

Serum cytoskeleton-associated protein 4 as a biomarker for the diagnosis of hepatocellular carcinoma

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Background: Alpha-fetoprotein (AFP) is the most commonly applied biomarker for diagnosis of hepatocellular carcinoma (HCC), but the low sensitivity and specificity limit its clinical application. Cytoskeleton-associated protein 4 (CKAP4) is a novel oncogenic protein involved in the development and progression of HCC. This study aimed to evaluate whether measurement of circulating CKAP4 could improve diagnostic accuracy for HCC.

Methods: We analyzed data for patients with HCC, chronic hepatitis B infection, and cirrhosis and healthy controls (n=100 in each group), recruited from two centers between July 2013 and December 2015. Circulating levels of CKAP4 were measured with commercial enzyme-linked immunosorbent assay kits. Receiver operating characteristics were used to evaluate diagnostic accuracy.

Results: Serum concentrations of CKAP4 were significantly elevated in the HCC group, in comparison with the three control groups (all $P < 0.001$). The combined biomarker panel (AFP and CKAP4), created by binary logistic regression, presented better performance (area under the curve [AUC] 0.936, 95% CI [0.908–0.965], sensitivity 0.800, specificity 0.963) than AFP (AUC 0.875 [0.835–0.914], sensitivity 0.930, specificity 0.430, $P = 0.001$) or CKAP4 (AUC 0.821 [0.776–0.866], sensitivity 0.790, specificity 0.670, $P < 0.001$) alone to identify HCC, even though CKAP4 alone was not better than AFP ($P = 0.093$). Furthermore, the combined panel also presented a better performance even in identifying early HCC (AUC 0.922 [0.833–0.961]).

Conclusion: Serum CKAP4 is a novel biomarker for HCC, and it could complement AFP in improving diagnostic accuracy.

Keywords: alpha-fetoprotein, biomarker, cytoskeleton-associated protein 4, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent causes of cancer-related death in the world.^{1,2} Approximately half of the global HCC morbidity and mortality occurs in China.^{1,3} Surgical resection and liver transplant only benefit patients with early-stage disease.^{4–6} Patients with more advanced disease are, unfortunately, only eligible for palliative therapy and usually present a poor prognosis, with median survival of 1–2 years.⁷ Given the situation, surveillance strategies for early tumor detection are urgently required in clinical practice.⁸

Imaging tools (eg, ultrasound and computed tomography) and serum biomarkers (eg, alpha-fetoprotein [AFP]) are commonly used to screen and diagnose HCC.⁹ However, it is still hard for imaging to differentiate small HCC from cirrhosis nodules. Meanwhile, evaluated levels of AFP are not only seen in patients with HCC but also in

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chronic hepatitis infections.^{3,10} Thus, there is an urgent need for the identification of novel biomarkers for HCC screening.

Cytoskeleton-associated protein 4 (CKAP4) is a 63-kDa nonglycosylated and reversibly palmitoylated type II transmembrane protein.^{11,12} CKAP4 has recently been located in the endoplasmic reticulum, and identified as a cell surface receptor of the plasminogen activator and surfactant protein A, as well as an anti-proliferative factor to adversely mediate AKT signaling.^{13–15} Recently, Li et al¹⁶ examined the expression of CKAP4 in patients with HCC. They found that CKAP4 showed higher expression in HCC tumor tissues than in adjacent normal tissues and this was correlated with tumor size, intrahepatic metastases, portal-venous invasion, and HCC stage. Moreover, elevated CKAP4 expression was associated with favorable overall survival and longer disease-free survival. Recently, Li et al¹⁷ also proposed a novel mechanism for the mediation of EGFR/Gab1/AKT signaling activity, suggesting that CKAP4 is involved in progression and metastasis of HCC. Thus, these findings support CKAP4 playing a role in the oncogenic process of HCC and may be a potential target for diagnosis and therapy.

Recently, Yanagita et al¹¹ designed an antibody that specifically recognized CKAP4 in serum. They found that the circulating levels of CKAP4 in patients with lung cancer were significantly higher than those in healthy controls. The hospital-based case–control study suggested serum CKAP4 as a potential biomarker for lung cancer.

In this study, our aim was to evaluate the serum levels of CKAP4 in patients with HCC and other liver diseases. Our team proposes that CKAP4 might be a potential diagnostic biomarker for HCC. The combination of serum biomarkers is recommended in cancer diagnosis,^{10,18} therefore we also tried to assess the diagnostic value of the combination of CKAP4 and AFP in the clinical management of HCC.

