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Serum Epidermal Growth Factor-like domain 7 Serves as a Novel Diagnostic Marker for Early Hepatocellular Carcinoma

Meng-Yuan Yang
Liver Cancer Laboratory, Xiangya Hospital, Central South University
Fan Wu
Liver Cancer Laboratory, Xiangya Hospital, Central South University
Feng Fang
Liver Cancer Laboratory, Xiangya Hospital, Central South University
Hao Yang
Liver Cancer Laboratory, Xiangya Hospital, Central South University
Jing-Fan Zhang
Liver Cancer Laboratory, Xiangya Hospital, Central South University
Guo-Dong Chen
Liver Cancer Laboratory, Xiangya Hospital, Central South University
Lian-Yue Yang (lianyueyang@hotmail.com)
Liver Cancer Laboratory, Xiangya Hospital, Central South University

Research Article

Keywords: Hepatocellular carcinoma, Eepidermal growth factor-like domain 7, Alpha-fetoprotein, Early diagnosis, Serum marker

Posted Date: December 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-128105/v1

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Abstract

Background: Epidermal growth factor-like domain 7 (Egfl7), a recently identified secreted protein, was significantly increased in patients with HCC by our previous studies. However, its efficacy in diagnosis of early HCC remains unknown. In this study we evaluate the efficacy of serum Egfl7 for early HCC diagnosis and compare it with alpha-fetoprotein (AFP).

Methods: Serum Egfl7 levels in testing cohort (1065 participants) and validation cohort (464 participants) were measured by a sandwich enzyme-linked immunoassay (ELISA). The efficacies of Egfl7 and AFP in diagnosing early HCC were estimated by receiver operating characteristic (ROC).

Results: Serum Egfl7 was significantly elevated in patients with early HCC than all non-HCC controls. At the cut-off value of 2610 ng/mL, the area under ROC, sensitivity, specificity and accuracy of Egfl7 were 0.800, 75.2 %, 71.7% and 73.5% in discriminating early HCC from chronic liver disease (CLD), compared with 0.675, 61.8%, 62.0% and 61.9% for AFP using 20 ng/mL as a cut-off value. Egfl7 also exhibited a significant higher sensitivity and accuracy than AFP in differentiating early HCC patients from non-HCC individuals including healthy individuals and patients with CLD (76.6% *vs.* 64.0%, 79.9% *vs.* 66.1%). Additionally, 70.8% of early HCC patients with negative AFP could be diagnosed by Egfl7 and the combined use of Egfl7 and AFP increased the sensitivity to 91.0%. These results were confirmed by a validation cohort.

Conclusion: Egfl7 is a valuable serum marker in early diagnosis of HCC with a better sensitivity and accuracy than AFP.

Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the second leading cause of male cancer death in developing countries, which resulting in about 782,000 global deaths each year, while China alone accounting for about 50% [1, 2]. Because of the large population of patients with hepatitis B- or C-related liver cirrhosis, the major risk factor for HCC especially in China, the incidence of HCC will increase over the next decade [1, 3, 4]. Despite the great improvements in treatments, the long-term survival of patients with HCC remains unsatisfactory, with a 5-year survival rate of less than 20%, mainly due to limited effective therapeutic options for HCC patients with late stage [5]. Therefore, the early detection of HCC, providing the opportunity for radical treatment, is crucial to reduce the mortality and improve the long-term prognosis of this lethal malignancy [6].

Currently, basing the limited early diagnostic modalities available, only 30–40% of patients with HCC could receive curative treatments [7]. The most widely- used early modalities for clinical diagnosis were ultrasound (US) and serum alpha- fetoprotein (AFP). The reported sensitivity and specificity of US for HCC ≥ 1 cm in diameter were 70% and 48%, respectively [8]. However, when HCCs were less than 1 cm in diameter, the detection rate of US dropped to 34% [9]. The high level of serum AFP can provide the first indication of HCC, which prompting further medical imaging and investigations [10, 11]. However, the sensitivity of AFP in the early detection of HCC is only 39–65%, which means that its false-negative rate is 35–61% [12–14].

To improve the early diagnosis of HCC, many serum markers of HCC have been identified in the past decades, such as Des-γ-carboxy-prothrombin (DCP), AFP lectin-3 fraction (AFP-L3), glypican-3 (GPC3), Golgi protein 73 (GP73), Dickkopf-1 (DKK1) and so on [15, 16]. DCP has been shown to be a diagnostic serum marker for HCC comparable with AFP [17]. However, only 15–30% of patients with early HCC present significant concentration of serum DCP [18]. AFP-L3, an isoform of AFP, displays a specificity of more than 95% for HCC and a sensitivity > 90% for HCC > 5 cm but it can only be detected in 35% of patients with small HCC of < 3 cm, which limited its application in early detection of HCC [19–21]. Though GPC3 and GP73 are recently reported to be better than AFP, their efficacies in the detection of early HCC are still in debate [22–24]. Shen et al. and we have all reported that Dkk1 display a relative high sensitivity and specificity in the detection of early HCC [25, 26]. However, these results need to be confirmed in more researches. The novel effective serum marker, which is better than AFP, for diagnosing early HCC are still required [25].

Epidermal growth factor-like domain 7 (Egfl7) is a recently identified secreted protein which binds the extracellular matrix surrounding the blood vessels and it appears to act in an autocrine manner to promote angiogenesis [27–29]. Egfl7 is expressed at high levels in early embryos of mice, but only high expressed in a few organs in adult mice such as the lung, heart and kidney [30]. However, Egfl7 is strongly upregulated in tumors [31]. Previously, we found that Egfl7 was significantly upregulated in HCC tissues and the high expression of Egfl7 protein was closely correlated with clinicopathological parameters (such as vein

invasion, multiple nodes and capsule formation) and poor prognosis of HCC [32, 33]. Importantly, we have also demonstrated that Egfl7 could promote the metastasis of HCC by enhancing cell motility through EGFR-dependent FAK (focal adhesion kinase) phosphorylation [32]. As a secreted protein, we subsequently explored the serum levels of Egfl7 in patients with a series of epithelial tumors including HCC as well as healthy individuals, the results documented a significantly higher level of serum Egfl7 in patients with HCC than those with other tumors and healthy individuals, shedding a light on the potential application of Egfl7 as a serum diagnostic marker for HCC [33]. However, the efficacy of serum Egfl7 in diagnosing early HCC remains unknown.

Therefore, we carried out the present study to measure the levels of serum Egfl7 in patients with early HCC and evaluate the efficacy of Egfl7 in the early detection of HCC by comparing with AFP.

Materials And Methods

Serum Samples

The Testing Cohort Serum samples were collected from the patients operated for early HCC (n = 314), metastatic liver cancer (n = 14), benign liver tumors (n = 19), and the patients with chronic liver disease including cirrhosis and chronic viral hepatitis (n = 300) in Xiangya Hospital, Central South University (CSU) from June 2006 to October 2009. At the same time, serum samples of healthy individuals (n = 432) were collected from the healthy blood donors of Changsha Blood Bank. The demographic and clinical characteristics of these individuals were collected prospectively (Table 1).

