

## Original Article

# Interleukin-8 is a prognostic indicator in human hilar cholangiocarcinoma

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**Abstract:** Interleukin-8 (IL-8), matrix metalloproteinase-9 (MMP-9) and neovascularization have been implicated to be associated with biological processes, especially cancer progression. However, few studies have investigated the role of IL-8 in human hilar cholangiocarcinoma. In this study we detected the expression of IL-8 combined with MMP-9 and microvessel density (MVD) in hilar cholangiocarcinoma to evaluate their clinicopathological significance and prognostic value. A total of 62 patients with hilar cholangiocarcinoma who underwent curative surgery were enrolled in this study. The expression of IL-8, MMP-9 and MVD were examined immunohistochemically. The correlation of IL-8 with MMP-9 expression, MVD, clinicopathological features and survival time of patients were then analyzed. Expression of IL-8 was observed in 56.5% tumors, which was related to advanced TNM stage ( $P = 0.026$ ) and tumor recurrence ( $P = 0.018$ ). IL-8 had a positive correlation with MMP-9 expression and MVD. Furthermore, patients with high IL-8 expression had a significantly shorter overall survival than those with low IL-8 expression ( $P = 0.01$ ). Multivariate analysis confirmed IL-8 as an independent prognostic factor ( $P = 0.005$ ). In conclusion, IL-8 expression significantly correlated with MMP-9 expression and MVD, and IL-8 was a valuable prognostic factor for human hilar cholangiocarcinoma.

**Keywords:** Cholangiocarcinoma, IL-8, immunohistochemistry, prognosis

## Introduction

Hilar cholangiocarcinoma, also called Klatskin tumor, is the most frequently identified cholangiocarcinoma arising at the confluence of the right and left hepatic ducts [1]. Proximity of the porta hepatis and local invasion contribute to the worse prognosis of hilar cholangiocarcinoma than intrahepatic or distal cholangiocarcinoma [2]. Radical surgery, either in the form of hepatic resection or liver transplantation, is the only curative treatment. However, due to the specific location at the biliary tree, this fatal disease is often advanced at presentation, which precludes the effectiveness of conventional therapies [3]. What is worse, patients who undergo surgery still have to face a high risk of recurrence with a 5-year survival rate of 30-42% [4]. Furthermore, there is a lack of effective adjuvant therapies. Therefore, a bet-

ter understanding of molecular regulation involved in hilar cholangiocarcinoma progression is beneficial to better prediction of the patient outcome and new treatment strategies.

Interleukin-8 (IL-8), a member of the Cysteine-X-Cysteine (CXC) motif chemokines, is a multifunctional chemoattractant secreted by multiple cell types including cancer cells. The biological action of IL-8 is mediated through binding to two relative receptors, CXCR1 and CXCR2, both of which belong to cell-surface G-protein-coupled receptor (GPCR) family [5]. As a potent angiogenic, mitogenic and motogenic factor, IL-8 induces angiogenesis and promotes tumor growth, invasion and metastasis [6, 7]. As an autocrine/paracrine growth factor, IL-8 has been demonstrated to be highly expressed in a variety of tumors relative to normal tissues. In addition, numerous studies have defined the

role of IL-8 in tumor progression involving activation of multiple signaling pathways within various human cancers, including melanoma, colon, cervical, gastric and breast cancer [8]. However, few studies have focused on the study of IL-8 in hilar cholangiocarcinoma.

Degradation of the extracellular matrix (ECM) is essential for tumor invasion and metastases, and matrix metalloproteinases (MMPs) are potent proteolytic enzymes which play an important role in this process. MMP-9 is characterized by the potency to degrade Type IV collagen, which is a major component of basement membrane penetrated by tumor cells when they become invasive [9]. Previous studies have suggested the vital role of MMP-9 in malignancies, including cholangiocarcinoma [10, 11]. In addition to proteolytic enzymes, tumor-associated neovascularization has been shown to have a critical influence on cancer progression. The microvessel density (MVD) has been analyzed in hilar cholangiocarcinoma, where the MVD could serve as an independent prognostic factor for survival after curative resection [12].

