under sub-optimal conditions, HCC diagnosis was confirmed by using two imaging techniques or liver biopsy. The Barcelona clinic liver cancer (BCLC) staging system was used for HCC staging [16]. Early HCC was defined as a single tumor $<$3 cm in diameter [17,18].

Measurements of tumor markers

Circulating levels of AFP, PIVKA-II, and AFP-L3 were measured in serum samples obtained from the enrolled patients. For HCC patients, samples were collected at the time of diagnosis, prior to commencing treatment. For cirrhotic patients without HCC, the samples were obtained at the time of diagnosis of cirrhosis; the absence of HCC was confirmed at 1 year from the time of tumor marker measurement. The measurements of AFP and AFP-L3 were performed using the μTAS assay (Wako Pure Chemical Industries, Ltd, Osaka, Japan), and PIVKA-II was analyzed by an enzyme immunoassay (Fujirebio Inc., Tokyo, Japan). The cutoff-values of AFP (20 and 200 ng/mL) and PIVKA-II (40 and 100 mAU/mL) were used in accordance with previous studies [18,19]. Furthermore, the cutoff-values of AFP-L3 were set at 5 and 10% [13,19].

Statistical analyses

Continuous variables were compared with Mann–Whiney U tests, and categorical variables were compared using the chi-square or Fisher's exact tests. Receiver operating characteristics (ROC) curves were constructed and the areas under the ROC curves (AUROC) were calculated. Logistic regression analysis was used to calculate the AUROC for the combined tumor markers.

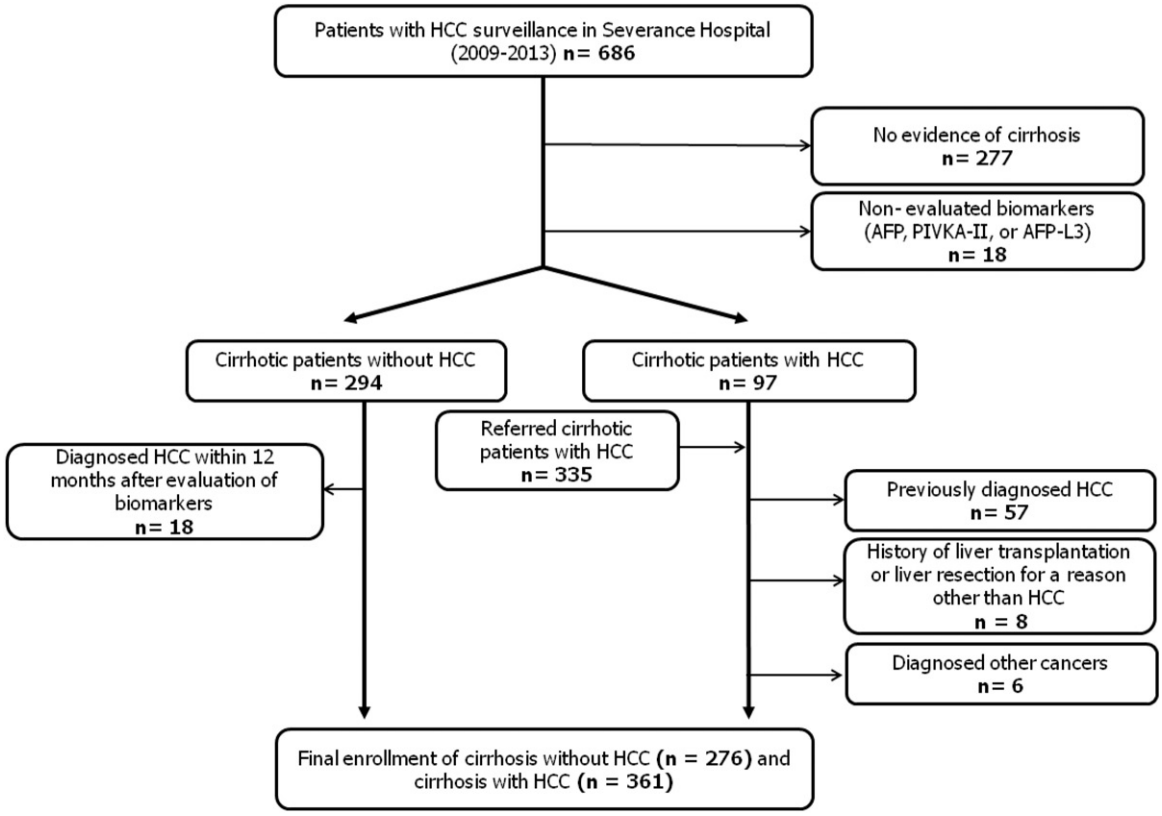

Figure 1. The flow of recruiting cirrhotic patients with or without hepatocellular carcinoma (HCC).

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also calculated. A probability (*p*) value of 0.05 was considered statistically significant. Statistical analyses were performed using the SAS program, version 9.1 (SAS Inc, Cary, NC).

