

# Double-Negative $\alpha$ -Fetoprotein and Carbohydrate Antigen 19-9 Predict a Good Prognosis in Intrahepatic Cholangiocarcinoma: A Propensity Score Matching Analysis

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**INTRODUCTION:** Carbohydrate antigen 19-9 (CA19-9) and  $\alpha$ -fetoprotein (AFP) are routinely tested in patients with liver malignancies before surgery. However, few reports have explored the relevance of the expression pattern of these 2 tumor markers regarding the prognosis of intrahepatic cholangiocarcinoma (ICC). We herein combined these 2 tumor markers to investigate the influence on ICC malignancy and patient prognosis.

**METHODS:** From March 2009 to December 2019, 519 consecutive patients with newly diagnosed ICC who underwent R0 resection were enrolled and followed. The relationships between clinicopathological parameters and these 2 tumor markers were analyzed. Propensity score matching was used to eliminate the baseline differences.

**RESULTS:** A lower proportion of patients with double-negative AFP and CA19-9 had advanced tumor-node-metastasis stage, larger tumor diameter, multiple tumors, lymph node metastasis, microvascular invasion, and perineural invasion. With propensity score matching, patients were divided into double-negative and non-double-negative groups, with 128 patients in each group, and the 5-year recurrence-free survival and overall survival rates were 33.8 vs 15.2 ( $P < 0.001$ ) and 45.3 vs 19.0, respectively ( $P < 0.001$ ). In the multivariate Cox analyses, double negativity for the 2 tumor markers was an independent factor for recurrence-free survival (hazard ratios, 0.578; 95% CI, 0.442–0.755,  $P < 0.001$ ) and overall survival (hazard ratios, 0.567; 95% CI, 0.434–0.741,  $P < 0.001$ ).

**DISCUSSION:** Double negativity for CA19-9 and AFP indicated less invasive tumor characteristics in patients with ICC. Patients with double-negative tumor markers achieved better outcomes than those with non-double-negative markers, which is meaningful for prognostic counseling and therapeutic triage.

SUPPLEMENTARY MATERIAL accompanies this paper at <https://links.lww.com/CTG/A720>

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## INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer after hepatocellular carcinoma (HCC) (1). On the basis of its anatomical origin, second-order bile ducts serve as the point of separation between ICC and perihilar cholangiocarcinoma or distal cholangiocarcinoma (2,3). Although the frequency of ICC worldwide is considerably less than that of HCC, several recent studies from around the world

have reported rapidly increasing rates of ICC over the past few decades (4–6). Surgical resection is the main treatment of ICC. However, the outcomes of surgical resection for ICC have remained relatively unsatisfactory, with the 5-year overall survival (OS) after surgical resection ranging from 15% to 40% in most series (7–9). Thus, efforts to identify prognostic factors to better stratify patients who are likely to benefit from resection remain necessary.

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Tumor markers, such as carbohydrate antigen 19-9 (CA19-9) and  $\alpha$ -fetoprotein (AFP), should be assessed in patients with possible ICC (10). Serum CA19-9 is a frequently used tumor marker for ICC diagnosis but has a sensitivity and specificity of only 62% and 63%, respectively (10,11). The elevated CA19-9 levels are associated with worse recurrence-free survival (RFS) after surgical resection (7,12–15). Interestingly, approximately 22.1%–35.8% of patients with ICC also have elevated AFP levels, so they are easily misdiagnosed with HCC before surgery (16–18). Therefore, it is not uncommon for patients with ICC to have elevated AFP levels, and clinicians must interpret these laboratory values with caution because the prognosis of HCC is significantly different from that of ICC. However, there are few reports of elevated AFP in patients with ICC, and the long-term outcome of these patients, based on large samples, remains largely unknown.

The objective of this study was to test our hypotheses that elevated serum AFP in ICC is significantly related to increased tumor malignancy and that the combination of preoperative AFP and CA19-9 can be used to predict the prognosis of patients with ICC.

## PATIENTS AND METHODS

### Patient selection

From March 2009 to December 2019, a total of 519 consecutive patients with newly diagnosed ICC who underwent R0 surgical resection at the Department of Liver Surgery and Liver Transplantation Center of the West China Hospital of Sichuan University were enrolled and followed. All patients underwent postoperative chest radiography and at least 2 dynamic imaging examinations (contrast-enhanced ultrasound, contrast-enhanced computed tomography, or magnetic resonance imaging). Hepatitis status, liver function, and hematological parameters were serologically examined within 1 week before surgery. Tumor-node-metastasis (TNM) staging was based on the 8th edition of the American Joint Committee on Cancer (AJCC) TNM staging system (19).

This study complied with the standards of the Declaration of Helsinki and current ethical guidelines and was approved by the Ethics Committee of West China Hospital, Sichuan University. All medical records from our prospectively maintained database were reviewed retrospectively.

### AFP and CA19-9

AFP and CA19-9 were also measured at the time of preoperative examinations. Serum AFP and CA19-9 levels were determined by a chemiluminescent enzyme immunoassay (Roche, Basel, Switzerland). Referencing the local population, the upper normal ranges of AFP and CA19-9 in our institution were 8 ng/mL and 22 U/mL, respectively. In this study, tumor markers that were higher than the upper normal range were defined as positive, and tumors were classified as double-negative and non-double-negative (single-positive and double-positive) according to the number of negative tumor markers.

### Pathological criteria for ICC

The diagnosis of ICC was confirmed by postoperative histopathologic examination (CL Lu, with more than 20 years of experience in the diagnosis of liver diseases). According to the 4th edition of the World Health Organization classification of liver tumors, ICC was defined as being located upstream of the second-degree bile ducts, with at least 1 cholangiocyte marker, such as

cytokeratin 19 or mucin core protein 1, being strongly positive (20). Any patients with HCC or combined hepatocellular and cholangiocarcinoma showing 2 distinctive HCC and ICC elements, with or without transitional or transformational zones between the areas showing more HCC-like or ICC-like features, or any tumors expressing hepatocyte and cholangiocyte markers in the separated cells or areas were excluded.

### Follow-up

All the patients received follow-up monitoring 1 month after the operation, every 3 months thereafter during the first 3 years, and then every 6 months in subsequent years. Physical examination, blood cell and differential counts, liver function tests, AFP and CA19-9 level measurements, and imaging examinations were included in the follow-up examinations when necessary. OS was defined as the interval between the operation and death or the last follow-up. RFS was defined as the interval between the operation and the first incidence of positive recurrence. The last follow-up date was the end of May 2020.

### Statistical analysis

Variables are expressed as ratios or medians (interquartile range). Continuous variables were compared between groups using a *t* test or the Mann-Whitney *U* test for variables with a nonnormal distribution. Categorical data were compared using the  $\chi^2$  test or Fisher exact test. OS rates were analyzed using the Kaplan-Meier method, and the differences were analyzed using the log-rank test. The Cox proportional hazards model was used for univariate and multivariate analyses of prognostic factors after surgery. Two-tailed *P* values  $\leq 0.05$  were considered statistically significant.