In view of the restricted expression in normal tissues and upregulated expression in malignancies, IL-8 might be a promising therapeutic target for cancer treatment. To the best of our knowledge, this is the first study performed to detect the expression of IL-8 and investigate its relationship with MMP-9 expression and MVD in this disease. In our study, IL-8, MMP-9 expression and MVD in human hilar cholangiocarcinoma were examined by immunohistochemistry. The correlation of IL-8 with clinicopathological features and prognosis were evaluated to determine whether IL-8 expression levels could be used to predict the prognosis in patients with hilar cholangiocarcinoma.

### Materials and methods

#### *Antibodies and reagents*

The polyclonal antibodies against IL-8 was obtained from Novus Biologicals, rabbit anti-MMP-9 monoclonal antibody from Epitomics (California, USA), monoclonal antibody antihuman CD31 from Dako (Glostrup, Denmark), biotinylated anti-mouse and anti-rabbit IgG from Dako (Copenhagen, Denmark), and 3,3-diaminobenzidine (DAB) staining reagent was obtained from Beyotime (Shanghai, China).

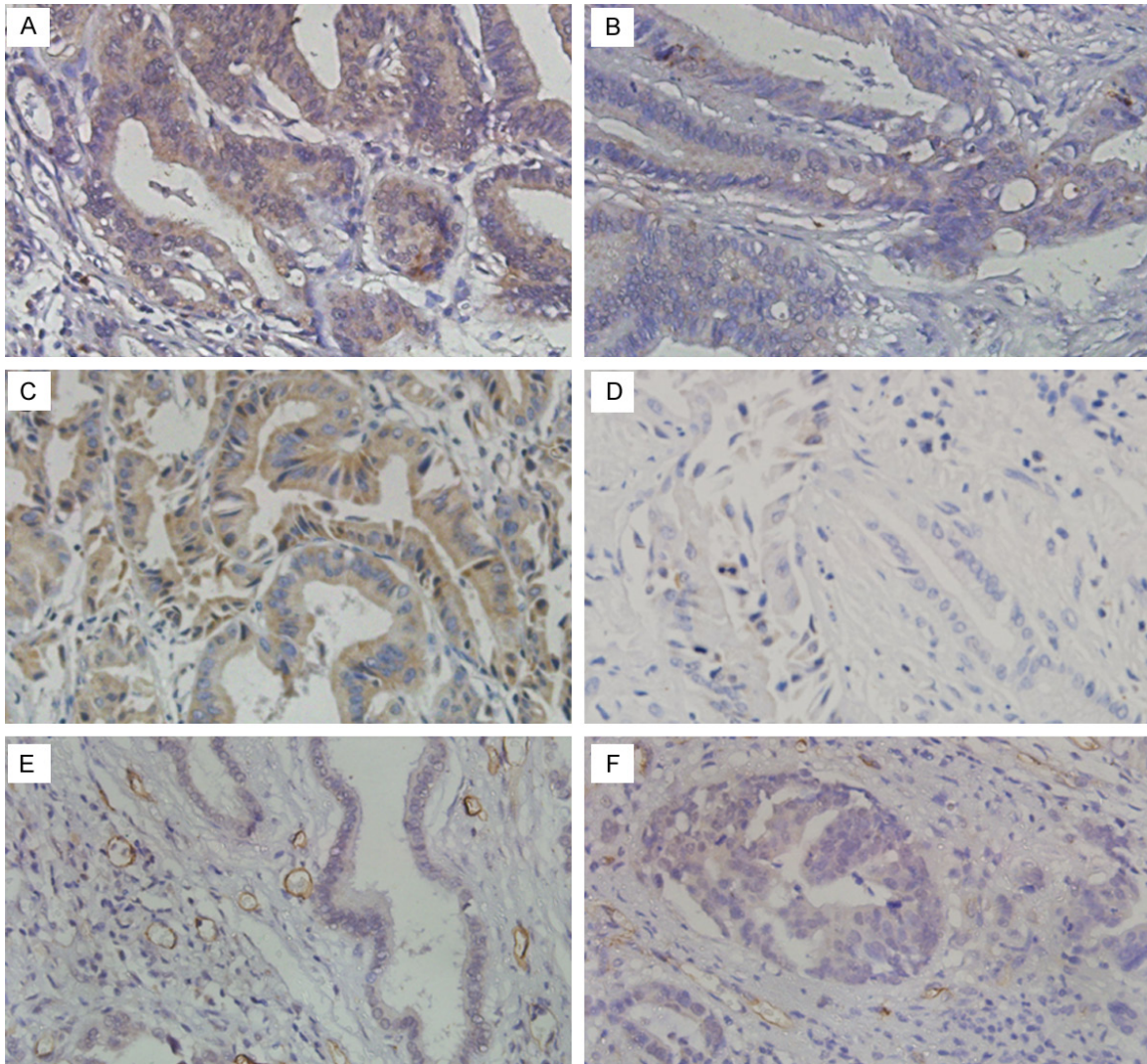
#### *Clinical samples*

The study was approved by The Committee of Ethics in Qilu Hospital, Shandong University. A total of 62 patients diagnosed as hilar cholangiocarcinoma who underwent curative surgery in Qilu Hospital of Shandong University were compiled consecutively from January 2006 to December 2012. There were 47 males and 15 females, and median age of the patients was 58 years old (range 37-79). No patient had received any pre- or post-surgery treatment, such as chemotherapy and radiotherapy. Patients who died within 90 days of surgery and died from other diseases were excluded. Medical records of these patients were reviewed for extraction of clinical and histopathological information. Follow-up began on the date of surgery and ended in July 2013. The median follow-up period of time for these patients was 15 months (range 5-98 months). Regular history and physical examinations of all patients were conducted on the outpatient clinic. Routine radiological examinations including abdominal CT and MRI were performed when necessary.