Table 1
Demographic data for the patients and healthy individuals in the Testing and the Validation Cohorts

Groups	Testing Cohort							Validation Cohort						
	n	Gender		Median age years (range)	Virus-positive subjects			n	Gender		Median age years (range)	Virus-positive subjects		
		M	F		HBV	HCV	Non-B-non-C		M	F		HBV	HCV	Non-B-non-C
Early Hepatocellular Carcinoma	314	275	39	49 (20-78)	237	0	77	158	142	16	50 (22-78)	119	0	39
Chronic Liver Disease	300	222	78	44 (17-83)	258	4	38	120	96	24	43 (17-77)	103	2	15
Metastatic Liver Cancers	16	8	8	59 (23-75)				12	7	5	61 (25-75)			
Benign Liver Tumors	19	12	7	49 (14-63)				14	9	5	47 (15-65)			
Healthy individuals	432	221	211	22 (18-54)				172	83	89	21 (18-53)			
M: male; F: female; HBV: with hepatitis B virus infection; HCV: with hepatitis C virus infection; Non-B- non-C: without hepatitis B or hepatitis C virus infection														

The Validation Cohort At the end of the first part of the present study, serum samples from a second set of individuals were collected from November 2009 to March 2011 to serve as the validation cohort. This Cohort consisted of healthy individuals (n = 172) and patients who were operated for early HCC (n = 158), metastatic liver cancer (n = 12), benign liver tumors (n = 14) or patients with chronic liver disease (n = 120). The demographic and clinical characteristics were collected prospectively (Table 1).

Early HCC was classified by Barcelona Clinic Liver Cancer (BCLC) criterion as stage 0 ~ A [34]. The diagnosis of HCC was confirmed by histopathological study after hepatic resection. The diagnosis of cirrhosis was based either on histopathology or on

the criteria used in the guidelines from American Association for the Study of Liver Diseases (AASLD). Patients diagnosed with chronic liver disease were limited to those patients who had no history of HCC, there was no ultrasonic evidence of HCC for more than 6 months from the day of serum collection, and their hepatic functions were in the compensated phase. The serum samples were spun, aliquoted and stored at -80 °C until testing.

Prior informed consent was obtained from the subjects for collection of serum samples in accordance with the guidelines of CSU and the study protocols were approved by the Ethics Committee of CSU.

Egfl7 and AFP detection

To avoid subjective bias and to reduce system error, a double-blind principle was followed in this study according to the criteria of STARD (standards for reporting of diagnostic accuracy studies) 2015 for reporting studies of diagnostic accuracy [35]. Individuals who collected the serum samples did not participate in the testing and the testing personnel did not know the sources and the groups of the samples. After testing was completed, the information on the samples and the corresponding results were disclosed, and the values of Egfl7 and AFP were analyzed.

Serum Egfl7 levels were measured quantitatively by a sandwich enzyme linked immunosorbent assay (ELISA) system. Firstly, a murine monoclonal antibody (Abnova, Taiwan, China) specific to human Egfl7 protein, as a capture antibody, was added into a 96-well microplate (Greiner bio-one, Germany) and incubated at room temperature for 2 h. Then these 96-well microplates were coated at 4℃ overnight and washed by 0.05% PBST (phosphate buffered saline with Tween-20) to remove any unbound antibody. And 4% BSA (bovine serum albumin) was added into these 96-well microplates for blocking and incubated at 4℃ for 2 h. Secondly, serum samples (250-fold dilution) were added into the 96-well microplates after washing with 0.05% PBST and incubated at room temperature for 2 h. Thirdly, a rabbit polyclonal antibody (Santa Cruz, USA) specific to human Egfl7 protein, as a detection antibody was added into the wells, after washing away any unbound substances with 0.05% PBST, and incubated at room temperature for 2 h. Fourthly, a goat anti-rabbit IgG antibody was added into the wells after washing again with 0.05% PBST and incubated at room temperature for 2 h. After washing with 0.05% PBST, TMB (tetramethyl benzidine) soluble reagent (Tiangen Biotech Co., Ltd., Beijing, China) was added into the wells and incubated to react at room temperature for 30 min. Lastly, H_2SO_4 (1M, Jingmei Biotech Co., Ltd., Beijing, China) was added to stop the reaction and a photometer was employed to determine the intensity of color at a wavelength of 450 nm, with a reference wavelength of 570 nm. We also tested recombinant protein Egfl7 (Abnova, Taiwan, China) in each assay as a standard sample. Serum AFP was detected in the same specimens by using commercially available immunoassays utilizing enhanced chemiluminescence. The upper normal limit of serum AFP level was set as 20.0 ng/mL.

Statistical analysis

SPSS 13.0 (Chicago, IL, USA) was employed to perform all the statistical analyses. The comparisons of multi-group for continuous variables were performed by one-way analysis of variance (ANOVA) with Tukey test as post hoc test. For the categorical variables, Chi-square test or Fisher's exact test (where appropriate) was used. Receiver operating characteristic (ROC) curve was established to get the cut-off values and compare the diagnostic efficacies of serum Egfl7 and AFP. The area under the ROC (AUROC) curves were constructed and compared using the Z test. Correlation between serum Egfl7 and AFP levels was evaluated by the Pearson correlation coefficient. All analyses were two-sided and $P < 0.05$ was considered as statistically significant.

Results

Serum Egfl7 levels in patients with early HCC

The demographic data of the Testing Cohort and Validation Cohort are shown in Table 1. The serum Egfl7 levels in the Testing Cohort are shown in Fig. 1. The median serum level of Egfl7 in early HCC was 4017.28 ng/mL (95% CI, 297.6-9271.7 ng/mL), and it was significantly higher than metastatic liver cancer (2707.6 ng/mL; 95% CI, 274.2-5234.8 ng/mL; $P < 0.05$), benign liver tumors (1756.6 ng/mL; 95% CI, 494.8-3343.8 ng/mL; $P < 0.05$), chronic liver disease (1600.3 ng/mL; 95% CI, 20.4-6232.1 ng/mL; $P < 0.05$) and healthy individuals (1082.80 ng/mL; 95% CI, 7.5-4906.1 ng/mL; $P < 0.05$). Although there was no significant difference

between the groups of benign liver tumors and chronic liver disease ($P > 0.05$), the serum Egfl7 levels in these two groups were higher than the healthy individuals ($P < 0.05$). The results from the Validation Cohort also showed that the serum Egfl7 level in early HCC was significantly higher than that the other groups ($P < 0.05$, Fig. 3).

The diagnostic efficacies of serum Egfl7 for early HCC in the Testing Cohort

ROC curve analyses and the area under the ROC (AUROC) were used to evaluate the diagnostic efficacies of serum Egfl7 with AFP. The cut-off value of Egfl7 and AFP was set as 2610.0 ng/mL and 20 ng/mL, respectively.

To differentiate early HCC from healthy individuals, Egfl7 showed a significantly higher sensitivity than AFP (77.4% *vs.* 65.3%, $P < 0.05$; Table 2), and a comparable AUROC (0.860 *vs.* 0.868, $P > 0.05$; Fig. 2A) and accuracy (80.2% *vs.* 83.8%, $P > 0.05$; Table 2) with AFP. However, the specificity of Egfl7 was lower than AFP (82.2% *vs.* 97.2%, $P < 0.05$; Table 2).

