Original Article

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Elevated serum CA19-9 indicates severe liver inflammation and worse survival after curative resection in hepatitis B-related hepatocellular carcinoma

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SUMMARY We explored the prognostic value of preoperative CA19-9 in α-fetoprotein (AFP)-positive and -negative HCC with hepatitis B virus (HBV) background (HBV-HCC), and explored the underlying mechanism. Recurrence-free survival (RFS) and overall survival (OS) were assessed in HBV-HCC patients who underwent curative resection (Cohort 1). Immunohistochemical staining of CA19-9 in HCC and liver parenchyma were quantified in another cohort of 216 patients with resected HCC (Cohort 2). Immunohistochemical staining of CA19-9 and serum CA19-9 level was also compared between patients with HCC and intrahepatic cholangiocarcinoma (ICC) (Cohort 3). In Cohort 1, CA19-9 ≥ 39 U/mL was an independent risk factor for RFS (HR = 1.507, 95% CI = 1.087-2.091, $p = 0.014$) and OS (HR = 1.646, 95% CI = 1.146-2.366, $p = 0.007$). CA19-9 ≥ 39 U/mL was also associated with significantly higher incidence of macrovascular invasion (MaVI) compared with CA19-9 < 39 U/mL (23.0% *vs*. 7.2%, $p = 0.002$), and elevated aminotransferase and aspartate aminotransferase to platelet ratio index (APRI), and lower albumin. Immunohistochemical staining of CA19-9 revealed that CA19-9 expression was found exclusively in the background liver but not in HCC tumor cells. In contrast, tumor tissue was the main source of CA19-9 in ICC patients. CA19-9 ≥ 39 U/mL was associated with worse OS and RFS in both AFP-positive and negative HCC patients. CA19-9 indicated more severe inflammation and cirrhosis in the liver of HCC patients.

Keywords carbohydrate antigen 19-9, hepatocellular carcinoma, α-fetoprotein, survival, EpCAM

1. Introduction

Primary liver cancer is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide (*1,2*). In general, primary liver cancer is classified into two types as hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), with HCC being more common, accounting for 75-85% of all cases. However, mixed HCC-ICC and other rare types have also been reported. Alpha-fetoprotein (AFP) and carbohydrate antigen 19-9 (CA19-9) are the most commonly used biomarkers for HCC and ICC. A higher AFP level is associated with poor outcome after curative resection or liver transplantation (*3*). CA19-9, also known as Sialyl-Lewis-a, is mainly used as a biomarker for malignancies of the hepatobiliary tract and pancreas (*4*). However, serum CA19-9 levels may also be elevated in gastric, esophageal, and colonic cancers and in a number of non-malignant conditions including jaundice (*5*). Meanwhile, the serum CA19-9 level is elevated in approximately 60% of cholangiocarcinoma patients and in 30% of HCC patients (*6*). It is also frequently elevated in patients with combined HCC-cholangiocarcinoma. Elevated preoperative serum CA19-9 levels have been reported to be associated with worse survival in HCC patients

who had undergone resection (> 27U/mL) or liver transplantation (> 100 U/mL) (*7-9*). However, most of these studies mainly included patients with HCV-related HCC who underwent resection or transplantation, and the underlying mechanism by which CA19-9 influences prognosis remains unclear.

Thus, this study aimed to investigate the prognostic value of preoperative serum CA19-9 according to AFP status in HCC patients and in ICC patients with HBV background who underwent curative resection. And we will further explore the mechanism of CA19-9 by immunostaining EpCAM, a molecular marker for stem cells.

2. Methods

2.1. Patients and study design

We retrospectively evaluated three patient cohorts as follows. Cohort 1 involved 380 patients diagnosed with HCC at Tianjin Medical University Cancer Institute & Hospital (Tianjin, People's Republic of China) between 2012 and 2013. In this cohort, CA19-9 (+) was defined as serum CA19-9 ≥ 39 U/mL, whereas CA19-9 (-) was defined as serum CA19-9 < 39 U/mL, according to the upper limit of serum CA19-9 in our hospital. AFP (+) was defined as serum AFP > 20 ng/mL, whereas AFP (-) was AFP ≤ 20 ng/mL. Cohort 2 involved 216 patients with resected HCC in whom tissue microarray (TMA) samples were obtained. Patients with lymph node metastasis or distant metastasis were excluded to reduce confounding factors. Cohort 3 included 136 ICC patients who underwent radical resection.

All patients underwent curative resection for HCC, defined as complete macroscopic removal of the tumor. All tumors of HCC were staged according to the TNM classification system of International Union Against Cancer (8th edition) and the Barcelona Clinic Liver Cancer guidelines.

2.2. Demographic and clinicopathological factors

Demographic and clinicopathological factors including tumor factors, systemic inflammation factors, and liver factors were evaluated. Demographic factors included sex and age. Tumor factors included tumor size, number of tumor lesions, macroscopic vascular invasion (MaVI), microscopic vascular invasion (MiVI), intrahepatic metastasis, and tumor differentiation according to Edmondson's grade. Systemic factors included the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR). Liver factors included intraoperative detection of liver cirrhosis; alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (rGT), alkaline phosphatase (ALP), total bilirubin, and albumin levels; prothrombin time (PT); and the aspartate aminotransferase-to-platelet ratio index (APRI) as the parameter most closely related to liver cirrhosis and fibrosis in both chronic hepatitis B (*10,11*) and hepatitis C (*12,13*). NLR and PLR are both indicators of systemic inflammation and its relationship with the prognosis of several cancers has been identified (*14-16*).

