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ORIGINAL ARTICLE

Serum CYFRA 21-1 in Biliary Tract Cancers: A Reliable Biomarker for Gallbladder Carcinoma and Intrahepatic Cholangiocarcinoma

Li Huang · Wei Chen · Peiwen Liang · Wenjie Hu · Kunsong Zhang · Shunli Shen · Jiancong Chen · Zhaohui Zhang · Bin Chen · Yuyan Han · Fanyin Meng · Sharon DeMorrow · Xiaoyu Yin · Jiaming Lai · Lijian Liang

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Abstract

Background Biliary tract cancers encompass gallbladder carcinoma, and intrahepatic, perihilar and distal cholangiocarcinoma. Upregulated serum CYFRA 21-1 has been reported in intrahepatic cholangiocarcinoma.

Aims The present study aimed to explore the clinical significance of serum CYFRA 21-1 in all biliary tract cancer subtypes.

Methods Serum CYFRA 21-1, carbohydrate antigen 19-9 and carcinoembryonic antigen were quantitated preoperatively, postoperatively and during follow-up in 134 malignant and 52 benign patients. Receiver operator characteristic curves of biomarkers were analyzed. Level of CYFRA 21-1 was correlated with patients' clinicopathological features and follow-up data.

Results CYFRA 21-1 was significantly upregulated in biliary malignancies, and expressional difference existed between these subtypes. Based on the maximal Youden's index, cutoff values were selected (ng/mL): 2.61 for biliary tract cancers (sensitivity 74.6 % and specificity 84.6 %); 3.27 for intrahepatic cholangiocarcinoma (75.6 and 96.2 %) and gallbladder carcinoma (93.7 and 96.2 %); 2.27 for perihilar cholangiocarcinoma (71.0 and 71.2 %); and 2.61 for distal cholangiocarcinoma (63.3 and 84.6 %). CYFRA 21-1 showed better diagnostic performance than other biomarkers in gallbladder carcinoma and intrahepatic cholangiocarcinoma; its performance was not inferior to that of the combination of these three biomarkers and declined after curative resection and re-elevated when tumor recurred, which was correlated with tumor aggressiveness and TNM stage; it was an independent predictor for 1-year recurrence-free survival and overall survival on multivariate analysis.

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L. Huang · W. Chen · P. Liang · W. Hu · K. Zhang · S. Shen · J. Chen · Z. Zhang · X. Yin · J. Lai (✉) · L. Liang (✉)
Department of Hepatobiliary Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
e-mail: jmlai2000@aliyun.com

L. Liang
e-mail: lianglj@medmail.com.cn

B. Chen
Department of Interventional Radiology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Y. Han
Department of Medicine, Baylor and Scott and White Digestive Disease Research Center, Texas A&M HSC College of Medicine and Scott and White Hospital, Temple, TX, USA

F. Meng · S. DeMorrow
Division of Research, Central Texas Veterans Health Care System, Temple, TX, USA

F. Meng · S. DeMorrow
Department of Medicine, Scott and White Healthcare and Texas A&M Health Science Center, College of Medicine, Temple, TX, USA

Conclusion Serum CYFRA 21-1 represents a reliable biomarker for gallbladder carcinoma and intrahepatic cholangiocarcinoma.

Keywords Biliary tract cancers · Biomarker · CYFRA 21-1 · Diagnosis · Prognosis

Introduction

Biliary tract cancers (BTCs) are malignancies originated from the neoplastic transformation of epithelium lining in bile ducts and gallbladder, and their incidence is increasing worldwide [1, 2]. Based on their anatomical locations, BTCs can be typically classified as intrahepatic and extrahepatic cholangiocarcinoma, and gallbladder carcinoma (ICC, EHCC and GBC, respectively), and EHCC can be subdivided into perihilar cholangiocarcinoma and distal cholangiocarcinoma (perihilar-CC and distal-CC, respectively) [2]. BTC patients have poor prognosis due to the lack of early diagnosis, and their symptoms usually become evident after blockage of the bile duct. Furthermore, BTCs are refractory to chemotherapy and radiotherapy, leaving surgical resection as the only potentially curative therapeutic option. Patients with advanced BTCs have a median survival of less than 1 year, while the 1- and 3-year survival rates for patients after curative resection are approximately 70 and 30 %, respectively [2]. Therefore, improvement on early diagnosis for BTCs remains scientific rationale in clinical practice [3].

Despite advances in imaging modalities, including contrast-enhanced ultrasound, CT, MRI and positron emission computed tomography (PET-CT), the cost–effect ratio and instrument requirements limit their routine applications. Albeit with unsatisfactory diagnostic sensitivity and specificity, serum biomarkers serve as cheaper and easier complementary diagnostic tools for BTCs. Among them, carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) are the most widely used in clinical practice, although with wide variation in diagnostic sensitivity (53–92 % for CA19-9; 33–68 % for CEA) and specificity (50–98 % for CA19-9; 79–100 % for CEA) [4]. Thus, it remains reasonable to seek other reliable biomarkers for BTCs.

Cytokeratin 19 (CK 19) is a constituent of the intermediate filament and widely expressed in epithelium of many organs, including cholangiocyte [5]; CYFRA 21-1 is a serum-soluble fragment of CK 19 [6]. Like other epithelial markers, only trace amounts of CYFRA 21-1 can be detected in peripheral blood under physiological conditions; abnormal elevation of serum CYFRA 21-1 has been reported in many types of cancer, especially in non-small cell lung cancer (NSCLC) [7]. Upregulation of serum CYFRA 21-1 in ICC was first reported in 1998 [8], which was followed by a few preliminary clinical studies to evaluate its clinical value [9, 10]. A pilot study had also been launched to explore the diagnostic performance of CYFRA 21-1 in primary sclerosing cholangitis (PSC), PSC-related BTCs and sporadic BTCs [11]. However, none of the above studies had included comprehensive analysis of CYFRA 21-1 in BTC subtypes. Therefore, clinical significance of serum CYFRA 21-1 in BTCs remains unaddressed.

The present study aimed to determine the diagnostic and prognostic performance of CYFRA 21-1 in BTCs and BTC subtypes.

Patients and Methods

Patients and Study Design

From October 2011 to December 2012, a cohort of 134 consecutive BTC patients with pathological confirmation

Table 1 Demographics and characteristics of benign biliary disease and biliary tract cancer patients

BTCs	$n = 134$
Median age, years (range)	61 (52–68)
Gender (men/women)	76:58
BTC subtypes (*n*)	
ICC	41
GBC	32
Perihilar-CC	31
Distal-CC	30
TNM stage (*n*)[a]	
0	3
I	24
II	43
III	35
IV	29
Benign biliary diseases	$n = 52$
Median age, years (range)	56 (33–84)
Gender (men/women)	28:24
Disease subtypes (*n*)	
Cholelithiasis (hepatolithiasis, cholecystolithiasis, choledocholithiasis)	28
Congenital cystic dilatation of bile duct	6
Polyp of gallbladder	5
Adenomyomatosis of gallbladder	5
Adenoma of gallbladder	4
Sclerosing cholangitis	2
Iatrogenic bile duct injury	2

[a] According to the American Joint Committee Cancer (AJCC) TNM staging (7th edition, 2010)

BTC biliary tract cancer, *ICC* intrahepatic cholangiocarcinoma, *GBC* gallbladder carcinoma, *perihilar-CC* perihilar cholangiocarcinoma, *distal-CC* distal cholangiocarcinoma

were analyzed retrospectively, including 41 ICC, 61 EHCC (31 perihilar-CC and 30 distal-CC) and 32 GBC patients who visited the Department of Hepatobiliary Surgery, the First Affiliated Hospital of Sun Yat-sen University. Fifty-two patients with benign biliary diseases were enrolled as controls (patients' demographics and clinicopathological characteristics are listed in Table 1 and Table S-1 to S-4). Exclusion criteria were as follows: previous chemotherapy or radiation therapy; concomitant malignancies of other organs; concomitant serious morbidity or preoperative mortality. Study protocol was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University, and the study was conducted in accordance with the Helsinki Declaration. Written informed consents were obtained from all patients.