Table 2
Efficacies of serum Egfl7, AFP or Parallel in differentiating early HCC from healthy individuals

Data	Testing Cohort			Validation Cohort		
	Egfl7	AFP	Parallel	Egfl7	AFP	Parallel
Sensitivity	77.4%	65.3%	88.9%	75.9%	58.9%	86.7%
Specificity	82.2%	97.2%	79.9%	79.1%	97.1%	76.2%
Accuracy	80.2%	83.8%	83.6%	77.6%	78.8%	81.2%
Omission diagnostic rate	22.6%	34.7%	11.1%	24.1%	41.1%	13.3%
Mistake diagnostic rate	17.8%	2.8%	20.1%	20.9%	2.9%	23.8%
Positive likelihood ratio	4.34	23.5	4.42	3.63	20.3	3.64
Negative likelihood ratio	0.28	0.36	0.14	0.30	0.42	0.17
Positive predict value	0.76	0.95	0.76	0.77	0.95	0.77
Negative predict value	0.83	0.79	0.91	0.78	0.72	0.86
Parallel: combined Egf17 and AFP.						

As the majority of patients with HCC (> 85%) had chronic liver disease (CLD) such as chronic viral hepatitis and/or cirrhosis, the diagnostic value of Egfl7 in the discrimination of early HCC from CLD was further investigated. The results showed that Egfl7 was superior to AFP in full-scale with a significantly higher AUROC (0.800 *vs.* 0.675, $P < 0.05$; Fig. 2B), sensitivity (75.2% *vs.* 61.8%, $P < 0.05$), specificity (71.7% *vs.* 62.0%, $P < 0.05$), accuracy (73.5% *vs.* 61.9%, $P < 0.05$), positive likelihood ratio (2.65 *vs.* 1.56, $P < 0.05$) and positive predict value (0.74 *vs.* 0.66, $P < 0.05$) than AFP (Table 3).

Table 3
Efficacies of serum Egfl7, AFP or Parallel in discriminating early HCC from chronic liver disease

Data	Testing Cohort			Validation Cohort		
	Egfl7	AFP	Parallel	Egfl7	AFP	Parallel
Sensitivity	75.2%	61.8%	88.9%	75.9%	36.1%	82.9%
Specificity	71.7%	62.0%	60.7%	71.7%	90.8%	66.7%
Accuracy	73.5%	61.9%	75.1%	74.1%	59.7%	75.9%
Omission diagnostic rate	24.8%	38.2%	11.1%	24.1%	63.9%	17.1%
Mistake diagnostic rate	28.3%	39.7%	39.3%	29.3%	9.2%	33.3%
Positive likelihood ratio	2.65	1.56	2.26	2.59	3.92	2.49
Negative likelihood ratio	0.32	0.62	0.18	0.34	0.70	0.26
Positive predict value	0.74	0.66	0.70	0.78	0.84	0.77
Negative predict value	0.73	0.61	0.84	0.69	0.52	0.75
Parallel: combined Egf17 and AFP.						

The high cut-off value of AFP, such as 100 ng/mL or even 400 ng/mL, was often used in distinguishing the patients with HCC from those with CLD to assure a satisfactory specificity [36, 37]. We also evaluated the efficacies of AFP at different cutoff values for the diagnosis of early HCC from healthy individuals or patients with CLD. The results showed that when the cutoff values of AFP increased from 20 ng/ml to 400 ng/ml, the sensitivity of AFP was dramatically decreased from 61.8%-65.3–38.4% (Table 4). More importantly, 70.8% (85 of 120) of early HCC with negative AFP could be diagnosed by Egfl7 (Table 5).

Table 4
Efficacies of serum AFP testing at different cutoff value in diagnosing early HCC

Group	Accuracy (%)	Sensitivity (%)	Specificity (%)
Early HCC vs. **non-HCC patients**			
Serum AFP			
Cutoff value: 20.0 ng/mL	83.8%	65.3%	97.2%
Cutoff value: 100.0 ng/mL	89.1%	55.1%	100%
Cutoff value: 400.0 ng/mL	85.1%	38.4%	100%
Early HCC vs. **chronic liver disease**			
Serum AFP			
Cutoff value: 20.0 ng/mL	61.9%	61.8%	62%
Cutoff value: 100.0 ng/mL	65.9%	55.1%	78.3%
Cutoff value: 400.0 ng/mL	62.8%	38.4%	90.8%

Table 5
Efficacies of serum Egfl7 in diagnosing early HCC with negative AFP

Testing Cohort				Validation Cohort			
Egfl7	AFP		Total	Egfl7	AFP		Total
	Positive	Negative			Positive	Negative	
Positive	151	85	236	Positive	76	44	120
Negative	43	35	78	Negative	17	21	38
Total	194	120	314	Total	93	65	158

Furthermore, there was no significant correlation between the serum Egfl7 and AFP levels (Spearman rank correlation coefficient: $r = 0.154$, $P > 0.05$) and their diagnosis results were not identical (kappa = 0.147, $P > 0.05$), suggesting Egfl7 and AFP combination could be helpful to improve the efficacy of early HCC diagnosis. Consist with the hypothesis, in differentiating the patients with early HCC from healthy individuals or the patients with CLD, the combination significantly improved the sensitivity to 88.9% and increase the AUROC to 0.967 or 0.819 (Tables 2 & 3, Fig. 2A & 4A).

To fully evaluate the diagnostic value of Egfl7 in the early detection of HCC, we pooled the healthy individuals and the patients with CLD into a single control group named as non-HCC individuals, and investigated the efficacies of Egfl7 in distinguishing the patients with early HCC from these individuals. Our results showed that the diagnostic efficacies of Egfl7 was much better than AFP, with a significantly higher sensitivity (76.6% *vs.* 64%, $P < 0.05$), specificity (82.7% *vs.* 68%, $P < 0.05$), accuracy (79.9% *vs.* 66.1%, $P < 0.05$), positive likelihood ratio (4.44 *vs.* 2.0, $P < 0.05$) and positive predictive value (0.80 *vs.* 0.64, $P < 0.05$) (Table 6). Moreover, the sensitivity of Egfl7 and AFP combination was 91.0%, which was dramatically higher than Egfl7 or AFP alone (Table 6).

Table 6
Efficacies of serum Egfl7, AFP or Parallel in distinguishing early HCC patients from non-HCC individuals

Data	Testing Cohort			Validation Cohort		
	Egfl7	AFP	Parallel	Egfl7	AFP	Parallel
Sensitivity	76.6%	64%	91%	75.9%	58.9%	86.7%
Specificity	82.7%	68%	66%	76.0%	81.2%	62.3%
Accuracy	79.9%	66.1%	77.6%	76.0%	73.3%	70.9%
Omission diagnostic rate	23.4%	36%	9%	24.1%	41.1%	13.3%
Mistake diagnostic rate	17.3%	32%	34%	24.0%	18.8%	37.7%
Positive likelihood ratio	4.44	2.0	2.6	3.16	3.13	2.30
Negative likelihood ratio	0.28	0.53	0.14	0.32	0.51	0.21
Positive predict value	0.80	0.64	0.70	0.63	0.63	0.55
Negative predict value	0.79	0.68	0.89	0.85	0.78	0.90
Parallel: combined Egf17 and AFP.						

