

## Original Article

# Cytoskeleton-linking membrane protein 63 as a serum biomarker for the early detection of hepatocellular carcinoma

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**Abstract:** The serum alpha-fetoprotein (AFP) is a common marker for diagnosis of hepatocellular carcinoma (HCC). But the disadvantage like low specificity and sensitivity of AFP detection in the diagnosis of HCC are problems in clinical practice. The aim of this study was to investigate the clinical value of serum cytoskeleton-linking membrane protein (CLIMP)-63 in the diagnosis of HCC. Liver and serum samples from DEN-induced hepatocarcinogenesis in Rats were used to detect CLIMP-63 level. Serum samples were collected from 40 healthy volunteers and 90 patients with HCC confirmed by pathology. The patients were divided into subgroups according to the baseline AFP level as follows:  $\leq 5$ ,  $\leq 10$ ,  $\leq 20$  ng/ml. The serum CLIMP-63 was determined with enzyme-linked immunosorbent assays (ELISAs). The area under the receiver operating characteristic curve (AuROC), sensitivity and specificity of AFP and CLIMP-63 levels were evaluated for diagnostic performance with positive biopsy. The liver CLIMP-63 levels tend to increase according to the progression of DEN-treatment rats. The serum CLIMP-63 level after DEN-injection 10 weeks was remarkably higher than it from the non DEN-treatment rats. The serum CLIMP-63 levels in HCC patients were much higher than that in healthy volunteers ( $P < 0.05$ ). Furthermore, the AuROC for serum CLIMP-63 in the diagnosis of HCC was 0.771 ( $P = 0.000$ ), and for serum AFP was 0.892 ( $P = 0.000$ ). In the subgroups, the AuROC of AFP level of  $\leq 5$ ,  $\leq 10$ ,  $\leq 20$  ng/ml in the diagnosis of HCC was 0.629 ( $P = 0.110$ ), 0.652 ( $P = 0.034$ ), 0.705 ( $P = 0.003$ ) respectively. While the AuROC of serum CLIMP-63 was 0.777 ( $P = 0.001$ ), 0.769 ( $P = 0.000$ ), 0.759 ( $P = 0.000$ ) respectively. CLIMP-63 may be a novel serum biomarker for clinical diagnosis of HCC in the early stage and testing serum CLIMP-63 and AFP may improve the detection rate of HCC.

**Keywords:** Cytoskeleton-linking membrane protein (CLIMP)-63, alpha-fetoprotein (AFP), hepatocellular carcinoma (HCC), diagnosis

## Introduction

Hepatocellular carcinoma (HCC) has become the most common solid tumor worldwide and the third leading cause of tumor-related death in many countries [1]. An estimated 748,300 new liver cancer cases and 695,900 cancer deaths occurred worldwide in 2008. Half of these cases and deaths was estimated to occur in China [2]. The prognosis of patients with HCC is generally very poor with a 5-year

survival rate of less than 10-15% owing to lack of effective and timely diagnostic methods [3]. Therefore, early diagnosis is extremely important for improving clinical outcomes. Currently, a-fetoprotein (AFP) is the most important tumor mark for HCC diagnosis. However, AFP level is insensitive for the detection of HCC at the commonly used cutoff of 20 ng/ml. Furthermore, the specificity of AFP is low due to many patients with hepatitis, hepatocirrhosis and gastrointestinal cancer also have elevated serum levels of

AFP [4]. Therefore, novel serum makers to complement AFP are needed to improve the diagnostic accuracy of HCC.

Cytoskeleton-linking membrane protein (CLIMP)-63 is a type II transmembrane protein that is located in the endoplasmic reticulum [5, 6]. Previous reports have shown that CLIMP-63 plays an important role to maintain endoplasmic reticulum structure [6-9]. Recent studies have shown that CLIMP-63 is closely related to the tumor growth, proliferation, invasion, and metastasis. It has been shown CLIMP-63 could inhibit the proliferation of bladder cancer cells by combining its ligand antiproliferative factor (APF) [10]. Our group reported that the expression of CLIMP-63 was significantly associated with tumor size, lymph node metastasis, and UICC and TNM stage features and was a prognostic marker in patients with intrahepatic cholangiocellular carcinoma (ICC) [11]. In addition, we also reported that CLIMP-63 could inhibit tumor intrahepatic metastasis and venous invasion, and was a prognostic marker in patients with HCC [12]. A study defined CLIMP-63 as a novel protein interactor and regulator of Dicer function, and it can be secreted by cultured human cells with Dicer [13]. Based on the biology role and previous studies of CLIMP-63 in tumors, we aimed to evaluate whether serum CLIMP-63 can be used as a serum biomarker in detection of HCC in this study.

