

# Increased expression of cystathionine beta-synthase and chemokine ligand 21 is closely associated with poor prognosis in extrahepatic cholangiocarcinoma

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## Abstract

The expression of Cystathionine beta-synthase (CBS) and Chemokine ligand 21 (CCL21) is associated with the tumorigenesis and progression of a variety of tumors, but whether alterations in their expression levels correlates with the carcinogenesis and progression of EHCC is still unknown. This study investigated the clinicopathological significance of CBS and CCL21 expression in EHCC.

We investigated the correlations between the expression of CBS and CCL21 and clinicopathological characteristics in EHCC using EnVision immunohistochemistry.

The expression of CBS and CCL21 was significantly higher in EHCC tumors than in nontumor tissues ( $P < .05$  and  $P < .01$ ). EHCC patients with CBS and CCL21 expression combined with lymph node metastasis, tumor cell invasion, and TNM III/IV stage had more severe conditions than those with no lymph node metastasis, distant invasion and TNM I/II stage ( $P < .01$ ). Kaplan-Meier survival analysis showed that the overall survival rates for EHCC patients with negative CBS or CCL21 reaction were significantly higher than those for patients with positive CBS or CCL21 reaction ( $P < .01$ ). CBS or CCL21 expression was revealed as an independent poor prognostic factor for EHCC patients by Cox multivariate analysis.

The present study indicates that CBS and CCL21 expression is closely associated with the pathogenesis of clinical, pathological and biological behaviors and poor prognosis in EHCC.

**Abbreviations:** CBS = cystathionine beta-synthase, CCL21 = chemokine ligand 21, CI = confidence interval, EHCC = Extrahepatic cholangiocarcinoma, RR = relative risk, TNM = tumor-node-metastasis.

**Keywords:** cystathionine beta-synthase, chemokine ligand 21, extrahepatic cholangiocarcinoma, immunohistochemistry, prognosis

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## 1. Introduction

Cholangiocarcinoma (CCA) is a malignant tumor originating from the epithelium of the bile ducts that has a poor prognosis and comprises 3% of all gastrointestinal carcinomas.<sup>[1]</sup> Globally, CCA is more prevalent in Asia. Compared to Western countries, which have only 2.1 new cases per 100,000, the incidence of CCA in Asia is approximately 113/100,000.<sup>[2]</sup> The risk factors for CCA are thought to be related to viral hepatitis, cirrhosis, hepatolithiasis, primary sclerosing cholangitis, parasitic infections, and gene mutations.<sup>[3]</sup> Anatomically, CCA can be distinguished into intrahepatic CCA (IHCC, 8%) and extrahepatic CCA (EHCC, 90%). Based on the cystic duct, EHCC can further be characterized as perihilar (Klatskin) tumors, originating in the bile ducts that are confluent in the liver hilum (50%), or tumors of the distal bile duct (42%).<sup>[4]</sup> Because of its anatomic location, the diagnosis of EHCC is often incidental, and many patients who receive the first diagnosis are in advanced stages; the clinical manifestations of most patients (>90%) are obstructive jaundice.<sup>[5]</sup> Unlike superficial tumors, it is always difficult to obtain pathological documentation of CCA. Endoscopic retrograde cholangiopancreatography (ERCP) provides an option for tumor sampling for cytology; however, it should be mentioned that fine-needle aspiration should not be used in order to avoid

disseminating the tumor.<sup>[6]</sup> Thus, in general, only a minority of patients receive a cytologic diagnosis (15%–30%), and the diagnosis of EHCC usually relies on multidisciplinary approaches, including laboratory findings and high-quality abdominal imaging, such as computed tomography (CT) scans or magnetic resonance imaging (MRI).<sup>[7]</sup> There are four histologic grades of EHCC: well-differentiated, moderately differentiated, poorly differentiated, and undifferentiated. In addition to tumor grade,<sup>[8]</sup> independent predictors of patient survival and disease recurrence include lymphovascular space invasion, lymph node metastasis, and periductal tumor involvement.<sup>[9]</sup> Surgical resection is the first choice for EHCC patients, but many patients present with late-stage, unresectable disease. Therefore, further research on new therapeutic targets of EHCC is necessary.

CBS (cystathionine beta-synthase) is one of the heme-containing enzymes and, more importantly, is the first rate-limiting enzyme that catalyzes homocysteine to form the cysteine precursor cystathionine and hydrogen sulfide (H<sub>2</sub>S) in the transsulfuration pathway.<sup>[10]</sup> The aberrant expression of CBS was discovered to be strongly involved in various pathological and physiological progressions in various tumors.<sup>[11]</sup> In a study of colon cancer, researchers found that the expression of CBS in tumor specimens was markedly higher than that in patient-matched normal mucosa tissue; after using short hairpin RNAs (shRNAs) to suppress CBS expression in HCT116 cells, they found that cell proliferation, migration, and invasion were markedly decreased.<sup>[12]</sup> Evaluated by patient-derived tissue arrays and immunoblot analysis, Sen et al found that significantly increased levels of CBS expression in breast cancer when compared with their normal counterparts.<sup>[13]</sup> Interestingly, no consensus has been reached on the function of CBS. In colorectal cancer, it was considered a promoter, as the hydrogen sulfide produced by CBS through the transsulfuration pathway could promote cell growth, proliferation, and peritumor angiogenesis. After inhibition or genetic silencing of CBS, Jin found significant inhibitory activity against tumor cells both *in vitro* and *in vivo*, which could enhance the antitumor efficacy of anticancer therapeutics.<sup>[14]</sup> Conversely, Takano et al found that decreased CBS expression could promote glioma tumor formation, as it increases HIF-2 $\alpha$  protein levels and HIF-2 target gene expression.<sup>[15]</sup> Zhao et al also believed CBS to be a tumor suppressor gene since CBS mRNA levels were downregulated through hypermethylation in gastric cancer.<sup>[16]</sup>