The diagnostic efficacies of serum Egfl7 for early HCC in the Validation Cohort

When Egfl7 was used to differentiate the patients with early HCC from healthy individuals, it showed a distinctly higher sensitivity than AFP (75.9% *vs.* 58.9%, $P < 0.05$). Though the specificity of Egfl7 was lower than AFP (79.1% *vs.* 97.1%, $P < 0.05$), their accuracy and AUROC were comparable (77.6% *vs.* 78.8%, 0.827 *vs.* 0.861, respectively; Fig. 2B and Table 2). When Egfl7 was used

to discriminate early HCC from CLD, the AUROC, sensitivity and accuracy of Egfl7 were all dramatically higher than AFP (0.787 *vs.* 0.563, 75.9% *vs.* 36.1%, 74.1% *vs.* 59.7%, respectively; $P < 0.001$; Fig. 4B and Table 3). Importantly, the combined use of Egfl7 and AFP significantly improved the sensitivity. And 67.7% (44/65) of patients with early HCC who had a negative AFP could be diagnosed by Egfl7 (Table 5). Furthermore, in distinguishing the early HCC patients from the non-HCC individuals, the sensitivity and accuracy of Egfl7 were all better than AFP (75.9% *vs.* 58.9%, 76.0% *vs.* 73.3%, $P < 0.05$; Table 6). The combined use of Egfl7 and AFP could improve the sensitivity to 86.7%, which was remarkably higher than Egfl7 or AFP alone (Table 6).

Discussion

Since HCC is usually asymptomatic for much of its natural history, most of HCC patients were detected at an intermediate or advanced stage, by which time surgical or oncological treatment options were limited [38]. Therefore, screening and diagnose HCC as early as possible, when it is curable, was critical important to improve the treatment of this deadly disease [9]. For this reason, surveillance in patients with high risks of developing HCC has been recommended, and US and serum AFP are commonly used [25]. Recently, studies have shown that surveillance program improved prognosis of HCC [26]. However, US examination depends on the experience of the sonographer and the technical quality of the US equipment, and is therefore subjective and nonrepetitive [24]. Furthermore, the use of AFP to screen a population with chronic liver disease who is at risk of developing HCC has been reported to have a poor sensitivity of only 20–30% at cutoff values > 100 ng/ml [27]. A better screening program is in urgent need for early HCC diagnosis.

Many novel serum diagnostic markers of HCC have been found, including DCP, GPC3, AFP-L3 and GP73 [31, 32]. Although many non-protein serum markers have been identified for HCC in the past decade such as mutated DNAs, methylated DNAs and RNAs [including microRNA, lncRNA (long non-coding RNA) and circRNA (circular RNA) or even these RNAs inside exosomes] [39–43], protein markers could be detected in serum are the most applicable for clinical routine assessments [22, 44], for their advantages including non-invasive, requiring less than 100μL serum, low dependence on operator expertise, low cost, high reproducibility, and no samples need pretreatment (such as extraction, purification or reverse transcription) [22]. Egfl7, a recently identified protein involved in the progression of HCC, maybe a satisfactory marker for HCC: it is a secretory protein, is specifically overexpressed in HCC cells instead of normal cells such as vascular endothelial cells or cholangiocytes within HCC tissues, and is hardly detectable in human adult normal liver tissues [32, 33]. More importantly, Egfl7 has already been evidenced to be upregulated in the serum of patients with HCC or other type of cancers [33, 45–47], suggesting its possible application in the diagnosis of HCC. However, the levels of serum Egfl7 in patients with early HCC are still unknown.

In the present study, we detected the serum levels of Egfl7 in the patients with early HCC. The results showed a significantly higher level of serum Egfl7 in these patients than healthy individuals (increased by 3.71 folds), which was consist with the elevated levels of serum Egfl7 in HCC patients [33, 45]. In differentiating early HCC from the healthy individuals, Egfl7 had a significantly higher sensitivity than AFP (75.9%-77.4% *vs.* 58.9%-65.3%%) and their accuracy and AUROC were similar indicating a generally superior of Egfl7 in the detection of early HCC to AFP as well as some other serum marker for early HCC, such as GPC3 (sensitivity ranged from 47.9–66.2%) [48] and DKK1 (sensitivity ranged from 70.9–73.8%) [25, 26].

Our results showed serum Egfl7 levels was modestly elevated in patients with CLD compared with healthy individuals (1600.3 ng/mL vs. 1082.80 ng/mL) but much lower than those with early HCC (4017.28 ng/mL). Furthermore, in surveillance of early HCC from CLD, Egfl7 had a much better AUROC (0.787-0.800 *vs.* 0.563–0.675), sensitivity (75.2%-75.9% vs. 36.1%-61.8%) and accuracy (73.5%- 74.1% vs. 59.7%-61.9%) than AFP, indicating Egfl7 as a better serum marker than AFP in the surveillance of early HCC from CLD. Moreover, Egfl7 also exhibited an advantage in the aspect of sensitivity compared with some recently reported serum markers for distinguishing early HCC from CLD such as GPC3 (sensitivity of 55%) [22], GP73 (sensitivity of 62%) [49] and DKK1 (sensitivity ranged from 54.8–73.8%) [25, 26].

Although AFP is often recommended as a serum marker for the surveillance of HCC, the use of AFP to screen a population with chronic liver disease who is at risk of developing HCC has been reported to have a poor sensitivity of only 20–30% at cutoff values > 100 ng/mL [40]. In this study, to distinguish early HCC from chronic liver disease, AFP had a specificity which ranged from 62–90.8% when the cutoff value increased from 20 ng/mL to 400 ng/mL but the sensitivity correspondingly decreased from 61.8–38.4%, which meaning 61.6% of early HCC was missed at a cutoff value of 400 ng/mL. Therefore, the recognition of AFP-

negative HCC is important to improve the efficacy of early detection of HCC. In the present study, 67.7%-70.8% of patients with early HCC who had a negative AFP could be diagnosed by Egfl7, which was slightly better than AFP-L3 (50%) [50], GP73 (66%-67%) [23, 50] and comparable with DKK1 (67.3%-73.1%) [25, 26]. In consideration of a high specificity of AFP in the surveillance and early detection of HCC, Egfl7 might be helpful to make up the deficiency of AFP in sensitivity and further improve the diagnostic efficacy of early HCC. The combination of Egfl7 and AFP showed a significantly increased sensitivity ranged from 88.9–91.0%, which was remarkably higher than the sensitivity of Egfl7 or AFP alone and also the sensitivity of AFP combined with other serum markers such as DKK1 (63.8%-90.8%) [25, 26] and GPC3 (81%-89%) [22].