2.3. TMA in ICC

We selected 158 consecutive patients with ICC who underwent surgical treatment at Tianjin Medical University Cancer Institute and Hospital between January 2012 and December 2017. Patients with combined HCC-CCA (*i.e.*, HCC and ICC) were excluded. The specimens of all patients were reviewed by two independent pathologists (Y.B. and Z.F.L.) to confirm the diagnosis of ICC and for restaging according to the 8th edition of the 2017 American Joint Committee on Cancer staging system. Of the 158 patients, we excluded 28 because of loss to follow-up (n = 11), non-R0 resection (n = 14), death from postoperative complications (n = 1), and death from non-tumor-related causes (n = 2). Thus, 130 patients (Cohort 3) were eventually included for comparison of clinical characteristics and survival analyses. The patients' formalin-fixed paraffin-embedded (FFPE) samples and hematoxylin-eosin (HE) staining slides from surgical specimens were then collected from the Department of Pathology in Tianjin Medical University Cancer Institute and Hospital. TMA samples comprising 2-mm cores of FFPE tumor tissue were prepared for various staining procedures by selecting representative tumor areas and a typical paratumoral region from each case. The Medical Ethics Committee of Tianjin Medical University Cancer Institute and Hospital approved this study, and informed consent was obtained from all patients.

2.4. Follow-up and postoperative treatment

All patients were monitored prospectively according to serum AFP and CA19-9 levels and using abdomen ultrasonography every 2 months in the first year and every 3 months after the first year. Recurrence was confirmed using computed tomography and/or magnetic resonance imaging based on typical imaging appearance in the imaging scan and an elevated AFP level. The treatment modality after relapse varied among individuals. Follow-up was concluded on July 10, 2019, with the patients followed up for a median of 56.6 months.

2.5. TMA and immunohistochemistry

TMAs were constructed as described previously (*17*). The mouse monoclonal antibodies used were anti-human CA19-9 (Zhongshan Company). Immunohistochemical

analysis was performed using a two-step protocol (Novolink Polymer Detection System, Novocastra) according to the manufacturer's instructions and as described previously (*17*). Briefly, paraffin sections were first deparaffinized and then hydrated. After microwave antigen retrieval, as required, endogenous peroxidase activity was blocked with incubation of the slides in 0.3% H_2O_2, and nonspecific binding sites were blocked with Protein Block (RE7102; Novocastra). After serial incubation with primary antibodies, Post Primary Block (RE7111; Novocastra), and secondary antibody (Novolink Polymer RE7112), the sections were developed in diaminobenzidine solution under a microscope and counterstained with hematoxylin. Negative control slides omitting the primary antibodies were included in all assays. CA19-9 immunoreactivity was evaluated in a semiquantitative manner on the basis of both labeling intensity and the percentage of immunopositive tumor cells for all antibodies. The score was calculated through multiplying staining intensity (0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = strong staining) by the percentage of immunoreactive tumor cells (0-100). The immunostaining result was considered negative (0) when the score was < 25; weak positive (1+) when the score was 26-100; moderate positive (2+) when the score was 101-200; or strong positive (3+) when the score was 201-300.

2.6. Statistical analysis

In univariate analyses of cumulative survival, survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Multivariate analyses were based on the Cox proportional hazards regression model. For the comparison of individual variables, χ^2 tests, Fisher's exact tests, and Student's *t*-tests were used as appropriate. All statistical analyses were performed using SPSS software (SPSS v22.0, Chicago, IL). A two-tailed *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. CA19-9 was an independent risk factor for RFS and OS

In Cohort 1, the 1-, 3-, 5-year overall survival (OS) was 80.3%, 37.7%, 34.4% for CA19-9 (+) patients and 90.3%, 62.7%, 51.4% for the CA19-9 (-) patients (Figure 1a). The 1-, 3-, 5-year RFS was 45.9%, 14.8%, 13.1% for the CA19-9 (+) patients, and 67.1%, 40.4%, 33.5%, respectively, for the CA19-9 (-) patients (Figure 1b). The 1-, 3-, 5-year OS was 84.0%, 47.1%, 38.3% for AFP (+) patients and 94.2%, 72.4%, 60.9% for the AFP (-) patients (Figure 1c). The 1-, 3-, 5-year RFS was 54.9%, 29.1%, 24.8% for the AFP (+) patients and 74.7%, 44.8%, 37.4% for the AFP (-) patients (Figure 1d).

In multivariate analysis, tumor size > 5 cm, presence of MaVI, AFP > 20 ng/mL, and CA19-9 ≥ 39 U/mL were independent risk factors for RFS (Table 1). Meanwhile, tumor size > 5 cm, presence of MaVI, AFP > 20 ng/mL, CA19-9 ≥ 39 U/mL, and albumin ≤ 35g/L were independent risk factors for OS (Table 2).

3.2. Positive CA19-9 predicted worse prognosis in both AFP (+) and AFP (-) HCC patients

The 1-, 3-, 5-year OS was 95.4%, 74.5%, 61.4% for patients with CA19-9 (-) and AFP (-), whereas they were 83.3%, 50.0%, 45.8% for patients with CA19-9 (+) and AFP (-) ($p < 0.05$, Figure 1e). The 1-, 3-, 5-year RFS was 77.1%, 47.1%, 39.2% for patients with CA19-9 (-) and AFP (-), whereas they were 45.8%, 20.8%, 16.7% in CA19-9 (+) and AFP (-) patients ($p < 0.05$, Figure 1f). These results showed that CA19-9 (+) predicted worse OS and RFS in AFP (-) patients.

The 1-, 3-, 5-year OS was 84.7%, 52.8%, and 42.9% for patients with CA19-9 (-) and AFP (+),and was 77.5%, 27.5%, 22.5% for patients with CA19-9 (+) and AFP (+) ($p < 0.05$, Figure 1e). The 1-, 3-, 5-year RFS was 58.9%, 34.4%, and 28.8% for patients with CA19-9 (-) and AFP (+) ($p < 0.05$, Figure 1f), whereas 40.0%, 10.0%, 7.5% for patients with CA19-9 (+) and AFP (+) ($p < 0.05$, Figure 1f). These results indicate that CA19-9 (+) predicted worse OS and RFS in AFP (+) patients. In summary, CA19-9 (+) predicted worse OS and RFS in both AFP (+) and AFP (-) HCC patients.