Rigorous adjustments for significant differences in the patients' baseline characteristics were performed using propensity score matching (PSM) (21,22). Using the variables derived from multivariable logistic regression analysis, a propensity score was estimated for each patient (21). One-to-one matching between the 2 groups was then performed using the nearest-neighbor matching method to be within a range of 0.05 SD (23). Data analysis was performed using SPSS 26.0 for Mac (IBM, Armonk, NY) and R software v.3.3.3 (R Foundation for Statistical Computing, Vienna, Austria; [www.r-project.org](http://www.r-project.org)).

The study was approved by the relevant ethical committee for West China Hospital, Sichuan University. All participants provided written informed consent.

## RESULTS

### Patient characteristics and tumor markers

Of the 519 patients who were included in this study, 52.8% were male, the median age was 59 years (interquartile range: 51–65 years), 69.2% had hepatitis B virus (HBV) infection, 56.3% had tumors  $>5$  cm in diameter, 27.2% had multiple tumors, 17.0% had lymph node metastases, and 86.1% had mass-forming (MF) type ICCs. The positivity rate of microvascular invasion (MVI) was 13.1%, and well, moderate, and poorly differentiated tumors were identified in 5.4%, 28.5%, and 66.1% of the patients, respectively.

The distribution of serum AFP and CA19-9 levels is presented as a dot plot (see Supplementary Figure 1, <http://links.lww.com/CTG/A720>). The analytical measurement range of CA19-9 is from 0.60 to 1000 U/mL. However, 102 patients had CA19-9 levels greater than 1,000, and 16 patients had CA19-9 levels less than 0.06; a cutoff value calculated from the median value and

**Table 1.** Baseline demographics between patients with elevated AFP and normal AFP

Variables	Elevated AFP (n = 92)	Normal AFP (n = 427)	P value
Sex (male:female)	58 (63.0):34 (37.0)	216 (50.6):211 (49.6)	0.030
Age (yr)	56.5 (48.25–63)	59 (51–65)	0.093
< 60 yr: ≥60 yr	55 (54.2):37 (45.8)	223 (52.2):204 (47.8)	0.187
Etiology (HBV:non-HBV <sup>a</sup> )	77 (83.7):15 (16.3)	282 (66.0):145 (34.0)	0.001
CA19-9 (ng/mL)			
< 22 U/mL: ≥22 U/mL	25 (27.2):67 (72.8)	142 (33.3):285 (66.7)	0.257
TBL (μmol/L)	13.6 (10.2–17.0)	12.6 (9.6–16.8)	0.158
ALT (IU/L)	26 (17–37.75)	25 (17–41)	0.284
ALB (g/L)	41.9 (38.4–46.2)	42.8 (40.2–45.5)	0.245
ALP (IU/L)	118 (88–151.5)	107 (82–157)	0.327
WBC (10 <sup>9</sup> /L)	6.52 (4.79–8.43)	6.46 (5.33–7.84)	0.068
PLT (10 <sup>9</sup> /L)	149 (120.75–209)	159 (121–210)	0.475
PT (s)	11.7 (11.1–12.78)	11.4 (10.9–12.0)	0.002
INR	1.04 (0.97–1.13)	1.01 (0.95–1.06)	0.001
AJCC 8th edition T stage			
T1a/T1b	23/27 (54.3)	164/134 (69.8)	0.004
T2/T3/T4	42 (46.7)	129 (30.2)	—
AJCC 8th edition TNM stage			
Ia/Ib	18 (19.6)/24 (26.1)	155 (28.9)/107 (24.9)	0.006
II/III/IV	50 (54.3)	165 (46.2)	—
Differentiation			
Well/moderate	28 (30.4)	148 (34.7)	0.446
Poor/undifferentiated	64 (69.6)	280 (55.3)	
Diameter of tumor (cm)	6.5 (4–9)	5.5 (4–7.8)	0.004
No. of tumors (single vs multiple)	56 (78.2):36 (21.8)	321 (70.8):106 (29.2)	0.005
Lymph node metastasis (yes vs no)	17 (18.5):75 (81.5)	71 (16.6):356 (83.4)	0.668
Morphologic type			
MF, IG	85 (92.4)	363 (85.0)	0.062
PI, MF + PI	7 (7.6)	64 (15.0)	—
MVI (yes:no)	20 (21.7):72 (78.3)	48 (11.2):379 (88.8)	0.007
PNI (yes:no)	10 (10.9):82 (89.1)	55 (12.8):372 (87.2)	0.597

AFP, α-fetoprotein; ALB, serum albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CA19-9, carbohydrate antigen 19-9; DFS, disease-free survival; HBV, hepatitis B virus; ICC, intrahepatic cholangiocarcinoma; IG, intraductal growth; INR, international normalized ratio; MF, mass forming; MVI, microvascular invasion; PI, periductal infiltrating; PLT, platelet; PNI, perineural invasion; PSM, propensity score matching; PT, prothrombin time; TBIL, total bilirubin; TNM, tumor node metastasis; WBC, white blood cell.

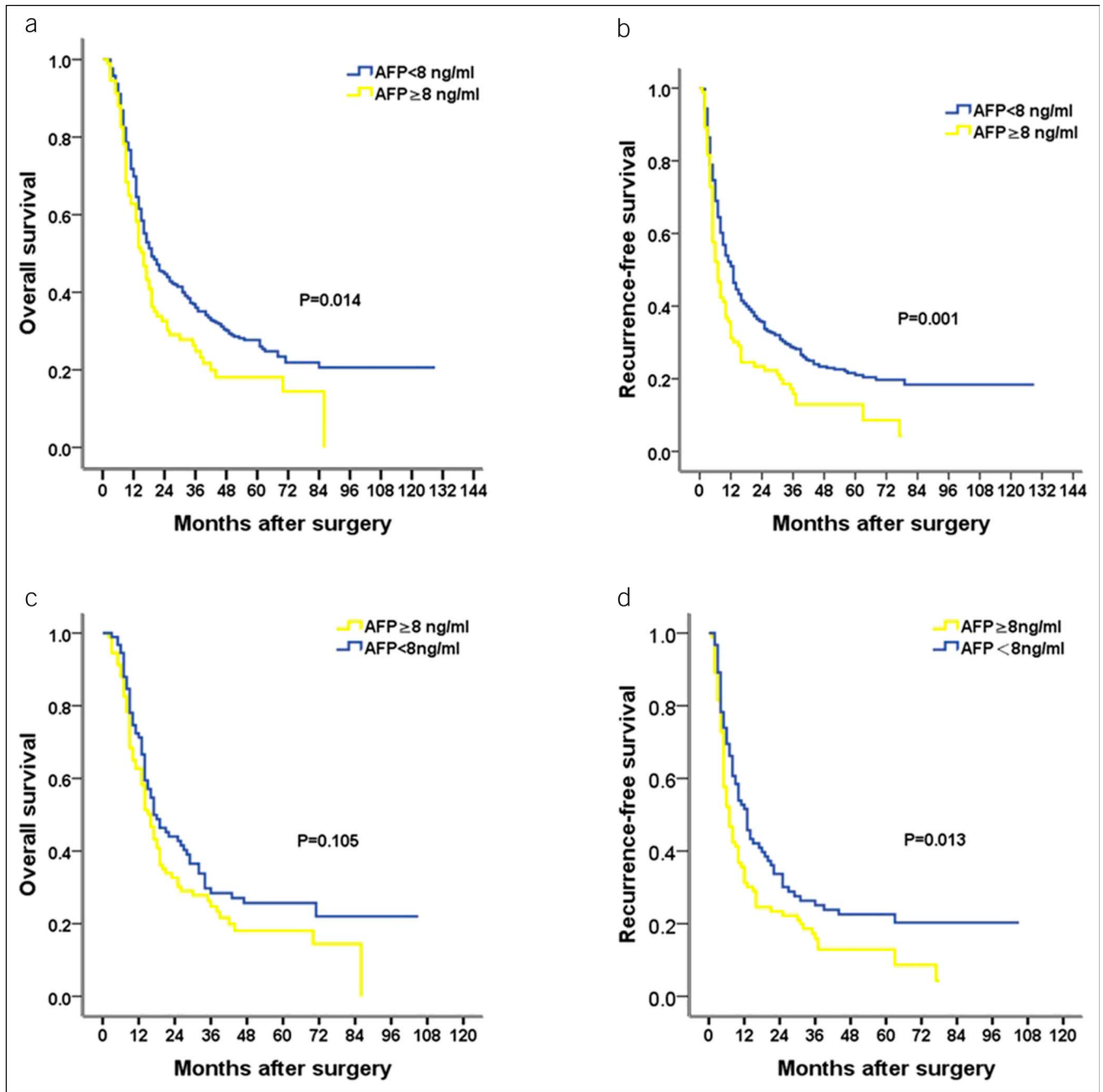
<sup>a</sup>Non-HBV, including lithiasis/parasitosis/fatty liver disease/cryptogenic.