In addition, we have found that the level of serum Egfl7 was also elevated in patients with metastatic liver cancer (increased by 2.50 folds) or benign liver tumors (increased by 1.62 folds) also had a moderately elevated compared with the healthy individuals, although the magnitude is smaller than that in early HCC (3.71 folds), suggesting serum Egfl7 might be helpful to determine the nature (benign tumor or HCC) and origin (primary or secondary) of liver tumors.

To our knowledge, this is the first large-scale study to report the performance of Egfl7 as a serum diagnostic marker for early HCC in a test cohort and an independent validation cohort. The results indicate Egfl7 as a novel and effective serological marker for the early detection of HCC, with a significantly higher sensitivity and accuracy than AFP, especially in the surveillance of a high-risk population with CLD. In addition, serum Egfl7 was positive in most early HCC patients with negative AFP and could be used combined with AFP to further improve the diagnostic efficacy.

Abbreviations

AASLD: American association for the study of liver diseases; AFP: Alpha-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive fraction of alpha- fetoprotein; AUROC: Area under the ROC curve; BCLC: Barcelona clinic liver cancer criterion; BSA: Bovine serum albumin; CI: confidence interval; circRNA: circular RNA; CLD: Chronic liver disease; CSU: Central south university; DCP: Des-gamma-carboxy prothrombin; DKK1: Dickkopf-1; Egfl7: Epidermal growth factor-like domain 7; ELISA: Enzyme linked immunosorbent assay; FAK: Focal adhesion kinase; GP73: Golgi protein 73; GPC3: Glypican-3; HCC: Hepatocellular carcinoma; lncRNA: long non-coding RNA; PBST: Phosphate buffered saline with Tween-20; ROC: Receiver operating curve; STARD: Standards for reporting of diagnostic accuracy studies; TMB: Tetramethyl benzidine; US: ultrasound

Declarations

- ### Ethics approval and consent to participate

This study was approved by the Ethics Committees of Central South University. Informed written consent was obtained from all subjects. All methods in this study were carried out in accordance with relevant guidelines and regulations.

- ### Consent for publication:

Not applicable.

- ### Availability of data and materials

All data generated or analysed during this study are included in this published article.

- ### Competing interests

All authors declare that there is no conflict of interests in this study.

- ### Funding

This work was supported by National Keystone Basic Research Program of China (No. 2004CB720303, No. 2009CB521801), National High Technology Research and Development Program of China (No. 2006AA02Z4B2), National Nature Science

Foundation of China (No. 81502539, 81572858), Clinical Subjects' Key Project of Ministry of Health of China (2010-2013), and National Science & Technology Major Projects (2008ZX10208, 2009ZX09103-681). The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

MYY, FW and LYY designed and coordinated the study. MYY, FW, FF, HY, JFZ and GDC performed the experiments and analyzed data. MYY and FW wrote the manuscript. LYY and FF revised the manuscript. All authors approved the final version of the article.

Acknowledgements:

Not applicable.

References

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68: 394-424.
2. Torre LA, Bray F, Siegel RL, et al. Global cancer statistics, 2012. CA Cancer J Clin. 2015; 65: 87-108.
3. MacLachlan JH, Cowie BC. Hepatitis B virus epidemiology. Cold Spring Harb Perspect Med. 2015; 5: a021410.
4. Thein HH, Isaranuwatchai W, Campitelli MA, et al. Health care costs associated with hepatocellular carcinoma: a population-based study. Hepatology. 2013; 58: 1375-84.
5. Fujiwara N, Friedman SL, Goossens N, et al. Risk factors and prevention of hepatocellular carcinoma in the era of precision medicine. J Hepatol. 2018; 68: 526- 49.
6. Kulik L, El-Serag HB. Epidemiology and Management of Hepatocellular Carcinoma. Gastroenterology. 2019;156:477-91.
7. Ghany MG, Strader DB, Thomas DL, et al. Diagnosis, management, and treatment of hepatitis C: An update. Hepatology. 2009;49:1335-74.
8. Chou R, Cuevas C, Fu R, et al. Imaging techniques for the diagnosis of hepatocellular carcinoma: a systematic review and meta-analysis. Ann Intern Med. 2015;162:697-711.
9. Burrel M, Llovet JM, Ayuso C, et al. MRI angiography is superior to helical CT for detection of HCC prior to liver transplantation: an explant correlation. Hepatology. 2003;38:1034-42.
10. **Debruyne EN, Delanghe JR. Diagnosing and monitoring hepatocellular carcinoma with alpha-fetoprotein: new aspects and applications. Clin Chim Acta. 2008; 395: 19- 26.**
11. **Tamura Y, Suda T, Arii S, et al. Value of highly sensitive fucosylated fraction of alpha-fetoprotein for prediction of hepatocellular carcinoma recurrence after curative treatment. Dig Dis Sci. 2013;58:2406-12.**
12. Gebo KA, Chander G, Jenckes MW, et al. Screening tests for hepatocellular carcinoma in patients with chronic hepatitis C: a systematic review. Hepatology. 2002; 36:S84-92.
13. Daniele B, Bencivenga A, Megna, AS, et al. α-Fetoprotein and ultrasonography screening for hepatocellular carcinoma. Gastroenterology. 2004; 127: S108-12.
14. Sun T, Tang Y, Sun D, et al. Osteopontin versus alpha-fetoprotein as a diagnostic marker for hepatocellular carcinoma: a meta-analysis. Onco Targets Ther. 2018; 11: 8925-35.
15. Sturgeon CM, Duffy MJ, Hofmann BR, et al. National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines for use of tumor markers in liver, bladder, cervical, and gastric cancers. Clin Chem. 2010; 56: e1-48.
16. Mao Y, Yang H, Xu H, et al. Golgi protein 73 (GOLPH2) is a valuable serum marker for hepatocellular carcinoma. Gut. 2010; 59: 1687-93.
17. Liebman HA, Furie BC, Tong MJ, et al. Des-gamma-carboxy (abnormal) prothrombin as a serum marker of primary hepatocellular carcinoma. N Engl J Med. 1984; 310: 1427-1431.