Diagnosis, Treatment and Follow-Up

Patients underwent initial investigations, including a full medical history and physical examination. Peripheral blood was collected for evaluating blood routine test, liver biochemistry, hepatitis virus series and other infectious diseases preoperatively. Measurements of serum CA19-9, CEA and CYFRA 21-1 were conducted preoperatively and on postoperative 7th day (POD7). Other routine tests included electrocardiogram, chest X-ray, abdominal ultrasound, contrast-enhanced ultrasound and computed tomography or magnetic resonance imaging; PET-CT was applied in suspected metastatic cases. A preoperative diagnosis was comprehensively based on above indirect diagnostic modalities.

Furthermore, 118 of the 134 BTC patients (37 ICC, 29 GBC, 26 perihilar-CC and 26 distal-CC patients) received curative surgery, while 16 patients (4 ICC, 3 GBC, 5 perihilar-CC and 4 distal-CC patients) received palliative drainage and biopsy according to their unresectable stages. Patients' specimens were pathologically confirmed by three independent pathologists in the Department of Pathology, the First Affiliated Hospital of Sun Yat-sen University (Table 1 and Table S-1 to S-4). Patients were categorized according to the American Joint Committee Cancer (AJCC) TNM staging (7th edition, 2010) [12].

After discharge, all patients were followed up once every month in the first 6 months and every 3 months thereafter. Physical examinations and history inquiries, as well as laboratory tests including blood routine test, liver biochemistry, serum biomarkers and abdominal ultrasound, were performed. When tumor recurrence was suspected, contrast-enhanced ultrasound, CT or MRI would be applied. Time to tumor recurrence was defined as interval between the date of surgery and the appearance of a neoplasm at surgical site or in the liver, which was confirmed by two types of radiologic modalities. Overall survival was defined as the interval between the date of surgery and death or the end point of follow-up (January 12, 2014). Follow-up period ranged from 3.0 to 26.7 months (median 11.6 months).

Measurement of Tumor Markers

Samples of fasting peripheral blood were collected in Vacutainer^{TM} tubes (SST II Advance, Cat#367957, BD, Plymouth, UK) from enrolled patients and sent to the Department of Clinical Laboratory, the First Affiliated Hospital of Sun Yat-sen University. Serums were obtained by centrifugation and measured by two automatic analyzers for clinical test according to the manufacturer's instruction: Cobas 6000 (Roche Diagnostics, IN, USA) for CYFRA 21-1 (Elecsys assay kit) and Architect i2000SR (Abbott Diagnostics, IL, USA) for CA19-9 (Ref code: 2K91) and CEA (Ref code: 7K68). Briefly, for quantitative measurement of CA19-9 or CEA, conventional chemiluminescent microparticle immunoassay (CMIA) was applied; for quantitative measurement of CYFRA 21-1, an electrochemiluminescent immunoassay (ECLIA) was performed with two mouse monoclonal antibodies targeting different epitopes of CYFRA 21-1 (Ks 19.1, a biotinylated antibody that recognized amino acids 311–335; BM 19.21, a ruthenium-labeled antibody recognized amino acids 346–368). Results are expressed as nanograms per milliliter (ng/mL) for CYFRA 21-1 and CEA for units per milliliter (U/mL) for CA19-9.

Levels of serum total bilirubin will not significantly disturb the measurement assay as per manufacturer's instruction (up to 1,112 μmol/L (65 mg/dL) for CYFRA 21-1 and 376 μmol/L (22 mg/dL) for CA19-9 and CEA). Recommended cutoff values of biomarkers provided by manufacturers were 3.30 ng/mL for CYFRA 21-1, 37.0 U/mL for CA19-9 and 5.0 ng/mL for CEA. The optimal cutoff values in the present study were determined by the maximal Youden's index [13].

Statistical Analysis

Heterogeneity of baseline data between two groups was assessed by Chi-square test or Mann–Whitney *U* test. As concentrations of CYFRA 21-1 among BTC patients failed the normality test (data not shown), data were presented as the median with interquartile ranges, as in other biomarkers and other quantitative variables. Mann–Whitney *U* test was performed in analysis between two independent groups; Kruskal–Wallis test was performed in comparison between multiple groups, and when results reached significance, subgroup analyses were performed by Mann–Whitney *U* test with significance levels corrected by the Holm–Bonferroni method. Wilcoxon-matched pairs test was

adopted in pairwise comparison. Spearman correlation analysis was performed to assess correlation between serum biomarkers and total bilirubin. Statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL). $P < 0.05$ indicated statistical significance.

Patients' diagnoses were pathologically confirmed after surgery or biopsy. To compare the efficacy of these biomarkers, receiver operating characteristic (ROC) curves were applied and respective areas under the ROC curve (AUC) with 95 % confidence interval (CI) and standard error (SE) were constructed by MedCalc 12.7 software (Frank Schoonjans, Mariakerke, Belgium). For comparing diagnostic efficacy of combination of these three biomarkers and CYFRA 21-1 alone, ROC curve for combination of these biomarkers was constructed based on the combined diagnostic probability for each patient. The combined diagnostic probability was calculated by logistic regression equation, which was established based on individual levels of three biomarkers. Optimal cutoff values were determined by the maximal Youden's index (Youden's index = sensitivity + specificity–1) [13]. Comparison of diagnostic efficacy of CYFRA 21-1 between any two BTC subtypes was made by the Z test: $Z = |AUC_1 - AUC_2|/sqrt (SE_1\text{^}2 + SE_2\text{^}2)$; significance was determined by the probability corresponding to the Z value in the two-tailed normal distribution table [14]; significance levels of multiple comparisons were corrected by the Holm–Bonferroni method. Recurrence-free survival and overall survival analyses were performed by the Kaplan–Meier method followed by the log-rank test. Covariates with $P < 0.1$ in univariate tests were chosen in multivariate Cox proportional hazards regression models; risk ratios were presented with 95 % CI.

Results

Patient Demographics and CYFRA 21-1 Distribution

Patient demographics are listed in Table 1. Heterogeneity analysis of baseline data did not reach significance between BTC patients and benign controls.

