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Original Article

Detection of early-stage extrahepatic cholangiocarcinoma in patients with biliary strictures by soluble B7-H4 in the bile

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Abstract: Increasing evidence has demonstrated that serum soluble B7-H4 (sB7-H4) is a useful tumour marker for cancer diagnosis and prognosis evaluation. Whether sB7-H4 is expressed in the bile of cholangiocarcinoma (CC) and is related to the progression of CC need to be explored. Bile sB7-H4 was obtained through endoscopic retrograde cholangiopancreatography (ERCP) from 213 patients with biliary strictures and detected was detected by a B7-H4 ELISA kit. Diagnostic value was compared among bile sB7-H4, CA19-9, CA12-5, CEA and ERCP-based cytological/tissue examination. Additionally, the correlations between the bile sB7-H4 concentration and the clinical characteristics of early-stage cholangiocarcinoma (ESCC) were studied. The bile sB7-H4 levels of patients with ESCC were significantly higher than in patients with benign biliary strictures (BBS) (P<0.001). The receiver operating characteristic (ROC) curves of CA19-9, CA12-5 and CEA were 0.713, 0.554 and 0.451, respectively, were significantly lower than the ROC curves of bile sB7-H4 (0.837), the sensitivity of ERCP-based cytological/tissue examination was 57.5% and 68.4%, which was lower than that of bile sB7-H4 (81.7%) at cut-off value. A high level of bile sB7-H4 in patients with ESCC was found to be correlated with vascular invasion (P<0.001), lymph node metastasis (P<0.001) and TNM stage (P=0.018), respectively. The overall survival rate (OS) of ESCC patients in the high sB7-H4 group was significantly lower than the OS of patients in the low sB7-H4 group (P=0.009). Bile sB7-H4 could serve as a valuable biomarker for patients with ESCC and high levels of bile sB7-H4 correlate with poor clinical outcomes.

Keywords: B7-H4, biliarystrictures, cholangiocarcinoma, ERCP, prognosis

Introduction

Cholangiocarcinoma (CC) is a malignant tumor of the biliary tract originating from the duct epithelium. In the region of Asia, China, Japan and India are countries with highest CC morbidity, the rate of 5-year overall survival rate is about 5-10% and 5-year survival rate after surgery is 25-30% [1-3]. Particularly, in metastatic cases who lost their surgical opportunities, median survival is no longer than 8-12 months even under combined adjuvant chemotherapy [4]. Early diagnosis is the key to the possibility of operation and improving the survival rate of patients with CC.

However, differential diagnosis between malignant biliary strictures (MBS), especially early-stage cholangiocarcinoma (ESCC) and benign biliary stricture (BBS) has been a problem. For patients with biliary strictures, endoscopic retrograde cholangiopancreatography (ERCP) is the first-line examination and ERCP-based brush cytology (ETBC) and ERCP-guided transpapillary forceps biopsy (ETFB) can accurately diagnose malignant biliary strictures [5], but their sensitivity is not ideal. In the past decade, endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is considered effective to improve the diagnostic accuracy in CC patients negative for malignancy by ETBC or ETFB [6]. However, there are some inherent disadvantages of EUS-FNA, such as time consuming and prone to bile leaks and tumour seeding. Therefore, it is necessary to exploit sensitive molecular biomarkers for ESCC with strictures to ensure early diagnosis.

B7-H4 is one member of the B7 family, as a transmembrane protein, B7-H4 plays an impor-

Table 1. The patient clinical characteristics

	BBS	ESCC	*P* value
Number	104	109	
Age, years	53.34±11.82	53.16±10.29	NS
Gender			NS
Male, n (%)	50 (51.7%)	57 (51.7%)	
Female, n (%)	54 (48.3%)	52 (48.3%)	
TBIL (μmol/l)	118.12±64.39	139.77±68.37	0.033
ALP (U/L)	178.17±61.85	312.37±74.82	0.002
ALT (U/L)	105.77±25.99	115.96±26.31	0.041
AST (U/L)	89.84±24.87	98.30±23.62	0.033
CA19-9 (U/ml)	112.60±35.91	148.39±49.58	<0.001
CA12-5 (U/ml)	83.02±41.46	94.93±47.97	<0.001
CEA (ng/ml)	14.62±6.65	16.58±6.30	0.011
Stricture location			NS
Proximal	39 (37.5%)	44 (40.4%)	
Middle	38 (36.5%)	36 (33.0%)	
Distal	27 (26.0%)	29 (26.6%)	