18. Soroida Y, Ohkawa R, Nakagawa H, et al. Increased activity of serum mitochondrial isoenzyme of creatine kinase in hepatocellular carcinoma patients predominantly with recurrence. J Hepatol. 2012; 57: 330-6.
19. Taketa K, Sekiya C, Namiki M, et al. Lectin-reactive profiles of alpha-fetoprotein characterizing hepatocellular carcinoma and related conditions. Gastroenterology. 1990; 99: 508-18.
20. Kumada T, Nakano S, Takeda I, et al. Clinical utility of Lens culinaris agglutinin-reactive alpha-fetoprotein in small hepatocellular carcinoma: special reference to imaging diagnosis. J Hepatol. 1999;30:125-30.
21. Yamashiki N, Seki T, Wakabayashi M, et al. Usefulness of Lens culinaris agglutinin A-reactive fraction of alpha-fetoprotein (AFP-L3) as a marker of distant metastasis from hepatocellular carcinoma. Oncol Rep. 1999;6:1229-32.
22. Xu D, Su C, Sun L, et al. Performance of serum Glypican 3 in diagnosis of hepatocellular carcinoma: a meta-analysis. Ann Hepatol. 2019; 18: 58-67.
23. Mao Y, Yang H, Xu H, et al. Golgi protein 73 (GOLPH2) is a valuable serum marker for hepatocellular carcinoma. Gut. 2010;59:1687-93.
24. Li B, Li B, Guo T, et al. The clinical values of serum markers in the early prediction of hepatocellular carcinoma. Biomed Res Int. 2017;2017:5358615.
25. Shen Q, Fan J, Yang XR, et al. Serum DKK1 as a protein biomarker for the diagnosis of hepatocellular carcinoma: a large-scale, multicentre study. Lancet Oncol. 2012;13:817-26.
26. Yang H, Chen GD, Fang F, et al. Dickkopf-1: as a diagnostic and prognostic serum marker for early hepatocellular carcinoma. Int J Biol Markers. 2013; 28: 286-97.
27. Fitch MJ, Campagnolo L, Kuhnert F, et al. Egfl7, a novel epidermal growth factor-domain gene expressed in endothelial cells. Dev Dyn. 2004;230:316-24.
28. Nikolic I, Stankovic ND, Bicker F, et al. EGFL7 ligates αvβ3 integrin to enhance vessel formation. Blood. 2013;121:3041-50.
29. **Schmidt M, Paes K, De Mazière A, et al.** EGFL7 regulates the collective migration of endothelial cells by restricting their spatial distribution. Development. 2007;134:2913-23.
30. Nichol D, Stuhlmann H. EGFL7: a unique angiogenic signaling factor in vascular. Blood. 2012;119:1345-52.
31. Parker LH, Schmidt M, Jin SW, et al. The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. Nature. 2004;428:754-8.
32. Wu F, Yang LY, Li YF, et al. Novel role for epidermal growth factor-like domain 7 in metastasis of human hepatocellular carcinoma. Hepatology. 2009;50:1839-50.
33. Fan C, Yang LY, Wu F, et al. The expression of Egfl7 in human normal tissues and epithelial tumors. Int J Biol Markers. 2013; 28: 71-83.
34. Bruix J, Reig M, Sherman M. Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma. Gastroenterology. 2016; 150: 835-853.
35. Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. Radiology. 2015;277:826- 32.
36. Luo P, Yin P, Hua R, et al. A large-scale, multi-center serum metabolite biomarkers identification study for the early detection of hepatocellular carcinoma. Hepatology. 2018; 67: 662-75.
37. Nguyen MH, Garcia RT, Simpson PW, et al. Racial differences in effectiveness of alpha-fetoprotein for diagnosis of hepatocellular carcinoma in hepatitis C virus cirrhosis. Hepatology. 2002; 36: 410-7.
38. Li D, Sedano S, Allen R, et al. Current Treatment Landscape for Advanced Hepatocellular Carcinoma: Patient Outcomes and the Impact on Quality of Life. Cancers (Basel). 2019; 11: 841.
39. García-Fernández N, Macher HC, Rubio A, et al. Detection of p53 mutations in circulating DNA of transplanted hepatocellular carcinoma patients as a biomarker of tumor recurrence. Adv Exp Med Biol. 2016; 924: 25-8.
40. Zhang C, Ge S, Wang J, et al. Epigenomic profiling of DNA methylation for hepatocellular carcinoma diagnosis and prognosis prediction. J Gastroenterol Hepatol. 2019; 34: 1869-77.

41. Zhou J, Yu L, Gao X, et al. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J Clin Oncol. 2011; 29: 4781-8.
42. Xu H, Chen Y, Dong X, et al. Serum exosomal long noncoding RNAs ENSG00000258332.1 and LINC00635 for the diagnosis and prognosis of hepatocellular carcinoma.. Cancer Epidemiol Biomarkers Prev. 2018;27:710-6.
43. Zhang X, Xu Y, Qian Z, et al. circRNA_104075 stimulates YAP-dependent tumorigenesis through the regulation of HNF4a and may serve as a diagnostic marker in hepatocellular carcinoma. Cell Death Dis. 2018;9:1091.
44. Malaguarnera G, Giordano M, Paladina I, et al. Serum markers of hepatocellular carcinoma. Dig Dis Sci. 2010; 55: 2744-55.
45. Liu XJ, Wang B, Jiang WG, et al. Multivariate analysis of molecular markers in peripheral blood associated with recurrence and metastasis of hepatocellular carcinoma. Genet Mol Res. 2015;14:1502-7.
46. Shen X, Han Y, Xue X, et al. Epidermal growth factor-like domain 7 promotes cell invasion and angiogenesis in pancreatic carcinoma. Biomed Pharmacother. 2016;77: 167-75.
47. Hansen TF, Andersen RF, Olsen DA, et al. Prognostic importance of circulating epidermal growth factor-like domain 7 in patients with metastatic colorectal cancer treated with chemotherapy and bevacizumab. Sci Rep. 2017;7:2388.
48. Jia X, Liu J, Gao Y, et al. Diagnosis accuracy of serum glypican-3 in patients with hepatocellular carcinoma: a systematic review with meta-analysis. Arch Med Res. 2014; 45: 580-8.
49. Marrero JA, Romano PR, Nikolaeva O, et al. GP73, a resident Golgi glycoprotein, is a novel serum marker for hepatocellular carcinoma. J Hepatol. 2005;43:1007-12.
50. Zhang Z, Zhang Y, Wang Y, et al. Alpha-fetoprotein-L3 and Golgi protein 73 may serve as candidate biomarkers for diagnosing alpha-fetoprotein-negative hepatocellular carcinoma. Onco Targets Ther. 2015; 9: 123-9.