Serum CYFRA 21-1 (ng/mL) was significantly higher in BTCs compared with benign group (3.82; 2.48–6.98 vs 1.81; 1.49–2.39; $Z = -7.423$, $P < 0.001$; Fig. 1a). Level of CYFRA 21-1 (ng/mL) varied among BTC subtypes (ICC 6.53, 3.22–12.80; GBC 5.26, 3.79–10.93; perihilar-CC 2.68, 1.79–4.06 and distal-CC 2.91, 2.01–3.70; $H = 36.714$, $P < 0.001$) and can be subdivided into two groups: (1) ICC and GBC; (2) perihilar-CC and distal-CC (ICC vs perihilar-CC or distal-CC, $Z = -3.611$, -3.731, $P < 0.001$, respectively; GBC vs perihilar-CC or distal-CC, $Z = -4.744$, -5.036, $P < 0.001$, respectively; ICC vs GBC, $Z = -0.395$, $P = 0.693$; perihilar-CC vs distal-CC, $Z = -0.346$, $P = 0.729$; Fig. 1b). CT images of represented patients are shown in Fig. 1c.

Because heterogeneity existed among BTC subtypes according to the constituent ratio of TNM stage ($X^2 = 16.293$, $P < 0.05$), CYFRA 21-1 was further stratified in different TNM stages, and comparisons between BTC subtypes showed that GBC and ICC had higher CYFRA 21-1 titer in advanced TNM stages (Table S-1 to S-4).

Diagnostic Efficacy of CYFRA 21-1 in BTCs and BTC Subtypes

Serum CYFRA 21-1 cutoff values (ng/mL) were selected as follows (Table 2): 2.61 for BTCs (sensitivity 74.6 % and specificity 84.6 %), 3.27 both for ICC (75.6 and 96.2 %) and GBC (93.7 and 96.2 %), 2.27 for perihilar-CC (71.0 and 71.2 %) and 2.61 for distal-CC (63.3 and 84.6 %). When a higher cutoff value of 3.30 ng/mL was applied, diagnostic specificity of CYFRA 21-1 reached 96.2 % in BTCs and each BTC subtype; diagnostic sensitivity was 55.2 % for BTCs, 70.7 % for ICC, 84.4 % for GBC, 29.1 % for perihilar-CC and 30.0 % for distal-CC, respectively.

Moreover, AUCs with 95 % CI and SE for CYFRA 21-1 in BTCs and BTC subtypes were calculated (Table 2): GBC > ICC > distal-CC > perihilar-CC (GBC vs distal-CC, GBC vs perihilar-CC, $Z = 3.752$, 4.141, respectively, $P < 0.001$; GBC vs ICC, $Z = 2.622$, $P < 0.008$; ICC vs perihilar-CC, $Z = 1.952$, $P = 0.051$; ICC vs distal-CC, $Z = 1.468$, $P = 0.142$; distal-CC vs perihilar-CC, $Z = 0.497$, $P = 0.619$). AUCs for CA19-9 and CEA were also analyzed (Table 2). When compared to CA19-9 and CEA, CYFRA 21-1 showed better discrimination performance in BTCs, especially in GBC and ICC ($P < 0.001$; Fig. 2a–e). The combined diagnostic probability for each patient was determined by the constructed logistic regression equation: combined probability $= 1/[1 + e^{-(2.683-1.137\times X_1+0.001\times X_2-0.140\times X_3)}]$, where X_1, X_2 and X_3 represent individual levels of CYFRA 21-1, CA19-9 and CEA, respectively. ROC curves of the predicted combined diagnostic probability for BTCs and BTC subtypes were plotted (Fig. 2a–e), and respective AUCs were calculated (Table 2). Discrimination capacity of combining three biomarkers was not superior to that of a solo marker CYFRA 21-1 in BTCs nor in BTC subtypes ($P > 0.05$).

Correlation Between CYFRA 21-1 and Clinicopathological Variables of BTCs

Distinct from CA19-9, neither serum CYFRA 21-1 nor CEA was correlated with total bilirubin (TB) in BTC