receiver operator characteristic (ROC) analysis could not be used in this study. The median serum AFP level was 3.01 ng/mL. Serum AFP and CA9-9 levels over the upper normal ranges were observed in 92 (17.7%) and 352 (67.8%) patients, respectively.

The median follow-up time was 16 months (range, 2–129 months). For the whole cohort, the 1-, 3-, 5-, 7-, and 10-year OS rates were 68.6%, 34.0%, 26.0%, 19.4%, and 18.3%, respectively, and the 1-, 3-, 5-, 7-, and 10-year RFS rates were 47.4%, 26.2%, 19.5%, 15.8%, and 15.8%, respectively (see Supplementary Figure 2, <http://links.lww.com/CTG/A720>).

#### Patients with elevated AFP had more malignant tumor characteristics and a poor prognosis

According to the AFP level, the patients were divided into the elevated AFP group (n = 92) and the normal AFP group (n = 427). The elevated AFP group had a higher number of men and more younger patients, and a higher proportion of patients in this group had HBV infection, a more advanced T stage and TNM stage, MVI, a larger tumor size, a longer prothrombin time (PT), and a higher international normalized ratio. No significant differences were observed for Child-Pugh score, serum



**Figure 1.** The long-term outcome of patients with ICC with different AFP levels before and after PSM. (a, b) Before PSM, the 1-, 3-, and 5-year OS rates in the elevated AFP group and normal AFP group were 62.7%, 24.8%, and 18.1% vs 69.8%, 36.0%, and 27.7%, respectively ( $P = 0.014$ ). The 1-, 3-, and 5-year DFS rates were 31.2%, 15.8%, and 12.9% vs 51.0%, 28.5%, and 21.0%, respectively ( $P = 0.001$ ). (c, d) After PSM, the 1-, 3-, and 5-year OS rates in the elevated AFP group and normal AFP group were 62.7%, 24.8%, and 18.1% vs 71.3%, 28.4%, and 25.7%, respectively ( $P = 0.105$ ). The 1-, 3-, and 5-year DFS rates were 35.7%, 15.8%, and 12.9% vs 51.6%, 25.1%, and 22.6%, respectively ( $P = 0.013$ ). AFP,  $\alpha$ -fetoprotein; DFS, disease-free survival; ICC, intrahepatic cholangiocarcinoma; OS, overall survival; PSM, propensity score matching.

concentration of CA19-9, total bilirubin, alanine aminotransferase (ALT), albumin, alkaline phosphatase, white blood cell and platelet counts, tumor differentiation, lymph node metastasis, morphologic type, or perineural invasion (PNI) (Table 1). Using Bonferroni correction, these different variables and 2 other variables, age and morphologic type, were included in the model. After PSM, the baseline characteristics between the 2 groups were

consistent (see Supplementary Table 1, <http://links.lww.com/CTG/A720>).

As shown in Figure 1, the 1-, 3-, and 5-year OS rates in the elevated AFP group and the normal AFP group were 62.7%, 24.8%, and 18.1% vs 69.8%, 36.0%, and 27.7%, respectively ( $P = 0.014$ ), and the 1-, 3-, and 5-year RFS rates were 31.2%, 15.8%, and 12.9% vs 51.0%, 28.5%, and 21.0%, respectively ( $P = 0.001$ ). Both

**Table 2.** Baseline demographics between patients with double-negative and non-double-negative before PSM

Variables	Double-negative (n = 142)	Non-double-negative (n = 377)	P value
Sex (male:female)	87 (61.3):55 (38.7)	187 (49.6):190 (50.4)	0.018
Age (yr)	58 (50–66)	59 (51–65)	0.229
< 60 yr: ≥60 yr	77 (54.2):65 (45.8)	201 (53.3):176 (46.7)	0.921
Etiology (HBV:non-HBV <sup>a</sup> )	105 (73.9):37 (26.1)	254 (67.4):123 (32.6)	0.166
AFP (ng/mL)			
< 8 ng/mL: ≥8 ng/mL	142:none	287 (76.1):92 (23.9)	—
CA19-9 (ng/mL)			
< 22 U/mL: ≥22 U/mL	142:none	38 (10.1):339 (89.9)	—
TBL (μmol/L)	11.65 (9.3–15.3)	13.5 (10.15–17.5)	0.046
ALT (IU/L)	21 (16–35.25)	26 (18–43)	0.037
ALB (g/L)	42.85 (40.38–46.03)	42.6 (39.8–45.45)	0.080
ALP (IU/L)	99.5 (79–130.5)	115 (86.5–165)	<0.001
WBC (10 <sup>9</sup> /L)	6.35 (5.26–7.9)	6.51 (5.25–7.92)	0.513
PLT (10 <sup>9</sup> /L)	156 (116.75–204.25)	159 (124.5–211.5)	0.259
PT (s)	11.4 (10.88–11.9)	11.5 (10.9–12.2)	0.158
INR	1.01 (0.95–1.05)	1.01 (0.96–1.07)	0.210
AJCC 8th edition T stage			
T1a/T1b	65/42 (73.6)	122/119 (63.9)	0.016
T2/T3/T4	35 (26.4)	136 (36.1)	—
AJCC 8th edition TNM stage			
Ia/Ib	64 (45.1)/37 (26.1)	109 (28.9)/94 (24.9)	<0.001
II/III/IV	41 (28.8)	174 (46.2)	—
Differentiation			
Well/moderate	51 (35.9)	125 (33.2)	0.535
Poor/undifferentiated	91 (64.1)	253 (66.8)	—
Diameter of tumor (cm)	5 (3.15–7.5)	6 (4–8)	0.004
≤5 cm	72 (50.7)	155 (41.1)	0.059
>5 cm	70 (49.3)	222 (58.9)	—
No. of tumors (single vs multiple)	111 (78.2):31 (21.8)	267 (70.8):110 (29.2)	0.098
Lymph node metastasis (yes vs no)	16 (11.3):126 (88.7)	72 (19.1):305 (80.9)	0.036
Morphologic type			
MF, IG	129 (90.8)	318 (84.4)	0.064
PI, MF + PI	13 (9.2)	59 (15.6)	—
MVI (yes:no)	9 (6.3):133 (93.7)	59 (15.6):318 (84.4)	0.005
PNI (yes:no)	11 (7.7):131 (92.3)	54 (14.3):323 (85.7)	0.052