Figures

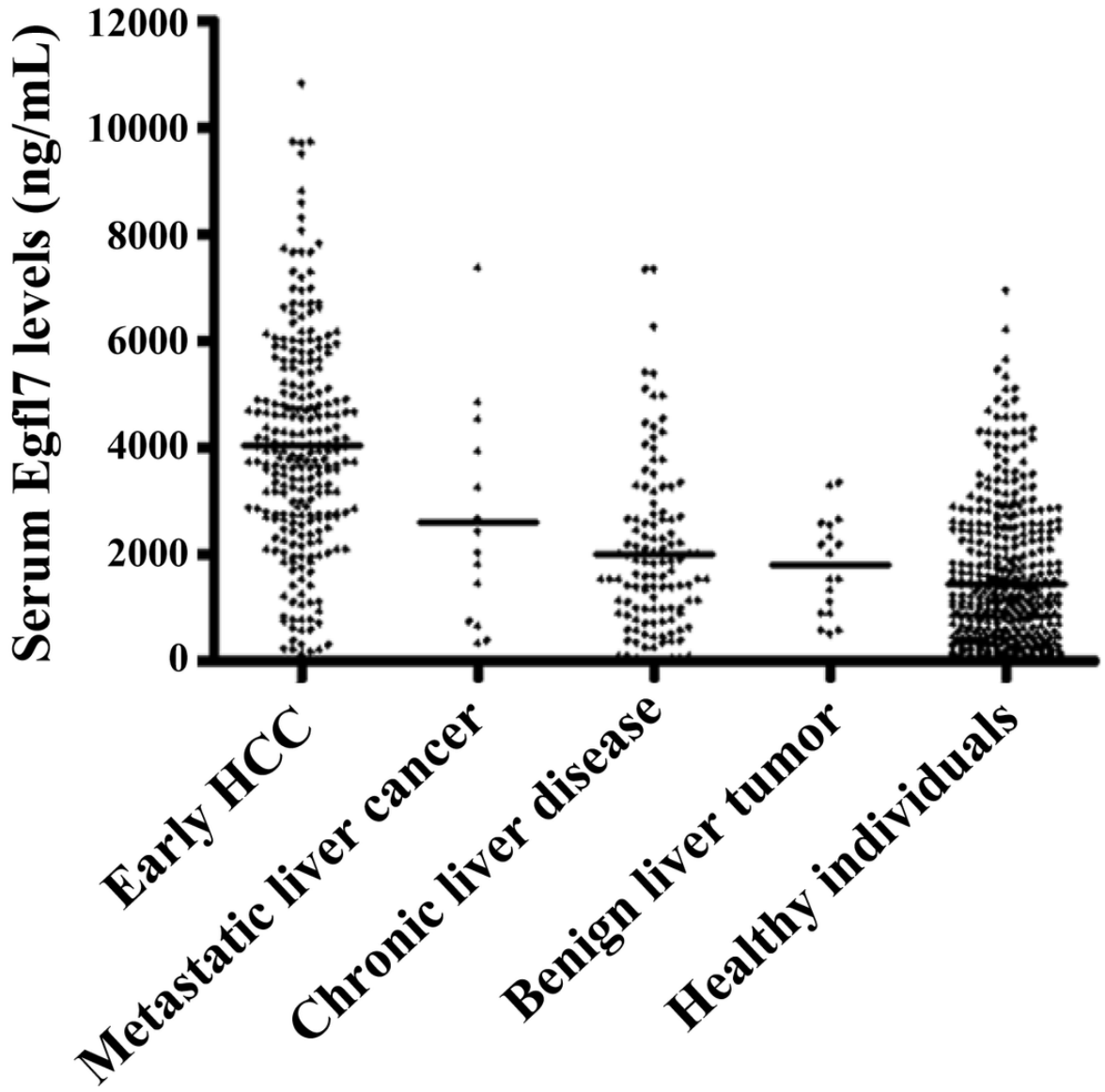

Figure 1

Serum Egfl7 levels in controls, early HCC and other patients in the Testing Cohort. The serum Egfl7 levels in patients with early HCC (n=314), metastatic liver cancer (n=16), benign liver tumors (n=19), or chronic liver disease (n=300) was determined by ELISA. The serum Egfl7 levels in healthy individuals (n=432) were measured as controls.

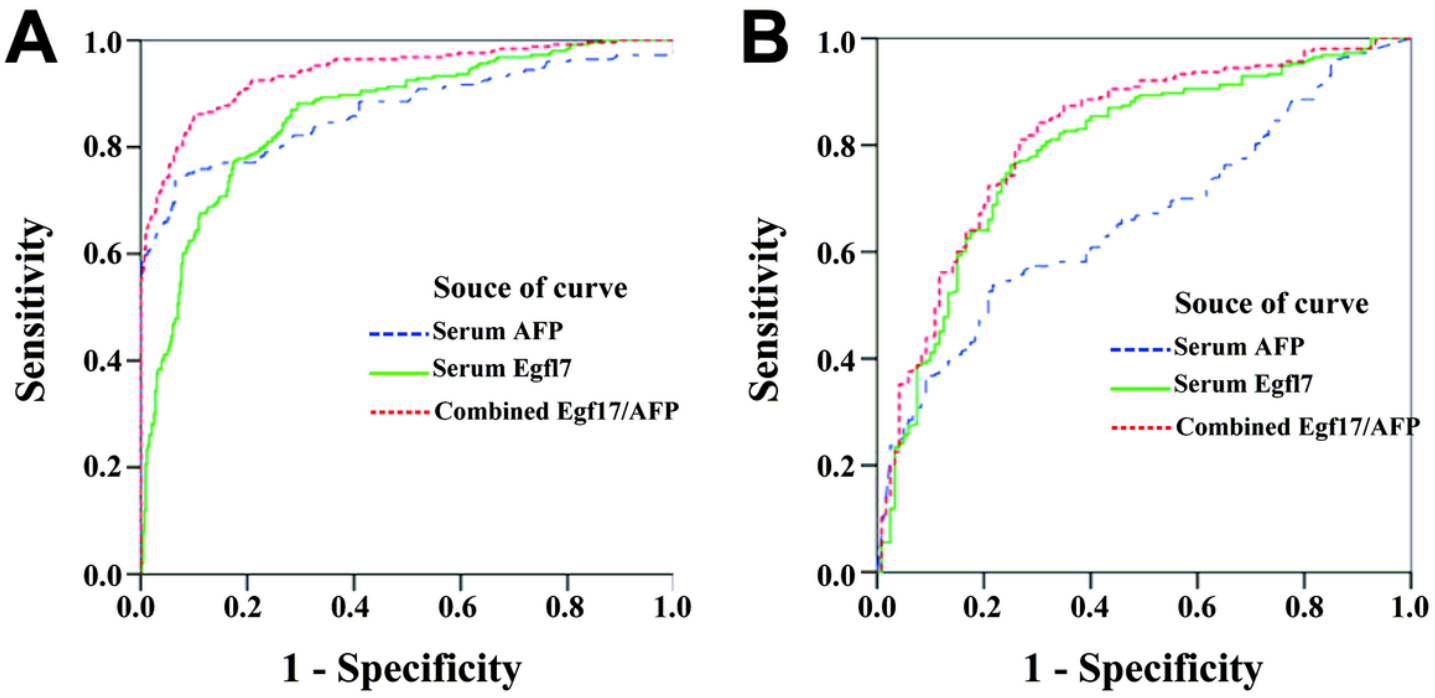

Figure 2

ROC curves of Egfl7, AFP or combined Egfl7 and AFP in the Testing Cohort. ROC curves of Egfl7, AFP or combined Egfl7 and AFP in differentiating early HCC patients from healthy individuals (A) or patients with chronic liver disease (B) in the Testing Cohort.