BBS: benign biliary strictures; ESCC: early-stage cholangiocarcinoma; TBIL: total bilirubin; ALP: alk phosphatase; ALT: alanine aminotransferase AST: aspartate aminotransferase; NS: not significant.

tant role in T cell-mediated immune inflammatory responses [7], and increasing studies found that highly expressed B7-H4 in tumour cells, including lung [8], gastric [9] and breast [10], is closely related to adverse clinical outcomes. Recently, soluble B7-H4 (sB7-H4) has been detected in peripheral blood from patients with cancer, including ovarian cancer [11, 12], gastric cancer [13] and renal cell carcinoma [14], and the expression level of serum sB7-H4 in tumour patients was significantly higher than healthy volunteers and was closely related to malignant progression and prognosis, which suggested sB7-H4 is a useful tumour marker for cancer diagnosis and prognostic evaluations [15, 16]. In the tumour microenvironment, whether CC cells that cause biliary strictures can release sB7-H4 into bile, the level of sB7-H4 in bile from ESCC is higher than BBS, and the expression of sB7-H4 in bile is associated with bile duct cancer malignant progression and prognosis remain unclear.

In the current study, bile was collected in patients with biliary strictures when patients were undergoing ERCP examination, and the diagnostic accuracy was compared bile sB7-H4, routine tumor markers, ETBC and ETFB between BBS and ESCC. Additionally, we collected the data of clinical parameters of ESCC and assessed correlations between bile sB7-H4 levels and clinical progression of ESCC.

Materials and methods

Consecutive patients diagnosed with biliary strictures and treated from June 2009 to June 2014 in the Union Hospital of Huazhong University of Science and Technology were enrolled prospectively in this study. Patients included in this study underwent routine diagnostic procedures, including abdominal ultrasound, abdominal CT scans and biliary tract MRI, and could not be ruled out. Then, ETBC or ETFB were performed first, and bile was collected during the ERCP. In patients with negative cytology/histology after the first attempt of ETBC or ETFB, EUS-FNAB was performed.

Unoperated patients considered negative for CC were noted on follow-up imaging studies for at least 6 months, if any deterioration of the patient's general health was found, a second attempt of ETFB was executed. Diagnostic accuracy is defined as the ratio of the total of ture-positive and ture-negative divided by the sum of specimens. After ETBC, ETFB or EUS-FNAB was performed, all patients received at least 1 day of follow-up examinations with laboratory and radiologic tests.

For patients with positive cytology/histology undergoing radical resection of tumour or palliative surgery, the tumour specimens were confirmed again by pathological examination. Patients diagnosed with CC were classified according to the tumour-node-metastasis (TNM) classification (UICC, 2010) [17], only CC patients with TNM stage I or II were included. The study was approved by the Ethics Committee of Huazhong University of Science and Technology for Clinical Investigation and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the patients were followed up for 3 years with detailed records of the clinical data.

Bile acquisition and storage

During ERCP, after the common bile duct cannulation and before the injection of contrast agent, the catheter was successfully passed by

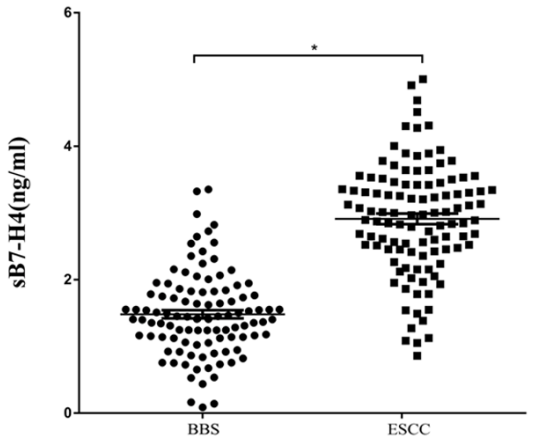

Figure 1. Detection of sB7-H4 levels in bile samples. The concentration of sB7-H4 in the bile of MBS was significantly higher than in BBS (*P<0.001).