Chemokines are small chemotactic cytokines that attract leukocytes for activation and migration and are currently correlated with the restriction of tumor growth and distant metastasis.<sup>[17]</sup> Chemokine ligand 21 (CCL21) and chemokine ligand 19 (CCL19) act as ligands interacting with C-C chemokine receptor 7 (CCR7), which plays a role in lymphocyte trafficking as well as an important role in the inflammatory response.<sup>[18,19]</sup> Regarding malignant diseases, CCR7 is highly expressed in non-small-cell lung cancer (NSCLC)<sup>[20]</sup>; moreover, once activated by CCL21 and CCL19, it could increase the tumorigenesis, proliferation, and invasion of tumor cells.<sup>[21]</sup> Accompanied by CCR7, CCL21 expression also correlates with poor prognosis and distant metastasis in breast cancer, thyroid cancer,<sup>[22]</sup> gastric cancer,<sup>[23]</sup> and colorectal cancer.<sup>[24]</sup>

CBS and CCL21 seem to have biological significance in the occurrence and development of many malignant diseases; nonetheless, their roles in EHCC remain to be clarified. Therefore, in our study, the expression of CBS and CCL21 was detected by immunohistochemistry in surgically resected

specimens, which contain EHCC, pericancerous tissues, adenoma, and normal biliary tract. Additionally, the clinicopathological significance and prognostic values of CBS and CCL21 expression were analyzed.

## 2. Material and methods

### 2.1. Case selection and clinical data

All research protocols were approved by the Ethics Committee for Human Research, Central South University. A total of 100 EHCC tissues, 30 peritumoral tissues, 10 biliary tract adenoma tissues, and 15 normal biliary tract tissues were randomly collected at the Second and Third Xiangya Hospitals, Central South University, from January 2001 to December 2014. All samples were obtained with informed consent. Based on the 7th TNM Classification of Malignant Tumors, the tumors were divided into clear staging classifications. All patients had a defined histological diagnosis of EHCC and were confirmed by two pathologists following the World Health Organization (WHO) tumor classification system. According to the World Health Organization criteria, the tumor differentiation degrees were defined as well, moderately and poorly differentiated. Fifteen normal biliary tract tissues were collected from voluntary organ donors of liver transplantation, and pathological examination revealed normal biliary tract tissues.

All tissues were fixed in neutral buffered formalin for 24 to 48 hours, embedded in paraffin, and cut into sections for micro examination and further processing. Survival data were calculated from the time of diagnosis to 30 months through letters and/or telephone calls, and patients who survived longer than 30 months were included in the analysis as censored cases.

### 2.2. Immunohistochemistry

Rabbit anti-human CBS and CCL21 polyclonal antibodies were purchased from Dako Corporation (Carpentaria, CA). The EnVision™ Detection Kit was purchased from Dako Laboratories (CA). Positive controls were provided with the EnVision™ Detection Kit. Immunohistochemistry (IHC) staining for CBS and CCL21 was performed using the EnVision™ Detection Kit (Dako Laboratories, CA). In brief, each section was deparaffinized and then incubated with 3% H<sub>2</sub>O<sub>2</sub> in the dark for 15 minutes. Heat-induced epitope retrieval was conducted with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 96°C for 30 minutes. The sections were incubated with rabbit anti-human CBS and CCL21 primary antibodies (1:100 dilution; Dako Corporation, Carpentaria, CA) at 37°C for 2 hours after they were soaked in PBS for 3 × 5 min. The sections were dehydrated, soaked in xylene, and mounted with neutral balsam. Five hundred cells from ten random fields were examined per section by 2 observers independently. An average of the percentages from these two observers was used for final evaluation. Cases with positive cells  $\geq 25\%$  were considered positive, whereas other cases were considered negative.<sup>[25–27]</sup>

### 2.3. Statistical analysis

SPSS 21.0 (Statistical Package for the Social Sciences, Version 21.0) was used to analyze the correlations between CBS and CCL21 expression and histological or clinical factors in EHCC by  $\chi^2$  test or Fisher exact test. Kaplan-Meier univariate survival analysis and the long-rank test were used to assess the overall

survival of patients with EHCC. To identify whether the expression of CBS and CCL21 was an independent prognostic factor of OS for EHCC, multivariate analysis was performed with the Cox proportional hazards model, and 95% confidence intervals were calculated.  $P < 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. The basic characteristics of the patients