Methods

Study population

A total of 100 consecutive patients with HCC were recruited in this study from the First Affiliated Hospital of Soochow University, Suzhou and Jintan Affiliated Hospital of Jiangsu University, Changzhou, China (from July 2013 to December 2015). Meanwhile, we also recruited patients with chronic hepatitis B (CHB) infection (n=100) or cirrhosis (n=100) from the two hospitals and healthy controls (n=100) from the Out-patient Department. Written informed consent was obtained from each participant. This study was approved by the Ethics Committee of each center, in accordance with

the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Inclusion criteria

HCC was firstly diagnosed by imaging (eg, ultrasound or computed tomography), and then the cases were further confirmed by histopathology, based on the guidelines of the American Association for the Study of Liver Diseases.¹⁹ HCC stages were classified according to the Barcelona Clinic Liver Cancer staging system.⁷ In this study, tumors at Barcelona Clinic Liver Cancer stage 0 and A were categorized as early-stage HCC.

The diagnosis of CHB infection was based on: 1) hepatitis B surface antigen-positive for the previous 6 months; 2) hepatitis B virus DNA >10³ copies/mL; and 3) elevated alanine aminotransferase, according to the guidelines for prevention and treatment of chronic HBV infection.²⁰

Cirrhosis was diagnosed based on the histopathology of liver biopsy samples, and on other evidence (eg, portal hypertension, splenomegaly, nodular liver contour, presence of ascites, varices, enlargement of the caudate lobe, and collateral portal-venous anastomoses).¹⁹

The inclusion criteria for healthy controls were the following: 1) normal liver biochemistry; 2) no history of liver disease; 3) no other systematic diseases; and 4) no malignant disease.

Laboratory examination

Serum samples of patients were collected at the time of diagnosis. All samples were centrifuged and stored at –80°C for further evaluation. Serum biochemical tests were performed using a Hitachi 7600 Auto-Analyzer (Hitachi Ltd., Tokyo, Japan). Circulating levels of CKAP4 were measured with commercial enzyme-linked immunosorbent assay kits (Catalog No CSB-EL005453HU; CUSABIO, Wuhan, China). The intra-assay and inter-assay coefficients of variation of serum CKAP4 measurement were 7.2% and 9.7%, respectively. Circulating levels of AFP were measured by chemiluminescence immunoassay with an Abbott-Architect Immunoanalyzer (Abbott Laboratories, Abbott Park, IL, USA). The examination was performed blinded and in duplicate.

Statistical analyses

Differences among various groups (more than two) used one-way analysis of variance or the Kruskal–Wallis test. Receiver operating characteristic curves were developed to evaluate

sensitivity, specificity, and areas under the curves (AUCs) with 95% CI. The optimum cutoff values for diagnosis were calculated according to the Youden Index. To investigate whether the combined utility of biomarkers was better than either alone, a new variable of predicted probability (p) for HCC was created on the basis of an equation obtained by binary logistic regression (all HCC vs Control + Cirrhosis + CHB). All statistical analyses and plotting were performed using Stata (version MP 11.2; StataCorp LP, College Station, TX, USA), Prism (version 6; GraphPad Software, Inc., LA Jolla, CA, USA), and SPSS (version 24.0; IBM Corporation, Armonk, NY, USA). A two-sided $P < 0.05$ was considered statistically significant.

Results

The characteristics of all participants and HCC patients are indicated in Tables 1 and 2.

Biomarker levels

As shown in Figure 1A, serum AFP in patients with HCC (median 148.2 [IQR 48.2–277.5] ng/mL) presented higher levels than those in controls (healthy control: 7.4 [4.8–267.3] ng/mL; CHB: 31.5 [22.7–52.2] ng/mL; cirrhosis: 31.3 [22.2–53.3] ng/mL; $P < 0.0001$).

Serum levels of CKAP4 were significantly higher in patients with HCC (345.9 [264.8–474.5] pg/mL) than in all controls (healthy control: 184.2 [111.1–267.3] pg/mL; CHB: 180.1 [97.2–283.5] pg/mL; cirrhosis: 228.1 [129.2–307.1] pg/mL; all $P < 0.001$), as shown in Figure 1B.