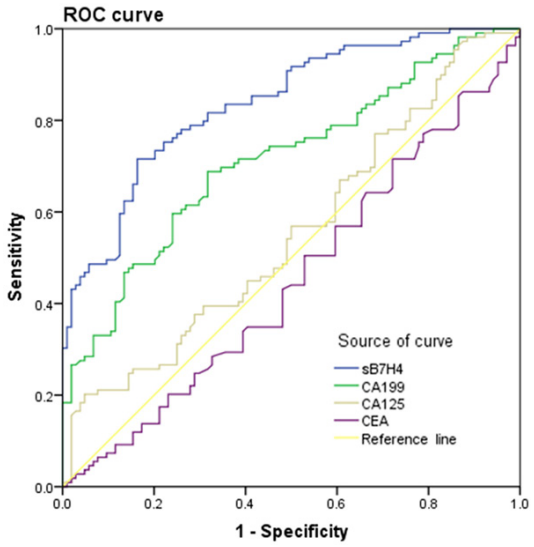

Figure 2. Comparison of the diagnostic value of bile sB7-H4, CA19-9, CA12-5 and CEA for MBS. The AUC values of bile sB7-H3, CA19-9, CA12-5 and CEA were 0.837, 0.713, 0.554 and 0.451, respectively.

the obstructing lesion into the proximal bile duct and collected 1-3 ml of bile through the sphincterotome and into a sterile syringe. Bile samples were centrifuged for 1000 g for 15 min at 4°C, then divided into 5 tubes and stored at -70°C. Every three months, the sB7-H4 concentration in the bile was detected and recorded.

Detection of sB7-H4

The level of sB7-H4 in the bile was detected using an sB7-H4 ELISA kit purchased from R&D Systems (Minneapolis, MN, USA) and in accordance with the operating instructions for processing. Briefly, all the bile samples were added in to the microplate strips and incubated for 2 h. We then aspirated each well and washed four times. We transferred 200 µl of B7-H4 conjugate to each well and incubated for another 2 h at room temperature, then removed the B7-H4 and washed. Next, we added 200 µl of substrate solution to each well and incubated for 30 min, avoiding light. Finally, we added 50 µL of Stop Solution to each well, and the sample's absorbance was detected at 450/540 nm.

Statistical analysis

Differences between the BBS and ESCC groups were evaluated by chi-square test, Fisher's exact test or Mann-Whitney U-test. The diagnostic value of sB7-H4 in differentiating between BBS and ESCC was assessed by receiver operating characteristic (ROC) curves. The Kaplan-Meier method was used to assess the survival curves, and the log-rank test was performed to detect the differences in survival rates. Related factors of prognosis in ESCC were analysed with univariate and multivariate analyses (Cox regression models), and covariates with *P* value less than 0.05 were included in the multivariate analysis. *P* values <0.05 were considered statistically significant. SPSS software version 20.0 (SPSS, Chicago, IL, USA) was used for the statistical analyses.

Results

Detection of sB7-H4 expression in bile from patients with bile duct strictures

A total of 213 patients with duct strictures were enrolled in the study. According to the ETBC and ETPB, EUS-FNAB, postoperative specimen pathological reports and the results of follow-up imaging, all enrolled patients reached an exact diagnosis and were divided into two groups BBS (n=104) and ESCC (n=109). The clinical characteristics of the two groups are shown in **Table 1**. The data of CA19-9, CA12-5, CEA and other blood biochemical examination results were obtained from the clinical laboratory before the patients received any treatment. The serum CA19-9 levels were the highest in the ESCC (148.39±49.58 U/ml) group followed by the CA12-5 levels (94.93±47.97 U/