AFP, α-fetoprotein; ALB, serum albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CA19-9, carbohydrate antigen 19-9; DFS, disease-free survival; HBV, hepatitis B virus; ICC, intrahepatic cholangiocarcinoma; IG, intraductal growth; INR, international normalized ratio; MF, mass forming; MVI, microvascular invasion; PI, periductal infiltrating; PLT, platelet; PNI, perineural invasion; PSM, propensity score matching; PT, prothrombin time; TBIL, total bilirubin; TNM, tumor node metastasis; WBC, white blood cell.

<sup>a</sup>Non-HBV, including lithiasis/parasitosis/fatty liver disease/cryptogenic.

the OS and RFS rates in the elevated AFP group were significantly lower than those in the normal AFP group (Figures 1a and b).

After PSM, the 1-, 3-, and 5-year OS rates in the elevated AFP group and the normal AFP group were 62.7%, 24.8%, and 18.1% vs 71.3%, 28.4%, and 25.7%, respectively ( $P = 0.105$ ), and the 1-,

3-, and 5-year RFS rates were 35.7%, 15.8%, and 12.9% vs 51.6%, 25.1%, and 22.6%, respectively ( $P = 0.013$ ). The RFS rates in the elevated AFP group were still significantly lower than those in the normal AFP group but differed from the OS rates (Figures 1c and d).

**Table 3. Baseline demographics between patients with double-negative and non-double-negative after PSM**

Variables	Double-negative (n = 128)	Non-double-negative (n = 128)	P value
Sex (male:female)	74 (57.8):54 (42.2)	87 (68.0):41 (32.0)	0.093
Age (yr)	59 (50.25–66)	58 (50–65)	0.815
< 60 yr: ≥60 yr	66 (51.6):62 (48.4)	55 (43.0):73 (57.0)	0.168
Etiology (HBV:non-HBV <sup>a</sup> )	94 (73.4):34 (26.6)	102 (79.7):26 (20.3)	0.238
AFP (ng/mL)			
< 8 ng/mL: ≥8 ng/mL	128:none	89 (69.5):39 (30.5)	—
CA19-9 (ng/mL)			
< 22 U/mL: ≥22 U/mL	128:none	17 (13.3):111 (86.7)	—
TBL (μmol/L)	11.7 (9.4–15.725)	12.7 (9.425–15.45)	0.664
ALT (IU/L)	21 (16–35.75)	25 (16.25–34.75)	0.557
ALB (g/L)	42.6 (40.225–45.775)	42.2 (39.725–45.975)	0.275
ALP (IU/L)	100.5 (81–132)	100.5 (74–135.5)	0.570
WBC (10 <sup>9</sup> /L)	6.405 (5.34–8.1525)	6.535 (4.925–8.3475)	0.886
PLT (10 <sup>9</sup> /L)	159 (118.25–205.00)	143.5 (115–193.75)	0.320
PT (s)	11.5 (10.9–11.9)	11.6 (10.8–12.1)	0.233
INR	1.01 (0.95–1.05)	1.01 (0.97–1.06)	0.594
AJCC 8th edition T stage			
T1a/T1b	53/40 (72.7)	52/40 (71.9)	0.889
T2/T3/T4	35 (27.3)	36 (28.1)	—
AJCC 8th edition TNM stage			
Ia/Ib	87 (26.1)	89 (69.5)	0.787
II/III/IV	41 (28.8)	39 (30.5)	—
Differentiation			
Well/moderate	44 (35.9)	39 (30.5)	0.504
Poor/undifferentiated	84 (64.1)	89 (69.5)	—
Diameter of tumor (cm)	5.5 (3.5–7.8)	6 (4–8)	0.593
≤5 cm	60 (46.9)	62 (48.4)	0.802
>5 cm	68 (53.1)	66 (51.6)	—
No. of tumors (single:multiple)	98 (76.6):30 (23.4)	96 (75.0):32 (25.0)	0.770
Lymph node metastasis (yes:no)	16 (12.5):112 (87.5)	13 (10.2):115 (89.8)	0.554
Morphologic type			
MF, IG	116 (90.6)	121 (94.5)	0.233
PI, MF + PI	12 (9.4)	7 (5.5)	—
MVI (yes:no)	9 (7.0):119 (93.0)	15 (11.7):113 (88.3)	0.198
PNI (yes:no)	11 (8.6):117 (91.4)	8 (6.3):120 (93.7)	0.474

AFP, α-fetoprotein; ALB, serum albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CA19-9, carbohydrate antigen 19-9; DFS, disease-free survival; HBV, hepatitis B virus; ICC, intrahepatic cholangiocarcinoma; IG, intraductal growth; INR, international normalized ratio; MF, mass forming; MVI, microvascular invasion; PI, periductal infiltrating; PLT, platelet; PNI, perineural invasion; PSM, propensity score matching; PT, prothrombin time; TBL, total bilirubin; TNM, tumor node metastasis; WBC, white blood cell.

<sup>a</sup>Non-HBV, including lithiasis/parasitosis/fatty liver disease/cryptogenic.