Cutoff values and AUCs

According to the guideline,²¹ we chose 20 ng/mL as the cutoff value for AFP in this study (AUC 0.875 [95% CI 0.835–0.914], sensitivity 0.930, specificity 0.430; Figure 2A and Table 3). Receiver operating characteristic curves showed that the optimum diagnostic cutoff value for CKAP4 was 250.15 pg/mL (AUC 0.821 [0.776–0.866], sensitivity 0.790, specificity 0.670). The comparison between AFP and CKAP4 presented no difference ($P = 0.093$).

Combined biomarkers

To assess the diagnostic value of the combined variables, a new variable of predicted probability (p) for HCC was developed on the equation obtained from the binary logistic regression (all HCC vs all controls). The equation was as follows:

$$\ln\left(\frac{p}{1-p}\right) = 0.021 \times \text{AFP} + 0.015 \times \text{CKAP4} - 6.927$$

In Figure 2A, the optimum cutoff value calculated from the combined variables was 0.404. This indicated a better predicting ability (AUC 0.936 [0.908–0.965], sensitivity 0.800, specificity 0.963), compared with AFP and CKAP4 ($P = 0.001$ and $P < 0.001$). Predictive values and likelihood ratios for AFP, CKAP4, and the combination in the diagnosis of HCC are shown in Table 3.

Early-stage HCC

Given that early detection is the key to improve prognosis of patients with HCC, the diagnostic ability of AFP, CKAP4,

Table 1 Characteristics of participants

Items	Control	CHB	Cirrhosis	HCC
Age (years)	57.2±6.8	54.1±6.4	57.0±7.2	55.0±6.2
Sex				
Male	84	84	88	84
Female	16	16	12	16
BMI	22.7±3.2	23.4±3.9	22.1±2.9	22.3±3.3
ALT (U/L)	22.0 (16.0–34.0)	48.0 (32.0–62.0)	51.0 (33.0–67.0)	67.0 (43.0–85.5)
GGT (U/L)	39.0 (12.0–48.0)	51.0 (29.0–56.0)	78.0 (40.0–98.0)	99.0 (64.0–127.0)
HBsAg				
Positive	0	100	84	85
Negative	100	0	16	15
Child–Pugh				
A	NA	NA	77	81
B			23	19
MELD score	NA	NA	11 (9–15)	11 (9–14)*

Notes: Data presented as mean ± SD, median (IQR), or number. Each group, $n = 100$. *MELD score only in HCC cirrhosis (+) patients ($n = 91$).

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CHB, chronic hepatitis B; GGT, gamma-glutamyltranspeptidase; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; MELD, Model for End-Stage Liver Disease; NA, not applicable.

Table 2 Tumor features of HCC patients

Items	HCC (n=100)
Tumor size (cm)	
<3	28
3–5	49
>5	23
Tumor number	
Single	71
Multiple	29
BCLC stage	
0	7
A	56
B	18
C	19
D	0

Abbreviations: BCLC, Barcelona Clinic Liver Cancer; HCC, hepatocellular carcinoma.

and the combination in early-stage HCC were further evaluated. As shown in Figure 2B and Table 3, the combination presented the best AUC (0.922 [0.833–0.961]), followed by AFP (0.834 [0.782–0.886], $P<0.001$) and CKAP4 (0.842 [0.792–0.893], $P=0.001$). The latter two still indicated no difference ($P=0.831$).

Discussion

Recent evidence reported that the hepatic expression of CKAP4 increased in patients with HCC, and that CKAP4 is associated with clinical outcome and prognosis.^{16,17} Kimura et al¹² reported that CKAP4 at the plasma membrane stimulates proliferation of several cell lines, including pancreatic

cancer cells and lung cancer cells, via activating the phosphoinositide 3-kinase–AKT pathway. Furthermore, they found that the expression of CKAP4 in pancreatic and lung cancers was related to a poor prognosis for patients. Shinno et al²² reported that overexpression of CKAP4 could be observed in about 40% of esophageal squamous cell carcinoma patients. The phosphoinositide 3-kinase–AKT pathway may be a downstream cascade of the Dickkopf WNT signaling pathway inhibitor 1–CKAP4 signaling axis in esophageal squamous cell carcinoma cases. The role and mechanisms of CKAP4 in cancers still need further investigation.

Circulating biomarkers are promising potential tools for screening and early detection of HCC, owing to their noninvasive and cost-efficient features.^{23,24} AFP is currently still the most widely used biomarker in HCC, even though it presents a series of disadvantages: 1) less specificity, especially in patients with chronic hepatitis or cirrhosis; and 2) associated with tumor size and not suitable for surveillance at an early stage.²³ Therefore, a new reliable biomarker to complement AFP is urgently required to improve early detection.