Results

Baseline characteristics and comparison of tumor markers in cirrhosis and HCC

During the study period, 637 patients with cirrhosis (276 patients without HCC and 361 patients with HCC) were enrolled. The patients' baseline characteristics are summarized in Tables I and II. The majority were men ($n = 431$, 67.7%), and the median age was 57.0 years. The main etiologies of chronic liver disease were chronic hepatitis B ($n = 463$, 73.3%), followed by non-B, non-C ($n = 100$, 15.7%) hepatitis, and chronic hepatitis C ($n = 70$, 11.0%). The median levels of AFP, PIVKA-II, and AFP-L3 were at 9.1 ng/mL (interquartile range [IQR], 3.3–75.7 ng/mL), 29.0 mAU/mL (IQR, 18.0–274.0 mAU/mL), and 4.3% (IQR, 0.0–13.3%), respectively. One-hundred and sixty-two (25.4%) patients had histologically confirmed cirrhosis by liver biopsy or resection, and 475 (74.6%) patients were diagnosed with cirrhosis by clinical criteria. Most patients ($n = 537$, 84.3%) had preserved liver function (Child-Pugh A). Patients with HCC had a median age of 58.0 years, and 71.5% were men. The median tumor size was 4.2 cm, and single tumors were identified in 201 (55.7%) patients. According to the BCLC staging system, 147 (40.7%) patients had stage A tumors, 51 (14.1%) had stage B, 52 (42.1%) had stage C, and 11 (3.1%) had stage D. In the HCC group, the number of patients having tumor size <3 cm with single or multiple lesions was 137 (38.0%), and there were 70 (19.4%) patients with early stage HCC defined as a single tumor <3 cm.

Comparisons between patients with and without HCC

The baseline characteristics of patients with and without HCC are compared in Table I. The number of male patients, median age, and serum AST levels were higher in HCC patients than in those without HCC, whereas albumin levels were significantly higher in patients without HCC. The comparison of tumor markers in patients with and without HCC is shown in Figure 2. The median levels of AFP, PIVKA-II, and AFP-L3 were significantly higher in patients with HCC compared to those

Table I. Baseline characteristics of the study population.

Variables	All patients ($n=637$)	Patients without HCC ($n=276$)	Patients with HCC ($n=361$)	p
Male sex (%)	431 (67.7%)	173 (62.7%)	258 (71.5%)	0.019
Age (years)	57.0 (50.5–65.5)	55.0 (48.0–64.0)	58.0 (52.0–73.0)	<0.001
Etiology				
HBV/HCV/non-B, non-C, *n* (%)	467 (73.3%)/70 (11.0)/100 (15.7%)	192 (69.6%)/37 (13.4%)/47 (17.0%)	275 (76.2%)/33 (9.1%)/53 (14.7%)	0.131
Child-Pugh class: A/B/C, *n* (%)	537 (84.3%)/87 (13.7%)/13 (2.0%)	247 (89.5%)/22 (8.0%)/7 (2.5%)	290 (80.3%)/65 (18.0%)/6 (1.7%)	<0.001
Platelet count ($\times 10^3$/mm^3)	123.0 (86.0–160.0)	122.0 (85.0–159.0)	124.0 (87.5–163.5)	0.561
AST (IU/L)	39.0 (27.0–62.5)	33.0 (26.0–50.0)	44.0 (29.0–79.0)	0.005
ALT (IU/L)	31.0 (20.5–47.5)	30.0 (20.0–42.0)	32.0 (21.0–53.0)	0.593
Albumin (g/dL)	4.0 (3.4–4.3)	4.2 (3.8–4.5)	3.8(3.3–4.2)	<0.001
Total bilirubin (mg/dL)	0.9 (0.7–1.2)	0.9 (0.7–1.2)	0.9 (0.6–1.2)	0.013
PT-INR	1.0 (1.0–1.1)	1.0 (1.0–1.1)	1.0 (1.0–1.1)	0.491
AFP (ng/mL)	9.1 (3.3–75.7)	3.9 (2.4–10.7)	31.8 (5.8–569.9)	<0.001
PIVKA-II (mAU/mL)	29.0 (18.0–274.0)	20.0 (15.3–29.0)	137.0 (25.0–2000.0)	<0.001
AFP-L3 (%)	4.3 (0.0–13.3)	0.0 (0.0–5.2)	8.7 (1.6–43.0)	<0.001

Data are expressed as the number (percentage) and median (interquartile range).
HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; HCV, Hepatitis C virus; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; PT-INR, Prothrombin time international normalized ratio; AFP, Alpha-fetoprotein; PIVKA-II, Protein induced by vitamin K absence-II; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein.

Table II. Characteristics of hepatocellular carcinoma.

Variables	*n* (%)
Vessel invasion (%)	121 (33.5%)
Portal vein thrombosis (%)	89 (24.6%)
Distant metastasis (%)	31 (8.6%)
Tumor number, ≥2 (%)	160 (44.3%)
Tumor size, ≥3 cm (%)	224 (62.0%)
BCLC staging: A/B/C/D, *n* (%)	147 (40.7%)/51 (14.1%)/52(42.1%)/11(3.1%)

without HCC (31.8 ng/mL versus 3.9 ng/mL for AFP, 137.0 mAU/mL versus 20.0 mAU/mL for PIVKA-II, and 8.7% versus 0.0% for AFP-L3; all $p<0.001$).

