

Expression of interleukin-6 is associated with epithelial-mesenchymal transition and survival rates in gallbladder cancer

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Abstract. The present study aimed to investigate the expression of interleukin-6 (IL-6) in gallbladder cancer (GBC) tissues and its correlation with survival rate. The association between IL-6 and epithelial-mesenchymal transition (EMT)-associated markers was also examined. Using immunohistochemistry, reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, the protein and mRNA expression levels of IL-6, Twist, E-cadherin and Vimentin in 20 GBC tissues were analyzed. The IL-6, Twist and Vimentin proteins were overexpressed in 40, 20 and 70% of the human GBC samples, respectively. The protein expression of E-cadherin was higher in only 5% of the GBC samples. These differences were significant ($P < 0.05$). Western blot analysis also revealed overexpression of IL-6, Twist and Vimentin and underexpression of E-cadherin in the GBC samples with poor differentiation, local invasion and a higher tumor-node-metastasis (TNM) stage ($P < 0.05$). Higher mRNA expression levels of IL-6, Twist and Vimentin and a reduced expression level of E-cadherin were also demonstrated in the GBC tissues ($P < 0.05$). The degree of differentiation, local invasion, lymph node metastasis and clinical stage were significantly associated with the mRNA expression levels of IL-6, Twist and E-cadherin. The increased

expression levels of IL-6 and Twist and the reduced expression of E-cadherin correlated with shorter median survival rates ($P < 0.05$). Line regression results revealed correlation among the mRNA expression levels of IL-6, Twist, E-cadherin and Vimentin. To the best of our knowledge, the present study is the first to demonstrate that IL-6 is associated with EMT-associated markers, tumor differentiation, local invasion, TNM stage and survival rates in GBC.

Introduction

Gallbladder cancer (GBC) is a relatively rare type of neoplasm, but is particularly life-threatening. It is the most common biliary tract tumor and the seventh most common type of malignancy of the digestive tract worldwide (1). As the clinical symptoms are subtle, the majority of patients are diagnosed at an advanced stage. The 5 year mortality rate is up to 90% (2), while the median survival prognosis for patients is 4-6 months. Due to the advanced stage at presentation, only a third of patients are potential candidates for surgery (3). GBC is highly invasive and spreads to regional lymph nodes at an early stage. In addition, it has a high rate of recurrence (4). Treatment with adjuvant therapy has been considered, however, no previous studies have provided conclusive evidence supporting the benefit of adjuvant treatment for GBC (5). Thus, the majority of patients present with metastasis at the time of diagnosis.

GBC is suspected in patients with a long history of chronic cholecystitis secondary to cholelithiasis who demonstrate a change in symptoms. Interleukin-6 (IL-6) is a pleiotropic cytokine involved in acute inflammation, hematopoiesis (6,7) and the proliferation of cancer cells (8). Increased expression of IL-6 has been detected and associated with an unfavorable prognosis and metastasis in patients with cancer (9). Therefore, targeting IL-6-mediated pathways can offer an effective treatment modality (10). Several studies have suggested the role of IL-6 in modulating the tumor microenvironment, which is triggered by inducing epithelial-to mesenchymal transition (EMT) followed by downregulation the expression of E-cadherin and upregulation the expression levels of Vimentin, N-cadherin,

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Snail and Twist (11,12). EMT is an important mechanism in tumor invasion and metastasis (13) and Twist is important in promoting EMT (14). However, the exact effect of the expression of IL-6 remains to be elucidated.

The present study examined the association between IL-6, Twist, EMT and GBC. The progression, invasion and metastasis of GBC was analyzed by investigating the expression of the epithelial marker E-cadherin and interstitial marker Vimentin. The results may improve understanding of GBC prognosis and targeted therapy.