In this study, we investigate whether measurement of circulating CKAP4 levels could improve diagnostic accuracy for HCC, in a hospital-based case–control study that included 100 HCC patients and 300 non-HCC controls (ie, 100 healthy controls, 100 controls with chronic HBV infection, and 100 controls with cirrhosis). Our study followed the widely accepted protocol for evaluation of diagnostic biomarkers.²⁵ It found that serum concentrations of

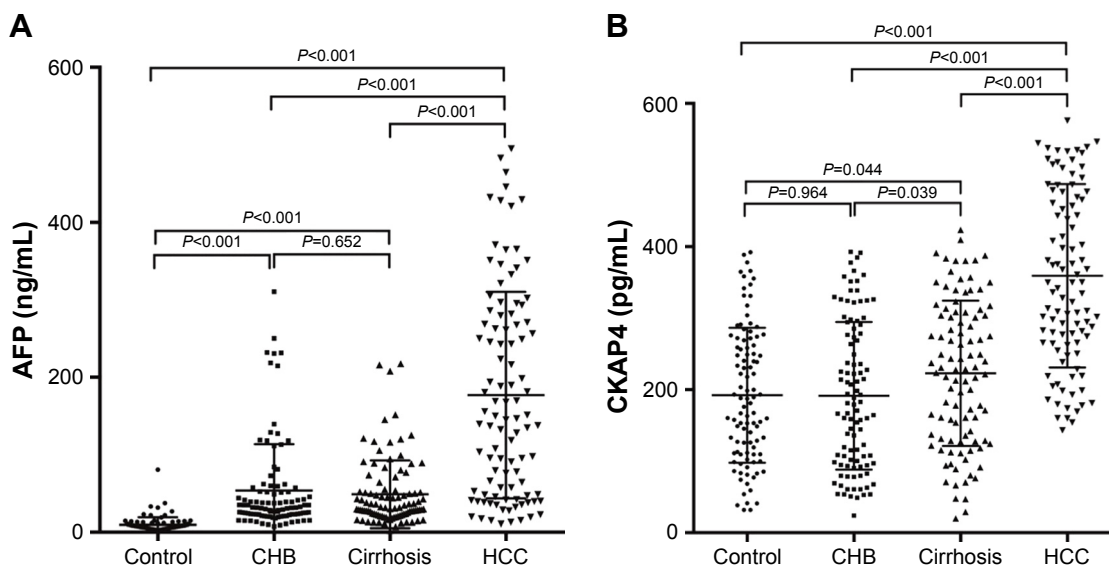


Figure 1 Circulating concentrations of AFP and CKAP4 in HCC, control, CHB, and cirrhosis groups.

Notes: (A) Levels of serum AFP (ng/mL, median [IQR]); (B) levels of serum CKAP4 (pg/mL, median [IQR]).

Abbreviations: AFP, alpha-fetoprotein; CHB, chronic hepatitis B; CKAP4, cytoskeleton-associated protein 4; HCC, hepatocellular carcinoma.