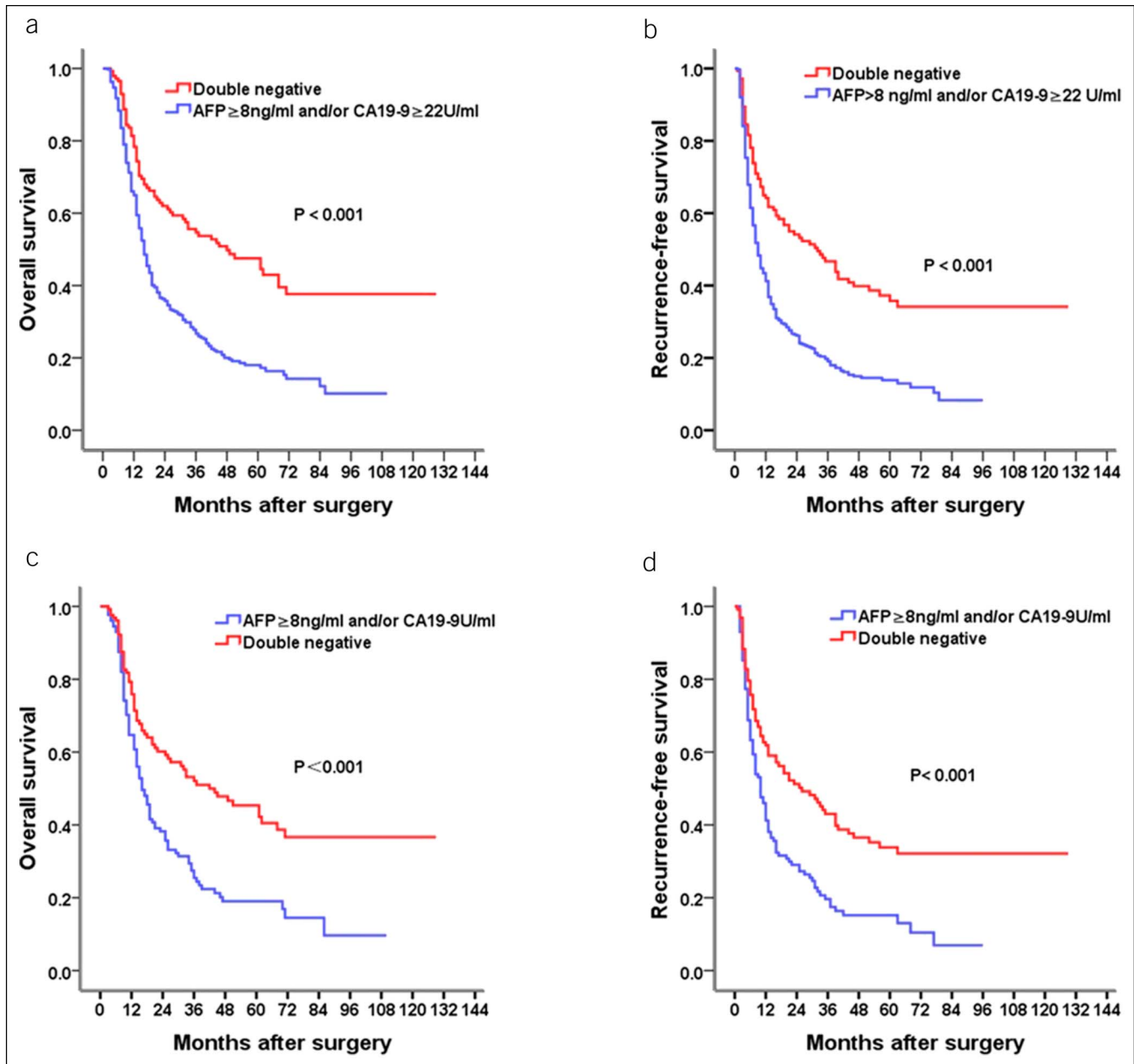
### Patients with double negativity for AFP and CA19-9 had a good prognosis

Previous studies reported that elevated CA19-9 is associated with worse survival after surgical resection (10,11). Thus, we wanted to determine whether patients with double negativity for AFP and

CA19-9 would have better long-term outcomes than patients with single-positive or double-positive expression of those 2 tumor markers.

The patients were subsequently divided into the double-negative group (n = 142) and the non-double-negative group





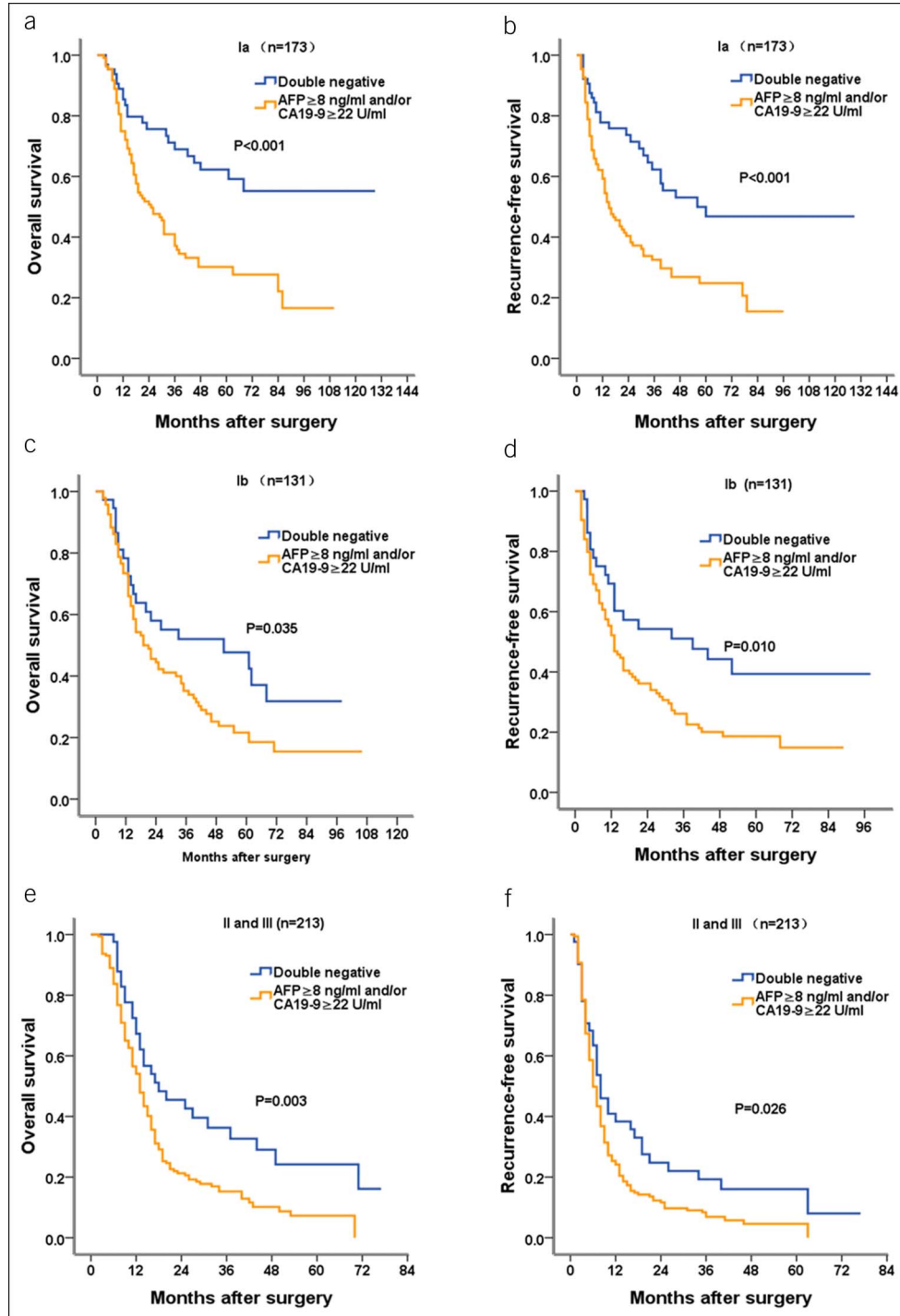
**Figure 2.** The long-term outcome of patients with ICC with different AFP and CA19-9 levels before and after PSM. (a, b) Before PSM, the 1-, 3-, and 5-year OS rates in the double-negative group and the non-double-negative group were 78.3%, 54.7%, and 47.5% vs 64.9%, 26.7%, and 18.0%, respectively ( $P < 0.001$ ). The 1-, 3-, and 5-year DFS rates were 64.2%, 46.7%, and 35.8% vs 41.2%, 19.0%, and 13.8%, respectively ( $P < 0.001$ ). (c, d) After PSM, the 1-, 3-, and 5-year OS rates in the double-negative group and the non-double-negative group were 75.9%, 52.1%, and 45.3% vs 64.7%, 25.4%, and 19.0%, respectively ( $P < 0.001$ ). The 1-, 3-, and 5-year DFS rates were 61.8%, 43.0%, and 33.8% vs 41.2%, 19.6%, and 15.2%, respectively ( $P < 0.001$ ). AFP,  $\alpha$ -fetoprotein; DFS, disease-free survival; ICC, intrahepatic cholangiocarcinoma; OS, overall survival; PSM, propensity score matching.