Diagnostic accuracy of tumor markers in detecting overall HCC

The ROC curves for tumor markers for diagnosing overall HCC are shown in Figure 3. The AUROCs for AFP, PIVKA-II, AFP-L3, and the combined tumor markers were 0.765 (95% confidence interval [CI], 0.728–0.801, $p<0.001$), 0.823 (95% CI, 0.791–0.854, $p<0.001$), 0.755 (95% CI, 0.718–0.792, $p<0.001$), and 0.877 (95% CI, 0.851–0.903, $p<0.001$), respectively. The AUROC for the combination of all three markers was superior to that of any one tumor marker alone (AFP versus combined markers: $p<0.001$; PIVKA-II versus combined markers: $p<0.001$; AFP-L3 versus combined markers: $p<0.001$). The sensitivity, specificity, PPV, and NPV for different cutoff values are presented in Table III. The sensitivity for AFP (cutoff: 20 ng/mL), PIVKA-II (cutoff: 40 mAU/mL), and AFP-L3 (cutoff: 5%) was 56.8, 62.9, and 61.2%, respectively, and the specificity was 82.8, 90.8, and 73.8%, respectively. The sensitivity of the three markers combined (cutoff values: 20 ng/mL for AFP, 40 mAU/mL for PIVKA-II, and 5% for AFP-L3) was enhanced to 87.0%, and the specificity was 60.1%.

Diagnostic accuracy of tumor markers in distinguishing early HCC

We analyzed the ROC curves (shown in Figure 4) in order to evaluate the diagnostic accuracy of tumor markers in distinguishing early HCC. The AUROC was 0.754 (95% CI, 0.691–0.816, $p<0.001$) for AFP, 0.701 (95% CI, 0.630–0.771, $p<0.001$) for PIVKA-II, 0.670 (95% CI, 0.596–0.744, $p<0.001$) for AFP-L3, and 0.773 (95% CI, 0.704–0.841, $P<0.001$) for the combined three tumor markers. The combination of all three markers showed the most superior AUROC, however, no statistical significance was observed between the combined markers and AFP (AFP versus combined markers: $p=0.434$; PIVKA-II versus combined markers: $p=0.018$, AFP-L3 versus combined markers: $p<0.001$). The sensitivity, specificity, PPV, and NPV for different cutoff values of tumor markers in distinguishing early HCC from cirrhosis are presented in Table IV. The sensitivity of AFP (cutoff: 20 ng/mL), PIVKA-II (cutoff: 40 mAU/mL), and AFP-L3 (cutoff: 5%) was 50.0, 37.1, and 47.1%, respectively, and the specificity was 83.7, 90.8, and 73.8%, respectively. The sensitivity of the three markers combined (cutoff values: 20 ng/mL for AFP, 40 mAU/mL for PIVKA-II, and 5% for AFP-L3) was enhanced to 75.7%, and the specificity was 60.1%.

Diagnostic utility of PIVKA-II and AFP-L3 in patients with low AFP (<20 ng/mL)

We analyzed 416 (260 with HCC and 156 without HCC) patients with AFP levels <20 ng/mL in order to evaluate the diagnostic role of PIVKA-II and AFP-L3 in patients with low AFP levels. The ROC curves for PIVKA-II and AFP-L3 in patients with AFP levels <20 ng/mL are shown in Figure 5. The AUROCs for PIVKA-II, AFP-L3 and the combined markers were 0.744 (95% CI, 0.692–0.796, $p<0.001$), 0.625 (95% CI, 0.567–0.684, $p<0.001$), and