### Patients and methods

#### *Patients and samples*

Serum samples were obtained before any treatment from 90 patients with HCC who underwent surgery from January 2013 to September 2013 at the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University in Shanghai, China. The blood samples were centrifuged at 3,000 rpm for 15 minutes and stored at -80°C until further assayed. The serum samples of 40 health people were obtained from the Health Check Center of Eastern Hepatobiliary Surgery Hospital from July 2013 to September 2013. This research was approved by the Institutional Review Board of the Second Military Medical University and all participants gave written informed consent.

#### *Animals and experimental procedures*

DEN was purchased from Wako Pure Chemical Co., Ltd. (Kyoto, Japan) and diluted with a 0.9% NaCl solution to a concentration of 0.1%. Male Sprague-Dawley rats (10 weeks old, average body weight  $98.7 \pm 6.3$  g) were provided by Animal center of The Second Military Medical University. Rats were randomly divided into two groups according to treating with or without DEN (given diethylnitrosamine 25 mg/kg/d by intraperitoneal injection) once a week for 20 weeks. The experiment was terminated at the end of 20 weeks. Rats were sacrificed under light ether anesthesia on weeks 0, 6, 8, 10, 12, 14, 16, 18, 20. Blood samples were taken from via cardiac puncture before sacrifice. The blood samples were centrifuged at 3,000 rpm for 15 min, serum was then separated and kept at -80°C until used for assays. Livers were immediately excised and processed for histological examination.