Materials and methods

Clinical specimens. Human GBC tissues were obtained with informed consent from the Eastern Hepatic Biliary Hospital affiliated with the Second Military Medicine University (Shanghai, China) and the procedures used in the present study were approved by the Protection of Human Subjects Committee of the Eastern Hepatic Biliary Hospital affiliated with the Second Military Medicine University. A total of 20 GBC specimens and their surrounding tissues were obtained from patients who underwent cholecystectomy. Immediately following surgical removal, half of the tissues were snap-frozen in liquid nitrogen for storage and the remaining half were fixed in 4% paraformaldehyde (DingGuo Biotech Co., Ltd, Shanghai, China) and embedded in paraffin (DingGuo Biotech Co., Ltd).

Immunohistochemical staining. The samples, prepared from the paraffin-embedded block, were rehydrated and then incubated in 3% hydrogen peroxide for 15 min to block endogenous peroxidase. For antigen retrieval, the samples were boiled in a pressure cooker for 10 min. Nonspecific binding was inhibited with 10% normal goat serum (Boshide Biological Engineering, Co., Ltd, Wuhan, China) for 20 min at 37°C. The samples were then incubated at 4°C overnight with the following primary antibodies: Rabbit anti-mouse polyclonal IL-6 (1:50; cat. no. ab6672; Abcam, Cambridge, MA, USA), mouse anti-goat polyclonal Twist (1:50; cat. no. ab50887; Abcam), rabbit anti-mouse polyclonal E-cadherin (1:100; sc-7870; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit anti-mouse polyclonal Vimentin (1:100; cat. no. sc-5565; Santa Cruz Biotechnology, Inc.). The sections were treated with goat anti-rabbit/anti-mouse polyclonal secondary antibodies conjugated to horseradish peroxidase (Abcam, Cambridge, MA, USA) for 30 min at room temperature and stained with diaminobenzidine (Beyotime Institute of Biotechnology, Shanghai, China) until brown granules appeared. The sections were then counterstained with hematoxylin (DingGuo Biotech Co., Ltd) for 2 min at room temperature.

Evaluation of immunohistochemical staining. The sections were evaluated by two pathologists in a blinded-manner using a light microscope (DMI3000B; Leica Microsystems AG, Solms, Germany). A semi-quantitative scoring criterion for immunohistochemistry was used, in which expression was determined based on the percentage of positive cells and staining intensity. The scores were interpreted as shown in Table I.

The final score was determined by the expression rate and intensity of proteins, graded as '-' for 0 point, '+' for 1-2 points, '++' for 3-4 points and '+++'' for 5-6 points. Immunoreactivity

'±' was used to denote overexpression and '++/+++'' was used to denote underexpression for statistical analysis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from 100 mg GBC tissues and the surrounding tissues. The tissues were added to 1 ml TRIzol reagent (Takara Bio, Inc., Tokyo, Japan) and homogenized according to the manufacturer's instructions. The first strand of cDNA was synthesized from 500 ng total RNA using PrimeScript® Reverse Transcriptase (Takara Bio, Inc.). The qPCR was performed in a reaction volume of 20 µl, including 2 µl cDNA. The primer sequences used are shown in Table II.

The PCR conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. The relative quantification of genes was analyzed using the comparative threshold cycle (C_t) method. To ensure that only a specific band was produced, melting curve analysis was performed at the end of each PCR experiment. The term $-\Delta C_t$ was used to describe the expression level of mRNA. The expression was subsequently divided into lower expression and higher expression groups, based on whether the mRNA levels were above or below the mean value.