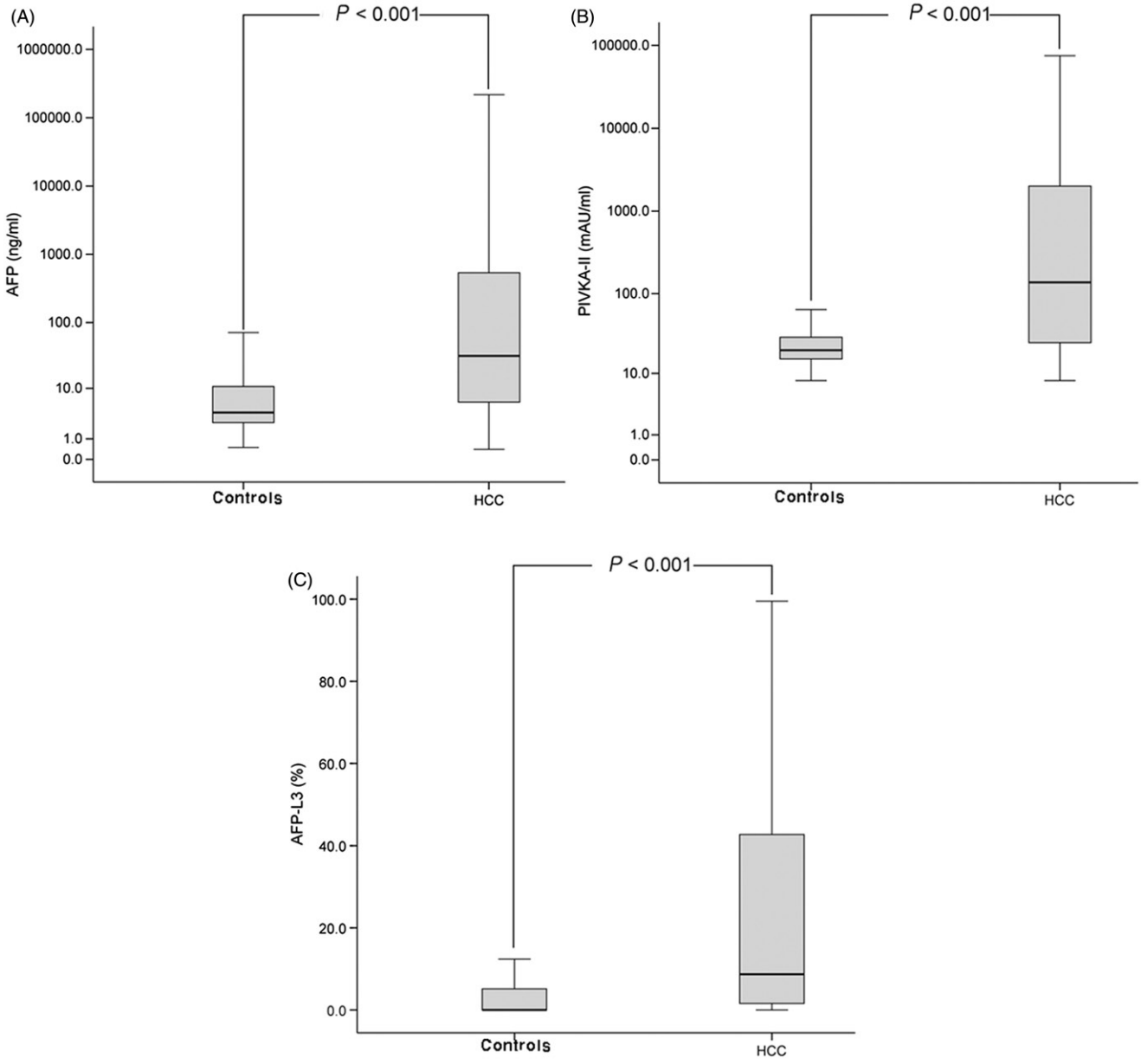

Figure 2. A comparison of serum alpha-fetoprotein (AFP), protein induced by vitamin K absence-II (PIVKA-II), and *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) values in patients with and without hepatocellular carcinoma (HCC). The values of AFP (A), PIVKA-II (B), and AFP-L3 (C) are shown as rectangles, where the line represents the median.

0.794 (95% CI, 0.746–0.841, $p < 0.001$), respectively. The AUROC for a combination of PIVKA-II and AFP-L3 was superior to that of either PIVKA-II or AFP-L3 alone (combination of PIVKA-II and AFP-L3 versus PIVKA-II: $p = 0.024$; combination of PIVKA-II and AFP-L3 versus AFP-L3: $p < 0.001$). The sensitivity, specificity, PPV, and NPV for PIVKA-II and AFP-L3 in patients with AFP levels < 20 ng/mL are presented in Table V. The sensitivity for PIVKA-II (cutoff: 40 mAU/mL) and AFP-L3 (cutoff: 5%) was 50.0 and 41.0%, respectively, and the specificity was 90.4 and 77.8%, respectively. The sensitivity for the combination of PIVKA-II and AFP-L3 (cutoff values: 40 mAU/mL for PIVKA-II, and 5% for AFP-L3) was enhanced to 69.9%, and the specificity was 71.9%.

Discussion

In this study, we evaluated the effectiveness of tumor markers in differentiating HCC in patients with cirrhosis. Tumor markers including AFP, PIVKA-II, and AFP-L3 were analyzed and compared when they were used either alone or in combination; as expected, all three markers were significantly elevated in patients with HCC, compared to those without.

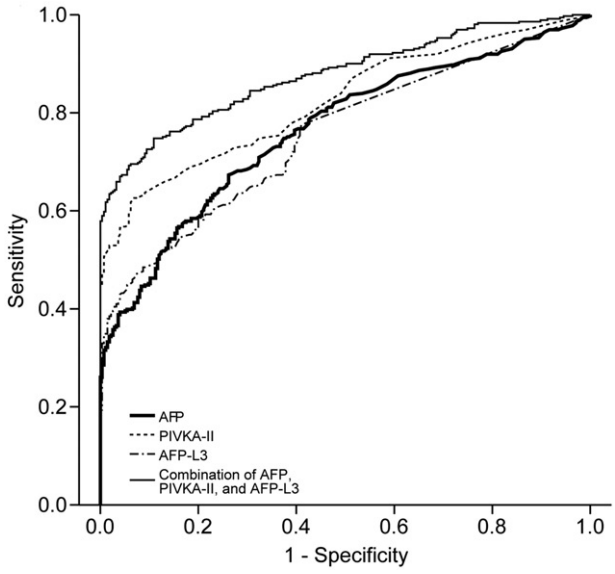

Figure 3. The receiver operating characteristic curves for alpha-fetoprotein (AFP), protein induced by vitamin K absence-II (PIVKA-II), and *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) for diagnosing hepatocellular carcinoma. The area under the receiver operating characteristic curve was 0.765 (95% confidence interval [CI], 0.728–0.801, $p < 0.001$) for AFP, 0.823 (95% CI, 0.791–0.854, $p < 0.001$) for PIVKA-II, 0.755 (95% CI, 0.718–0.792, $p < 0.001$) for AFP-L3, and 0.877 (95% CI, 0.851–0.903) for the combined AFP, PIVKA-II, and AFP-L3 markers.