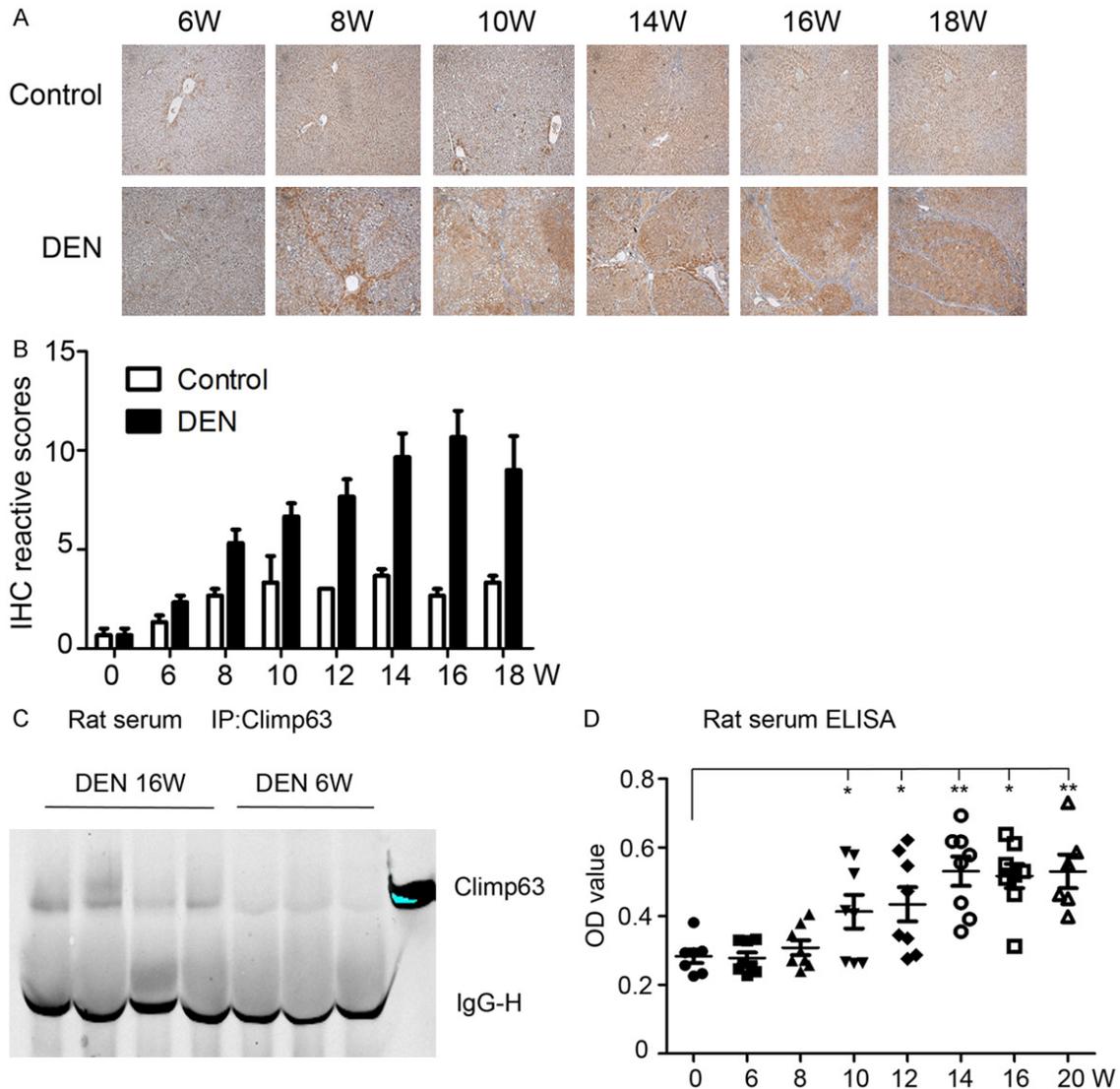
#### *ELISA agents*

CLIMP-63 monoclonal and polyclonal antibodies were purchased from Lifespan and Sigma. Horseradish peroxidase (HRP) labeled anti-mouse secondary antibody was from Pik days biotechnology Research Institute. TMB reagent was from sigma. Coating solution:  $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$  buffer (PH 9.6). Washing solution: PBS (PH 7.2) plus 0.05% tween-20. Blocking solution: 1% of BSA was added PBS containing 0.1% tween-20. Antibody dilution: PH 7.2 of PBS plus 0.1% BSA. Stop solution:  $\text{H}_2\text{SO}_4$  (concentration: 2 mol/L). ELISA method was described before [14].

#### *Coimmunoprecipitation assays*

Serum samples were prepared in radioimmuno-precipitation assay (RIPA) buffer and incubated with anti-CLIMP-63 antibody (sigma) for 8 h at 4°C, followed by addition of Protein A/G Plus-Agarose (Santa Cruz Biotechnology) for another 4 h. The samples from these assays were centrifuged at 12,000 g for 15 min. Immunoblotting was performed using anti-CLIMP-63 antibody, and immune-complexes were incubated with the fluorescein-conjugated secondary antibody and then detected using Odyssey fluorescence scanner (Li-Cor, Lincoln, NE).