#### *Immunohistochemistry*

Representative formalin-fixed, paraffin-embedded tissue was retrieved from the archives of the Department of Pathology, Qilu Hospital of Shandong University. Tissue samples were sectioned successively into 5 µm thick for staining. Immunohistochemical staining was performed with the streptavidin peroxidase complex method. Briefly, the slides were dewaxed and rehydrated with xylene and graded alcohol. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 20 min at room temperature. Optimal antigen retrieval was done in citrate buffer (pH 6.0) at 98°C for 15 min in the microwave. The slides were treated with 5% blocking serum for 30 min at 37°C to block nonspecific reactions. After that, slides were incubated with primary antibody anti-IL-8 (dilution 1:200), anti-MMP-9 (dilution 1:50) antibody or antihuman CD31 (dilution 1:100) overnight at 4°C. The following day, incubated with biotinylated rabbit anti-mouse or goat anti-rabbit IgG for 30 min at 37°C, the slides were subsequently treated with streptavidin peroxidase complex reagents. The desired staining of antibody-specific binding was achieved with DAB solution for 2 minutes. Finally, the sections

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**Figure 1.** Immunostaining of IL-8, MMP-9 and MVD in human hilar cholangiocarcinoma (magnification,  $\times 400$ ). A. High IL-8 expression. B. Low IL-8 expression. C. High MMP-9 expression. D. Low MMP-9 expression. E and F. High and low MVD (corresponding “vascular hot spot”).

were counterstained with hematoxylin, dehydrated and counted. The slides were routinely examined under a microscope and evaluated separately by two independent observers blinded to patients' clinical information. Disagreements were resolved simultaneously by observers using a double-headed microscope to make a conclusive judgment.