Table 2. Diagnostic performance of sB7-H4, CA199, CA125, and CEA for ESCC

Group	Cut off value	Sensitivity (%)	Specificity (%)	AUC	*P* Value*	95% CI
sB7-H4	1.86 ng/ml	81.7	69.1	0.837		0.785-0.889
CA19-9	126.91 U/ml	66.1	68.3	0.713	<0.001	0.645-0.782
CA12-5	104.34 U/ml	37.6	70.2	0.554	<0.001	0.477-0.631
CEA	17.87 ng/ml	20.2	76.0	0.451	<0.001	0.374-0.528

*P<0.05 for comparison of AUC values between sB7-H4 and CA199, sB7-H4 and CA125, sB7-H4 and CEA.

Table 3. Diagnostic performance of ETBC or ETFB on ESCC, either alone or combined with sB7-H4

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
BC	54.2	100	100	67.5	76.5
TFB	66.9	100	100	74.3	83.1
sB7+BC	83.4	100	100	85.2	91.5
sB7+TFB	88.1	100	100	98.9	93.9

ETBC: brush cytology; ETFB: transpapillary forceps biopsy; sB7:sB7-H4; NPV: negative predictive value; PPV: positive predictive value.

ml), and CEA levels (16.58±6.30 ng/ml) compared to the BBS group. The other biochemical indexes of ALT, AST, ALP and total bilirubin of ESCC also showed significant differences compared to BBS. According to the narrow point of bile duct strictures, the patients were divided into the following 3 groups: proximal, middle and distal. The numbers of BBS in the three groups were 41, 39 and 29, respectively, whereas the numbers of ESCC were 39, 37 and 28. There was no significant difference between BBS and ESCC.

We extracted the sB7-H4 data of the related patients from the database and analysed these data. As shown in **Figure 1**, the sB7-H4 levels in the bile of patients with ESCC (2.912± 0.079 ng/mL, with a range of 0.859 to 5.006 ng/mL) were significantly higher than in patients with BBS (1.483±0.061 ng/mL, with a range of 0.084 to 3.355 ng/mL, *P*<0.001).

The diagnostic value of bile sB7-H4 in ESCC

To estimate the diagnostic and differential diagnosis value of sB7-H4 in BBS and ESCC, ROC curves analyses were performed to assess. As shown in **Figure 2**, the area under the ROC curves of bile sB7-H4 was 0.837, and the 95% confidence interval (CI) reached 0.785-0.889. The highest efficacy was detected when 1.86 ng/ml was adopted as the cut-off, the sensitivity was 81.7% and specificity was 69.1%. The negative predictive value (NPV) was 78.3%, and the positive predictive value (PPV) was 73.5% according to the EUS-FAN and postoperative pathological results.

Furthermore, when we compared the bile sB7-H4 levels and conventional tumour markers including serum CA19-9, CA12-5 and CEA levels, we found the areas under the ROC curves of CA19-9, CA12-5 and CEA were 0.713, 0.554 and 0.451, respectively, which were significantly lower than that of bile sB7-H4. The overall sensitivity and specificity of the diagnosis of ESCC were significantly lower than bile sB7-H4 (**Table 2**).

Diagnostic power of the bile sB7-H4 level, ETBC and ETFB in MBS

ETBC or ETFB is routine practice in patients with suspected MBS, and although it is easy to perform, its lower sensitivity limits its clinical value. According to the set of diagnostic criteria, the sensitivity, specificity, NPV, PPV and accuracy of ETBC or ETFB alone or combined with sB7-H4 in differentiating MBS patients from BBS was analysed (**Table 3**). We determined that the sensitivity of ETBC and ETPB was 54.1% and 66.9%, which was far below than that of ETBC or ETPB combined with sB7-H4 in bile (83.4% and 88.1%), respectively. These results indicate that ETBC or ETFB combined with sB7-H4 was better than ETBC or ETFB alone in differentiating ESCC patients from BBS patients.