## CLIMP-63 as a serum biomarker for HCC

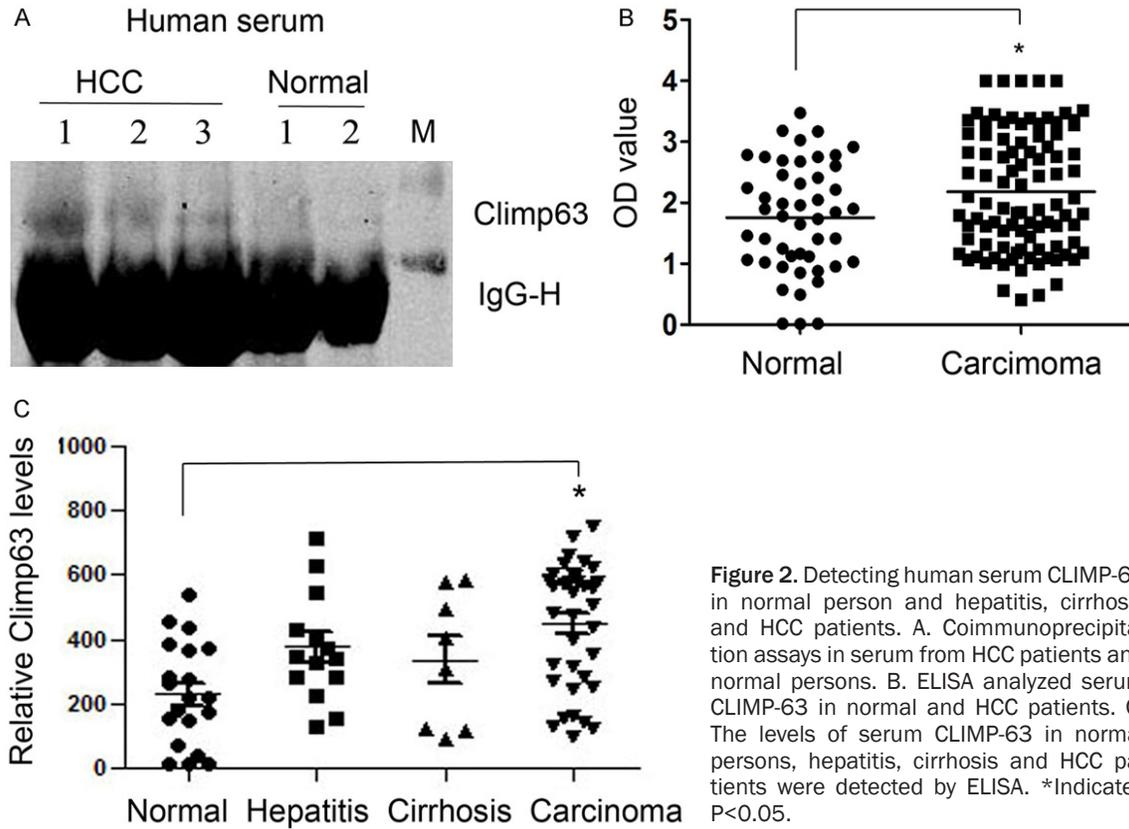


**Figure 1.** Liver tissue and serum level of CLIMP-63 on DEN-induced hepatocarcinogenesis in rats. A, B. Immunohistochemical staining CLIMP-63 expression in DEN induced hepatocarcinogenesis tissues of rats and the immunoreactive score of the protein in specimens. C. Serum CLIMP-63 in DEN-induced HCC was detected by coimmunoprecipitation assays in rats serums of DEN-injection 16 weeks and 6 weeks. D. Serum CLIMP-63 was examined in rats with 0-20 weeks DEN treatment by ELISA. \*Indicates  $P < 0.05$ , \*\*indicates  $P < 0.01$ .

### Immunohistochemistry

The tissue sections were dewaxed, rehydrated and then immersed in methanol containing 0.3% hydrogen peroxide (Sinopharm Chemical Reagent Co., Ltd., 10011218) for 30 min to block endogenous peroxidase activity. Pressure cooker filled with 10 mM ethylenediaminetetra acetic buffer (EDTA, pH 8.0) boil heated for 2 min. After blocking, the sections were incubated in a primary polyclonal antibodies against CLIMP-63 (Sigma, HPA000792) with 1:100 dilution. Finally, the visualization signal was

developed with diaminobenzidine and the slides were counterstained in hematoxylin. Stained sections were evaluated in a blinded manner without prior knowledge of the clinical information using the German immunoreactive score (IRS). Briefly, the IRS assigns sub-scores for immunoreactive distribution (0-4) and intensity (0-3). The percent positivity was scored as "0" (<5%), "1" (5-25%), "2" (25-50%), "3" (50-75%) or "4" (>75%). The staining intensity was scored as "0" (no staining), "1" (weakly stained), "2" (moderately stained) or "3" (strongly stained).



**Figure 2.** Detecting human serum CLIMP-63 in normal person and hepatitis, cirrhosis and HCC patients. **A.** Coimmunoprecipitation assays in serum from HCC patients and normal persons. **B.** ELISA analyzed serum CLIMP-63 in normal and HCC patients. **C.** The levels of serum CLIMP-63 in normal persons, hepatitis, cirrhosis and HCC patients were detected by ELISA. \*Indicates  $P < 0.05$ .