As listed in Table 2, there were 100 cases of EHCC (61 males and 39 females, aged from 35 to 80 with a median age of 58.8 years), with 31% well-differentiated, 34% moderately differentiated and 35% poorly differentiated tumors. Surgery included radical resection for 54 patients (54.0%), palliative resection for 36 patients (36.0%) and biopsy for 10 patients (10.0%). The clinical stage of EHCC was partitioned as follows: 35 cases of stage I+II, 38 cases of stage III, and 27 cases of stage IV, which included 67 (67.0%) cases of EHCC with distant metastasis, 38 (38%) cases of EHCC with regional lymph node metastasis, and 31 (31%) EHCC patients with gallstones. Fifty-nine (59%) patients died within 12 months, 24 (24%) patients died within 24 months, 9 (9%) patients died within 30 months, and 8 (8%) patients survived longer than 30 months and were included in the analysis as censored cases.

The pathological examination of 30 pericancerous tissues showed that 12 cases were normal tissues, 8 were mild dysplasia, 6 were moderate dysplasia and 4 were severe dysplasia. The biliary tract adenoma tissues collected from six males and four females showed 6 simple adenoma tissues, 2 mild dysplasia and 2 moderate to severe dysplasia.

#### 3.2. CBS and CCL21 protein expression in EHCC, peritumoral tissues, adenoma, and normal tissues

We evaluated the expression and cellular locations of CBS and CCL21 by IHC. As shown in Figures 1 and 2, CBS and CCL21 showed strong positive granule expression in the cytoplasm. In the 100 cases of EHCC, a positive percentage of CBS or CCL21 was 55.0% and 56.0%, respectively, as shown in Figure 1A, B and Figure 2A, B. For the 30 peritumoral tissues, 9 were CBS positive (30.0%) and 8 were CCL21 positive (26.7%), as shown in Figure 1C and Figure 2C. In adenoma, the expression of CBS and CCL21 was found in 2 of 10 cases (20.0%), as shown in Figure 1D and Figure 2D. For normal tissues, CBS and CCL21 expression was negative. Compared with peritumoral, adenoma, and normal tissues, the positive rates of CBS or CCL21 were significantly higher in EHCC ( $P < .05$  and  $P < .01$ , respectively) (Table 1). Moreover, peritumoral tissues and adenoma with positive CBS and/or CCL21 expression exhibited moderate to severe dysplasia (Table 1).

#### 3.3. CBS and CCL21 protein expression was associated with the clinicopathological characteristics of EHCC

We further investigated the associations between the expression of CBS and CCL21 in EHCC and clinicopathological characteristics, such as age, histological type, and clinical stage, using the chi-square test. As shown in Table 2, the expression of CBS and CCL21 exhibited no significant association with sex, age or tumor site ( $P > .05$ ). Positive rates of CBS and CCL21 expression in patients with advanced clinical stages (TNM stage III/IV) and

no resection (biopsy only) were markedly higher than that of patients in early clinical stages (TNM stage I+II) and in those who underwent radical resection ( $P < .005$ ). Compared to cases with lymph node metastasis, the expression of CBS and CCL21 was significantly lower in cases with no lymph node metastasis ( $P < .01$ ). Similarly, distant organ invasion also caused a higher expression of CBS and CCL21 in EHCC. CBS expression was significantly lower in cases with good differentiation than in those with poor differentiation ( $P < .01$ ). Interestingly, compared with EHCC patients with no bile stones, there was a higher expression of CBS in EHCC patients with bile stones ( $P = .01$ ). EHCC patients with tumor diameters  $\leq 3$  cm showed a significantly higher expression of CCL21 than those with tumor diameters  $> 3$  cm ( $P < .05$ ).