( $n = 377$ ). Variables including sex, total bilirubin, ALT, albumin, alkaline phosphatase, TNM stage, tumor diameter, number of tumors, lymph node metastasis, MVI, and PNI were significantly different between the 2 groups (Table 2). Therefore, after matching, 128 patients were included in each group (Table 3).

Before PSM, the 1-, 3-, and 5-year OS rates in the double-negative group and the non-double-negative group were 78.3%, 54.7%, and 47.5% vs 64.9%, 26.7%, and 18.0%, respectively ( $P < 0.001$ ), and the 1-, 3-, and 5-year RFS rates were 64.2%, 46.7%, and 35.8% vs 41.2%, 19.0%, and 13.8%, respectively ( $P < 0.001$ ). Both

the OS and RFS rates in the double-negative group were significantly better than those in the non-double-negative group (Figure 2a,b).

After PSM, the 1-, 3-, and 5-year OS rates in the double-negative group and the non-double-negative group were 75.9%, 52.1%, and 45.3% vs 64.7%, 25.4%, and 19.0%, respectively ( $P < 0.001$ ), and the 1-, 3-, and 5-year RFS rates were 61.8%, 43.0%, and 33.8% vs 41.2%, 19.6%, and 15.2%, respectively ( $P < 0.001$ ). The OS and RFS rates in the double-negative group were still significantly better than those in the non-double-negative group (Figure 2c,d).



**Figure 3.** The long-term outcome of patients with ICC with different AFP and CA19-9 levels in different TNM stages. (a, b) For patients with stage IIA disease, the 1-, 3-, 5-year OS rates in the double-negative group were 85.4%, 68.9%, and 62.3%, respectively, and in the non-double-negative group, they were 73.4%, 35.2%, and 21.6%, respectively ( $P < 0.001$ ). The 1-, 3-, 5-year RFS rates of patients in the double-negative group were 77.8%, 62.3%, and 46.8%, respectively, and were 59.3%, 32.5%, and 24.8% in the non-double-negative group, respectively ( $P < 0.001$ ). (c, d) For patients with stage IIB disease, the 1-, 3-, 5-year OS rates in the double-negative group were 78.3%, 52.0%, and 47.7%, respectively, and in the non-double-negative group, they were 73.4%, 35.2%, and 21.6%, respectively ( $P = 0.035$ ). The 1-, 3-, 5-year RFS rates in the double-negative group were 69.3%, 51.0%, and 39.3%, respectively, and they were 52.1%, 26.1%, and 18.6% in the non-double-negative group, respectively ( $P = 0.010$ ). (e, f) For patients with stage II or III disease, the 1-, 3-, and 5-year OS rates in the double-negative group were 67.3%, 36.3%, and 24.2%, respectively, and were 54.1%, 15.3%, and 7.3% in the non-double-negative group, respectively ( $P = 0.003$ ). The 1-, 3-, and 5-year RFS rates in the double-negative group were 38.3%, 19.3%, and 16.1%, respectively, and were 24.1%, 6.9%, and 4.6% in the non-double-negative group, respectively ( $P = 0.026$ ). AFP,  $\alpha$ -fetoprotein; ICC, intrahepatic cholangiocarcinoma; OS, overall survival; RFS, recurrence-free survival.



**Table 4. Univariate and multivariate analyses of prognostic factors for overall survival in patients with ICC**

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Tumor marker (NDG vs DG)	0.567 (0.431–0.746)	<0.001	0.567 (0.434–0.741)	<0.001
Sex (male vs female)	1.103 (0.869–1.401)	0.420	—	—
Age ( $\geq 60$ vs $< 60$ yr)	0.917 (0.738–1.140)	0.435	—	—
Etiology (non-HBV <sup>a</sup> vs HBV)	1.139 (0.889–1.461)	0.304	—	—
TBIL	0.996 (0.978–1.015)	0.680	—	—
ALT	1.002 (0.998–1.005)	0.451	—	—
ALB	0.980 (0.956–1.005)	0.121	—	—
WBC	0.999 (0.987–1.010)	0.844	—	—
PLT	1.001 (1.000–1.003)	0.090	—	—
ALP	1.000 (0.999–1.001)	0.358	—	—
PT	1.113 (1.018–1.218)	0.019	1.115 (1.029–1.208)	0.008
TNM stage (III–IV vs 0–2)	1.673 (1.174–2.283)	0.006	1.898 (1.519–2.371)	<0.001
Differentiation (low vs well/moderate)	1.425 (1.126–1.804)	0.003	1.429 (1.131–1.805)	0.003
Tumor size ( $> 5$ vs $\leq 5$ cm)	1.240 (0.983–1.564)	0.070	1.228 (0.984–1.533)	0.069
Tumor number (multiple vs single)	1.180 (0.834–1.669)	0.351	—	—
Lymph node metastasis (yes vs no)	1.110 (0.795–1.551)	0.540	—	—
Morphologic type (PI, MF + PI vs MF, IG)	1.637 (1.156–2.420)	0.004	1.864 (1.392–2.495)	<0.001
MVI (yes vs no)	1.567 (1.178–2.086)	0.002	1.598 (1.206–2.118)	0.001
PNI (yes vs no)	1.505 (1.096–2.068)	0.012	1.610 (1.185–2.188)	0.002

ALB, serum albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; DG, double-negative; HBV, hepatitis B virus; HR, hazard ratio; IG, intraductal growth; MF, mass forming; MVI, microvascular invasion; NDG, nondouble-negative; PI, periductal infiltrating; PLT, platelet; PNI, perineural invasion; PT, prothrombin time; TBIL, total bilirubin; TNM, tumor node metastasis; WBC, white blood cell.

<sup>a</sup>Non-HBV, including lithiasis/parasitosis/fatty liver disease/cryptogenic.