Previous studies evaluating tumor markers for early detection of HCC have used chronic liver disease as the control group [20,21]. In the current study, only patients with cirrhosis were enrolled because cirrhosis is the most common cause of HCC, irrespective of its etiology; moreover, in the cirrhotic liver, it is difficult to detect early HCC by US examination owing to its coarse background [4,5].

In the current study, PIVKA-II was found to be superior to AFP or AFP-L3 in detecting overall HCC; this finding is consistent with those from several earlier studies [14,22,23]. Liver diseases such as active hepatitis or cirrhosis are rarely characterized by false elevation of PIVKA-II levels, which is thus considered to be more specific than total AFP in the diagnosis of HCC [24,25]. However, this tumor marker is recognized for its limitations in the detection of small HCC, and is often considered an indicator of advanced HCC with vascular invasion or poor prognosis [26]. This suggests that PIVKA-II alone may not be a good screening candidate. In this study, the proportion of early HCC, which is defined as a single tumor <3 cm in diameter, was 19.4%, while the proportion of intermediate-advanced HCC was 80.6%. Although PIVKA-II demonstrated the best accuracy as a sole marker in differentiating overall HCC from cirrhosis, this finding might be because of the relatively higher number of intermediate-advanced HCC cases.

Table III. Sensitivity, specificity, PPV, and NPV for different cut-off values of tumor markers in distinguishing overall hepatocellular carcinoma from cirrhosis.

Variables	AUROC (95% CI)	Cut-off value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	NPV (%) (95% CI)	PPV (%) (95% CI)
AFP	0.765 (0.728–0.801)	20 ng/mL	56.8 (51.7–61.9)	82.8 (79.3–88.1)	59.7 (54.8–64.6)	82.0 (77.2–86.8)
		200 ng/mL	32.1 (27.3–37.0)	98.9 (97.7–100.0)	52.7 (48.4–57.0)	97.5 (94.7–100.0)
PIVKA-II	0.823 (0.791–0.854)	40 mAU/mL	62.9 (57.9–67.9)	90.8 (87.2–94.5)	61.9 (56.9–67.0)	91.2 (87.7–94.7)
		100 mAU/mL	52.9 (47.8–58.1)	97.1 (95.0–99.2)	57.8 (53.0–62.6)	96.5 (93.9–99.0)
AFP-L3	0.755 (0.718–0.792)	5%	61.2 (56.2–66.2)	73.8 (68.6–79.0)	59.2 (54.0–64.4)	75.4 (70.5–80.4)
		10%	47.1 (41.9–52.2)	92.7 (89.7–95.8)	57.2 (52.6–61.8)	89.5 (85.1–93.8)
AFP + PIVKA-II	0.851 (0.822–0.880)	20 ng/mL for AFP or 40 mAU/mL for PIVKA-II	78.1 (73.9–82.4)	77.5 (72.6–82.5)	73.0 (68.0–78.1)	82.0 (77.9–86.0)
PIVKA-II + AFP-L3	0.868 (0.841–0.895)	40 mAU/mL for PIVKA-II or 5% for AFP-L3	80.9 (76.8–84.9)	68.5 (63.0–74.0)	73.3 (67.9–78.7)	77.0 (72.8–81.3)
		40 mAU/mL for PIVKA-II or 10% for AFP-L3	74.8 (70.3–79.3)	87.0 (83.0–90.9)	72.5 (67.7–77.3)	88.2 (84.6–91.8)
AFP + AFP-L3	0.785 (0.750–0.820)	20 ng/mL for AFP or 5% for AFP-L3	74.8 (70.3–79.3)	65.1 (59.5–70.7)	66.3 (60.7–71.9)	73.8 (69.3–78.3)
		20 ng/mL for AFP or 10% for AFP-L3	67.0 (62.2–71.9)	78.6 (73.8–83.5)	64.6 (59.5–69.7)	80.4 (75.9–84.9)
AFP + PIVKA-II + AFP-L3	0.877 (0.851–0.903)	20 ng/mL for AFP, 40 mAU/mL for PIVKA-II, or 5% for AFP-L3	87.0 (83.5–90.5)	60.1 (54.4–65.9)	77.9 (72.4–83.5)	74.1 (69.9–78.2)
		20 ng/mL for AFP, 40 mAU/mL for PIVKA-II, or 10% for AFP-L3	82.8 (78.9–86.7)	73.2 (68.0–78.4)	76.5 (71.4–81.6)	80.2 (76.1–84.2)

AUROC, Area under the receiver operating characteristic curve; CI, Confidence interval; PPV, Positive predictive value; NPV, Negative predictive value; AFP, Alpha-fetoprotein; PIVKA-II, Protein induced by vitamin K absence-II; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein.