### Assessment of IL-8 immunostaining

Staining for IL-8 was evaluated according to the modified methods described previously [13, 14]. In brief, the intensity of staining was scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong). The extent of staining was scored as 0

(0%), 1 (1-10%), 2 (11-50%), 3 (51-80%), or 4 (81-100%). The median immunohistochemical staining score was used as the cut-off value to divide the patients into low and high IL-8 expression groups.

### Assessment of MMP-9 immunostaining

MMP-9 staining was assessed using the methods described previously [15]. Immunopositivity was considered to be as MMP-9 (-) when no more than 10% of the cells were positive, as MMP-9 (+) when more than 10% to 50% of the cells were positive, and as MMP-9 (++) when more than 50% of the cells were positive. Samples with MMP (-) and MMP-9 (+) were clas-



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**Table 1.** Relationships between the expression of IL-8 and clinicopathological features in human hilar cholangiocarcinoma

Clinicopathological features	n	IL-8		P†
		Low	High	
Gender				0.780
Male	47	20	27	
Female	15	7	8	
Age (years)				0.442
< 60	31	12	19	
≥ 60	31	15	16	
Differentiation				0.600
Well/moderately	51	23	28	
Poorly	11	4	7	
Bismuth-Corlette classification				0.191
Type I	16	7	9	
Type II	7	2	5	
Type IIIa	9	6	3	
Type IIIb	11	2	9	
Type IV	19	10	9	
Tumor size (cm)				0.121
< 3	22	11	11	
3-5	13	8	5	
≥ 5	27	8	19	
Tumor stage, pT				0.779
T1	15	9	6	
T2	18	11	7	
T3	29	7	22	
Lymph node metastasis				0.480
No	43	20	23	
Yes	19	7	12	
TNM stage				0.026*
I	9	7	2	
II	12	8	4	
IIIa	7	1	6	
IIIb	13	4	9	
IVa	21	7	14	
Tumor recurrence				0.018*
No	33	19	14	
Yes	29	8	21	

†X<sup>2</sup> test.

sified as low expression group, and those with MMP (++) were high expression group.

### Assessment of MVD

MVD of the tumor sections was determined by quantification of tumor infiltration with CD31-positive vessels using the modified method

**Table 2.** Correlation between IL-8 and MMP-9 expression in human hilar cholangiocarcinoma ( $r = 0.289$ ,  $P = 0.023$ )

MMP-9	IL-8		total
	Low	High	
-	13	7	20
+	5	8	13
++	9	20	29
total	27	35	62

**Table 3.** Correlation between IL-8 expression and MVD in human hilar cholangiocarcinoma ( $r = 0.304$ ,  $P = 0.016$ )

MVD	IL-8		total
	Low	High	
Low	15	9	24
High	12	26	38
total	27	35	62

described previously [12]. In brief, three areas of the highest vascular density (vascular hot spot) within the tumor were identified at low magnification (100 ×) and counted under 400 × magnification. MVD represents the average number of six counts. Whenever the average number was lower than or equal to 20, the sample was considered to have a low MVD; otherwise, it was included in the high MVD group for analysis.