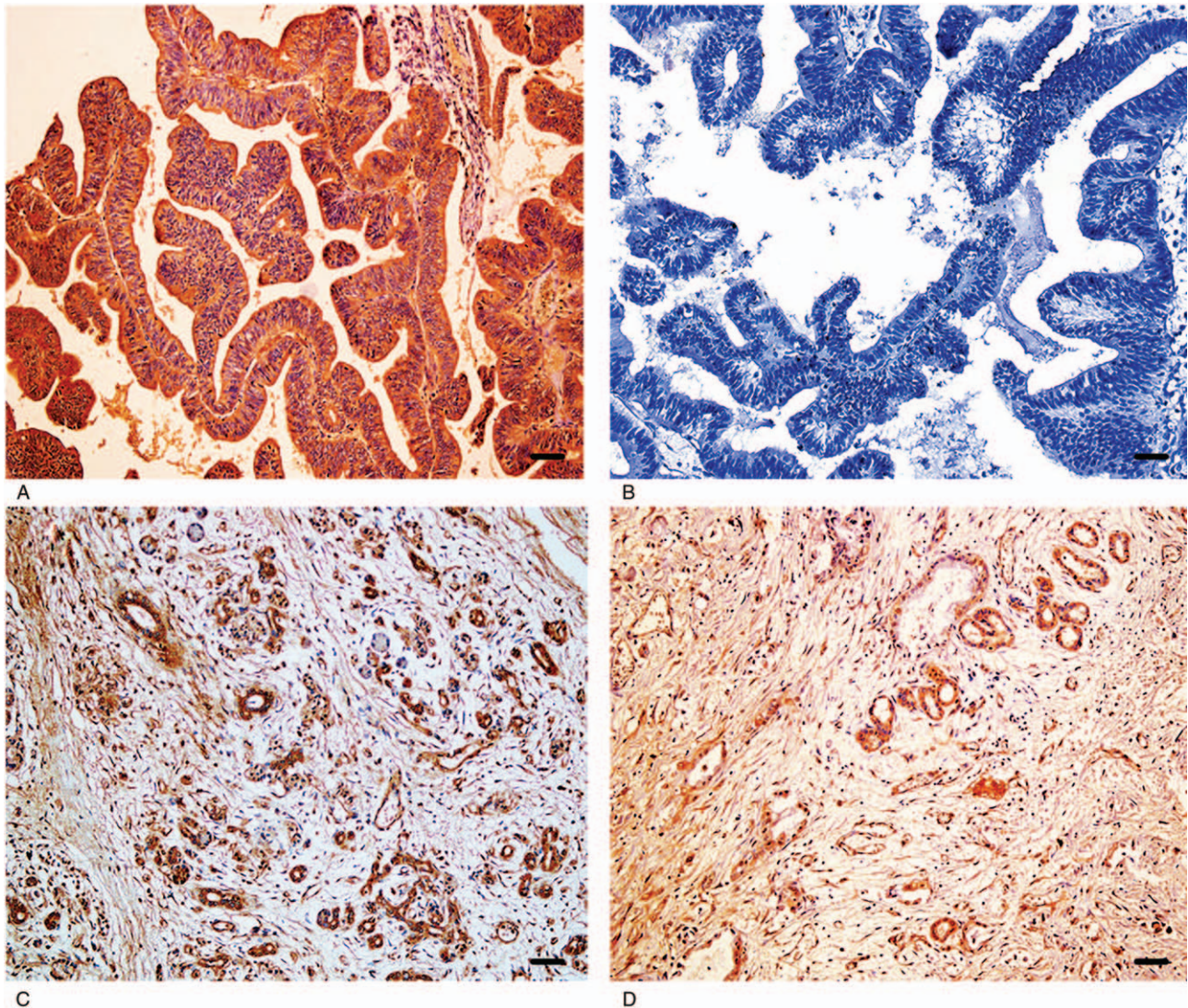
We further analyzed the relationship between CBS expression and CCL21 expression in EHCC. Among the 55 cases with positive CBS staining, 41 cases had positive CCL21 staining. Among the 45 cases with negative CBS staining, thirty cases had negative CCL21 staining. The expression of CBS was positively correlated with CCL21 ( $\chi^2 = 17.060$ ,  $P < .001$ ).

#### 3.4. CBS and CCL21 protein expression correlated with the poor overall survival rates of EHCC patients

Through careful follow-up, the detailed survival information of all 100 EHCC patients was collected. Fifty-nine patients died within 12 months, 24 patients died within 24 months, 9 patients died within 30 months, and 8 patients who survived longer than 30 months were analyzed as censored cases. Table 3 illustrates the Kaplan-Meier survival analysis for EHCC patients. There was a significant difference in the survival time of EHCC patients between those with negative and reaction of CBS and CCL21 (CBS: negative vs positive, 17.43 months vs 8.27 months; CCL21: negative vs positive, 16.34 months vs 9.30 months,  $P < .001$ ). Accompanied by the expression of CBS and CCL21, pathological characteristics such as differentiation ( $P < .001$ ), TNM stage ( $P < .001$ ), lymph node metastasis ( $P < .001$ ), invasion ( $P < .001$ ), and surgical procedure ( $P < .001$ ) were significantly associated with the average overall survival time of patients with EHCC (Table 3). Figure 3 also illustrates that the overall survival time for CBS- or CCL21-positive reaction EHCC patients was markedly lower than for those with negative reaction. To further investigate whether CBS or CCL21 are independent prognostic factors, we also carried out Cox multivariate analysis. The results showed that along with the expression of CBS and CCL21, poor differentiation, lymph node metastasis, invasion, and high TNM stage (III or IV) were positively correlated with mortality and negatively correlated with overall survival. The results revealed that positive CBS and CCL21 expression can be used as independent prognostic factors for EHCC patients ( $P = .02$  and  $P = .012$ , respectively) (Table 4). Finally, we depicted receiver operating characteristic (ROC) curves to evaluate the diagnostic efficacy of CBS expression and CCL21 expression. The AUC for CBS expression was 0.675 (95% CI: 0.588–0.762), and the AUC for CCL21 expression was 0.689 (95% CI: 0.604–0.774) (Fig. 4).

### 4. Discussion

Although the expression of CBS and CCL21 was associated with the progression and prognosis of a variety of tumors, no studies have investigated their expression and biological roles in EHCC.