Western blot analysis. Western blot analysis was performed, as previously described (15). For the total protein extraction, 100 mg of frozen tissue samples, previously stored in liquid nitrogen, were ground and homogenized using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Technology). The total protein concentrations of the cell extracts were determined using a bicinchoninic acid assay system (Beyotime Institute of Biotechnology) with bovine serum albumin as the standard. For electrophoresis, 80 µg of the total protein was added to each lane on SDS-PAGE gels (8, 10 and 15%; Beyotime Institute of Biotechnology) and the protein was then blotted onto a polyvinylidene difluoride membrane (DingGuo Biotech Co., Ltd) by wet transfer (200 mA, 1-2 h). The membranes were inhibited with 5% skimmed milk and incubated with anti-IL-6 (cat. no. ab6672; Abcam, US), anti-Twist (cat. no. ab50887; Abcam), anti-E-cadherin (cat. no. sc-7870; Santa Cruz Biotechnology, Inc.), anti-Vimentin (cat. no. sc-5565; Santa Cruz Biotechnology, Inc.) and anti-β-actin antibodies (cat. no. #4970; Cell Signaling Technology, Inc.), respectively, at 4°C overnight. This was followed by incubation with goat anti-rabbit/anti-mouse secondary antibody conjugated to horseradish peroxidase (1:1,000). The stain was visualized using an enhanced chemiluminescent (ECL) detection reagent from Millipore (Rockford, IL, USA). Images were captured and the optical densities of the bands were quantified using a Gel Doc 2000 system (BioRad, Hercules, CA, USA).

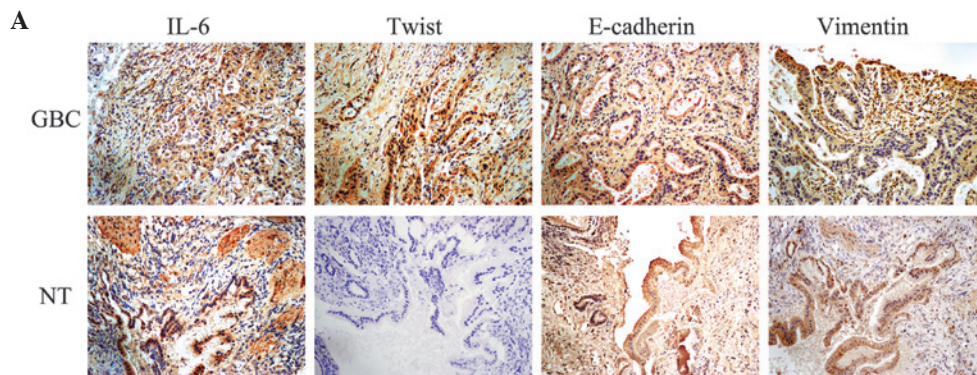
Statistical analysis. The results of all the assays are expressed as the mean ± standard deviation. All the assays were performed independently in triplicate. The data were analyzed using Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The survival curves were generated using the Kaplan-Meier method. The significance of the observed differences were determined using Student's t-test

Table I. Immunohistochemical staining score system.

Score	Description
0	≤5% of positive cells or negative staining
1	5-25% of positive cells or weak staining
2	25-50% of positive cells or moderate staining
3	50% of positive cells or strong staining

Table II. Primer sequences.

Gene	Forward/Reverse	Sequence (5'-3')
β-actin	Forward	CTGGGACGACATGGAGAAAA
	Reverse	AAGGAAGGCTGGAAGAGTGC
Interleukin-6	Forward	CCACACAGACAGCCACTCAC
	Reverse	GATGATTTTCACCAGGCAAGTC
Twist	Forward	AGTCCGCAGTCTTACGAGGAG
	Reverse	GACCTGGTAGAGGAAGTCGATG
E-cadherin	Forward	GTCTCTCTCACCACCTCCACAG
	Reverse	CTCGGACACTTCCACTCTCTTT
Vimentin	Forward	GAAGAGAACTTTGCCGTTGAAG
	Reverse	GAAGGTGACGAGCCATTTC



B

Marker	Group	Cases	Expression of protein				χ^2	P
			-	+	++	+++		
IL-6	Adjacent tissue	20	11	7	2	0	8.329	0.040
	Cancer tissue	20	4	8	6	2		
Twist	Adjacent tissue	20	14	5	1	0	6.918	0.031
	Cancer tissue	20	6	10	4	0		
E-cadherin	Adjacent tissue	20	5	10	3	2	9.957	0.019
	Cancer tissue	20	14	5	1	0		
Vimentin	Adjacent tissue	20	8	7	3	2	8.455	0.037
	Cancer tissue	20	3	3	8	6		