Carcinoembryonic antigen (CEA), a well-known tumor marker for colorectal cancer, has also gained attention as a potential tumor marker for ICC (24). Patients with both low preoperative CA19-9 and CEA levels have a much better prognosis. Similarly, patients in the double-negative (negative CA19-9 and negative CEA) group also had better OS and RFS than those of patients in the non-double-negative (positive CA19-9 and/or positive CEA) group in this study (5-year OS rate: 50.0% vs 18.6%,  $P < 0.001$ ; 5-year RFS rate: 34.8% vs 15.1%,  $P < 0.001$  (see Supplementary Figure 3, <http://links.lww.com/CTG/A720>). However, both the area under the curve and the C-index of CA19-9 plus AFP are better than those of CA19-9 plus CEA (see Supplementary Table 2 and Figure 4, <http://links.lww.com/CTG/A720>).

#### Subgroup analysis of patients with different TNM stages

To further verify the effectiveness of double negativity for AFP and CA19-9 in predicting the prognosis of patients with ICC, a subgroup analysis was performed for different TNM stages. Based on the TNM staging of the 8th edition AJCC TNM staging system, patients with stage IA ( $n = 173$ ), IB ( $n = 131$ ), II (112), and III ( $n = 101$ ) disease had different long-term outcomes (see Supplementary Figure 5, <http://links.lww.com/CTG/A720>).

In the double-negative group, patients with stage IIA disease had 1-, 3-, and 5-year OS rates of 85.4%, 68.9%, and 62.3%, respectively; for those with stage IB disease, the 1-, 3-, and 5-year OS

rates were 78.3%, 52.0%, and 47.7%, respectively; and for those with stage II–III disease, they were 67.3%, 36.3%, and 24.2%, respectively. For patients in the non-double-negative group, patients with stage IIA had 1-, 3-, and 5-year OS rates of 74.9%, 41.0%, and 30.2%, respectively; for those with stage IIB disease, the 1-, 3-, and 5-year OS rates were 73.4%, 35.2%, and 21.6%, respectively; and for those with stage II–III disease, they were 54.1%, 15.3%, and 7.3%, respectively. The OS rates of the patients in the non-double-negative group were significantly poorer than those of the patients in the double-negative group ( $P < 0.001$ ,  $P = 0.035$ , and  $P = 0.003$ , respectively; Figure 3).

Similarly, for patients in the double-negative group, those with stage IIA disease had 1-, 3-, and 5-year RFS rates of 77.8%, 62.3%, and 46.8%, respectively; for those with stage IIB disease, the 1-, 3-, and 5-year RFS rates were 69.3%, 51.0%, and 39.3%, respectively; for those with stage II–III disease, they were 38.3%, 19.3%, and 16.1%, respectively. However, for patients in the non-double-negative group, those with stage IIA disease had 1-, 3-, and 5-year RFS rates of 59.3%, 32.5%, and 24.8%, respectively; for those with stage IIB disease, the 1-, 3-, and 5-year RFS rates were 52.1%, 26.1%, and 18.6%, respectively; and for those with stage II–III disease, they were 24.1%, 6.9%, and 4.6%, respectively. The RFS rates of the patients in the non-double-negative group were also significantly poorer than those of the patients in the double-negative group ( $P < 0.001$ ,  $P = 0.010$ , and  $P = 0.026$ , respectively).

**Table 5. Univariate and multivariate analyses of prognostic factors for recurrence-free survival in patients with intrahepatic cholangiocarcinoma**

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Tumor marker (DG vs NDG)	0.602 (0.465–0.780)	<0.001	0.578 (0.442–0.755)	<0.001
Sex (male vs female)	1.045 (0.832–1.314)	0.704	—	—
Age (≥60 vs <60 yr)	0.899 (0.731–1.106)	0.314	—	—
Etiology (non-HBV <sup>a</sup> vs HBV)	1.023 (0.806–1.298)	0.854	—	—
TBIL	1.007 (0.989–1.025)	0.440	—	—
ALT	1.000 (0.996–1.025)	0.863	—	—
ALB	0.988 (0.964–1.025)	0.317	—	—
WBC	0.997 (0.986–1.008)	0.607	—	—
PLT	1.001 (0.999–1.002)	0.471	—	—
ALP	1.000 (0.999–1.001)	0.627	—	—
PT	1.093 (1.003–1.191)	0.043	1.107 (1.020–1.200)	0.014
TNM stage (III–IV vs 0–2)	1.755 (1.241–2.483)	0.001	1.685 (1.259–2.257)	<0.001
Differentiation (low vs well/moderate)	1.258 (1.010–1.568)	0.041	1.397 (1.107–1.764)	0.005
Tumor size (>5 vs ≤5 cm)	1.241 (0.995–1.549)	0.056	1.242 (0.996–1.548)	0.055
Tumor number (multiple vs single)	1.382 (0.999–1.912)	0.051	1.267 (0.932–1.722)	0.131
Lymph node metastasis (yes vs no)	1.035 (0.754–1.420)	0.832	—	—
Morphologic type (PI, MF + PI vs MF, IG)	1.589 (1.149–2.200)	0.005	1.762 (1.315–2.362)	<0.001
MVI (yes vs no)	1.592 (1.206–2.100)	0.001	1.711 (1.291–2.267)	<0.001
PNI (yes vs no)	1.388 (1.018–1.893)	0.038	1.530 (1.125–2.081)	0.007

ALB, serum albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; DG, double-negative; HBV, hepatitis B virus; HR, hazard ratio; IG, intraductal growth; MF, mass forming; MVI, microvascular invasion; NDG, nondouble-negative; PI, periductal infiltrating; PLT, platelet; PNI, perineural invasion; PT, prothrombin time; TBIL, total bilirubin; TNM, tumor node metastasis; WBC, white blood cell.

<sup>a</sup>Non-HBV, including lithiasis/parasitosis/fatty liver disease/cryptogenic.

### Independent predictors of OS and RFS in patients with ICC

Among the 519 patients with ICC, the factors associated with OS were evaluated by univariate and multivariate analyses (Table 4). The univariate analysis revealed that double-negative status for the 2 tumor markers, PT, TNM stage, differentiation, morphologic type, MVI, and PNI were significant variables. The multivariate analysis revealed that double-negative status for the 2 tumor markers (hazard ratio [HR], 0.567), PT (HR, 1.115), TNM stage (HR, 1.898), differentiation (HR, 1.429), morphologic type (HR, 1.864), MVI (HR, 1.598), and PNI (HR, 1.610) were independent factors for OS.

The factors associated with RFS were also evaluated by univariate and multivariate analyses (Table 5). The univariate analysis revealed 7 significant variables for ICC: double-negative status for the 2 tumor markers, PT, TNM stage, differentiation, morphologic type, MVI, and PNI. In the multivariate analysis, double-negative status for the 2 tumor markers (HR, 0.578), PT (HR, 1.017), TNM stage (HR, 1.685), differentiation (HR, 1.397), morphologic type (HR, 1.762), MVI (HR, 1.711), and PNI (HR, 1.530) were independent factors for RFS.