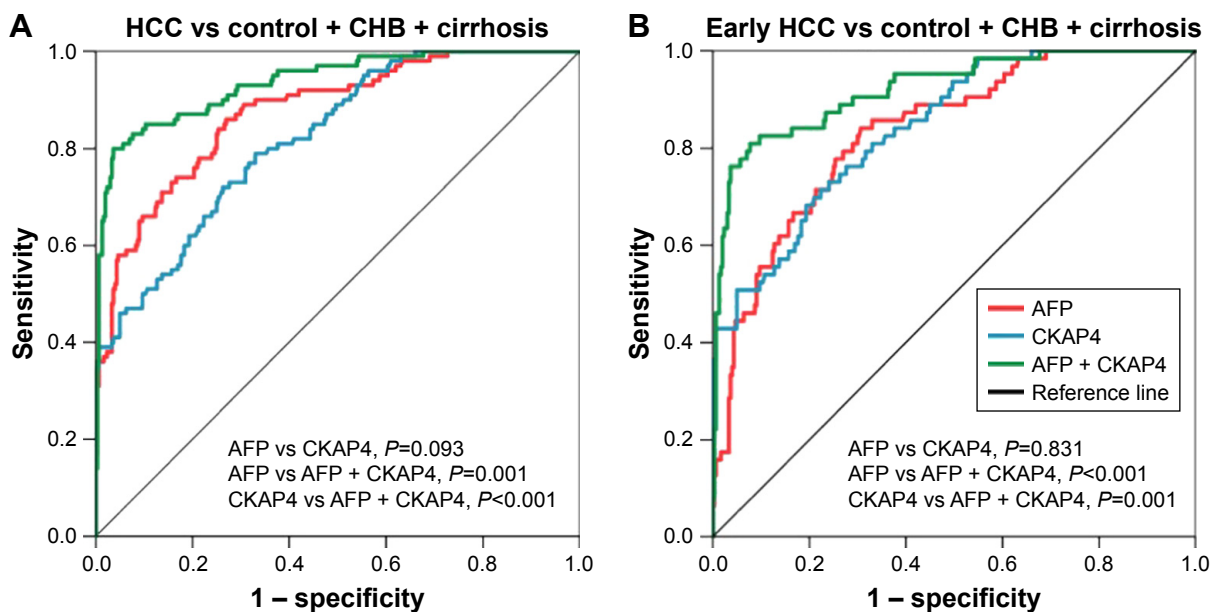


Figure 2 ROC curves for AFP, CKAP4, and the combination in the diagnosis of HCC and early-stage HCC.

Note: (A) ROC curves for AFP, CKAP4, and the combinations for patients with HCC vs all controls; (B) ROC curves for AFP, CKAP4, and the combinations for patients with early-stage HCC vs all controls.

Abbreviations: AFP, alpha-fetoprotein; CHB, chronic hepatitis B; CKAP4, cytoskeleton-associated protein 4; HCC, hepatocellular carcinoma; ROC, receiver operating characteristic.

CKAP4 were significantly elevated in the HCC group, in comparison with the three control groups. Besides, 86% of AFP-negative patients with HCC also indicated increased serum levels of CKAP4. The combined biomarker panel (AFP and CKAP4), created by binary logistic regression, outperformed stand-alone assays of AFP or CKAP4 to identify HCC, but CKAP4 alone was not better than AFP. The combined panel presented good diagnostic accuracy, even in identifying early HCC. However, the molecular mechanism and clinical importance of CKAP4 in HCC still need to be further explored.

Our study has some limitations. To begin with, patients with other digestive cancers (eg, cholangiocarcinoma and gastric cancer) should be included to rule out potential false-positive results. Given the previous findings, the serum levels

of CKAP4 in lung cancer,¹¹ renal cancer,²⁶ and esophageal cancer²² should be evaluated in further studies to assess the specificity. Also, validation studies should be conducted in patients with HCV-induced or alcohol-induced HCC, which are prevalent in western countries. Furthermore, this study was of a retrospective design and thus large prospective studies to assess the role of CKAP4 in the development of HCC are needed.

Conclusion

This is the first study to report the levels of serum CKAP4 in liver diseases. Our result indicates that serum CKAP4 could potentially be used to identify HCC. The result also shows that the combination of CKAP4 and AFP could enhance accuracy and specificity in the diagnosis of HCC,

Table 3 Performance of AFP, CKAP4, and the combination in the diagnosis of HCC and early-stage HCC

Items	AUC	95% CI	Sensitivity	Specificity	PPV	NPV	LR+	LR-
HCC vs control/CHB/cirrhosis								
AFP	0.875	0.835–0.914	0.930	0.430	0.352	0.949	1.632	0.163
CKAP4	0.821	0.776–0.866	0.790	0.670	0.444	0.905	2.394	0.313
Combination	0.936	0.908–0.965	0.800	0.963	0.879	0.935	21.818	0.208
Early HCC vs control/CHB/cirrhosis								
AFP	0.834	0.782–0.886	0.905	0.430	0.250	0.956	1.587	0.221
CKAP4	0.842	0.792–0.893	0.810	0.670	0.340	0.944	2.453	0.284
Combination	0.922	0.883–0.961	0.762	0.963	0.814	0.951	20.779	0.247

Abbreviations: AFP, alpha-fetoprotein; AUC, area under receiver operating characteristic curve; CHB, chronic hepatitis B; CKAP4, cytoskeleton-associated protein 4; HCC, hepatocellular carcinoma; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

even early-stage HCC, and may provide a new diagnostic strategy for HCC patients.

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Disclosure

The authors report no conflicts of interest in this work.

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