### Statistical analyses

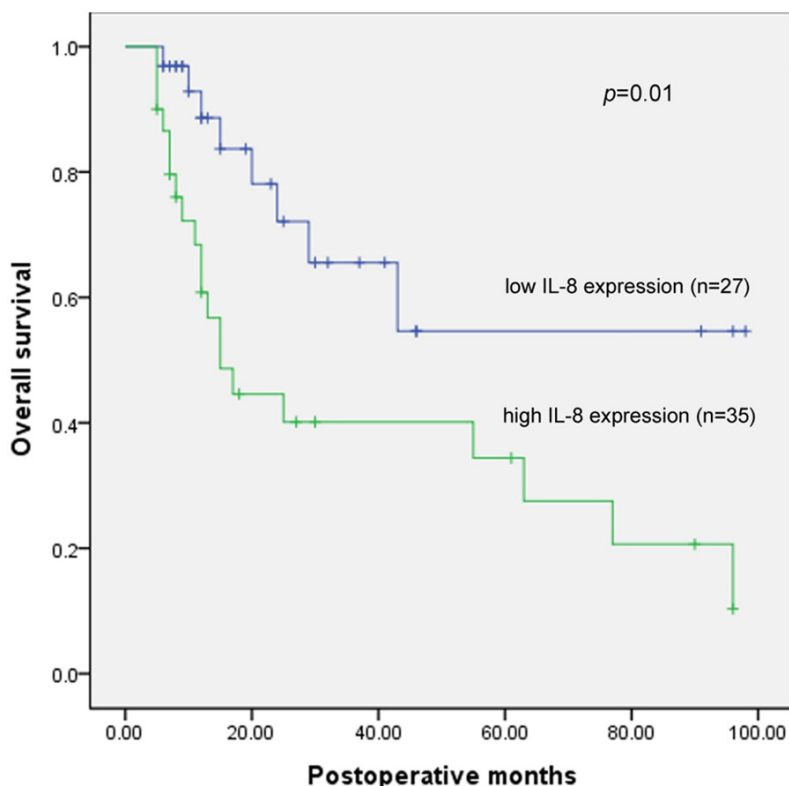
Statistical analyses were performed with the SPSS 18.0 software. Chi-square test and Fisher exact test were used to examine the correlation between the IL-8 expression and clinicopathological characteristics. Kaplan-Meier estimates and log-rank tests were used for overall survival (OS) analysis. Spearman correlation was applied to evaluate the correlation of IL-8 expression with MMP-9 expression and MVD. Cox analyses were used to evaluate IL-8 expression and other prognostic factors with respect to OS. For all analyses,  $P < 0.05$  was considered statistically significant.

## Results

### Expression of IL-8 in hilar cholangiocarcinoma

IL-8 staining was observed predominantly in the cytoplasm of tumor cells. Immunohistoche-

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**Figure 2.** Overall survival curves of patients for hilar cholangiocarcinoma with different IL-8 expression. Patients with high expression of IL-8 had a significantly poorer survival rate than patients with low IL-8 expression ( $P = 0.01$ ).

mical analysis of 62 samples suggested that high IL-8 expression was found in 35 tumors (56.5%) and low IL-8 expression in 27 tumors (43.5%, **Figure 1A** and **1B**). **Table 1** summarizes the relationship between the IL-8 expression and clinicopathological characteristics. There was no significant difference between IL-8-high and -low expression regarding gender, age, differentiation, Bismuth-Corlette classification, tumor size, and lymph node metastasis. However, significant statistical difference existed with respect to advanced TNM stage ( $P = 0.026$ ). More importantly, IL-8 expression was closely related to tumor recurrence ( $P = 0.018$ ).

### *Correlation of IL-8 with MMP-9 expression and tumor angiogenesis*

MMP-9 expression was prominent in the cytoplasm (**Figure 1C** and **1D**). In the 62 carcinoma samples, MMP-9 expression in tumor cells was as follows: MMP-9 (-) expression was seen in 20 patients (32.3%), MMP-9 (+) expression was seen in 13 patients (21.0%), and MMP-9 (++) expression was seen in 29 patients (46.7%). High IL-8 expression was observed in 68.9% of

MMP-9 (++) expression tissue, while the rates in MMP-9 (+) and MMP-9 (-) expression tissues were 61.5% and 35%, respectively. Based on the Spearman correlation analysis, IL-8 expression had a positive correlation with MMP-9 expression ( $r = 0.289$ ,  $P = 0.023$ , **Table 2**).

The abundance of vessels varied significantly among the carcinoma specimens (**Figure 1E** and **1F**). According to the foregoing definition of high and low MVD, 24 of 62 patients were allocated into low MVD group, whereas 38 patients had tumors with high MVD. Spearman correlation analysis showed that there was more MVD in tumors with high IL-8 expression than that in those with low IL-8 expression ( $r = 0.304$ ,  $P = 0.016$ , **Table 3**).