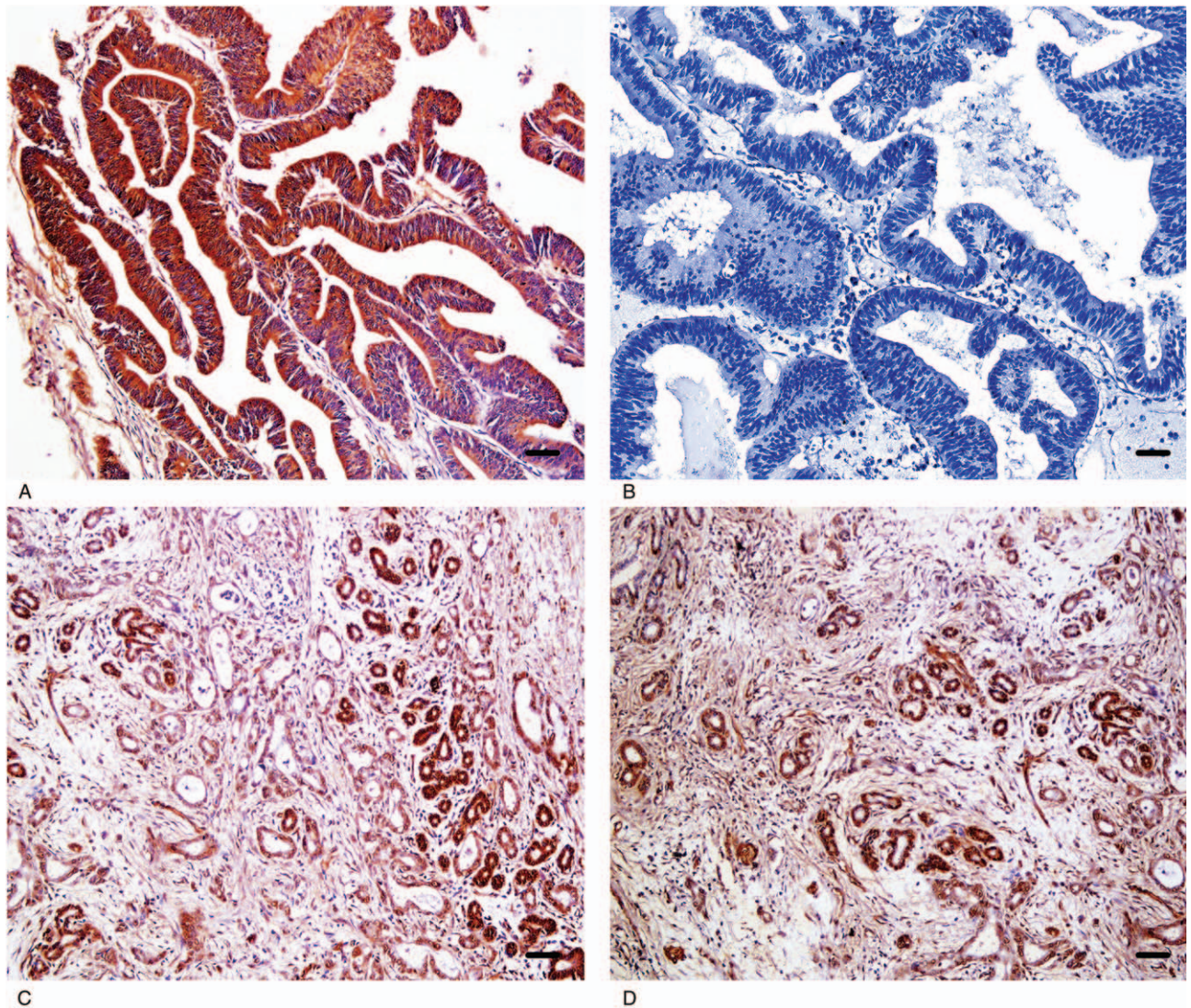


**Figure 1.** Immunohistochemical staining of CBS.  $\times 200$ . (A) Positive reaction of CBS in moderately differentiated EHCC. (B) Negative reaction of CBS in well-differentiated EHCC. (C) Positive reaction of CBS in pericancerous tissues. (D) Positive reaction of CBS in adenoma. Scale bars correspond to  $50\ \mu\text{m}$ .

As stated above, EHCC is a highly malignant tumor with poor prognosis. In our study, the mean survival time of patients with well differentiated tumors was much longer than that of patients with poorly differentiated tumors. Additionally, patients in early TNM stages (I/II) had a longer average survival time than those in advanced TNM stages (III or IV). Moreover, the mean survival time of patients who underwent radical surgery was drastically longer than that of patients who underwent biopsy surgery. This result indicates that early diagnosis and treatment can help improve patient outcomes. Therefore, it is urgent to find new specific diagnostic markers for the early diagnosis of EHCC. As the first analysis of the expression of CBS in EHCC, we found a significant increase in CBS and CCL21 expression in EHCC tumors. Moreover, CBS and CCL21 expression are associated with TNM stage, poor differentiation, lymph node metastasis, distant organ invasion and poor prognosis in EHCC.