Figure 1. Expression levels of IL-6 protein and epithelial-mesenchymal transition-associated proteins in GBC tissues and in paired adjacent tissues. (A) Representative immunostaining of IL-6, Twist, E-cadherin and Vimentin in the GBC and adjacent tissues (magnification, x200). (B) Different protein expression levels of IL-6, Twist, E-cadherin and Vimentin in 20 paired samples graded based on protein expression intensity as: - (0 point); + (1-2 points); ++ (3-4 points) and +++ (5-6 points). GBC gallbladder cancer; NT, normal tissues; IL-6, interleukin-6.

or χ^2 test. Associations among the IL-6, Twist, E-cadherin and Vimentin mRNA were analyzed by correlation coefficients and linear regression analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of IL-6 and EMT-associated protein in the GBC tissues. The expression levels of IL-6 and the Twist, E-cadherin

Table III. mRNA expression levels of IL-6, Twist, E-cadherin and Vimentin and the association with the clinical pathological features of gallbladder cancer.

Variable	Cases	%	Mean \pm SD expression of IL-6	P-value	Mean \pm SD expression of Twist	P-value	Mean \pm SD expression of E-cadherin	P-value	Mean \pm SD expression of Vimentin	P-value
Normal tissue	20		1.39 \pm 2.12	0.002	2.52 \pm 2.39	0.026	3.66 \pm 1.34	0.035	7.57 \pm 1.77	0.026
Carcinoma tissue	20		-0.23 \pm 2.37		1.26 \pm 2.55		4.72 \pm 1.89		6.23 \pm 2.03	
Gender				0.558		0.569		0.725		0.903
Male	6	30	0.95 \pm 1.18		2.04 \pm 2.50		3.49 \pm 1.40		7.65 \pm 0.61	
Female	14	70	1.58 \pm 2.43		2.73 \pm 2.40		3.73 \pm 1.36		7.54 \pm 2.11	
Age (years)				0.992		0.368		0.901		0.872
\leq 60	13	65	1.39 \pm 2.54		2.89 \pm 2.35		3.69 \pm 1.14		7.52 \pm 2.15	
$>$ 60	7	35	1.40 \pm 1.16		1.85 \pm 2.49		3.61 \pm 1.76		7.66 \pm 0.85	
Tumor size (cm)				0.806		0.314		0.939		0.889
\leq 5	11	55	1.50 \pm 1.85		2.02 \pm 2.18		3.68 \pm 1.33		7.62 \pm 1.30	
$>$ 5	9	45	1.26 \pm 2.53		3.13 \pm 2.61		3.63 \pm 1.44		7.50 \pm 2.31	
Degree of differentiation				0.021		0.026		0.012		0.062
Well and moderately differentiated	18	90	0.70 \pm 1.09		1.77 \pm 1.60		4.13 \pm 1.08		7.09 \pm 1.40	
Poorly differentiated	2	10	3.02 \pm 3.08		4.29 \pm 3.1		2.56 \pm 1.33		8.69 \pm 2.16	
Local invasion				0.009		0.007		0.001		0.18
Positive	7	35	2.70 \pm 2.36		4.03 \pm 2.84		2.69 \pm 1.25		8.16 \pm 1.82	
Negative	13	65	0.32 \pm 1.14		1.29 \pm 0.81		4.45 \pm 0.81		7.08 \pm 1.66	
Lymph-node metastasis				0.025		0.003		0.006		0.064
Positive	12	60	2.66 \pm 2.58		4.32 \pm 2.45		2.71 \pm 1.36		8.46 \pm 1.96	
Negative	8	40	0.55 \pm 1.27		1.33 \pm 1.46		4.29 \pm 0.92		6.97 \pm 1.42	
TNM stage				0.014		0.025		0.003		0.144
I-II	4	20	0.15 \pm 1.20		1.24 \pm 0.62		4.58 \pm 0.82		6.92 \pm 1.66	
III-IV	16	80	2.41 \pm 2.21		3.58 \pm 2.79		2.91 \pm 1.23		8.10 \pm 1.76	