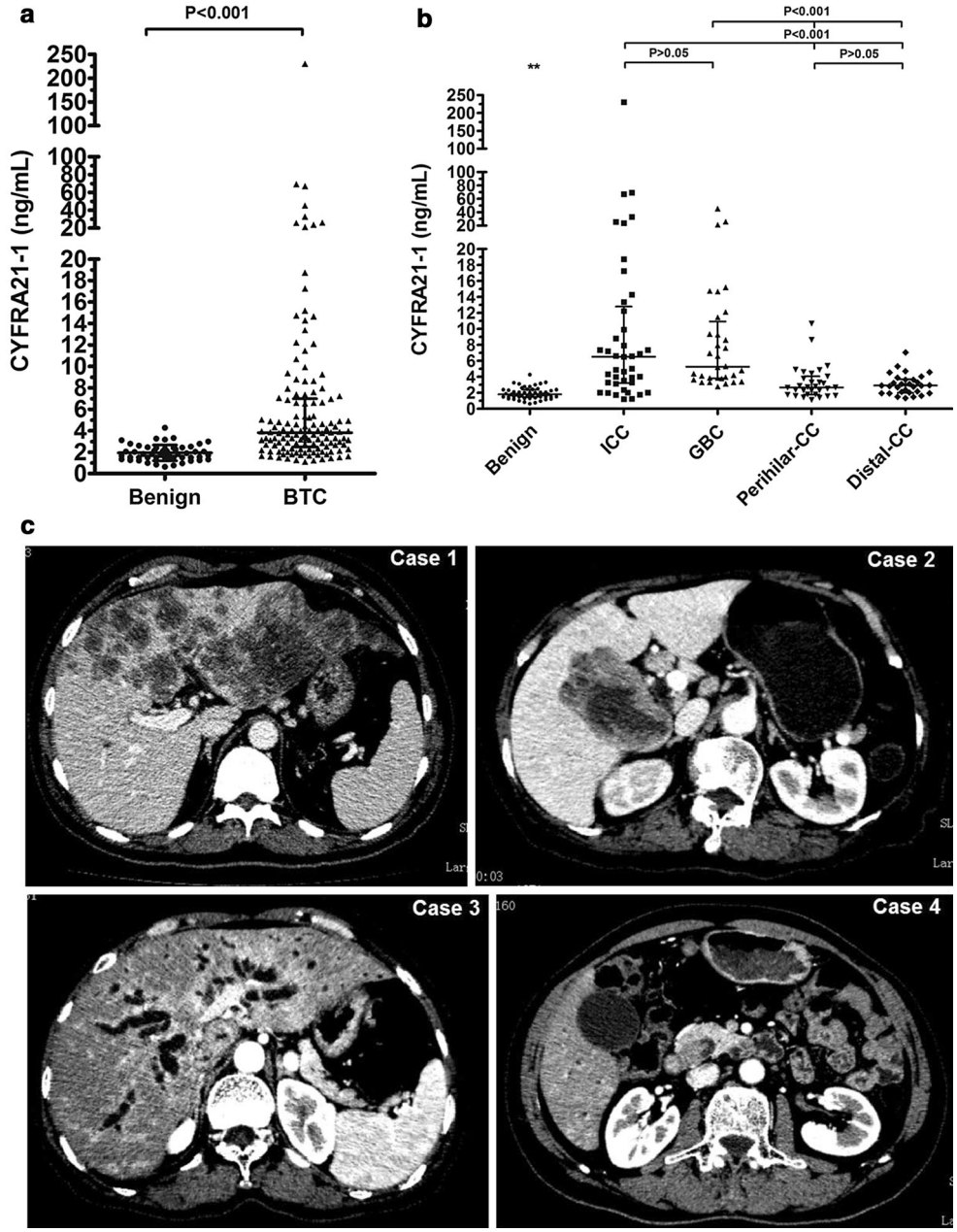

Fig. 1 **a** Distribution of individual serum CYFRA 21-1 in benign biliary disease and biliary tract cancer patients: Data are presented as median (*horizontal line*) and interquartile range. Serum CYFRA 21-1 is significantly elevated in biliary tract cancers compared with benign group (3.82; 2.48–6.98 ng/mL vs 1.81; 1.49–2.39 ng/mL; Mann–Whitney U test, $Z = -7.423$, $P < 0.001$). **b** Distributions of individual serum CYFRA 21-1 in biliary tract cancer subtypes: Data are presented as median (*horizontal line*) and interquartile range. Analysis of serum CYFRA 21-1 (ng/mL) in biliary tract cancer (BTC) subtypes reveals significant difference (Kruskal–Wallis test, $H = 36.714$, $P < 0.001$): intrahepatic cholangiocarcinoma (ICC), 6.53; 3.22–12.80; gallbladder carcinoma (GBC), 5.26; 3.79–10.93; perihilar cholangiocarcinoma (perihilar-CC), 2.68; 1.79–4.06 and distal cholangiocarcinoma (distal-CC), 2.91; 2.01–3.70; furthermore, BTC subtypes can be subdivided into two groups: (1) ICC and GBC; (2) perihilar-CC and distal-CC (Mann–Whitney U test, significance levels are corrected by the Holm–Bonferroni method, $P < 0.001$). **c** CT images of represented patients in each biliary tract cancer subtype: (1) *Case 1* Intrahepatic cholangiocarcinoma (adenocarcinoma, G3, periductal infiltrating type, stage IVa [T4 N1 M0]) had the highest serum CYFRA 21-1 titer of 230.20 ng/mL in present cohort. (2) *Case 2* Gallbladder carcinoma (adenocarcinoma, G2, mass-forming type, stage IIIb [T3 N1 M0]) had the highest serum CYFRA 21-1 titer of 45.21 ng/mL in gallbladder carcinoma group. (3) *Case 3* perihilar cholangiocarcinoma (adenocarcinoma, G2, nodular sclerosing type, stage IIIb [T2b N1 M0]) had serum CYFRA 21-1 titer of 5.37 ng/mL. (4) *Case 4* Distal cholangiocarcinoma (adenocarcinoma, G2, nodular sclerosing type, stage IIb [T3 N1 M0]) had serum CYFRA 21-1 titer of 3.63 ng/mL. *G2* moderate differentiation, *G3* poor differentiation

Table 2 Diagnostic efficacy of CYFRA 21-1, CA19-9, CEA and their combination in biliary tract cancers and all subtypes

Subtypes	CYFRA 21-1 (ng/mL)	CA19-9 (U/mL)	CEA (U/mL)	Combined
BTCs				
Cutoff value	2.61	76.53	2.70	0.2629
Sensitivity	74.6 %	61.2 %	59.0 %	75.4 %
Specificity	84.6 %	82.7 %	76.9 %	88.5 %
AUC (95 % CI)	0.851 (0.797–0.905)	0.725 (0.647–0.804)	0.720 (0.643–0.797)	0.866 (0.815–0.917)
SE	0.0275	0.0399	0.0392	0.0260
P value*		0.0085	0.0014	0.1107
ICC				
Cutoff value	3.27	76.53	2.70	0.1078
Sensitivity	75.6 %	63.4 %	63.4 %	75.6 %
Specificity	96.2 %	82.7 %	76.9 %	98.1 %
AUC (95 % CI)	0.879 (0.802–0.956)	0.739 (0.632–0.846)	0.723 (0.614–0.831)	0.904 (0.834–0.974)
SE	0.0392	0.0547	0.0553	0.0357
P value*		0.0249	0.0109	0.0997
GBC				
Cutoff value	3.27	144.10	2.39	0.2456
Sensitivity	93.7 %	53.1 %	84.4 %	100 %
Specificity	96.2 %	88.5 %	69.2 %	88.5 %
AUC (95 % CI)	0.985 (0.965–1.000)	0.681 (0.558–0.805)	0.809 (0.707–0.910)	0.981 (0.959–1.000)
SE	0.0099	0.0631	0.0517	0.0113
P value*		<0.0001	0.0006	0.4754
Perihilar-CC				
Cutoff value	2.27	36.86	1.41	0.3406
Sensitivity	71.0 %	77.4 %	87.1 %	61.3 %
Specificity	71.2 %	69.2 %	38.5 %	82.7 %
AUC (95 % CI)	0.743 (0.631–0.856)	0.778 (0.676–0.880)	0.661 (0.540–0.782)	0.753 (0.640–0.866)
SE	0.0576	0.0522	0.0616	0.0575
P value*		0.6431	0.2246	0.5788
Distal-CC				
Cutoff value	2.61	76.53	2.17	0.3599
Sensitivity	63.3 %	63.3 %	70.0 %	70.0 %
Specificity	84.6 %	82.7 %	61.5 %	80.8 %
AUC (95 % CI)	0.782 (0.678–0.886)	0.699 (0.570–0.828)	0.683 (0.561–0.806)	0.806 (0.704–0.907)
SE	0.0532	0.0657	0.0625	0.0517
P value*		0.3227	0.1763	0.2178

BTC biliary tract cancer, *ICC* intrahepatic cholangiocarcinoma, *GBC* gallbladder carcinoma, *perihilar-CC* perihilar cholangiocarcinoma, *distal-CC* distal cholangiocarcinoma, *AUC* areas under the ROC curve, *95 % CI* 95 % confidence interval, *SE* standard error

* Comparison of AUC between CYFRA 21-1 and other biomarkers

patients (correlation coefficients: CA19-9 and TB, 0.240, $P = 0.005$; CYFRA 21-1 and TB, −0.159, $P > 0.05$; CEA and TB, 0.098, $P > 0.05$). Furthermore, CYFRA 21-1 was related to tumor stage of each BTC subtype (Table S-1 to S-4). Briefly, serum CYFRA 21-1 was correlated with aggressive tumor behavior (vascular invasion, regional lymph node metastasis, adjacent organ invasion and distant metastasis) and higher tumor burden in BTCs as a tendency of increased CYFRA 21-1 was demonstrated from TNM stage I to IV (Figure S1a-e and Table S-1 to S-4).

Prognostic Prediction Value of CYFRA 21-1 in BTCs

Postoperative death occurred in 1 ICC patient who received curative resection and died from liver failure during hospital stay. In all other 61 patients with elevated preoperative CYFRA 21-1, the level of CYFRA 21-1 declined significantly on POD7 after curative resection (Fig. 3a).