As one of the “biologic gases”, the function of hydrogen sulfide has attracted much attention. It is mainly catalyzed by cystathionine- $\beta$ -synthase (CBS) and cystathionine- $\gamma$ -lyase (CSE), using cystathionine and homocysteine as zymolytes.<sup>[10]</sup>

In a study of ovarian cancer, Bhattacharyya et al found that CBS can serve as a potential therapeutic target for relapsing and drug-resistant tumors.<sup>[28]</sup> Chen et al also believe that the inhibition of  $\text{H}_2\text{S}$  synthesis by reducing the expression of CBS may be a potential target for the treatment of the acquired resistance of colorectal cancer to 5-FU.<sup>[29]</sup> Gai et al found that in human urothelial cell carcinoma of the bladder (UCB),  $\text{H}_2\text{S}$  and its synthase CBS increased as the degree of malignancy increased.<sup>[30]</sup> Similarly, our study showed that the expression of CBS was significantly increased in EHCC compared with noncancerous tissues and adenoma, which indicates that CBS could serve as a tumor promoter in EHCC. However, some authors found that the high expression of CBS might suppress the progression of some malignant lesions, such as gastrointestinal carcinoma and hepatocellular carcinoma, and lead to a better prognosis.<sup>[16,31]</sup> Kim et al found that the expression level of CBS in HCC specimens is markedly lower than that in noncancerous liver tissues.<sup>[31]</sup> Zhao et al also believed CBS to be a tumor suppressor gene since CBS mRNA levels were downregulated through hypermethylation in gastric cancer.<sup>[16]</sup> Thus, for now, no



**Figure 2.** Immunohistochemical staining of CCL21 × 200. (A) Positive reaction of CCL21 in moderately differentiated EHCC. (B) Negative reaction of CCL21 in well-differentiated EHCC. (C) Positive reaction of CCL21 in pericancerous tissues. (D) Positive reaction of CCL21 in adenoma. Scale bars correspond to 50 μm.

consensus has been reached on the function of CBS in malignant diseases. CBS may function to either promote or suppress tumor growth, depending on the cancer cell type, and its potential mechanism needs further study.

CCL21 is one of the chemokines secreted by secondary lymphoid organs, lymph nodes, and the spleen, sharing a common receptor, CCR7, with CCL19.<sup>[32]</sup> The binding of CCL21 and

CCR7 mediates T cell recruitment and differentiation. CCL21 is involved in immunosurveillance and plays an important role in antitumor immunity. Previous studies have shown that CCL21/CCR7 is closely related to lymph node metastasis and is involved in many events related to the tumorigenesis of malignant solid tumors, such as angiogenesis, adhesion, and proliferation.<sup>[20,33–35]</sup> However, no studies have investigated whether it has the same function in EHCC. Our study first demonstrated that the expression of CCL21 was significantly higher in EHCC patients with lymph node metastasis than in patients with no lymph node metastasis. In terms of its mechanism, Tutunea-Fatan et al found that the CCL21/CCR7 chemokine axis could promote the expression of the lymphangiogenic factor VEGF-C. The study suggested that it has direct lymphangiogenic potential by means of the VEGFR-3 signaling pathway, and blocking VEGFR-3 would reduce lymphatic endothelial cell (LEC) proliferation and tube formation.<sup>[36]</sup> Moreover, it promotes tumor-induced lymphovascular recruitment. Interestingly, the study found that the expression of CCR7 was significantly upregulated in breast cancer tissues compared to nontumor samples, while no obvious

**Table 1**  
**Comparison of CBS and CCL21 expression in normal tissue, adenoma, peritumoral tissue and EHCC.**

Tissue Type	Case No.	CBS Positive (%)	CCL21 Positive (%)
EHCC	100	55 (55.0)	56 (56.0)
Peritumoral tissues	30	9 (30.0)*	8 (26.7)**
Adenoma	10	2 (20.0)*	2 (20.0)*
Normal tissues	15	0 (0.0)**	0 (0.0)**

Compared to EHCC

\*  $P < .05$ .

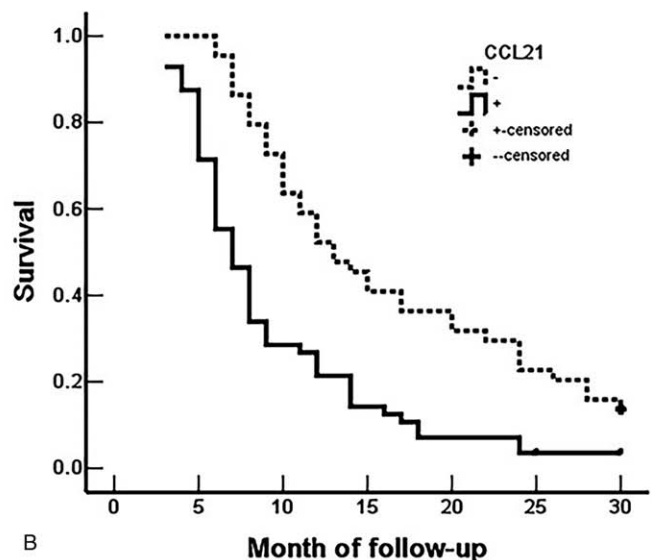
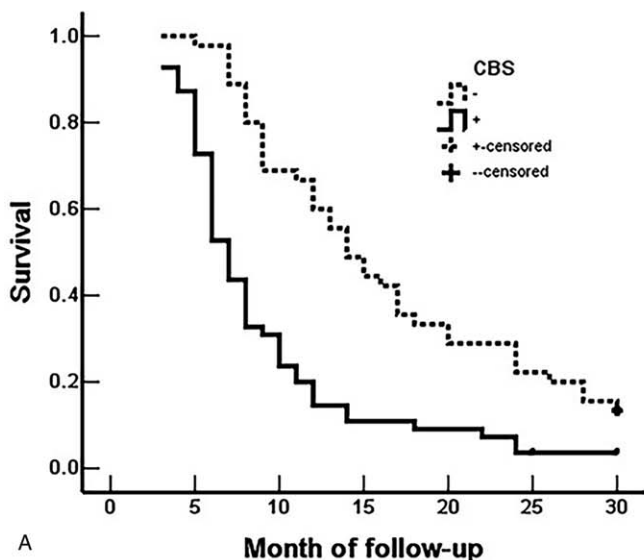
\*\*  $P < .01$ .