### *Prognostic value of IL-8 expression in hilar cholangiocarcinoma*

Kaplan-Meier curves of overall survival based on IL-8 expression were shown in **Figure 2**. Patients with high IL-8 expression had a poorer OS than those with low IL-8 expression ( $P = 0.01$ ). In addition to IL-8 expression, univariate analysis demonstrated that MVD, MMP-9 expression and clinicopathological characteristics including differentiation, and lymph node metastasis were also significantly associated with OS, while others were not (**Table 4**).

To obtain a more precise estimate, the Cox proportional hazard regression model was performed to establish the independent prognostic factors. Multivariate analysis confirmed IL-8 high expression as an independent prognostic factor for hilar cholangiocarcinoma ( $P = 0.005$ , **Table 5**), suggesting that high IL-8 expression was a high-risk factor for poor prognosis. Moreover, lymph node metastasis ( $P = 0.006$ ), MMP-9 expression ( $P = 0.016$ ) and MVD ( $P = 0.008$ ) remained the independent prognostic

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**Table 4.** Univariate analysis of clinicopathological features for overall survival of 62 patients with hilar cholangiocarcinoma

Characteristics	n	Survival rate (%)	P*
Gender			0.554
Male	47	40.4	
Female	15	46.6	
Age (years)			0.327
< 60	31	45.1	
≥ 60	31	38.7	
Differentiation			0.013
Well/moderately	51	45.1	
Poorly	11	27.2	
Bismuth-Corlette classification			0.282
Type I	16	37.5	
Type II	7	71.4	
Type IIIa	9	55.6	
Type IIIb	11	36.4	
Type IV	19	31.2	
Tumor size (cm)			0.312
< 3	22	36.4	
3-5	13	61.5	
> 5	27	34.7	
Tumor stage, pT			0.339
T1	15	53.3	
T2	18	44.4	
T3	29	34.5	
Lymph node metastasis			0.009
No	43	44.2	
Yes	19	36.8	
TNM stage			0.452
I	9	44.4	
II	12	58.3	
IIIa	7	28.6	
IIIb	13	15.4	
IVa	21	42.9	
IL-8 expression			0.010
Low	27	59.3	
High	35	28.6	
MMP-9 expression			0.022
-	20	55.0	
+	13	53.8	
++	29	27.6	
MVD			0.018
Low	24	58.3	
High	38	31.6	

\*Log-rank test.

indicator as well, while tumor differentiation did not.

## Discussion

Considerable data have proved that abnormal levels of IL-8 originated from tumor cells facilitate tumor progression. IL-8 exerts its action as a growth factor through an autocrine and paracrine loop by binding to CXCR1 and CXCR2 [16]. Originally discovered as a leukocyte chemoattractant, IL-8 has been shown to play an important role in angiogenesis, tumor growth, metastasis and chemoresistance [7, 17]. Yet so far little information exist dealing with the role of IL-8 in cholangiocarcinoma.

Our current data showed that high expression ratio of IL-8 in hilar cholangiocarcinoma is 56.5%. The high IL-8 expression is associated with advanced TNM stage, and more closely with tumor recurrence. Furthermore, the expression of IL-8 was correlated with MMP-9, which was consistent with previous studies [18, 19]. IL-8 induces MMP-9 secretion through distinct pathways within various cell types [19-21]. Tumor-derived IL-8 has the capacity to exert profound effects on the tumor microenvironment, where IL-8 could induce tumor cells and adjacent stromal cells to express increased levels of MMP-9 to facilitate cell growth, invasion, and metastases. In addition, degradation and remodeling of ECM by MMPs is required in endothelial cell migration, organization, and hence, angiogenesis [22, 23]. Angiogenesis is a crucial step for promoting tumor growth and metastasis. IL-8 activates endothelial cells in the tumor vasculature to promote angiogenesis, and induces a chemotactic infiltration of neutrophils into the tumor site, which secrete additional growth factors to further increase the rate of tumor proliferation and invasion [5, 24].