IL-6, interleukin-6; TNM, tumor-node-metastasis; SD, standard deviation.

and Vimentin EMT-associated proteins were determined in 20 human GBC tissues and 20 surrounding tissues using immunohistochemistry (Fig. 1A). The results revealed overexpression of IL-6, Twist and Vimentin and underexpression of E-cadherin in the GBC tissues compared with the adjacent tissues (Fig. 1A). The results also revealed that the IL-6, Twist and Vimentin proteins were overexpressed in 8, 4 and 14 (40, 20 and 70%) of the GBC samples, respectively. The underexpression of E-cadherin was observed in a single GBC sample (5%). By contrast, the expression levels of IL-6, Twist, Vimentin in the GBC tissues were significantly higher compared with those in the surrounding tissues ($\chi^2=8.329$, $P=0.040$; $\chi^2=6.918$, $P=0.031$ and $\chi^2=8.455$, $P=0.037$, respectively). However, the expression of E-cadherin was significantly lower in the GBC tissues ($\chi^2=9.957$, $P=0.019$).

Western blot analysis also revealed the overexpression of IL-6, Twist and Vimentin and underexpression of E-cadherin in the GBC tissues compared with the normal adjacent tissues ($P<0.05$; Fig. 2Aa and b). The decrease in the differentiation level of the GBC tissue (Fig. 2Ac and d), increase in tumor-node-metastasis (TNM) stage (Fig. 2Ae and f) and positive local invasion (Fig. 2A, g and h) were correlated with increased expression levels of IL-6, Twist and Vimentin ($P<0.05$). The expression of E-cadherin in the GBC tissues was significantly lower compared with that in the surrounding tissues ($P<0.05$) and was lower in patients with GBC exhibiting high grade differentiation, local invasion and a high TNM stage ($P<0.05$; Fig. 2A).

IL-6 and EMT-associated mRNA expression in gastric cancer tissues is associated with advanced clinical stage, lymph node metastasis and poor patient prognosis. Consistent with the above data, the results confirmed that the expression levels of IL-6, Twist and Vimentin in the GBC tissues were significantly higher ($P<0.05$; Fig. 3A, B and D). However, the mRNA expression of E-cadherin was significantly lower in the GBC tissues ($P=0.0265$; Fig. 3C). The correlations between the IL-6 and EMT-associated mRNA expression and the clinicopathologic characteristics of GBC are summarized in Table III. The differentiation, local invasion, lymph node status and clinical stages were correlated with the expression of IL-6. The median expression level of IL-6 was 2.41 ± 2.21 in the 20 cases with advanced stage (stage III and IV) and 0.15 ± 1.20 ($P=0.014$) in cases with early-stage (stage I and II) disease. In the 20 cases of GBC with either local invasion or lymph node metastasis, the median expression levels of IL-6 were 2.70 ± 2.36 and 2.66 ± 2.58 , respectively. This was significantly higher compared with the expression levels in the 20 adjacent tissues (0.32 ± 1.14 ; $P=0.009$ and 0.55 ± 1.27 ; $P=0.025$, respectively). The expression of IL-6 in the GBC patients did not correlate with gender, age or tumor size. A statistically significant correlation was observed between the degree of differentiation, local invasion, lymph node metastasis, clinical stage and Twist/E-cadherin expression. However, no statistically significant correlation was observed between the expression of Vimentin and the clinicopathologic characteristics. In addition, the present study examined whether the mRNA expression levels of IL-6, Twist, E-cadherin and Vimentin were associated with survival rate in patients with GBC. Based on the mean expression level of IL-6 (1.394), Twist (2.524), E-cadherin (3.660) and Vimentin (7.568), as shown in Fig. 3A-D, the GBC specimens were divided into a higher and a