3.3. CA19-9 was associated with higher incidence of MaVI and a trend toward multiple tumors

CA19-9 was not associated with tumor size (6.1 ± 4.8 cm *vs*. 5.6 ± 3.8 cm, $p = 0.404$), MiVI (62.3% *vs*. 54.9%, $p = 0.225$) and AFP (5488.9 ± 28616.1 ng/mL *vs*. 5401.4 ± 40162.5 ng/mL, $p = 0.987$). However, CA19-9 was related to higher incidence of MaVI (23.0% *vs*. 7.2%, $p = 0.002$), and a trend toward more multiple tumors with marginal significance (23.0% *vs*. 13.8%, $p = 0.068$) (Table 3).

3.4. CA19-9 was associated with more severe liver cirrhosis and liver inflammation but not with systemic inflammation

Comparison of clinicopathological factors between CA19-9 (+) and CA19-9 (-) patients revealed that CA19-9 (+) patients tend to be older (mean age: 58.4 ± 10.4 years *vs*. 55.4 ± 10.6 years, $p = 0.048$), have higher incidence of liver cirrhosis (70.5% *vs*. 56.1%, $p = 0.037$), higher APRI (1.53 ± 1.61 *vs*. 0.72 ± 0.96, $p < 0.001$), elevated ALT (75.4 ± 77.3 U/L *vs*. 43.9 ± 59.5 U/L, $p = 0.004$), elevated AST (75.1 ± 69.0 U/L *vs*. 41.5 ± 46.0

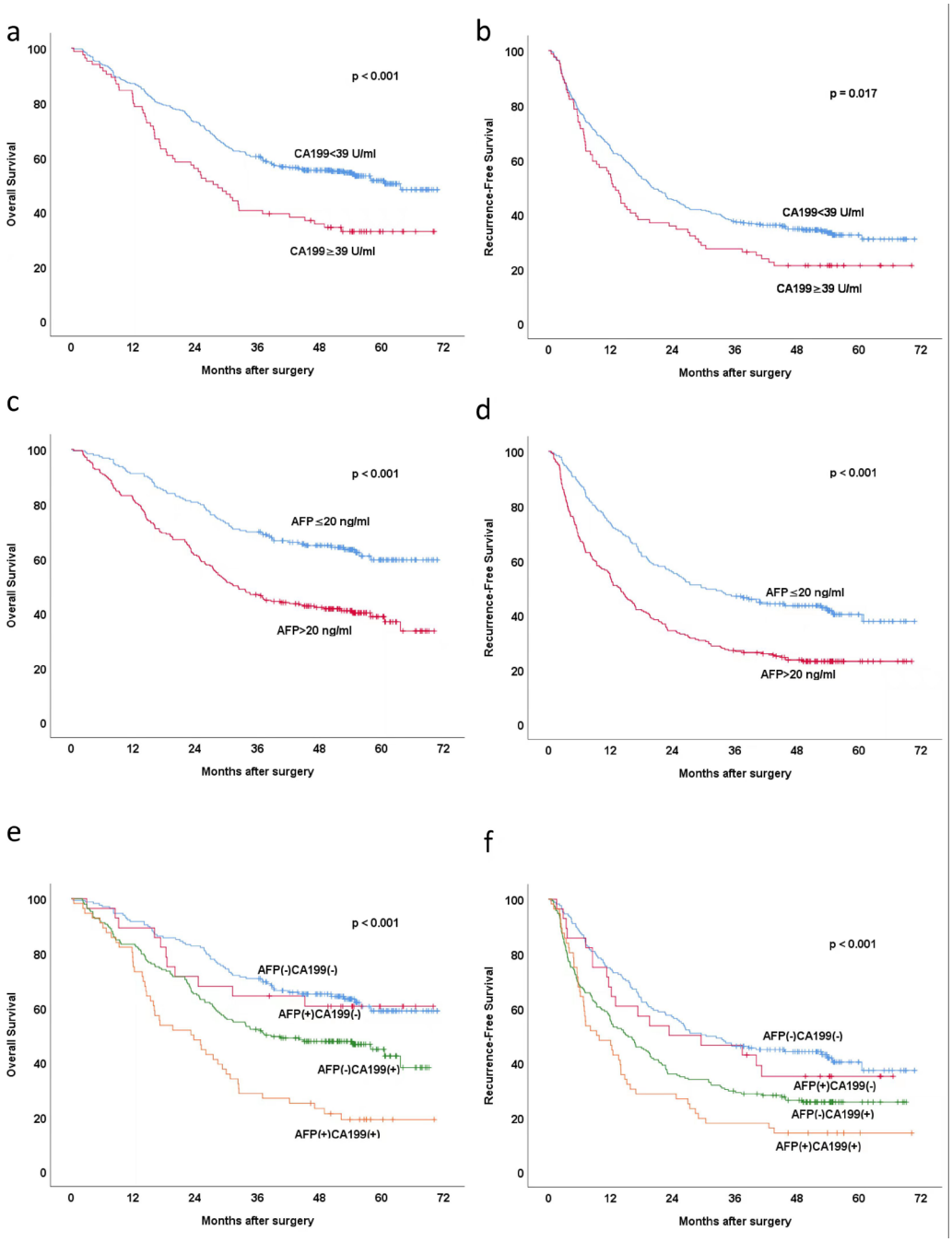


Figure 1. AFP, CA19-9 and combination to predict OS and RFS for HCC patients after curative resection.