Correlation between bile sB7-H4 concentration and clinical characteristics in ESCC

Based on the ELISA result of sB7-H4, we used sB7-H4 (1.86 ng/ml) as the cut-off value and divided patients with ESCC into groups with high (n=80) and low (n=29) sB7-H4 levels. The correlation between bile sB7-H4 levels and various clinicopathologic features and tumour

Table 4. Clinicopathological characteristics of ESCC

Characteristics	Number	sB7-H4 level		*P* value
		High, n (%)	Low, n (%)	
Age, years				NS
<60	57	45 (78.9%)	12 (21.1%)	
≥60	52	35 (67.3%)	17 (32.7%)	
Gender				NS
Male	53	39 (73.6%)	14 (26.4%)	
Female	56	41 (73.2%)	15 (26.7%)	
Stricture location				NS
Proximal	41	29 (70.7%)	12 (29.3%)	
Middle	39	28 (71.8%)	11 (28.2%)	
Distal	29	23 (79.3%)	6 (20.7%)	
Vascular invasion				<0.001
Negative	28	9 (32.1%)	19 (67.9%)	
Positive	81	71 (87.7%)	10 (12.3%)	
Lymph node metastasis				<0.001
Without	32	12 (37.5%)	20 (62.5%)	
With	77	68 (88.3%)	9 (11.7%)	
TNM stage				0.018
I	51	32 (62.7%)	19 (37.3%)	
II	58	48 (82.8%)	10 (17.2%)	

NS: not significant.

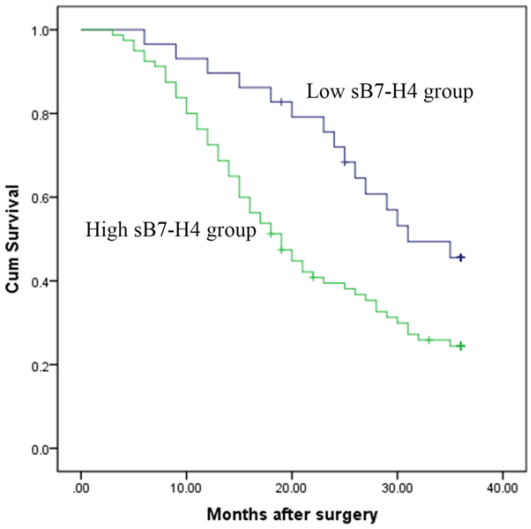

Figure 3. OS rates for MBS patients in relation to the bile sB7-H4 level. All MBS patients were divided into high (n=124) and low (n=25) sB7-H4 groups. The survival rate of the MBS patients in the highsB7-H4 group was significantly lower than that of the patients in the low sB7-H4 group (P=0.009).

related factors was evaluated. Patients diagnosed with ESCC were classified according to TNM classification. As shown in **Table 4**, a high level of bile sB7-H4 in patients with ESCC was found to be correlated with vascular invasion (*P*< 0.001), lymph node metastasis (*P*<0.001) and TNM stage (*P*= 0.018). However, there is no statistically significant correlation between sB7-H4 level and age or gender, which suggests that sB7-H4 levels might be closely related to the vascular invasion and lymph node metastasis of patient with ESCC.

Correlation between bile sB7-H4 levels and prognosis of ESCC

The median survival times of patients with ESCC with high and low sB7-H4 levels were 20.37 months and 27.24 months, respectively. The overall survival (OS) of patients in the high sB7-H4 group was significantly lower than patients in the low sB7-H4 group (**Figure 3**, *P*=0.009). As shown in **Figure 4**, the univariate analysis revealed that the factors impacting the OS were vascular invasion, lymph node metastasis and TNM stage and bile sB7-H4 levels (*P*= 0.021, *P*=0.015, *P*=0.001, *P*=0.012, respectively). The multivariate Cox regression analysis showed that the death risk of patients in the high sB7-H4 group was significantly higher (*P*=0.025), which was the same in patients with vascular invasion, lymph node metastasis and higher TNM stage (*P*=0.024, *P*=0.021, *P*=0.011, respectively).