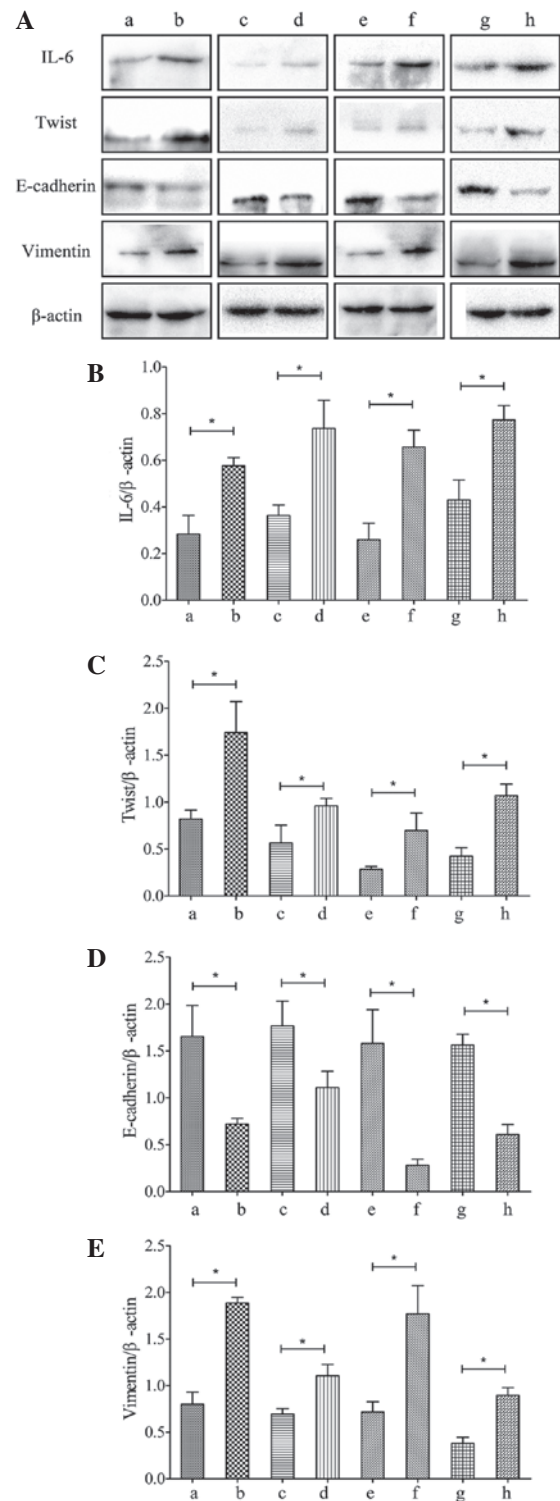


Figure 2. (A) Protein expression levels of IL-6, Twist, E-cadherin and Vimentin in gallbladder cancer tissues. (B-E) Relative protein expression levels of IL-6, Twist, E-cadherin and Vimentin in gallbladder cancer tissues. a, adjacent tissue; b, gallbladder cancer tissue; c, well and moderately differentiated; d, poorly differentiated; e, TNM stage I-II; f, TNM stage III-IV; g, without local invasion; h, local invasion. Values are presented as the mean \pm standard deviation. * $P<0.05$. IL-6, interleukin-6; TNM, tumor-node-metastasis.

lower expression group. Kaplan-Meier survival analysis revealed that patients whose tumors exhibited increased expression of IL-6 or Twist compared with that of the lower expression group or reduced expression of E-cadherin compared with that of the