U/L, $p < 0.001$), increased rGT (147.1 ± 162.4 U/L *vs*. 81.4 ± 99.4 U/L, $p = 0.003$), and lower level of albumin (39.8 ± 5.5 g/L *vs*. 42.0 ± 5.3 g/L, $p = 0.002$) (Table 3). All the factors except MaVI are related to liver cirrhosis.

To exclude the confounding effect of MaVI, we excluded patients with MaVI. The results showed that CA19-9 was still correlated with liver cirrhosis, APRI, ALT, AST, rGT and albumin (data not shown). Furthermore, multivariate analysis showed that CA19-9 (+) and MaVI (+) were both independent risk factors for RFS.

In the current study, CA19-9 was not correlated to NLR or PLR, indicating that CA19-9 was not correlated to systemic inflammation.

3.5. Immunohistochemical staining of CA19-9 in both HCC and ICC

To determine the source of CA19-9, we examined its expression in TMA samples of HCC patients. Immunohistochemical staining of CA19-9 in both tumor tissue and non-tumor liver parenchyma specimens from HCC patients was also assessed. The results showed that none of the HCC tumor cells express CA19-9, and CA19-9 was only expressed in non-tumor liver parenchyma (Figure 2).

Immunohistochemical staining of CA19-9 in both tumor and non-tumor liver parenchyma samples from Cohort 3 (Figure 2) revealed that CA19-9 was expressed

Table 1. Univariate and multivariate analysis for RFS in Cohort 1

Recurrence-free Survival, Variable	Comparison	Univariate, *P*-value	Multivariate, *P*-value	Hazard Ratio (95.0% CI)
Gender	Male *vs*. Female	0.268		
Age	≤ 50 *vs*. > 50 years	0.957		
Tumor size	≤ 5 *vs*. > 5 cm	< 0.001	0.001	1.569 (1.204-2.045)
Number	Solitary *vs*. Multiple	0.075		
MaVI	Yes *vs*. No	< 0.001	0.023	1.586 (1.065-2.361)
Differentiation	I/II *vs*. III/IV	0.482		
MiVI	Yes *vs*. No	0.025		
IHM	Yes *vs*. No	0.022		
Cirrhosis	Yes *vs*. No	0.588		
HBeAg	Yes *vs*. No	0.142		
AFP	≤ 20 *vs*. > 20 ng/mL	< 0.001	0.012	1.373 (1.071-1.759)
CA19-9	≥ 39 *vs*. < 39 U/ml	< 0.001	0.014	1.507 (1.087-2.091)
ALT	≤ 40 *vs*. > 40 U/L	0.046		
AST	≤ 40 *vs*. > 40 U/L	< 0.001		
Albumin	≤ 35 *vs*. > 35 g/L	0.114		
NLR	≤ 5 *vs*. > 5	0.019		
PLR	≤ 300 *vs*. > 300	0.072		
rGT	≤ 60 *vs*. > 60 U/L	< 0.001		
HKLC	0/1/2/3	< 0.001	NA	
BCLC	A/B/C	< 0.001	NA	

Table 2. Univariate and multivariate analysis for OS in Cohort 1

Overall Survival, Variable	Comparison	Univariate, *P*-value	Multivariate, *P*-value	Hazard Ratio (95.0% CI)
Gender	Male *vs*. Female	0.222		
Age	≤ 50 *vs*. > 50 years	0.246		
Tumor size	≤ 5 *vs*. > 5 cm	< 0.001	< 0.001	1.931 (1.430-2.607)
Number	Solitary *vs*. Multiple	0.029		
MaVI	Yes *vs*. No	< 0.001	0.003	1.871 (1.230-2.847)
Differentiation	I/II/III/IV	0.216		
MiVI	Yes *vs*. No	< 0.001		
IHM	Yes *vs*. No	0.002	0.009	1.483 (1.104-1.992)
Cirrhosis	Yes *vs*. No	0.158		
HBeAg	Yes *vs*. No	0.798		
AFP	≤ 20 *vs*. > 20 ng/mL	< 0.001	0.003	1.558 (1.163-2.089)
CA19-9	≥ 39 *vs*. < 39 U/ml	0.001	0.007	1.646 (1.146-2.366)
ALT	≤ 40 *vs*. > 40 U/L	0.142		
AST	≤ 40 *vs*. > 40 U/L	< 0.001		
NLR	≤ 5 *vs*. > 5	< 0.001		
PLR	≤ 300 *vs*. > 300	0.004	0.029	2.920 (1.118-7.624)
Albumin	≤ 35 *vs*. > 35 g/L	0.174		
rGT	≤ 60 *vs*. > 60 U/L	< 0.001		
TB	≤ 19 *vs*. > 19 μmol/L	0.056		
HKLC	0/1/2/3	0.011	NA	
BCLC	A/B/C	< 0.001	NA	

Table 3. Comparison of clinicopathological factors between patients with CA19-9 (+) and CA19-9 (-)