In recent years, it has also been demonstrated that a link exists between IL-8, tumor epithelial-mesenchymal transition (EMT) and tumor stemness [25]. The secretion of IL-8 could be induced during the EMT mediated by TGF- $\beta$  or SNAIL overexpression [26, 27]. IL-8 released by tumor cells could play following roles in tumor progression: maintenance of the mesenchymal, promotion of tumor invasion via an autocrine loop; induction of EMT through a paracrine effect on adjacent epithelial tumor cells, angiogenesis and chemoattractant of immune cells to the tumor site to create an inflammatory environment that further facilitate tumor dissemination and metastasis.

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**Table 5.** Multivariate analysis of clinicopathological features for overall survival of 62 patients with hilar cholangiocarcinoma

Factors	Category	P	HR	95% CI
MMP-9 expression	-	0.016	3.269	1.224-6.942
	+			
	++			
IL-8 expression	Low	0.005*	2.457	0.921-8.537
	High			
MVD	Low	0.008*	2.431	0.662-6.783
	High			
Differentiation	Well/moderately	0.625	0.744	0.287-2.665
	Poorly			
Lymph node metastasis	No	0.006*	1.112	0.284-3.532
	Yes			

HR, hazard ratio; CI, confidence interval.

Tumor-associated neovascularization is known to be critically involved in the progression of solid tumors, especially involved in the malignancies of the biliary system [12, 28]. Our results demonstrated that IL-8 expression was also associated with MVD, which was the most widely accepted marker of tumor angiogenesis. This confirmed that IL-8 could promote angiogenesis, which was a necessary step for tumor progression as tumor ceased to grow beyond a certain size without the nutrient and oxygen supply from the new blood vessels [29]. On the other hand, a lot of cancer cells are still exposed to a hypoxic environment, although angiogenesis activity accelerates in the majority of tumors, either due to immature functional tumor neovasculature or too rapid proliferation of cancer cells [30]. A local decrease in oxygen tension may lead to the induction of many angiogenic factors, including IL-8, which has been proved by previous studies [31-33]. Additionally, IL-8 has been proved to increase integrin  $\alpha\beta 3$ , which is a pivotal proangiogenic factor [34]. And our group has recently found that IL-8 could dose-dependently upregulated integrin  $\alpha\beta 6$  expression in colorectal cancer, which plays a vital role in IL-8-mediated migration in tumor [35].

The most important conclusion in these findings is that after analyzing the potential relationship between the IL-8 expression and survival time, we observed that patients with high IL-8 expression had a poorer overall survival. The Cox analysis confirmed IL-8 expression as an independent prognostic indicator, which agreed with the results of many other studies

that IL-8 indicated a poor outcome in the cancers [13, 36-39]. It should be mentioned here that the samples enrolled in this study were not big enough since hilar cholangiocarcinoma is an uncommon malignancy. Future study with a larger sample size and more diversified tumor stages will be needed to independently verify our current findings.

Our study is the first report on the relationship between IL-8 expression and the prognosis of hilar cholangiocarcinoma; it would help to develop some

new therapeutic strategies to improve the clinical treatments for hilar cholangiocarcinoma and prolong the patients' survival. The multiple effects of IL-8 signaling upon different cell types present within the tumor microenvironment indicates that targeting of CXC-chemokine signaling (including but not limited to IL-8) might have important implications to defer tumor progression. In conclusion, the present study suggested that IL-8 expression was related to MMP-9 and MVD. Meanwhile, IL-8 was correlated with unfavorable clinical prognostic factors and decreased survival for human hilar cholangiocarcinoma. Considering the contributions of IL-8 to tumor progression, targeting IL-8 signaling pathways might be a potential therapeutic strategy.

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### Disclosure of conflict of interest

None.

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