**Table 2**  
Correlations of CBS and CCL21 protein expression with the clinicopathological characteristics of EHCC.

Characteristics	Case No.	CBS			CCL21		
		Positive (%)	$\chi^2$	P value	Positive (%)	$\chi^2$	P value
Age (yr)							
≤45 years	17	7 (41.2)	1.581	.209	9 (52.9)	0.078	.780
>45 years	83	48 (57.8)			47 (56.6)		
Sex							
Male	61	35 (57.4)	0.357	.550	34 (55.7)	0.004	.947
Female	39	20 (51.3)			22 (56.4)		
Differentiation							
Well	31	9 (29.0)	12.891	.002	13 (41.9)	3.615	.164
Moderately	34	21 (61.8)			21 (61.8)		
Poorly	35	25 (71.4)			22 (62.9)		
Tumor size							
≤3cm	62	37 (59.7)	1.442	.230	40 (64.5)	4.802	.028
>3cm	38	18 (47.4)			16 (42.1)		
Tumor site							
Hilar site	27	17 (63.0)	6.687	.010	19 (70.4)	0.960	.619
Hepatic duct	4	4 (100.0)			2 (50.0)		
Distal duct	69	34 (49.3)			35 (50.7)		
Bile stone							
No	69	32 (46.4)	6.687	.010	36 (52.2)	1.322	.250
Yes	31	23 (74.2)			20 (64.5)		
Lymphnode metastasis							
No	62	23 (37.1)	21.130	.000	24 (38.7)	19.796	.000
Yes	38	32 (84.2)			32 (84.2)		
Invasion							
No	33	8 (24.2)	18.826	.000	12 (36.4)	7.708	.005
Yes	67	47 (70.1)			44 (65.7)		
TNM stage							
I+II	35	7 (20.0)	29.050	.000	12 (34.3)	16.040	.000
III	38	25 (65.8)			21 (55.3)		
IV	27	23 (85.2)			23 (85.2)		
Surgery							
Radical	54	20 (37.0)	15.496	.000	23 (42.6)	12.706	.002
Palliative	36	28 (77.8)			23 (63.9)		
Biopsy	10	7 (70.0)			10 (100.0)		

**Table 3**  
Correlations of clinicopathological characteristics, CBS and CCL21 expression with the mean survival in patients with EHCC.

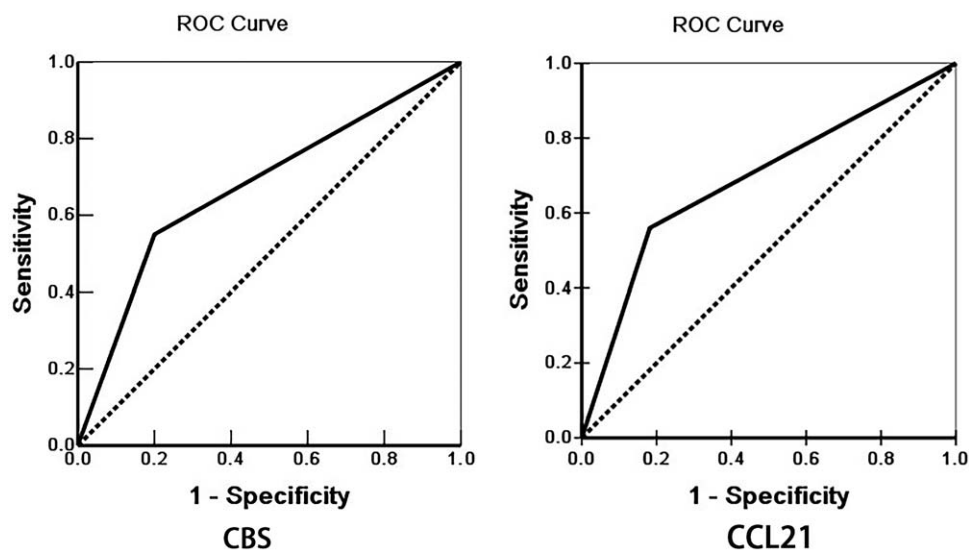
Group	Case No. (n)	Mean survival (month)	Chi-square	P value
Sex				
Male	61	12.67 (3–30)	0.001	.980
Female	39	12.59 (4–30)		
Age (yr)				
≤45	17	13.82 (3–30)	0.667	.414
>45	83	12.10 (3–30)		
Differentiation				
Well	31	18.46 (5–30)	27.655	.000
Moderately	34	11.41 (3–30)		
Poorly	35	7.97 (3–30)		
Tumor size				
≤3cm	62	12.62 (3–30)	0.235	.628
>3cm	38	12.03 (5–30)		
TNM stage				
I+II	35	18.57 (7–30)	57.569	.000
III	38	11.05 (3–30)		
IV	27	6.26 (3–13)		
Lymph node metastasis				
No	62	15.52 (4–30)	39.001	.000
Yes	38	7.18 (3–25)		
Invasion				
No	33	17.52 (4–30)	17.399	.000
Yes	67	9.87 (3–30)		
Surgery				
Radical	54	16.62 (3–30)	48.388	.000
Palliative	36	7.58 (4–24)		
Biopsy	10	6.90 (3–14)		
CBS				
-	45	17.43 (6–30)	37.125	.000
+	55	8.27 (3–24)		
CCL21				
-	44	16.34 (6–30)	18.503	.000
+	56	9.30 (3–30)		