*Statistical analysis*

To compare the concentrations of CLIMP-63 and AFP in HCC samples and controls, we calculated *P*-values for ELISA results by an independent-samples t-test. We constructed receiver operating characteristic (ROC) curves for serum CLIMP-63 and AFP, to assess their diagnostic accuracy in distinguishing HCC patients from normal control subjects. Using the ROC method, we calculated the sensitivity, specificity, and area under the receiver operating characteristic curve (AuROC) to determine the diagnostic accuracy of our findings. Statistical Package for Social Sciences software (SPSS, Inc., Chicago, IL, USA version 17.0) was used for data support and analysis and *p* values  $< 0.05$  were considered as statistically significant differences.

**Results**

*Liver tissue and serum level of CLIMP-63 on DEN-induced hepatocarcinogenesis in rats*

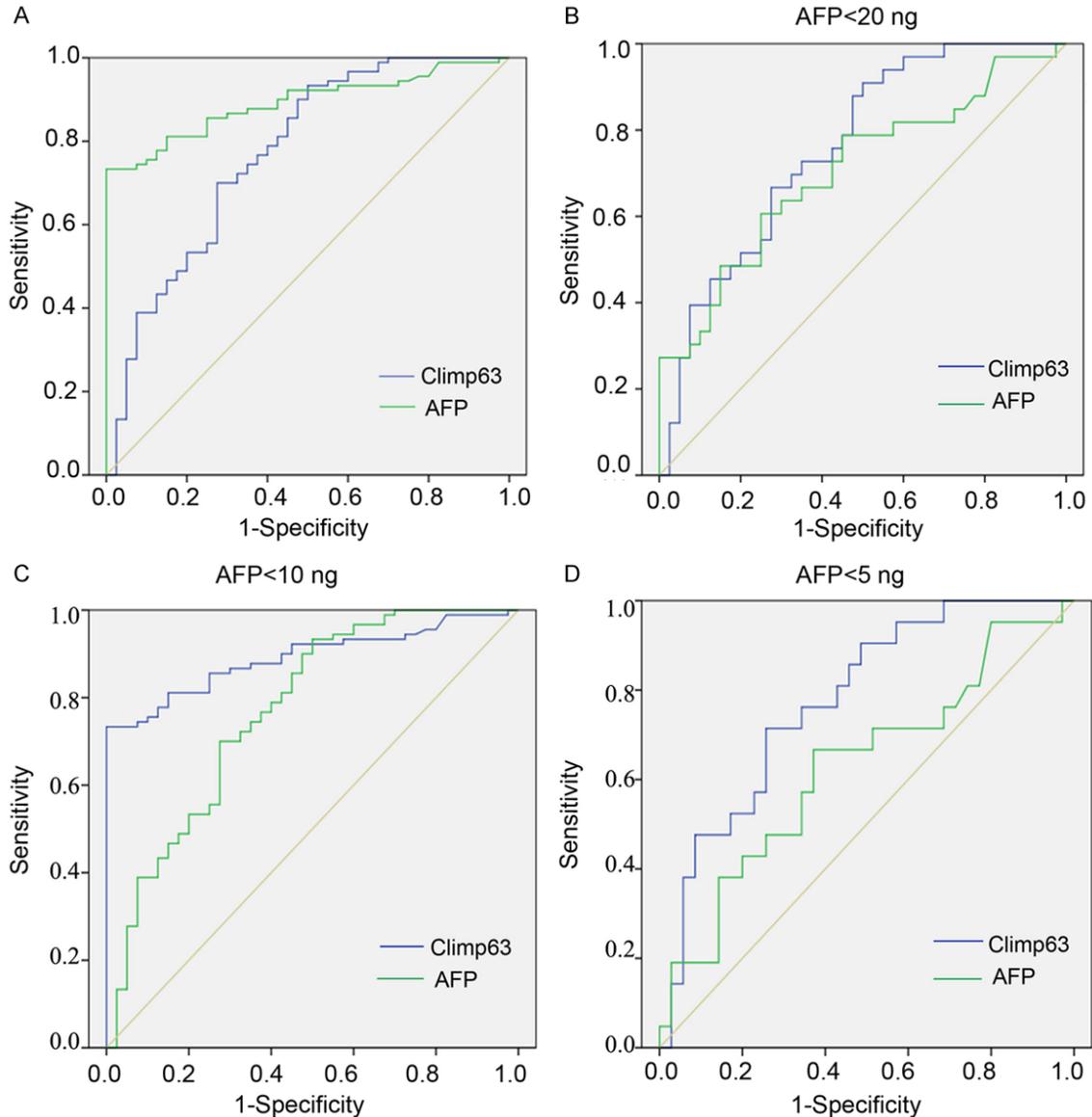
Immuohistochemical studies indicated that CLIMP-63 were significantly higher after DEN-