During follow-up, 69 of 118 patients who received curative surgeries had developed tumor recurrence (25 ICC, 18 GBC, 14 perihilar-CC and 12 distal-CC patients);

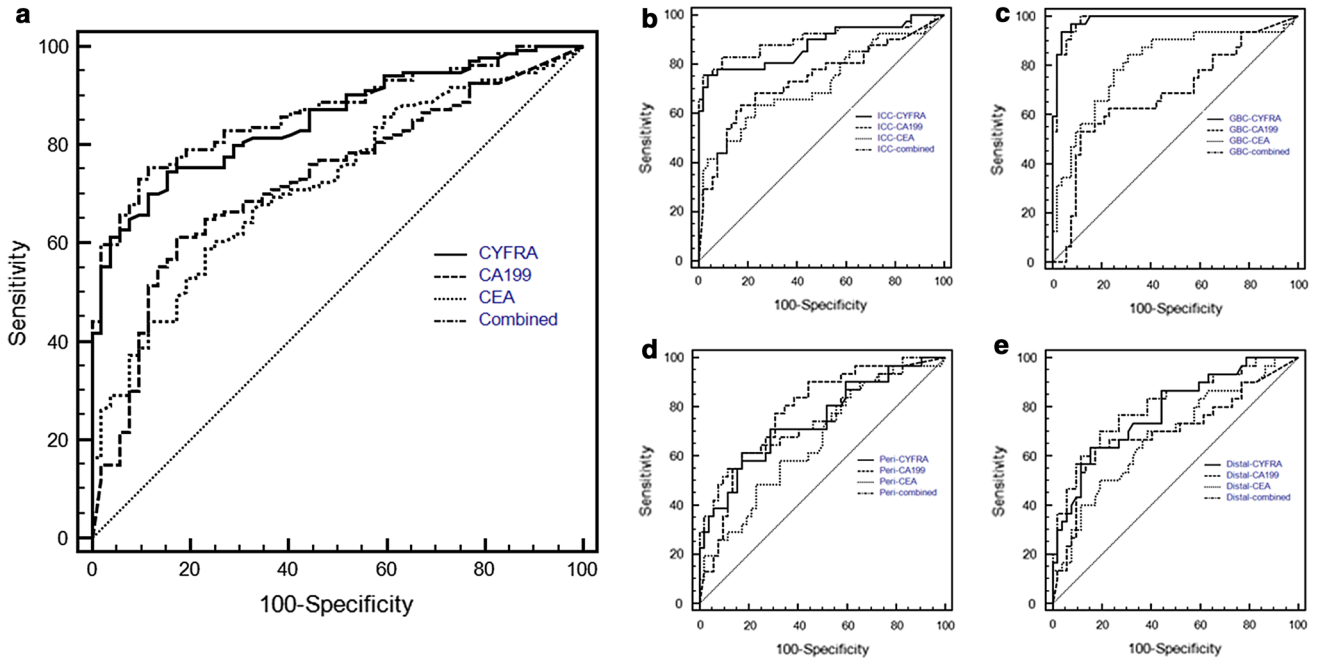

Fig. 2 Diagnostic efficacy of CYFRA 21-1 in biliary tract cancers and all subtypes. Receiver operating characteristic (ROC) curves of CYFRA 21-1, CA19-9, CEA and their combination: **a** biliary tract cancers (BTCs) and benign biliary diseases; **b** intrahepatic cholangiocarcinoma (ICC) and benign biliary diseases; **c** gallbladder carcinoma (GBC) and benign biliary diseases; **d** perihilar cholangiocarcinoma (perihilar-CC) and benign biliary diseases; **e** distal cholangiocarcinoma (distal-CC) and benign biliary diseases. When compared to CA19-9 and CEA, CYFRA 21-1 shows better discrimination performance in BTCs and BTC subtypes GBC and ICC ($P < 0.001$); combination of these three biomarkers does not show superior discrimination capacity to a solo biomarker CYFRA 21-1 in BTCs nor in BTC subtypes ($P > 0.05$)

41 of them (18 ICC, 16 GBC, 4 perihilar-CC and 3 distal-CC patients) had re-elevated serum CYFRA 21-1 (Fig. 3b; CT images of represented patients are shown in Fig. 3c). The median recurrence-free survival (RFS) of low-CYFRA 21-1 group were significantly longer than those of high-CYFRA 21-1 group in ICC and GBC; the respective 1-year RFS rates were also higher in low-CYFRA 21-1 group in each BTC subtype (Fig. 4a–d). Sixty-one of these 134 BTC patients died at the end of follow-up (21 ICC, 18 GBC, 11 perihilar-CC and 11 distal-CC patients, except for 1 ICC patient died from postoperative liver failure during hospital stay). The cause of death in this cohort includes tumor-related deaths ($n = 42$), other malignancies ($n = 8$) and cardio-cerebrovascular diseases ($n = 11$). The median overall survival (OS) of low-CYFRA 21-1 group were significantly longer than those of high-CYFRA 21-1 group in ICC, but not significant in GBC; the respective 1-year OS rates were also higher in low-CYFRA 21-1 group in each BTC subtype (Fig. 4e–h).

Details of univariate analyses are listed in Table S-5 to S-8; in multivariate analyses, regional lymph node metastasis, resection margin, CYFRA 21-1 and TNM stages were demonstrated as independent risk factors for RFS and OS in BTC subtypes (Table S-9). Low serum CYFRA 21-1 (<3.27 ng/mL for ICC, <2.27 ng/mL for perihilar-CC and <2.61 ng/mL for distal-CC) predicted higher 1-year RFS rate and OS rate in BTC patients, whereas the predictive efficacy did not reach significance in GBC.

Discussion

The major finding of the present study reveals that serum CYFRA 21-1 can be regarded as a reliable diagnostic biomarker for BTCs, especially for GBC and ICC. Titer of serum CYFRA 21-1 correlates with BTCs' tumor stage, and higher preoperative CYFRA 21-1 predicts earlier recurrence in patients who received curative resection and shorter overall survival. Collectively, these results support CYFRA 21-1 as a sufficient diagnostic and prognostic biomarker for BTCs.

Despite advances in modern diagnostic modalities, early diagnosis of BTCs remains challenging in clinical practice as the majority of BTC patients develop symptoms at advanced stage when they refer to hospitals [3]. Although significant advances have been achieved in identifying serologic biomarkers for BTCs, the majority of these markers remain in laboratory or preclinical trials [15]. Presently, CA19-9 and CEA are the most widely applied in clinical practice although with unsatisfied diagnostic