Cohort 1. Variable	CA19-9 < 39 U/mL (*n* = 319)	CA19-9 ≥ 39U/mL (*n* = 61)	*P*-value
Gender (Male/Female)	255/64 (78.0%)	48/13 (78.7%)	0.824
Age (year) (Mean ± SD)	55.4 ± 10.6	58.4 ± 10.4	0.048
Tumor size (Mean ± SD)	5.6 ± 3.8	6.1 ± 4.8	0.404
Number (Solitary *vs*. Multiple)	275/44 (13.8%)	47/14 (23.0%)	0.068
MaVI (Yes *vs*. No)	23/296 (7.2%)	12/49 (23.0%)	0.002
MiVI (Yes *vs*. No)	175/144 (54.9%)	38/22 (62.3%)	0.225
IHM (Yes *vs*. Bo)	111/208 (34.8%)	24/36 (39.3%)	0.440
AFP (ng/mL) (Mean ± SD)	5,401.4 ± 4,0162.5	5,488.9 ± 2,8616.1	0.987
HBeAg (Yes *vs*. no)	45/274	12/49	0.265
Cirrhosis (Yes *vs*. No)	179/140 (56.1%)	43/18 (70.5%)	0.037
APRI (Mean ± SD)	0.72 ± 0.96	1.53 ± 1.61	< 0.001
Ascites (Yes *vs*. No)	32/287	7/54	0.733
rGT (Mean ± SD)	81.4 ± 99.4	147.1 ± 162.4	0.003
ALT (U/L) (Mean±SD)	43.9 ± 59.5	75.4 ± 77.3	0.004
AST (U/L) (Mean ± SD)	41.5 ± 46.0	75.1 ± 69.0	< 0.001
Albumin (g/L) (Mean ± SD)	42.0 ± 5.3	39.8 ± 5.5	0.002
TB (μmol/L) (Mean ± SD)	18.5 ± 9.8	26.4 ± 37.8	0.109
NLR (Mean ± SD)	2.3 ± 1.5	2.1 ± 1.4	0.388
PLR (Mean ± SD)	134.3 ± 368.7	95.5 ± 47.8	0.412

in 64% (87/136) of ICC tumors and 4.4% (6/136) of non-tumor liver parenchyma. Serum CA19-9 was positive (≥ 39 U/mL) in 58.1% and negative (< 39 U/mL) in 41.9% of the patients with ICC. The results that immunohistochemical staining of CA19-9 was positive only in 4.4% of ICCs indicate that serum CA19-9 mainly derives from the tumor tissue of patients with ICC, which is distinct from the dominant expression of CA19-9 in the background liver in HCC patients (Figure 3).

3.6. relationship between EpCAM and serum CA19-9 and AFP

Positive and negative stain of EpCAM in tumor tissue was detected by immunohistochemistry (Figure 4a-b). The positive ratio of EpCAM staining was similar in patients with CA19-9 ≥ 39 U/mL and CA19-9 < 39 U/mL(Figure 4c). While more patients had elevated HBVDNA in patients with serum CA19-9 ≥ 39 U/mL than CA19-9 < 39 U/mL (Figure 4d).

It is quite the opposite for AFP. The proportion of

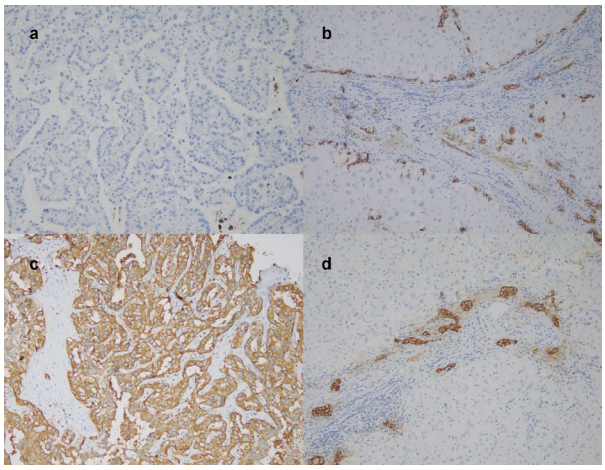


Figure 2. Immunohistochemical staining of CA19-9 in HCC and ICC in Cohort 2 and 3. (a), HCC tumor tissue was negative for CA19-9. **(b)**, HCC non-tumor liver parenchyma was positive for CA19-9 in the portal area. **(c)**, ICC tumor tissue was positive for CA19-9. **(d)**, ICC non-tumor liver parenchyma was positive for CA19-9 in the portal area.

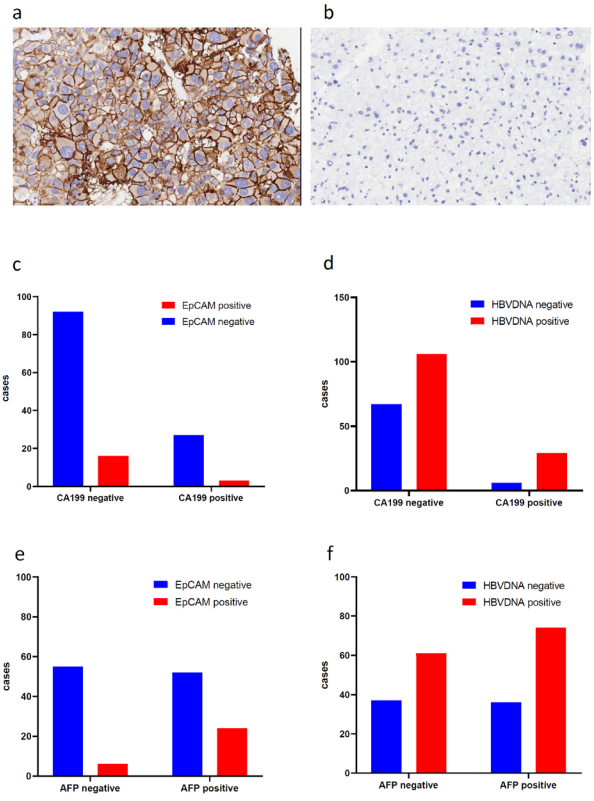
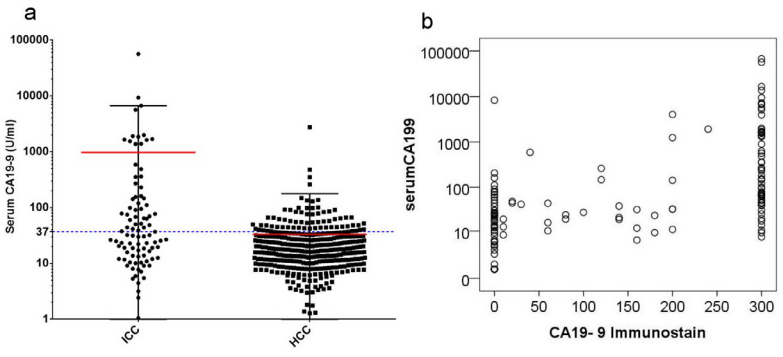