injection 6-8 weeks compared with control (**Figure 1A, 1B**). For analysis the serum concentrations of CLIMP-63 in DEN-induced HCC, we performed coimmunoprecipitation assays in serums of DEN-injection 16 weeks and 6 weeks rats. Endogenous CLIMP-63 was immunoprecipitated by CLIMP-63 antibody and CLIMP-63 protein, which were determined by western blot assay (**Figure 1C**). The results showed that CLIMP-63 could be detected in serum of HCC rats. The serum CLIMP-63 was verified by enzyme-linked immunosorbent assays (ELISA) (**Figure 1D**). The CLIMP-63 levels tended to increase according to the progression of DEN-induced hepatocarcinogenesis in rats. The serum CLIMP-63 level after DEN-bearing 10 weeks was remarkably high than that of non DEN-bearing rats ( $P < 0.05$ , **Figure 1D**).

*Serum level of CLIMP-63 in human specimen*

We performed coimmunoprecipitation assays in serum from HCC patients and normal persons. The results showed that CLIMP-63 could be detected in serum of HCC patients (**Figure 2A**). Further analysis by ELISA revealed that the CLIMP-63 serum level was obviously higher in

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**Figure 3.** Comparing diagnostic accuracy of serum CLIMP-63 and AFP for HCC. A. ROC curve for CLIMP-63 or AFP for all patients with HCC versus all controls in the test cohort. The blue line indicates the CLIMP-63 and the green line indicates the AFP. B-D. ROC curves showed the performance of the CLIMP-63 in patients with AFP levels between 20 to 5 ng per milliliter from a total of 130 samples (90 from patients with HCC and 40 from controls).

HCC blood samples than it in normal ones ( $P < 0.05$ , **Figure 2B**). We also detected the levels of serum CLIMP-63 in normal persons, hepatitis, cirrhosis and HCC patients (**Figure 2C**). The results show that the CLIMP-63 level tends to increase according to the progression of hepatocarcinogenesis.

### *Compare diagnostic accuracy of serum CLIMP-63 and AFP for HCC*

The ROC curve of CLIMP-63 was close to AFP curve, which indicated that CLIMP-63 had com-

parable diagnosis accuracy to AFP for HCC in our study cohort (**Figure 3A**). The sensitivity, specificity and AuROC (95% CI) of serum CLIMP-63 for the diagnosis of HCC were 70%, 67.5%, 0.771 (0.678-0.863), respectively. For AFP, the sensitivity, specificity and AuROC (95% CI) were 75.6%, 87.5%, 0.892 (0.838-0.945), respectively (**Figure 3A; Table 1**). When the AFP concentration  $\leq 20$  ng/ml, the sensitivity, specificity and AuROC (95% CI) of serum CLIMP-63 for the diagnosis of HCC were 69.7%, 67.5%, 0.759 (0.651-0.868), respectively. While, for AFP, they

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**Table 1.** Comparison of AUC, Sensitivity, and Specificity between serum AFP and CLIMP-63 levels for the screening of HCC according to the different AFP Cut-off Values