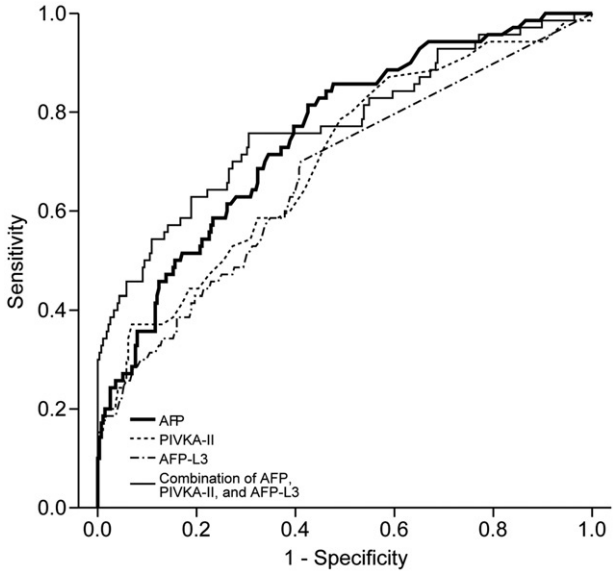

Figure 4. The receiver operating characteristic curves of alpha-fetoprotein (AFP), protein induced by vitamin K absence-II (PIVKA-II), and *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) for distinguishing early hepatocellular carcinoma (single tumor <3 cm in size) from cirrhosis. The area under the receiver operating characteristic curve was 0.754 (95% confidence interval [CI], 0.691–0.816, $p < 0.001$) for AFP, 0.701 (95% CI, 0.630–0.771, $p < 0.001$) for PIVKA-II, 0.670 (95% CI, 0.596–0.744, $p < 0.001$) for AFP-L3 and 0.773 for the combined AFP, PIVKA-II, and AFP-L3 markers.

It is still controversial whether combining AFP or AFP-L3 with PIVKA-II can improve the diagnostic performance of PIVKA-II as a marker; a few studies have reported the enhanced accuracy of PIVKA-II when it was combined with AFP and AFP-L3 [22,27], whereas another study reported different results [14]. In this study, PIVKA-II combined with AFP and AFP-L3 showed better accuracy than PIVKA-II alone. This result is not consistent with the study in the United States which suggested that PIVKA-II alone is superior to combined tumor markers [14]. This discrepancy might be due to different etiologies and study population. Durazo et al. included similar number of hepatitis B and hepatitis C, and defined chronic viral hepatitis with or without cirrhosis as a control group [14], while the dominant etiology in our study was hepatitis B, and only patients with cirrhosis were enrolled.

Although PIVKA-II had superior AUROC values compared to AFP or AFP-L3 in this study, its sensitivity (cutoff: 40 mAU/mL) was only 62.9%. The low sensitivity of PIVKA-II was enhanced to 87.0% upon combining with AFP (cutoff: 20 ng/mL) and AFP-L3 (cutoff: 5%). Moreover, our data demonstrate that AFP has better accuracy than AFP-L3 in detecting overall HCC. Accordingly, the order of AUROC superiority

Table IV. Sensitivity, specificity, PPV, and NPV for different cut-off values of tumor markers in distinguishing early hepatocellular carcinoma from cirrhosis.

Variables	AUROC (95% CI)	Cut-off value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	NPV (%) (95% CI)	PPV (%) (95% CI)
AFP	0.754 (0.691–0.816)	20 ng/mL	50.0 (38.3–61.7)	83.7 (79.3–88.1)	86.8 (82.8–90.9)	43.8 (32.9–54.6)
		200 ng/mL	18.6 (9.5–27.7)	98.9 (97.7–100.0)	82.7 (78.6–86.8)	81.2 (62.1–100.0)
PIVKA-II	0.701 (0.630–0.771)	40 mAU/mL	37.1 (25.8–48.5)	90.8 (87.2–94.5)	83.2 (78.7–87.7)	54.2 (70.1–68.3)
		100 mAU/mL	20.0 (10.6–29.4)	97.1 (95.0–99.2)	80.6 (76.1–85.2)	66.7 (46.5–86.8)
AFP-L3	0.670 (0.596–0.744)	5%	47.1 (35.4–58.8)	73.8 (68.6–79.0)	84.6 (80.0–89.2)	31.4 (22.5–40.3)
		10%	28.6 (18.0–39.2)	92.7 (89.7–95.8)	83.6 (79.5–87.8)	50.0 (34.5–65.5)
AFP + PIVKA-II	0.744 (0.673–0.815)	20 ng/mL for AFP or 40 mAU/mL for PIVKA-II	60.0 (48.5–71.5)	77.5 (72.6–82.5)	88.4 (84.4–92.5)	40.4 (31.0–49.8)
PIVKA-II + AFP-L3	0.743 (0.671–0.814)	40 mAU/mL for PIVKA-II or 5% for AFP-L3	61.4 (50.0–72.8)	68.5 (63.0–74.0)	87.5 (83.1–91.9)	33.1 (25.0–41.2)
		40 mAU/mL for PIVKA-II or 10% for AFP-L3	51.4 (39.7–63.1)	87.0 (83.0–90.9)	87.6 (83.7–91.5)	50.0 (38.5–61.5)
AFP + AFP-L3	0.738 (0.673–0.803)	20 ng/mL for AFP or 5% for AFP-L3	68.6 (57.7–79.4)	65.1 (59.5–70.7)	89.1 (84.7–93.4)	33.3 (25.6–41.0)
		20 ng/mL for AFP or 10% for AFP-L3	58.6 (47.0–70.1)	78.6 (73.8–83.5)	88.2 (84.2–92.2)	41 (31.4–50.6)
AFP + PIVKA-II + AFP-L3	0.773 (0.704–0.841)	20 ng/mL for AFP, 40 mAU/mL for PIVKA-II, or 5% for AFP-L3	75.7 (65.7–85.8)	60.1 (54.4–65.9)	90.7 (86.5–94.9)	32.5 (25.3–39.7)
		20 ng/mL for AFP, 40 mAU/mL for PIVKA-II, or 10% for AFP-L3	67.1 (56.1–78.1)	73.2 (68.0–78.4)	89.8 (85.8–93.7)	38.8 (30.2–47.5)