Discussion

Stenosis is a common disease in the bile duct system, the main cause of malignant stenosis is CC, and the biological characteristics of infiltration along the bile duct wall make it difficult to distinguish it from benign stenosis. Routine examination methods such as transabdominal ultrasound, CT, MRI/ERCP and other methods were also poor ability to distinguish, often not be used as a clear basis for diagnosis [18], and the sensitivity of ERCP or EUS-FNA is also low [19], seriously affecting the accuracy of clinical diagnosis. Patients with suspected MBS have to undergo ERCP examination repeatedly or fur-

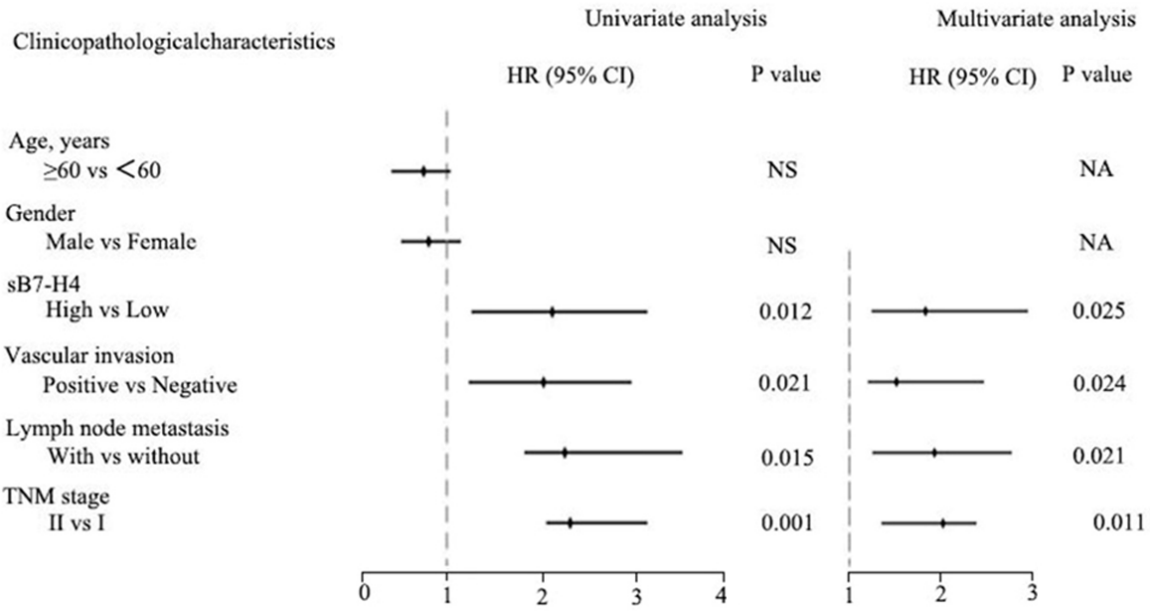

Figure 4. Univariate and multivariate analyses of OS in ESCC patients. The death risk of patients with high bile sB7-H4 level, vascular invasion, lymph node metastasis and higher TNM stage was significantly higher than patients without that through univariate and multivariate analyses. OS: overall survival; HR: hazard ratio; NS: not significant; NA: not adopted.

ther receive EUS-FNA, which increases patients' medical costs and suffering, and clinical decisions might be delayed for several weeks to months at a time [20]. Therefore, improving the sensitivity and accuracy of patients with suspected MBS, especially the ESCC, will be conducive to our clinical decisions and treatment.

Biomarkers (detected in the serum or other body fluids) are under intense scrutiny throughout numerous clinical studies. Their diagnostic and prognostic values are studied with the hope to identify early-stage carcinoma or to differentiate benign and malignant disease, as well as to guide treatment [21]. Serum or bile CA19-9 concentration is the most used tumor markers in clinical practice for CC detection. However, a wide range of sensitivity (50-90%) and specificity (54-98%) of this biomarker for CC has been reported [22-24], and the elevated serum or bile CA19-9 has also been detected in patients with benign diseases, including calculus of bile duct, cholangitis and nonmalignant jaundice, therefore, the diagnosis accuracy of CA19-9 for differentiating CC and BBS is not reliable [25, 26]. Other biomarkers including CA12-5 and CEA in serum or bile have also been applied to detect CCA, but both of them are not satisfactory for CCA diagnosis due to low specificity and sensitivity [27, 28]. In addition, several studies have shown that CA19-9, CA12-5 and CEA are not satisfied with the diagnosis of ESCC [29, 30]. Hence, explore new tumor markers would be beneficial in the diagnosis and management of CC.