**Figure 3.** CBS and CCL21 expression and survival in patients with EHCC. (A) Kaplan–Meier plots of overall survival in patients with CBS-positive and CBS-negative tumors. (B) Kaplan–Meier plots of overall survival in patients with CCL21-positive and CCL21-negative tumors.

**Table 4****Multivariate Cox regression analysis of survival rate in patients with EHCC.**

Groups	Factors	B	SE	wald	P	RR	95% CI	
							Lower	Upper
Differentiated degree	Well/moderately/poorly	0.575	0.155	13.762	.000	1.777	1.312	2.408
Tumor size	≤3 cm/>3 cm	0.501	0.246	4.148	.042	1.650	1.019	2.673
Lymph node metastasis	No/yes	0.690	0.311	4.922	.027	1.994	1.084	3.668
Invasion	No/Yes	0.769	0.354	4.719	.030	2.158	1.078	4.318
TNM stage	I/II/III/IV	0.781	0.270	8.367	.004	2.184	1.286	3.707
Surgery	Radical/ Palliative/ Biopsy	0.443	0.185	5.734	.017	1.557	1.084	2.238
CBS	-/+	0.661	0.284	5.417	.020	1.937	1.110	3.379
CCL21	-/+	0.704	0.281	6.277	.012	2.022	1.166	3.507

**Figure 4.** Multivariate analysis. ROC curves of diagonal segments are produced by ties of CBS (left) and CCL21 (right) in EHCC.

difference in CCL21 expression was observed.<sup>[36]</sup> Conversely, during tumor development, inhibition of the CCL21/CCR7 axis by a specific antagonist of CCL21 diminishes metastatic tumor formation.<sup>[37]</sup> The expression of CCL21 is detectable and associated with poor prognosis and distant metastasis in various human cancer types. Shields found a higher expression of CCL21 in human breast invasive ductal carcinoma than in normal tissue.<sup>[38]</sup> In a study of 48 circulatory inflammation markers, Henrik et al found that CCL21 was closely related to the pathophysiological characteristics of prostate cancer. Additionally, Zhong et al found that CCL21/CCR7 can trigger epithelial-mesenchymal transition (EMT) by activating the ERK1/2 signaling pathway, thereby promoting human lung cancer cell migration and invasion. In agreement with these reports, our study found that CCL21 expression was higher in cases with poor differentiation, lymph node metastasis, invasion, and advanced TNM stages, which means that CCL21 may contribute to the metastasis and development of EHCC.

In our present study, the results showed that the rates of CBS and CCL21 expression were significantly higher in EHCC than in cancerous tissues and adenoma. Adenoma and pericancerous tissues with CBS and/or CCL21 protein expression exhibited atypical hyperplasia. The CBS and CCL21 expression levels in EHCC patients with lymph node metastasis, tumor cell invasion, and TNM stage III/IV were higher than those in patients with no

lymph node metastasis and distant invasion and TNM stage I/II. Moreover, patients with positive reaction of CBS and CCL21 might have a lower survival probability than patients with negative CBS and CCL21 reaction, and multivariate analysis indicated that CBS and CCL21 expression are independent poor prognostic factors in patients with EHCC.

## 5. Conclusions

In conclusion, this is the first study to show that high expression levels of CBS and CCL21 are involved in the tumorigenesis and progression of EHCC and are positively related to poor OS rates in EHCC patients. Moreover, CBS and CCL21 expression was associated with poor prognosis in patients with EHCC. Therefore, it could be used as a novel prognostic marker for EHCC.

## Author contributions

Lingxiang Wang, Qiong Zou, Zhengchun Wu carried out the study and wrote the paper; Zhulin Yang designed the study and revised the paper; Shu Xu and Daiqiang Li performed the statistical analysis; Yuan Yuan and Jun He collected the specimens and experimental materials. All authors contributed to the data analysis, drafted or revised the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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