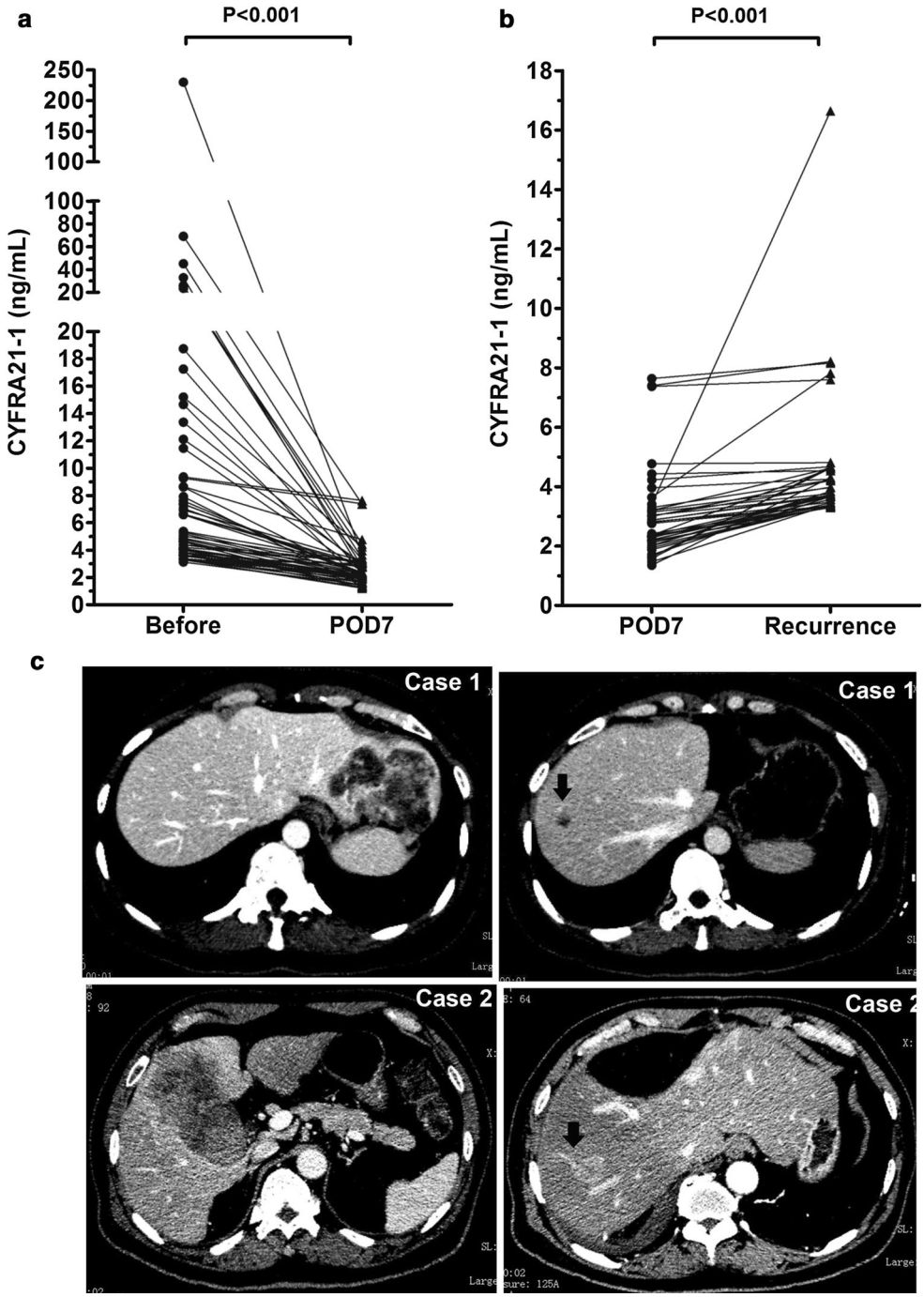

Fig. 3 **a** Marker response of serum CYFRA 21-1 in biliary tract cancer patients after curative surgery: In 61 patients with elevated preoperative serum CYFRA 21-1 who received curative resection, measurements of individual serum CYFRA 21-1 were repeated on postoperative 7th day (POD7) and shown a significant decline (baseline before operation vs POD7, 5.04; 3.82–9.31 ng/mL vs 2.33; 1.99–3.04 ng/mL; Wilcoxon-matched pairs test, $Z = -6.791$, $P < 0.001$). **b** Marker response of serum CYFRA 21-1 in biliary tract cancer patients with tumor recurrence after curative surgery: 69 patients who received curative surgery and developed tumor recurrence during follow-up. Forty-one of them had re-elevated serum CYFRA 21-1 when compared to postoperative 7th day (POD7): (POD7 vs tumor recurrence, 2.76; 2.14–3.25 ng/mL vs 3.72; 3.39–4.62 ng/mL; Wilcoxon-matched pairs test, $Z = -5.579$, $P < 0.001$). **c** Computed tomography images of represented biliary tract cancer patients developed tumor recurrence after curative surgery: (1) *Case 1* Intrahepatic cholangiocarcinoma patient (adenocarcinoma, G2, periductal infiltrating type, stage II [T2 N0 M0]; *upper left panel*) received left hemihepatectomy and developed tumor recurrence as a mass in hepatic segment VIII (*upper right panel*). She had serum CYFRA 21-1 titer of 4.62 ng/mL. (2) *Case 2* Gallbladder carcinoma patient (adenocarcinoma, G2, mass-forming type, stage IIIb [T3 N1 M0]; *lower left panel*) received curative en bloc resection of tumor and hepatic segment IVb plus V and developed tumor recurrence as a mass in hepatic segment VI (*lower right panel*). He had serum CYFRA 21-1 titer of 16.64 ng/mL