The study showed that the sB7-H4 concentration in the bile of ESCC was significantly higher than that in BBS ($P<0.001$). Compared to conventional the serum tumour markers CA19-9, CA12-5 and CEA, the diagnostic and differential diagnosis performance of bile sB7-H4 at the cut-off levels were significantly higher in differentiating BBS and ESCC than the first three. These results indicate that the bile sB7-H4 has a good diagnostic performance in the differential diagnosis of BBS and ESCC, and sB7-H4 in the bile might serve as a potential biomarker for patients with ESCC. Collecting bile and testing the sB7-H4 concentration when the bile duct stenosis patients are undergoing ERCP, and then using these findings separately or combined with ERCP for diagnosis and differential diagnosis in patients suspected ESCC makes a simple method and does not increase pain for the patients.

ERCP-based cytological/tissue examination is the basic and primary method of diagnosing patients with suspected MBS [31], observing

the narrow point and the degree of stenosis and obtaining cell and tissue samples with a cytology brush or forceps biopsy to obtain a definite diagnosis has the advantage of high specificity in patients with suspected MBS [32]. However, the diagnostic yield of ETBC or ETFB for biliary stricture has been reported to be unsatisfactory, mainly because of the following reasons: size and type of stricture, location, cytology preparation and interpretation, and the experience and skill of the endoscopist [33, 34]. In this study, bile was gathered through ERCP, and bile sB7-H4 was detected for the diagnosis of ESCC, overcoming the above shortcomings and enhancing the efficiency of the ESCC initial diagnosis; the diagnostic sensitivity and accuracy were well above ETBC or ETFB. Additionally, this method could reduce the incidence of further EUS-FNA detection after ETBC or ETFB and save expenses for patients.

The present study revealed a link between the bile sB7-H4 level and clinicopathological characteristics of patients with ESCC. We found that the level of sB7-H4 in bile was closely related to vascular invasion, lymph node metastasis and TNM staging of the ESCC, suggesting a full estimate the difficulty of surgical risk and possibility of palliative operation and indicates that the high sB7-H4 group could select strong targeted chemotherapy after surgery, and the low sB7-H4 group could choose modest postoperative chemotherapy. Additionally, our study investigated the relationship between the level of bile sB7-H4 and the OS in patients with ESCC, and the result showed that the 3-year OS in high sB7-H4 group was significantly lower than that in the low sB7-H4 group. Moreover, the multivariable analysis confirmed that sB7-H4 from bile is an independent factor influencing postsurgical survival time in patients with ESCC. These results indicate that measuring sB7-H4 levels in bile might provide insight into the prognosis of patients with ESCC.

It is worth noting that in this study, we did not evaluate B7-H4 expression for the correlation between bile sB7-H4 expression levels and B7-H4 expression level in tumour tissues. Therefore, it is unclear whether tumour cells could secrete sB7-H4 into bile. However, the results that the level of bile sB7-H4 in the ESCC patients was significantly higher than in BBS patients, suggesting that bile sB7-H4 in ESCC patients is released in large amounts from tumour cells. In addition, all ETBC or ETFB examinations were performed by two highly experienced endoscopists, and results could be different in other institutions. These research findings are based on a single prospective study centre, and the diagnostic and prognostic value of bile sB7-H4 for patients with ESCC needs a further multicentre study to be confirmed.

The present study demonstrated that bile sB7-H4 levels can be considered an index for the differential diagnosis of BBS and ESCC. Moreover, sB7-H4 can be used as a biomarker for diagnosis and prognosis in patients with ESCC.

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Disclosure of conflict of interest

None.

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