Figure 4. (a), positive staining for EpCAM. **(b)**, negative staining for EpCAM. **(c)**, the positive ratio of EpCAM staining were similar in patients with CA19-9 ≥ 39 U/mL and CA19-9 < 39 U/mL (10% *vs.* 14.8%, $p > 0.05$). **(d)**, more patients had elevated HBVDNA in patients with serum CA19-9 ≥ 39 U/mL than CA19-9 < 39 U/mL (82.9% *vs.* 61.3%, $p < 0.01$). **(e)**, the proportion of positive EpCAM staining in AFP positive group is much higher than that in AFP negative group (31.6% *vs.* 9.8%, $p < 0.001$). **(f)**, the proportion of positive elevated HBVDNA in AFP negative group is similar to that in AFP positive group (62.2% *vs.* 67.3%, $p > 0.05$).



c

ICC 136 cases	Tumor	Non-tumor Liver parenchyma
IHC CA19-9 (-)	49 (36%)	130 (96.3%)
IHC CA19-9 (+)	87 (64%)	6 (4.4%)

d

ICC 136 cases	IHC CA19-9 (-)	IHC CA19-9 (+)	
serum CA19-9 (-)	35	22	57 (41.9%)
serum CA19-9 (+)	14	65	79 (58.1%)

Figure 3. CA19-9 in HCC and ICC in Cohort 2 and 3. (a), comparison of serum CA19-9 level in ICC and HCC patients. **(b)**, Serum CA19-9 correlated with IHC CA19-9 ($p < 0.001$). **(c)**, Immunohistochemical staining of CA19-9 in ICC tumor and non-tumor liver parenchyma showed that CA19-9 is positive in 64% in tumor tissue and 4.4% in non-tumor liver parenchyma. **(d)**, comparison between serum CA19-9 and immunochemical staining of CA19-9.

positive EpCAM staining in AFP negative group is much more than that in AFP positive group (Figure 4e). While the proportion of positive elevated HBVDNA in AFP negative group is similar to that in AFP positive group (Figure 4f).

This indicated that AFP is related to the stemness of hepatocellular carcinoma as indicated by EpCAM. While CA19-9 is related to hepatitis and inflammation of the background liver as indicated by a higher proportion of elevated HBVDNA.

4. Discussion

Elevated serum CA19-9 levels had been reported to predict poor prognosis in HCC, even in AFP negative HCC. Chen *et al*. (*8*) found that a preoperative CA19-9 value of >27 U/mL was associated with poor prognosis after resection for HCC. Wan *et al*. (*9*) also showed that preoperative serum AFP levels of > 400 ng/mL and CA19-9 >100 U/mL predicted survival after liver transplantation in patients with HCC. Hsu *et al*. (*7*) found that an elevated serum CA19-9 level of ≥ 100 U/mL was an independent predictor of poor OS in HCV-related HCC. Lu *et al*. (*18*) found that a preoperative CA19-9 level of > 32.6 U/mL predicted poor prognosis and can be used as a prognostic marker in AFP-negative HCC. However, only one study evaluated patients with only HBV-related HCC. The current study included patients with exclusively HBV-related HCC. At a cut-off value of 39 U/mL, 16.1% of patients (61/380) were found to be CA19-9 positive, and CA19-9 ≥ 39 U/mL predicted worse OS and RFS in both AFP (-) and AFP (+) patients. CA19-9 positivity was revealed to be closely related to more severe liver cirrhosis and liver inflammation, as indicated by elevated rGT, ALT, AST and APRI (*19*).

CA19-9 is synthesized by normal biliary epithelium or by malignant tumors (*20*), and it is frequently elevated in biliary obstruction and biliary tract cancers (*21*). Furthermore, an elevated CA19-9 serum level is reported to be associated with mixed HCC-ICC, which tends to have more aggressive behavior than pure HCCs (*22*). In HCC, the source and implication of CA19-9 is still unclear. In the current study, we excluded the possibility of mixed HCC-ICC by two independent pathologists, and we applied immunohistology staining to confirm that the only source of serum CA19-9 in HCC patient is the background liver parenchyma. Furthermore, CA19-9 ≥ 39 U/mL was associated with elevated rGT, ALT, AST, APRI and higher incidence of MaVI. Previous reports have confirmed that elevated ALT, AST, and rGT levels are correlated to liver cirrhosis (*23,24*) and recurrence (*25,26*). Thus, we confirmed that CA19-9 is a liver biomarker, which indicated more severe liver inflammation and liver cirrhosis in HCC. To confirm this finding, we performed immunohistochemical staining of EpCAM, a biomarker for stemness of hepatocellular carcinoma (*27,28*). And we found that the positive ratio of EpCAM staining was similar between patients with CA19-9 ≥ 39 U/mL and CA19-9 < 39 U/mL. In contrast, elevated AFP was associated with positive EpCAM staining, indicating the stemness of tumor was associated with positive AFP rather than positive CA19-9. More impressively, more patients had elevated HBVDNA in patients with serum CA19-9 ≥ 39 U/mL than patients with CA19-9 < 39 U/mL. This finding confirms that CA19-9 is an indicator of hepatitis and liver inflammation.

Macrovascular invasion and multiple tumor nodules were also more common in CA19-9 (+) patients. These can be attributed to two reasons. First, chronic inflammation and cirrhosis of the liver are the key etiological risk factors for HCC (*29,30*), and an elevated ALT/AST/APRI in patients with elevated CA19-9 indicated more severe liver cirrhosis and an inflamed liver background (*31,32*), which is closely related to de novo tumor pathogenesis and multicentric recurrence (*33,34*). Second, liver inflammation has been reported as an independent risk factor for early tumor recurrence in patients with HCC (*35-37*), and preclinical studies have revealed that the inflammatory microenvironment of fibrotic liver promotes hepatocellular carcinoma metastasis by STAT3 activation (*38*).