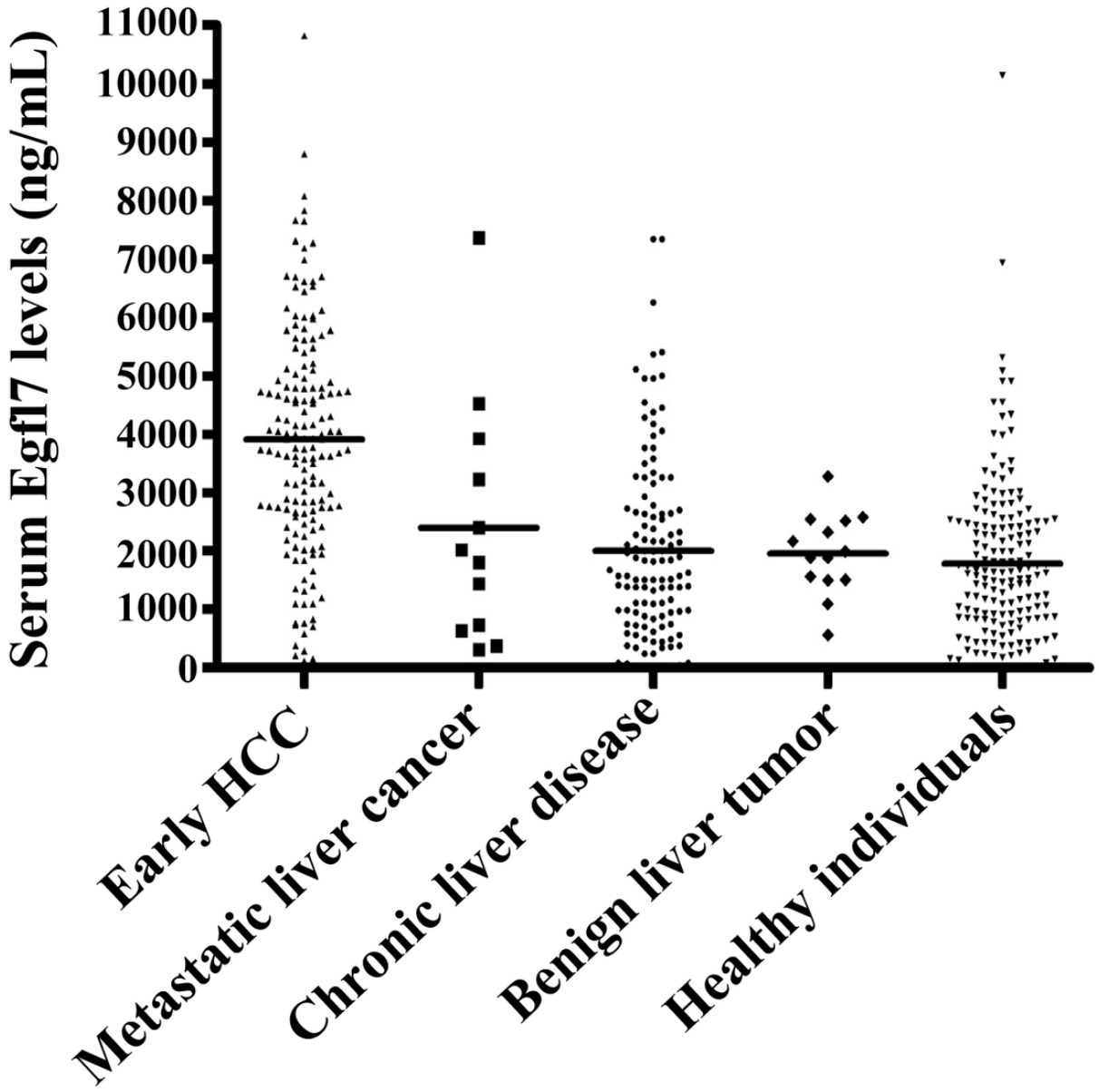

Figure 3

Serum Egfl7 levels in controls, early HCC and other patients in the Validation Cohort. The serum Egfl7 levels in patients from the Validation Cohort with early HCC (n=158), metastatic liver cancer (n=12), benign liver tumors (n=14), or chronic liver disease (n=120) were determined by ELISA. Serum Egfl7 levels in healthy individuals (n=172) were also measured as controls.

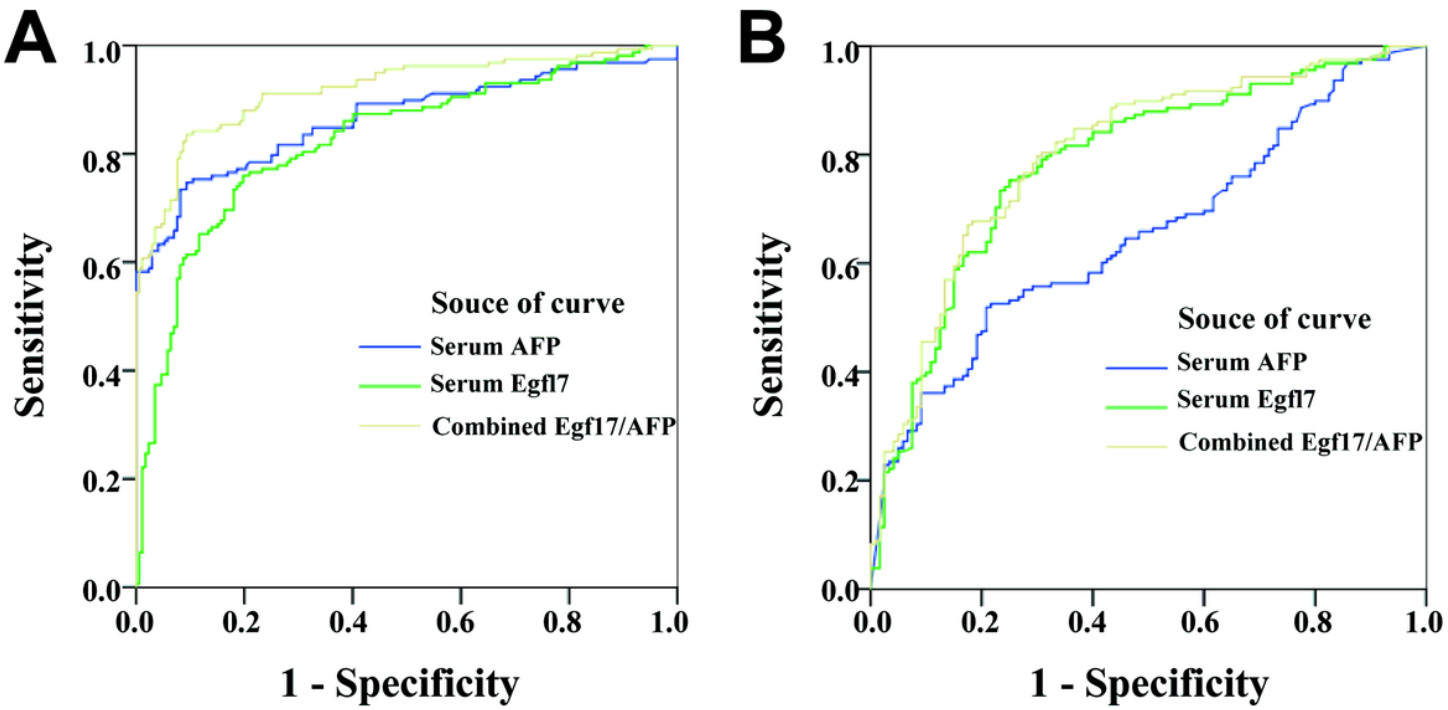

Figure 4

ROC curves of Egfl7, AFP or combined Egfl7 and AFP in the Validation Cohort. ROC curves of Egfl7, AFP or combined Egfl7 and AFP in differentiating early HCC patients from healthy individuals (A) or patients with chronic liver disease (B) in the Validation Cohort.