AUROC, Area under the receiver operating characteristic curve; CI, Confidence interval; PPV, Positive predictive value; NPV, Negative predictive value; AFP, Alpha-fetoprotein; PIVKA-II, Protein induced by vitamin K absence-II; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP.

(PIVKA-II > AFP > AFP-L3) in this study is in agreement with those of other studies [14,22,23].

In the detection of early HCC, a combination of AFP, PIVKA-II, and AFP-L3 showed the best AUROC. The second highest diagnostic accuracy was demonstrated by AFP alone, which had a better AUROC than either PIVKA-II or AFP-L3 alone, or a combination of the two markers. The findings that PIVKA-II and AFP had the best AUROC values for overall HCC and early HCC as sole markers, was similar to that reported in a previous Japanese study, where the diagnostic accuracy of PIVKA-II was lower than that of AFP in diagnosis of HCC <3 cm, while the opposite results were obtained for tumors >5 cm [18]. An American study reported that AFP was superior to PIVKA-II in detecting early stage HCC, while PIVKA-II had better AUROC values than AFP in intermediate-advanced stage HCC [15]. In our study, a combination of AFP, PIVKA-II, and AFP-L3 showed a modest increase in AUROC values in comparison with AFP alone, for detecting early HCC. Despite the lack of statistical significance, we propose that this finding is important because few studies have investigated the superiority of combined tumor markers, or included AFP-L3 in detecting early HCC in cirrhotic patients. Furthermore, the combination of all three tumor markers showed better sensitivity of 87.0% in overall HCC and 75.7% in early HCC, compared to either tumor marker alone. The sensitivity is regarded more important than specificity in HCC surveillance because the purpose of screening HCC is to detect early HCC more, and false-positive patients can be verified with subsequent ultrasonography. In addition, it is also important to identify very low-risk patients to have HCC for saving ultrasonographic investigation. In our study, NPV in combined three markers for early HCC diagnosis for early HCC diagnosis was increased to 90.7%. These remarkablely high values of sensitivity and NPV support the potential role of combined tumor markers as HCC surveillance.

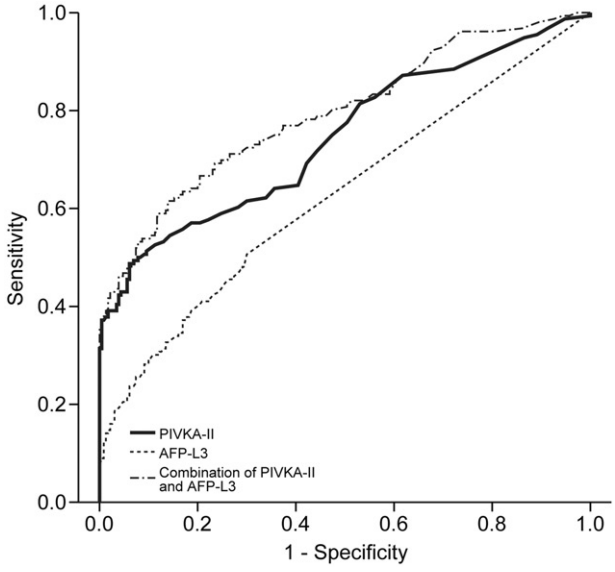

Figure 5. The receiver operating characteristic curves of protein induced by vitamin K absence-II (PIVKA-II), and *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) for diagnosing hepatocellular carcinoma in patients with AFP levels < 20 ng/mL. The area under the receiver operating characteristic curve was 0.744 (95% confidence interval [CI], 0.692–0.796, $p < 0.001$) for PIVKA-II, 0.625 (95% CI, 0.567–0.684, $p < 0.001$) for AFP-L3 and 0.794 (95% CI, 0.746–0841, $p < 0.001$) for a combination of PIVKA-II and AFP-L3.