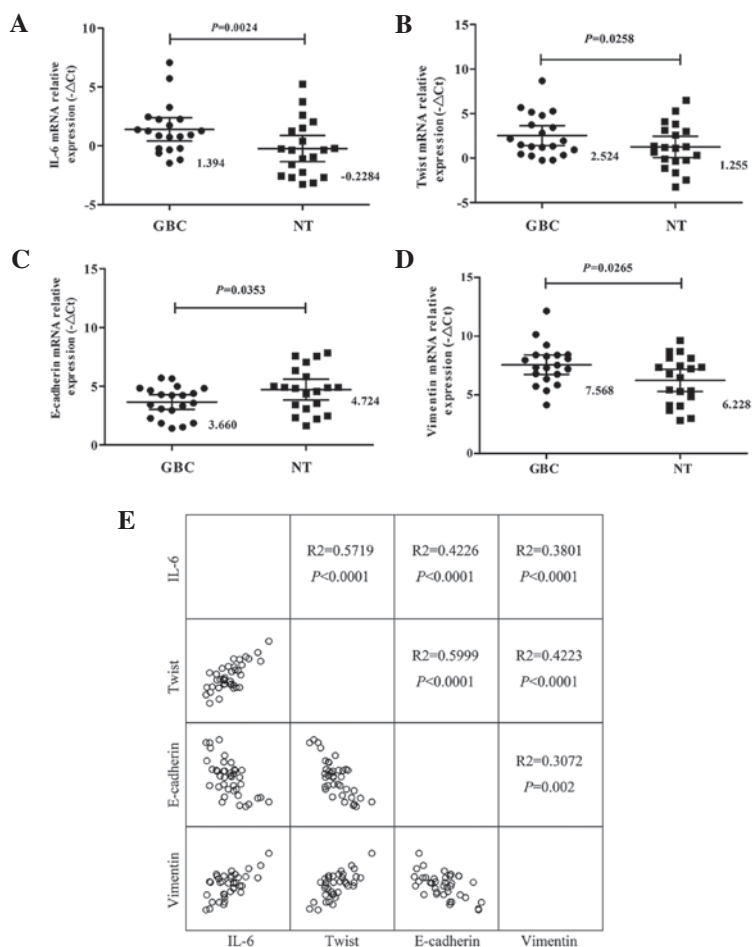


Figure 3. Results of the expression analysis of IL-6, Twist, E-cadherin and Vimentin in 20 matched GBC tissues and adjacent tissues. (A-D) mRNA expression levels of IL-6, Twist, E-cadherin and Vimentin in GBC tissues and in adjacent normal tissues. (E) Scatter plot analysis of all the GBC and NT samples of IL-6, Twist, E-cadherin and Vimentin with R^2 and P-values. IL-6, interleukin-6; GBC, gall bladder cancer; NT, normal tissue.

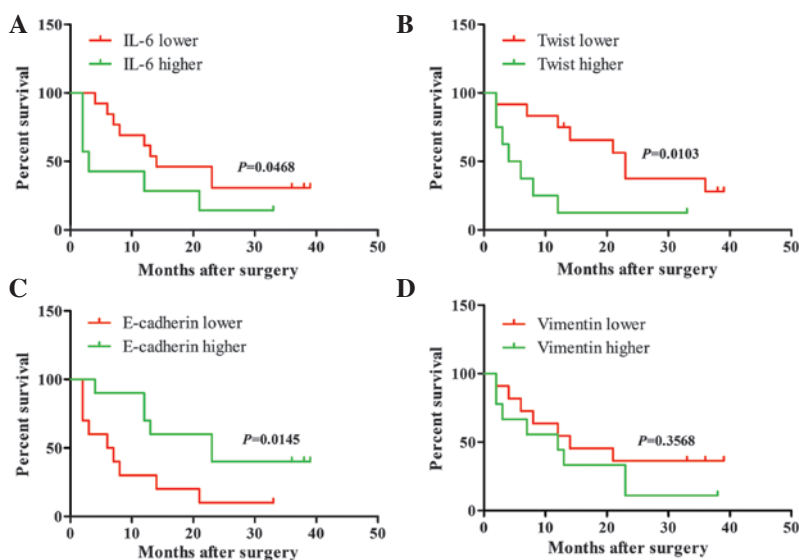


Figure 4. Comparison of Kaplan-Meier survival curves for gallbladder cancer patients between higher and lower expression levels of (A) IL-6, (B) Twist, (C) E-cadherin and (D) Vimentin. IL-6, interleukin-6.