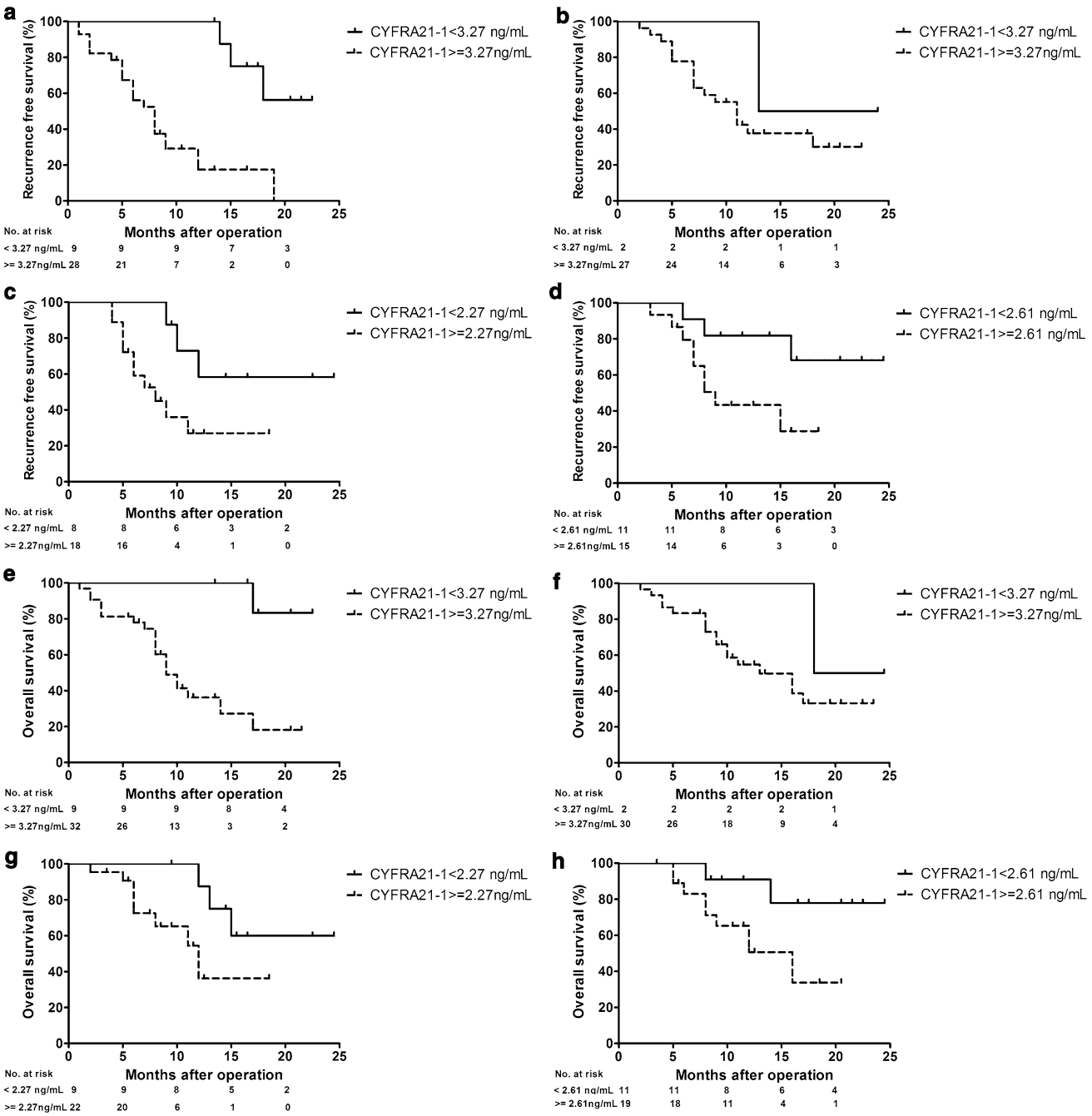

Fig. 4 Prognostic prediction value of CYFRA 21-1 in biliary tract cancers. Recurrence-free survival (RFS) curves of CYFRA 21-1: **a** intrahepatic cholangiocarcinoma (ICC); **b** gallbladder carcinoma (GBC); **c** perihilar cholangiocarcinoma (perihilar-CC); **d** distal cholangiocarcinoma (distal-CC). RFS (months) for ICC and GBC with low and high CYFRA 21-1 was as follows: in ICC, 19.5 (95 % CI, 17.3–21.7) vs 7.8 (5.9–9.6), $P < 0.01$; in GBC, 18.8 (10.8–26.7) vs 12.7 (9.8–15.6), $P = 0.437 > 0.05$. The respective 1-year recurrence-free survival rates for each BTC subtype with low and high CYFRA 21-1 are as follows: ICC, 100 and 13.1 %; GBC, 50.0 and 37.7 %; perihilar-CC, 58.3 and 27.1 %; distal-CC, 81.8 and 43.3 %. Overall survival (OS) curves of CYFRA 21-1: **e** ICC; **f** GBC; **g** perihilar-CC; **h** distal-CC. OS (months) for ICC and GBC with low and high CYFRA 21-1 were as follows: in ICC, 21.7 (20.3–23.1) vs 10.3 (8.1–12.6), $P < 0.01$; in GBC, 21.3 (16.7–25.8) vs 14.4 (11.5–17.2), $P = 0.408 > 0.05$. The respective 1-year overall survival rates for each BTC subtype with low and high CYFRA 21-1 are as follows: ICC, 100 and 33.6 %; GBC, 100 and 54.7 %; perihilar-CC, 87.5 and 36.3 %; distal-CC, 90.9 and 50.7 %

performance. Upregulated CA19-9 has also been reported in benign cholestatic diseases like primary sclerosing cholangitis or hepatolithiasis [4]. As shown in the present study, the level of CA19-9 was positively correlated with serum TB. Thus, there still remains scientific rationale to seek other reliable biomarkers for BTCs.

Cytokeratins (CKs) are intermediate filaments present in epithelium. To date, 28 kinds of type I and 26 kinds of type II CKs have been identified; paired combinations of these two types of CKs are expressed in tissue- and differentiation-specific patterns [16]. Concerning the liver, hepatocytes contain CK 8 and 18, while cholangiocytes contain CK 7 and 19 [5]. Thus, CK 19 has been widely adopted as a discriminatory marker between cholangiocarcinoma and hepatocellular carcinoma (HCC) [9], although with exception in advanced HCC or HCC with biliary transdifferentiation [17–20]. CYFRA 21-1, a serum-soluble fragment of CK 19, was first identified in 1993 and had been shown to be a product of CK 19 cleaved by caspase 3 [6, 21]. CYFRA 21-1 has been shown as a sufficient biomarker in many types of cancer and routinely applied in diagnosis and surveillance for non-small cell lung cancer (NSCLC). In the present study, serum CYFRA 21-1 was shown to be upregulated in BTCs. The level of serum CYFRA 21-1 in benign group may represent the basal metabolic turnover of CK 19 in the body, while the net increase in CYFRA 21-1 in BTC patients may arise from tumor apoptosis (cleaved by caspase 3) or, more likely, from tumor invasion or metastasis [19, 22, 23]. There were 3 patients (2 hepatolithiasis and 1 adenomyomatosis of gallbladder) with higher CYFRA 21-1 titer (beyond 3.27 ng/mL) in the benign group, although they did not reveal any overt malignancies even after thorough pathological sectioning. This result may reflect transforming potentials of these diseases, which have been recognized as risk factors for BTCs, and imply the importance of close surveillance for these patients [1].

Recently, CYFRA 21-1 has been demonstrated as a useful biomarker for ICC [8, 10] and PSC-related BTCs [11]. However, to our knowledge, the present study represents the first comprehensive report concerning diagnostic and prognostic value of CYFRA 21-1 in BTCs as well as in BTC subtypes. We showed that CYFRA 21-1 sufficiently discriminated BTCs from benign biliary diseases and had a better performance in GBC and ICC; CYFRA 21-1 was superior to both CA19-9 and CEA in the diagnosis of ICC, which was consistent with previous results [9, 11]. Furthermore, diagnostic efficacy of combining these three biomarkers did not surpass CYFRA 21-1 alone. Thus, these results supported CYFRA 21-1 as a bona fide diagnostic biomarker for BTCs, especially for GBC and ICC. A prospective trial to validate serum CYFRA 21-1 as a screening biomarker for GBC may be warranted to distinguish incidental GBC in suspected cases [24]; we also recommend that CYFRA 21-1 should be applied together with CA19-9 and CEA in diagnosing perihilar-CC or distal-CC. In the present study, the expressional difference of CYFRA 21-1 between BTC subtypes may reflect the biological heterogeneities of these tumors. As the presence of CK 19 mRNA-positive cells in peripheral blood represented circulating tumor cells and predicted a poor prognosis in malignancy, higher levels of CYFRA 21-1 in ICC and GBC than in their counterparts may reflect a fact in clinical practice that ICC or GBC generally had a worse prognosis than EHCC; GBC tended to recur more distantly than EHCC [25]. Moreover, as CK 19 and other CK-like antigens can be released from tumor cell during tumor invasion or metastasis [19, 22, 23, 26], higher level of CYFRA 21-1 in ICC and GBC may reflect more aggressiveness of these tumors. The mechanism involved in the expressional pattern of CYFRA 21-1 in BTCs is an ongoing research topic in our groups.