Our study has several limitations. First, it is a single-center study of retrospective cohorts, and only the serum level and immunohistochemical expression of CA19-9 were evaluated. Second, the precise mechanism by which CA19-9 promotes macroscopic vascular invasion is still unclear and thus further studies are needed to elucidate the underlying mechanism.

5. Conclusions

In conclusion, CA19-9 is associated with lower OS and RFS in both AFP (+) and AFP (-) patients. Importantly, CA19-9 is secreted by the background liver, but not by tumor cells in patients with HCC. Thus, CA19-9 is not a tumor biomarker, but a biomarker for liver cirrhosis and inflammation and a risk factor for worse OS and RFS in HCC.

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References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021; 71:209-249.
2. Sugawara Y, Hibi T. Surgical treatment of hepatocellular carcinoma. Biosci Trends. 2021; 15:138-141.
3. Borzio M, Dionigi E, Rossini A, *et al*. External validation of the ITA.LI.CA prognostic system for patients with hepatocellular carcinoma: A multicenter cohort study. Hepatology. 2018; 67:2215-2225.
4. Singh S, Tang SJ, Sreenarasimhaiah J, Lara LF, Siddiqui A. The clinical utility and limitations of serum carbohydrate antigen (CA19-9) as a diagnostic tool for pancreatic cancer and cholangiocarcinoma. Dig Dis Sci. 2011; 56:2491-2496.
5. Marrelli D, Caruso S, Pedrazzani C, Neri A, Fernandes E, Marini M, Pinto E, Roviello F. CA19-9 serum levels in obstructive jaundice: clinical value in benign and malignant conditions. Am J Surg. 2009; 198:333-339.
6. Tsuji M, Kashihara T, Terada N, Mori H. An immunohistochemical study of hepatic atypical adenomatous hyperplasia, hepatocellular carcinoma, and cholangiocarcinoma with alpha-fetoprotein, carcinoembryonic antigen, CA19-9, epithelial membrane antigen, and cytokeratins 18 and 19. Pathol Int. 1999; 49:310-317.
7. Hsu CC, Goyal A, Iuga A, Krishnamoorthy S, Lee V, Verna EC, Wang S, Chen FN, Rodriguez R, Emond J, Berk P, Lefkowitch J, Dove L, Brown RS Jr, Siegel AB. Elevated CA19-9 is associated with increased mortality in a prospective cohort of hepatocellular carcinoma patients. Clin Transl Gastroenterol. 2015; 6:e74.
8. Chen YL, Chen CH, Hu RH, Ho MC, Jeng YM. Elevated preoperative serum CA19-9 levels in patients with hepatocellular carcinoma is associated with poor prognosis after resection. ScientificWorldJournal. 2013; 2013:380797.
9. Wan P, Zhang J, Long X, Li Q, Xu N, Zhang M, Chen X, Han L, Xia Q. Serum levels of preoperative alpha-fetoprotein and CA19-9 predict survival of hepatic carcinoma patients after liver transplantation. Eur J Gastroenterol Hepatol. 2014; 26:553-561.
10. Lebensztejn DM, Skiba E, Sobaniec-Lotowska M, Kaczmarski M. A simple noninvasive index (APRI) predicts advanced liver fibrosis in children with chronic hepatitis B. Hepatology. 2005; 41:1434-1435.
11. Shin WG, Park SH, Jang MK, Hahn TH, Kim JB, Lee MS, Kim DJ, Jun SY, Park CK. Aspartate aminotransferase to platelet ratio index (APRI) can predict liver fibrosis in chronic hepatitis B. Dig Liver Dis. 2008; 40:267-274.
12. Snyder N, Gajula L, Xiao SY, Grady J, Luxon B, Lau DT, Soloway R, Petersen J. APRI: an easy and validated predictor of hepatic fibrosis in chronic hepatitis C. J Clin Gastroenterol. 2006; 40:535-542.
13. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Ledinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology. 2005; 128:343-350.
14. Amaral SR, Casal Moura M, Carvalho J, Chaves A, Jesus E, Sousa G. Prognostic significance of neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) in non-small cell lung cancer (NSCLC) treated with immune checkpoint inhibitors. Ann Oncol. 2019; 30 Suppl 1:i3.
15. Russo A, Russano M, Franchina T, *et al*. Neutrophil-to-Lymphocyte Ratio (NLR), Platelet-to-Lymphocyte Ratio (PLR), and outcomes with nivolumab in pretreated non-small cell lung cancer (NSCLC): A large retrospective multicenter study. Adv Ther. 2020; 37:1145-1155.
16. Tanaka H, Tamura T, Toyokawa T, Muguruma K, Miki Y, Kubo N, Sakurai K, Hirakawa K, Ohira M. Clinical relevance of postoperative neutrophil-lymphocyte ratio (NLR) to recurrence after adjuvant chemotherapy of s-1 for gastric cancer. Anticancer Res. 2018; 38:3745-3751.
17. Ma B, Meng H, Tian Y, Wang Y, Song T, Zhang T, Wu Q, Cui Y, Li H, Zhang W, Li Q. Distinct clinical and prognostic implication of IDH1/2 mutation and other most frequent mutations in large duct and small duct subtypes of intrahepatic cholangiocarcinoma. BMC cancer. 2020; 20:318.
18. Lu LH, Zhang YF, Wei W, Shi M, Guo RP. Preoperative carbohydrate antigen 19-9: its neglected role in alpha-fetoprotein-negative hepatocellular carcinoma patients. J Gastrointest Surg. 2017; 21:2025-2032.
19. Liu W, Li X, Zheng W, Yao R, Zheng J. Preoperative evaluation of the degree of liver fibrosis based on matter-element analysis using serological indicators in patients with hepatocellular carcinoma. Biosci Trends. 2019; 13:70-76.
20. Yuan RH, Jeng YM, Hu RH, Lai PL, Lee PH, Cheng CC, Hsu HC. Role of p53 and beta-catenin mutations in conjunction with CK19 expression on early tumor recurrence and prognosis of hepatocellular carcinoma. J Gastrointest Surg. 2011; 15:321-329.
21. Strom BL, Iliopoulos D, Atkinson B, Herlyn M, West SL, Maislin G, Saul S, Varello MA, Rodriguez-Martinez HA, Rios-Dalenz J, Soloway RD. Pathophysiology of tumor progression in human gallbladder: flow cytometry, CEA, and CA 19-9 levels in bile and serum in different stages of gallbladder disease. J Natl Cancer Inst. 1989; 81:1575-1580.
22. Lu XY, Xi T, Lau WY, Dong H, Zhu Z, Shen F, Wu MC, Cong WM. Hepatocellular carcinoma expressing cholangiocyte phenotype is a novel subtype with highly aggressive behavior. Ann Surg Oncol. 2011; 18:2210-2217.
23. Li Q, Lu C, Li W, Huang Y, Chen L. The gamma-glutamyl transpeptidase-to-albumin ratio predicts significant fibrosis and cirrhosis in chronic hepatitis B patients. J Viral Hepat. 2017; 24:1143-1150.
24. Lemoine M, Shimakawa Y, Nayagam S, *et al*. The gamma-glutamyl transpeptidase to platelet ratio (GPR) predicts significant liver fibrosis and cirrhosis in patients with chronic HBV infection in West Africa. Gut. 2016; 65:1369-1376.
25. Cheung YS, Chan HL, Wong J, Lee KF, Poon TC, Wong N, Lai PB. Elevated perioperative transaminase level predicts intrahepatic recurrence in hepatitis B-related