higher expression group had a shorter median survival rates at 10.71 ± 12.19 , 8.75 ± 10.38 and 9.8 ± 10.20 months, respectively ($P=0.0486$, $P=0.0103$ and $P=0.0145$; Fig. 4A-D).

IL-6 is associated with the expression of twist and can accurately discriminate between GBC and adjacent tissues. Line regression results produced R^2 values to compare the mRNA

expression levels of IL-6, Twist, E-cadherin and Vimentin, respectively, in the GBC and adjacent tissues. Significant correlations were observed among these four mRNAs ($P < 0.05$; Fig. 3E).

Discussion

The present study demonstrated that IL-6 protein and mRNA were overexpressed in GBC tissues (Figs. 1-3) and IL-6, Twist and E-cadherin were associated with local invasion, lymph node metastasis, poor differentiation and poor clinical prognosis in GBC. This is the first study, to the best of our knowledge, to examine the correlation between IL-6 and EMT and prognosis.

Although the mRNA expression of IL-6 was increased in the 20 GBC tissues, its source remains to be elucidated. Several cancer patients exhibit increased serum levels of IL-6, which can originate from a number of sources, including tumor cells and macrophages (16). If cancer cells increasingly secrete IL-6, it may act in an autocrine manner to enhance the metastatic ability (17) and resistance of the tumor to treatment (18). The increased levels of IL-6 have been correlated with poor prognosis and survival rate in a variety of types of cancer (19-22). Previous studies on solid tumors, including gastric, renal cell, colorectal, prostate, non-small cell lung, melanoma and head and neck cancer and hematologic malignancies, including myeloma and non-Hodgkin's lymphoma, have indicated the potential prognostic significance of IL-6 levels (19-22).

IL-6 signaling activates STAT3 (23), which is required for malignant transformation. It also has multiple protumorigenic functions, including the promotion of tumor cell proliferation, survival, invasion, metastasis and angiogenesis (24,25). IL-6 induces EMT changes in tumor cells via activation of the STAT3 signaling pathway and STAT3-knockdown reverses these changes (26). The key activity of EMT (27) is hypothesized to be the reduction of cell-to-cell adhesion and induction of cell motility through downregulation of E-cadherin and may be associated with the expression of Twist and Vimentin (14).

Consistent with the data of the present study, certain studies have identified that IL-6 induces an EMT phenotype (11,12). Additionally, increased expression of Twist and reduced expression of E-cadherin are correlated with poor differentiation and local invasion. Twist and E-cadherin are statistically significant prognostic factors in several types of cancer (28-32).

In the present study, linear correlation analysis revealed that the mRNA expression of IL-6 was positively correlated with the EMT-associated markers (Fig. 3E). These results suggested that the four genes have synergistic effects in tumorigenesis and metastasis in GBC. It also indicated that the malignant transformation process of GBC is accompanied by EMT.

In conclusion, the expression of IL-6 correlated with EMT-associated mRNA and protein expression, local invasion, lymph node metastasis, shorter survival time, poor clinical stage and differentiation. GBC is often diagnosed at an advanced stage and is associated with poor prognosis. It is possible to downregulate the expression of IL-6 through adjuvant therapy and several clinical studies have supported

the use of IL-6 as a therapeutic target (33,34). However, further studies are required to fully define the association between IL-6 and EMT in GBC.

Acknowledgements

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