The AFP-L3 concentration may be measured by automated analysis, and has been in commercial use in many countries. While it is well-established that the AFP-L3 concentration correlates with AFP, the percentage of AFP-L3 is not correlated with AFP [28,29]. As a marker, the AFP-L3 percentage is independent of AFP, therefore, the AFP-L3 percentage was selected for this analysis [21]. However, the assessment of AFP-L3 has been unreliable in patients with AFP levels <20 ng/mL owing to the low sensitivity of the instrument [30]. The recently developed highly sensitive AFP-L3 assay, which adopts a novel, on-chip electrokinetic reaction and separation by affinity electrophoresis, can be utilized for screening patients with AFP levels <20 ng/mL [12]. A recent Japanese study investigated the clinical utility of the highly sensitive AFP-L3 assay in HCC patients with AFP levels <20 ng/mL [13]. However, the study only compared the performance of the conventional assay

Table V. Sensitivity, specificity, PPV, and NPV for PIVKA-II and AFP-L3 in patients with AFP levels < 20 ng/ml.

Variables	AUROC (95% CI)	Cut-off value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	NPV (%) (95% CI)	PPV (%) (95% CI)
PIVKA-II	0.744 (0.692–0.796)	40 mAU/mL	50.0 (42.2–57.8)	90.4 (86.2–94.5)	69.5 (63.9–75.2)	80.4 (72.5–88.3)
		100 mAU/mL	39.1 (31.4–46.8)	97.0 (94.6–99.4)	66.8 (61.3–72.2)	91.0 (84.2–97.9)
AFP-L3	0.625 (0.567–0.684)	5%	41.0 (33.3–48.7)	77.8 (72.5–83.2)	66.1 (60.4–71.7)	55.7 (46.6–64.7)
		10%	23.7 (17.0–30.4)	93.9 (90.8–97.0)	64.5 (59.4–69.6)	72.5 (60.3–84.8)
PIVKA-II + AFP-L3	0.794 (0.746–0.841)	40 mAU/mL for PIVKA-II or 5% for AFP-L3	69.9 (62.7–77.1)	71.9 (66.1–77.7)	77.9 (72.4–83.5)	62.6 (55.5–69.8)
		40 mAU/mL for PIVKA-II or 10% for AFP-L3	60.9 (53.2–68.6)	87.4 (83.2–91.7)	76.8 (71.7–81.9)	76.6 (69.2–84.1)

AUROC, Area under the receiver operating characteristic curve; CI, Confidence interval; PPV, Positive predictive value; NPV, Negative predictive value; AFP, Alpha-fetoprotein; PIVKA-II, Protein induced by vitamin K absence-II; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein.

with the highly sensitive method for AFP-L3 measurement. In the present study, in patients with AFP levels < 20 ng/mL, a combination of PIVKA-II and AFP-L3 showed better AUROC values than either PIVKA-II or AFP-L3 alone, even though AFP-L3 had a lower AUROC value than PIVKA-II. The combination of PIVKA-II (cutoff: 40 mAU/mL) and AFP-L3 (cutoff: 5%) showed better sensitivity (69.9%) than PIVKA-II alone (50.0%); the AUROC value for the combined markers increased to 0.794 from 0.744 for PIVKA-II alone.

The optimal cutoff value for the AFP-L3 percentage remains controversial. Earlier studies have suggested 10% as the cutoff value of AFP-L3 [14,31]. However, more recent studies have presented various cutoff values between 5 and 10% [13,19]. In this study, we assessed the cutoff values of 5 and 10% for AFP-L3. The sensitivity of the AFP-L3 percentage in differentiating early HCC from cirrhosis was 47.1% for the cutoff value of 5%, and 28.6% for the cutoff value of 10%; similarly, the specificity of the AFP-L3 percentage was 73.8% for the cutoff value of 5%, and 92.7% for the cut-off value of 10%. As the role of biomarkers is to identify more early HCCs, our data indicate that the cutoff value of 5% should be recommended for HCC detection, instead of 10%.

We are aware that there are a few limitations in this study. First, there may be selection bias due to the retrospective nature of the study, despite the enrollment of consecutive patients. Second, liver disease had heterogeneous etiology although hepatitis B was the main cause of cirrhosis. The cost-effectiveness of using a combination of tumor markers might be another issue, and warrants further studies.

In conclusion, a combination of three tumor markers including AFP, PIVKA-II, and AFP-L3 showed better accuracy than either marker alone in differentiating overall and early HCC from cirrhosis. Moreover, a combination of PIVKA-II and AFP-L3 showed enhanced accuracy compared to PIVKA-II alone, in patients with AFP levels < 20 ng/mL. However, because the combined tumor markers showed only modest improvement over AFP in diagnosing early HCC, the use of other novel tumor markers should be evaluated for the differentiation of early HCC in cirrhotic patients.

Declaration of interest: The authors report no conflicts of interest. The authors are responsible for the content and writing of the paper.

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