Consistent with previous studies [9, 11], we showed that serum CYFRA 21-1 was significantly correlated with tumor aggressiveness and tumor burden in BTCs; even at early stage, upregulated CYFRA 21-1 had already been noticed (TNM stage 0-I in GBC and stage I in ICC; Table S-1 to S-4). These results supported that CYFRA 21-1 can serve as a sufficient tool for BTC staging. In the majority of our BTC patients who received curative resection, CYFRA 21-1 declined significantly on POD7 and re-elevated when tumor recurred during follow-up. As tumor recurrence represents the major obstacle for long-term survival in BTC patients after curative resection, CYFRA 21-1 can be applied in BTC surveillance. In addition to the well-recognized prognostic predictors like regional lymph node metastasis, histologic status of resection margin and TNM stage [27–30], we showed that CYFRA 21-1 was an independent risk factor for 1-year RFS and OS in ICC, perihilar-CC and distal-CC, while it did not reach significance in GBC. We attributed this paradox to relatively small proportion of GBC patients with lower serum CYFRA 21-1 (2 of 32). For validation studies to extend these results into broader clinical use, a prospective multicenter cohort study of measuring serum CYFRA 21-1 in suspected BTC patients, as well as in patients who received curative surgery during follow-up, is guaranteed to further validate the diagnostic and prognostic efficacy of this promising biomarker. Moreover, we also need to explore the diagnostic performance of combining CYFRA 21-1 with conventional radiologic examinations in BTC patients.

As higher serum CYFRA 21-1 may indicate the existence of circulating tumor cells, BTCs with elevated CYFRA 21-1 should be regarded as a systemic disease rather than a local malignancy, and curative resection alone may not sufficiently prevent tumor recurrence [10]. In parallel with improved surgical procedures, promising chemotherapy regimens (gemcitabine combined with a platinum-based agent) and targeted agents have evolved, and their survival benefit for BTC patients has been validated [31]. Therefore, it is conceivable that adjuvant therapy should be

regarded as a reasonable component of treatment, besides surgery, in BTC patients with higher serum CYFRA 21-1.

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References

1. Hezel AF, Zhu AX. Genetics of biliary tract cancers and emerging targeted therapies. *J Clin Oncol*. 2010;28:3531–3540.
2. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. *Gastroenterology*. 2013;145:1215–1229.
3. Liu LN, Xu HX, Lu MD, et al. Contrast-enhanced ultrasound in the diagnosis of gallbladder diseases a multi-center experience. *PLoS ONE*. 2012;7:e48371.
4. Alvaro D. Serum and bile biomarker for cholangiocarcinoma. *Curr Opin Gastroenterol*. 2009;25:279–284.
5. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell*. 1982;31:11–24.
6. Stieber P, Bodenmüller H, Banauch D, et al. Cytokeratin 19 fragments: a new marker for non-small-cell lung cancer. *Clin Biochem*. 1993;26:301–304.
7. Rastel D, Ramaioli A, Cornillie F, Thirion B. CYFRA 21-1, a sensitive and specific new tumour marker for squamous cell lung cancer. Report of the first European multicentre evaluation. CYFRA 21-1 Multicentre Study Group. *Eur J Cancer*. 1994;30A:601–606.
8. Kashihara T, Ohki A, Kobayashi T, et al. Intrahepatic cholangiocarcinoma with increased serum CYFRA 21-1 level. *J Gastroenterol*. 1998;33:447–453.
9. Uenishi T, Kubo S, Hirohashi K, et al. Cytokeratin-19 fragments in serum (CYFRA 21-1) as a marker in primary liver cancer. *Br J Cancer*. 2003;88:1894–1899.
10. Uenishi T, Yamazaki O, Tanaka H, et al. Serum cytokeratin 19 fragment (CYFRA21-1) as a prognostic factor in intrahepatic cholangiocarcinoma. *Ann Surg Oncol*. 2008;15:583–589.
11. Chapman MH, Sandanayake NS, Andreola F, et al. Circulating CYFRA 21-1 is a Specific Diagnostic and Prognostic Biomarker in Biliary Tract Cancer. *J Clin Exp Hepatol*. 2011;1:6–12.
12. Edge S, Byrd D, Compton C, Fritz A, Greene F, Trotti A, eds. *AJCC Cancer Staging Manual*. 2010.
13. Youden WJ. Index for rating diagnostic tests. *Cancer*. 1950;3:32–35.
14. Wu JC, Martin AF, Raghu NK. Measures, uncertainties, and significance test in operational ROC analysis. *J Res Natl Inst Stand Technol*. 2011;116:517–537.
15. Malaguarnera G, Paladina I, Giordano M, Malaguarnera M, Bertino G, Berretta M. Serum markers of intrahepatic cholangiocarcinoma. *Dis Markers*. 2013;34:219–228.
16. Schweizer J, Bowden PE, Coulombe PA, et al. New consensus nomenclature for mammalian keratins. *J Cell Biol*. 2006;174:169–174.
17. Wu PC, Fang JW, Lau VK, Lai CL, Lo CK, Lau JY. Classification of hepatocellular carcinoma according to hepatocellular and biliary differentiation markers. Clinical and biological implications. *Am J Pathol*. 1996;149:1167–1175.
18. Uenishi T, Kubo S, Yamamoto T, et al. Cytokeratin 19 expression in hepatocellular carcinoma predicts early postoperative recurrence. *Cancer Sci*. 2003;94:851–857.
19. Ding SJ, Li Y, Tan YX, et al. From proteomic analysis to clinical significance: overexpression of cytokeratin 19 correlates with hepatocellular carcinoma metastasis. *Mol Cell Proteomics*. 2004;3:73–81.
20. Uenishi T, Yamazaki O, Yamamoto T, et al. Clinical significance of serum cytokeratin-19 fragment (CYFRA 21-1) in hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg*. 2006;13:239–244.
21. Dohmoto K, Hojo S, Fujita J, et al. The role of caspase 3 in producing cytokeratin 19 fragment (CYFRA21-1) in human lung cancer cell lines. *Int J Cancer*. 2001;91:468–473.
22. Karantza V. Keratins in health and cancer: more than mere epithelial cell markers. *Oncogene*. 2011;30:127–138.
23. Alix-Panabières C, Vendrell JP, Slijper M, et al. Full-length cytokeratin-19 is released by human tumor cells a potential role in metastatic progression of breast cancer. *Breast Cancer Res*. 2009;11:R39.
24. Fuks D, Regimbeau JM, Le Treut YP, et al. Incidental gallbladder cancer by the AFC-GBC-2009 study group. *World J Surg*. 2011;35:1887–1897.
25. Jarnagin WR, Ruo L, Little SA, et al. Patterns of initial disease recurrence after resection of gallbladder carcinoma and hilar cholangiocarcinoma: implications for adjuvant therapeutic strategies. *Cancer*. 2003;98:1689–1700.
26. Brabon AC, Williams JF, Cardiff RD. A monoclonal antibody to a human breast tumor protein released in response to estrogen. *Cancer Res*. 1984;44:2704–2710.
27. Ito F, Agni R, Rettammel RJ, et al. Resection of hilar cholangiocarcinoma: concomitant liver resection decreases hepatic recurrence. *Ann Surg*. 2008;248:273–279.
28. Ito K, Ito H, Allen PJ, et al. Adequate lymph node assessment for extrahepatic bile duct adenocarcinoma. *Ann Surg*. 2010;251:675–681.
29. Li SQ, Liang LJ, Hua YP, et al. Long-term outcome and prognostic factors of intrahepatic cholangiocarcinoma. *Chin Med J (Engl)*. 2010;122:2286–2291.
30. Pilgrim CH, Groeschl RT, Turaga KK, Gamblin TC. Key factors influencing prognosis in relation to gallbladder cancer. *Dig Dis Sci*. 2013;58:2455–2462.
31. Jensen LH. Biliary-tract cancer: improving therapy by adding molecularly targeted agents. *Lancet Oncol*. 2012;13:118–119.