## DISCUSSION

At present, hepatectomy is still the most effective treatment for patients with ICC to achieve long-term survival, although its overall efficacy may not be as good as that for patients with HCC

(25,26). Thus, it is necessary to identify prognostic factors to better stratify patients who are likely to benefit from hepatectomy. This study demonstrated that patients with ICC with a double-negative status for 2 highly accessible tumor markers, namely, AFP and CA19-9, had better long-term outcomes.

Elevated AFP is not uncommon in ICC. Zhou et al. demonstrated that HBV-associated ICC shares many clinicopathological similarities with HBV-associated HCC and that these patients have a higher proportion of serum AFP levels (12); these findings were also confirmed in the studies by Zhou and Wang (17,27). Therefore, many ICC cases are misdiagnosed as HCC before surgery. Yamamoto reported that 35.8% (24 of 67) of MF ICCs received a diagnosis of HCC preoperatively based on preoperative imaging and AFP levels (15). Sapisochin and Lee reported that 66 of 2,301 and 44 of 618 patients who were initially diagnosed with HCC were subsequently found to have either ICC or combined hepatocellular carcinoma-cholangiocarcinoma on explants after liver transplantation (28,29).

Moreover, the effect of elevated AFP on the prognosis of patients with ICC is unclear. Only 1 previous study reported that patients with positive AFP had better 1- and 3-year survival rates than patients with negative AFP. However, the sample size was small, and the follow-up time was short, which may be the reason for the discrepancy between their results and the results of our study. By contrast, rigorous adjustments for significant differences in the baseline characteristics were performed using PSM in

our study. As shown in Supplementary Table 1 (<http://links.lww.com/CTG/A720>), a higher proportion of patients with elevated AFP had large tumors, multiple tumors, MVI, and an advanced TNM stage. This leads to the following question: How do the recurrence and survival curves compare between patients with elevated AFP and those with normal AFP? Before PSM, the OS and RFS of the patients with elevated AFP were significantly worse than those of the patients with normal AFP. After PSM, the RFS rates in the elevated AFP group were still significantly poorer than those in the normal AFP group, although a significant difference in the OS rates was not found. In addition, an elevated AFP level is associated with liver regeneration and serves as a specific serological biomarker for HCC. However, in our study, the ALT (a well-known marker for liver parenchymal injury) levels of most patients were normal and the ALT levels between the 2 groups were comparable. Therefore, elevated AFP is due to the characteristics and biology of the tumor itself, not liver regeneration. Thus, we demonstrated that patients who were AFP-positive had shorter survival and more pathologically invasive tumor characteristics.

CA19-9 is an established biomarker for the diagnosis and prognostic prediction of hepatobiliary malignancies (30). CA19-9 elevation and preoperative CA19-9 values higher than 100 U/mL or 1000 U/mL have been associated with worse survival after surgical resection (7,11). In this study, we defined the cutoff values of the 2 tumor markers as the upper limit of normal instead of the median value, the value calculated from ROC analysis, or values based on other reports. First, the analytical measurement range of CA19-9 was from 0.60 to 1000 U/mL. Among the 519 patients, 102 patients had CA19-9 levels higher than 1000 U/mL and 16 patients had CA19-9 levels lower than 0.06 U/mL. Thus, a cutoff value calculated from the median value and ROC analysis could not be used in this study. Second, referencing other reports is a good strategy for determining a cutoff value. However, different cutoff values have been used in various reports. Moreover, referring to the upper limit of normal of the local population and setting it as a cutoff value are easy to promote in clinical practice. For the reasons stated earlier, the cutoff values of these 2 tumor markers were defined as the upper normal limits in this study.

The results of this study revealed that a lower proportion of patients with double negativity for AFP and CA19-9 had invasive tumor characteristics, such as an advanced TNM stage, larger tumor diameters, multiple tumors, lymph node metastasis, MVI, and PNI. Before and after PSM, patients with double negativity for AFP and CA19-9 had significantly better OS and RFS rates than patients with nondouble negativity for AFP and CA19-9. In addition, in the univariate and multivariate analyses, double-negative status for those 2 tumor markers was found to be an independent predictor of the prognosis of patients with ICC. To the best of our knowledge, this is the first report to combine AFP and CA19-9 for the prediction of ICC prognosis. Our results are informative for stratifying patients with ICC who are likely to benefit from resection.

It has been suggested that the AJCC 8th edition TNM staging system can be used to stratify the survival of patients with ICC (31,32). Indeed, as shown in Supplementary Figure 5 (<http://links.lww.com/CTG/A720>), patients with different TNM stages had different long-term outcomes, except for the RFS rates for patients with stage II and III disease. The subgroup analysis showed that both OS and RFS of patients with double-negative tumor markers were significantly better than those of patients with non-double-negative tumor markers. Accordingly, we proposed that the combination of

AFP and CA19-9, as markers of biologically aggressive disease, should be considered in the TNM staging system. Patients with elevated AFP and/or CA19-9 levels are less likely to undergo surgery than those with normal levels, which is meaningful for prognostic counseling, therapeutic triage, and treatment sequencing.

Our study has a few limitations. First, it was a single institutional study. Second, it was a retrospective and non-randomized study, which prevents the elimination of selection bias between the treatment groups. We attempted to adjust for intergroup differences with PSM and subgroup analysis to ensure that the cohorts were as homogenous as possible.

In conclusion, we found that patients with elevated AFP levels had more aggressive tumor characteristics and a poor prognosis. The double-negative status of CA19-9 and AFP in patients with ICC was found to be an independent factor for better survival and indicated fewer malignant tumor features, which is meaningful for prognostic counseling and therapeutic triage.

## CONFLICTS OF INTEREST

**Guarantor of the article:** Tian-fu Wen, MD, PhD.

**Specific author contributions:** X.Z., T.W., and J.Y.: conceptualized and designed the study and reviewed and revised the manuscript. T.W., J.Y., and L.Y.: administrative support. W.P., C.L., and Y.Z.: collection and assembly of data. X.Y., W.P., and C.L.: data analysis and interpretation. All authors: provision of study materials or patients, manuscript writing, and final approval of the manuscript. **Financial support:** This work was in part supported by grant from the National Natural Science Foundation of China (81900463 and 81800449), the Science and Technological Supports Project of Sichuan Province (2020YFS0133), and Post-Doctor Research Project, West China Hospital, Sichuan University (2019HXBH004). **Potential competing interests:** None to report.

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## Study Highlights

### WHAT IS KNOWN

- ✓ Carbohydrate antigen 19-9 (CA19-9) and  $\alpha$ -fetoprotein (AFP) are routinely tested for patients with liver malignancies to distinguish hepatocellular carcinoma and intrahepatic cholangiocarcinoma (ICC) before surgery.
- ✓ Elevated AFP in patients with ICC is not uncommon, but the relationship between AFP and the prognosis for patients with ICC is not clear.

### WHAT IS NEW HERE

- ✓ Patients with elevated AFP had increased tumor malignancy and poor prognosis.
- ✓ This study reveals that patients with double-negative AFP and CA19-9 preoperatively had less invasiveness and better survival.
- ✓ This finding is substantial for prognostic counseling and therapeutic triage.

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