AFP cut-off values (ng/ml)	AUC (CI, 95%) (AFP vs CLIMP-63)	P (AFP vs CLIMP-63)	Sensitivity (%) (AFP vs CLIMP-63)	Specificity (%) (AFP vs CLIMP-63)
>20	0.892 (0.838-0.945) vs 0.771 (0.678-0.863)	0.000 vs 0.000	75.6 vs 70.0	87.5 vs 67.5
<20	0.705 (0.583-0.826) vs 0.759 (0.651-0.868)	0.003 vs 0.000	63.6 vs 69.7	65.0 vs 67.5
<10	0.652 (0.517-0.786) vs 0.769 (0.658-0.879)	0.034 vs 0.000	60.7 vs 71.4	60.0 vs 67.5
<5	0.629 (0.474-0.783) vs 0.777 (0.655-0.898)	0.110 vs 0.000	66.7 vs 76.2	60.0 vs 65.7

were 65.6%, 65%, 0.705 (0.583-0.826), respectively (**Figure 3B; Table 1**). Among patients with AFP levels of less than 10 ng/ml in the set, the serum CLIMP-63 had better discriminatory power ( $P=0.000$ ) as compared with AFP ( $P=0.034$ ) in distinguishing serum samples from those controls HCC patients (**Figure 3C; Table 1**). When the low limit of AFP was decreased to 5 ng/ml, the discriminatory power of the CLIMP-63 was maintained ( $P=0.000$ ), with an area under the curve of 0.78 (95 percent confidence interval, 0.655 to 0.898), whereas that for AFP decreased to 0.63 (95 percent confidence interval, 0.47 to 0.78) (**Figure 3D; Table 1**). These results indicated that the serum CLIMP-63 could improve diagnostic accuracy of HCC as a supplement for AFP, when serum AFP was less than 20 ng.

### Discussion

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world [15]. Prognosis for this disease is poor since HCC can be mostly only diagnosed at an advantaged stage. Over the past few decades, it has drawn attention worldwide to screen for an early diagnosis of HCC. In order to conveniently detect tumors at an early stage, serum biomarkers play important roles in HCC diagnosis. However, the limitation of AFP from the low sensitivity as the major serum marker for diagnosis of HCC in clinical practice, reliable and novel markers is urgently needed to improve the diagnosis accuracy for HCC. Our recent work indicates that CLIMP-63 is a prognostic marker for intrahepatic cholangiocellular carcinoma and HCC patients and clinicopathologically associated with tumor progression and metastasis [11, 12], making CLIMP-63 an attractive target as a tumor biomarker. To date, the serological diagnosis value of CLIMP-63 for HCC has not been reported.

In this study, for the first time, we evaluated the validity of CLIMP-63 as a serological biomarker for HCC and demonstrated that serum CLIMP-63 levels are sequentially increased in HCC according to progression of disease based on the theory of hepatocarcinogenesis (hepatitis-cirrhosis-carcinoma sequence). Serum CLIMP-63 levels were significantly increased in patients with HCC, compared to those of patients without cancer. The finding in this study may provide a potential marker of CLIMP-63 in diagnosing HCC. The sensitivity of the assay is 70% and the specificity 67.5%, which is similar to AFP with the cutoff value of 20 ng/ml in HCC detection. While, when the AFP cut-off value less than 20 ng/ml, the sensitivity and specificity of CLIMP-63 are much better than AFP in HCC detection. The data presented here indicated that examination of serum CLIMP-63 can increase the diagnostic efficacy when AFP is low or negative in HCC patients.

To our knowledge, this is the first report demonstrating higher serum CLIMP-63 levels in HCC patients and the serum CLIMP-63 could improve diagnostic accuracy of HCC as a supplement for AFP. The previous studies indicated that CLIMP-63 located in the endoplasmic reticulum, which evoked a question why CLIMP-63 could be detected in serum. Genevieve and coworkers reported that Dicer transits through the ER and can be secreted by cultured human cells with CLIMP-63 [13]. But the mechanism by which CLIMP-63 was secreted need to be further investigated.

Our study also found that serum CLIMP-63 was elevated when disease was in the stage of hepatitis and cirrhosis compared to normal in human and rat model. The data shown here revealed that detecting the serum level of CLIMP-63 could evaluate the disease sequence of hepatitis-cirrhosis-HCC, and provide evi-

dence for early intervention treatment. However, in this study, there was the relatively small sample size and clinical data collection was not comprehensive, especially for patients with hepatitis and cirrhosis. Further studies with larger sample size from different stage of HCC will be performed to confirm and validate whether CLIMP-63 can be used as a diagnostic marker in hepatitis and cirrhosis.

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#### Disclosure of conflict of interest

None.

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