hepatocellular carcinoma after curative hepatectomy. Asian J Surg. 2008; 31:41-49.
26. Zhou L, Wang SB, Chen SG, Qu Q, Rui JA. Prognostic value of ALT, AST, and AAR in hepatocellular carcinoma with b-type hepatitis-associated cirrhosis after radical hepatectomy. Clin Lab. 2018; 64:1739-1747.
27. Zeng SS, Yamashita T, Kondo M, Nio K, Hayashi T, Hara Y, Nomura Y, Yoshida M, Hayashi T, Oishi N, Ikeda H, Honda M, Kaneko S. The transcription factor SALL4 regulates stemness of EpCAM-positive hepatocellular carcinoma. J Hepatol. 2014; 60:127-134.
28. Chan AW, Tong JH, Chan SL, Lai PB, To KF. Expression of stemness markers (CD133 and EpCAM) in prognostication of hepatocellular carcinoma. Histopathology. 2014; 64:935-950.
29. Jiang K, Centeno BA. Primary Liver Cancers, Part 2: Progression pathways and carcinogenesis. Cancer Control. 2018; 25:1073274817744658.
30. Liu S, Zhou Z, Jia Y, Xue J, Liu Z, Cheng K, Cheng S, Liu S. Identification of portal vein tumor thrombus with an independent clonal origin in hepatocellular carcinoma *via* multi-omics data analysis. Cancer Biol Med. 2019; 16:147-170.
31. Tarao K, Rino Y, Takemiya S, Tamai S, Ohkawa S, Sugimasa Y, Miyakawa K, Morinaga S, Yoshida M, Shibuya A, Kokubu S, Kakita A, Endo O. Close association between high serum ALT and more rapid recurrence of hepatocellular carcinoma in hepatectomized patients with HCV-associated liver cirrhosis and hepatocellular carcinoma. Intervirology. 2000; 43:20-26.
32. Tarao K, Takemiya S, Tamai S, Sugimasa Y, Ohkawa S, Akaike M, Tanabe H, Shimizu A, Yoshida M, Kakita A. Relationship between the recurrence of hepatocellular carcinoma (HCC) and serum alanine aminotransferase levels in hepatectomized patients with hepatitis C virus-associated cirrhosis and HCC. Cancer. 1997; 79:688-694.
33. Shirabe K, Takenaka K, Taketomi A, Kawahara N, Yamamoto K, Shimada M, Sugimachi K. Postoperative hepatitis status as a significant risk factor for recurrence in cirrhotic patients with small hepatocellular carcinoma. Cancer. 1996; 77:1050-1055.
34. Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. Gastroenterology. 2013; 144:512-527.
35. Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, Kammula US, Chen Y, Qin LX, Tang ZY, Wang XW. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer cell. 2006; 10:99-111.
36. Matsumoto K, Yoshimoto J, Sugo H, Kojima K, Futagawa S, Matsumoto T. Relationship between the histological degrees of hepatitis and the postoperative recurrence of hepatocellular carcinoma in patients with hepatitis C. Hepatol Res. 2002; 23:196-201.
37. Liu Y, Wang ZX, Cao Y, Zhang G, Chen WB, Jiang CP. Preoperative inflammation-based markers predict early and late recurrence of hepatocellular carcinoma after curative hepatectomy. Hepatobiliary Pancreat Dis Int. 2016; 15:266-274.
38. Jiang Y, Chen P, Hu K, Dai G, Li J, Zheng D, Yuan H, He L, Xie P, Tu M, Peng S, Qu C, Lin W, Chung RT, Hong J. Inflammatory microenvironment of fibrotic liver promotes hepatocellular carcinoma growth, metastasis and sorafenib resistance through STAT3 activation. J Cell Mol Med. 2021; 25:1568-1582.

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