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Predictive value of intratumour inflammatory cytokine mRNA levels of hepatocellular carcinoma patients and activation of two distinct pathways govern IL-8 induced epithelial-mesenchymal transition in human hepatic cancer cell lines

Siqi Peng[a,1], Yutong Chen[a,1], Yihang Gong[b], Zizi Li[c], Rongzhi Xie[a], Yujing Lin[c], Baojia Zou[b], Jian Li[b,*], Linjuan Zeng[a,*]

[a] Department of Abdominal Oncology, The Cancer Center of the Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai 519000, China
[b] Department of Hepatobiliary Surgery, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, China
[c] Department of Pathology, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, China

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ABSTRACT

Hepatocellular carcinoma (HCC) is always accompanied by persistent inflammation of liver tissues, which is considered to exert protumourigenic effects by promoting cancer growth, progression, and metastasis. However, the tumour-promoting roles and predictive value of intratumoural inflammatory cytokines remain unclear. In the present study, we used database analysis, clinical pathological studies, and *in vitro* biological experiments on human hepatic cancer cell lines to assess the prognostic potential of the primary tumour cytokine mRNA levels and underlying mechanisms in HCC. First, we assessed the prognostic value of several cytokines from the TCGA database and found that IL-8 is a unique cytokine that is associated with poor overall survival of HCC patients. Then, we collected 87 HCC tumour and adjacent non-tumour specimens from patients and confirmed that patients with low IL-8 expression exhibited less intrahepatic invasion or distant metastasis, a lower recurrence rate and longer overall survival time compared to patients with high IL-8 expression. Wound healing, transwell, and western blotting assay results showed that IL-8 promotes the migration and invasion of Huh-7 and HepG2 cells, and the underlying mechanism is that IL-8 induces the EMT of HCC cells via the IL-8/ERK1/2/SNAI1 and IL-8/STAT3/TWIST1 signalling pathways. These results provide valuable biological IL-8 information which needs to be further investigated in liver cancer target therapy research. Furthermore, the intratumoural cytokine expression at the mRNA level may provide insight into hepatocarcinoma prognosis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent causes of cancer-related death worldwide. The 5-year recurrence rate after radical resection is up to 70% [1]. Although many novel treatments have been identified and great progress has been made in conquering recurrence in the last two decades via the use of therapies such as transcatheter arterial chemoembolization/transarterial chemoembolization (TACE), sorafenib and systemic chemotherapy, unfortunately, the prognosis of HCC has apparently not improved [2]. One of the essential keys to unlocking this paradox is to identify more precise biomarkers that are not only predictable for judging tumour recurrence but also candidate therapeutic targets for HCC.

High-risk factors for HCC include chronic liver cirrhosis background, hepatitis virus infection, alcohol abuse, metabolic syndrome, and non-alcoholic fatty liver disease. These pretumoural situations always lead to persistent inflammation in the hepatic parenchyma [3]. Increasing evidence has indicated that high levels of inflammatory

Abbreviations: EMT-TFs, epithelial-mesenchymal transition transcription factors; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, foetal bovine serum; HCC, Hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; H&E, haematoxylin and eosin; HRP, horseradish peroxidase; IL, interleukin; OS, overall survival; STAT3, transducer and activator of transcription 3; TACE, transcatheter arterial chemoembolization/transarterial chemoembolization; TCGA, The Cancer Genome Atlas; VM, vascular mimicry

* Corresponding authors at: 52 Mei Hua East Road, Zhuhai, Guangdong 519000, China.
E-mail addresses: lijian5@mail.sysu.edu.cn (J. Li), zenglinj@mail.sysu.edu.cn (L. Zeng).
[1] These authors contributed equally to this work.

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cytokines, such as IL-6, IL-8, IL-1β, and IL-10, exert protumourigenic effects specifically in promoting cancer growth, progression, and metastasis [4,5]. Tumour microenvironment components, including immune cells, fibroblasts, and malignant cells themselves, are the main source of inflammatory cytokines [6]. Other cells, such as adipocytes, epithelial cells, endothelial cells, and smooth muscle cells, may also produce inflammatory cytokines [7–10]. Consequently, inflammatory cytokines drive protumour effects on tumour cells and the tumour microenvironment in an autocrine and paracrine manner [11]. In addition, crosstalk between inflammatory cytokines may exist and mutually enhance their expression; for example, IL-21 stimulation upregulates the production of IL-2 and IFN-γ [12]. Although many studies have focused on the role of inflammatory cytokines in several types of cancer, the tumour-promoting roles of inflammatory cytokines in HCC progression and the predictive value that cytokine levels may contribute to prognosis improvement remain unclear.

Cytokine levels are commonly detected in bodily fluids, such as serum [13], and tumour interstitial fluid [14], and cytokine receptor levels can be detected in the tumour [7]. However, cytokine protein levels can be affected by chemical treatment, tumour metastasis, immune inflammation, and environmental hypoxia; hence, there are contrary outcomes and OS in different types of cancers. Whether intratumoural cytokine mRNA levels, which reflect the natural course of HCC, are valuable prognostic biomarkers remains unknown. The purpose of identifying prognostic markers using primary tumour tissues is to stratify the recurrence risk with high accuracy at the time of diagnosis without influence from other exogeneous factors. Moreover, the exact mechanisms by which inflammatory cytokines exert protumourigenic effects on HCC cells needs to be further elucidated.

In the present study, we analysed database information, collected clinical pathological features, and performed *in vitro* biological experiments using human hepatic cancer cell lines to assess the prognostic value of cytokine mRNA levels in the primary tumour and elucidated the underlying mechanisms in HCC.

2. Materials and methods

2.1. Human tissues.

Human liver cancer tissues were collected immediately from patients who underwent surgical resection at the Fifth Affiliated Hospital of Sun Yat-sen University (Zhuhai, China) from March 2010 to July 2017. Diagnosis was confirmed with haematoxylin and eosin (H&E) staining, and the pathological diagnosis of HCC was independently identified by two pathologists. The paired adjacent non-tumoural tissues without histopathological cancer cells were collected from at least 3 cm away from the tumour margin. The protocol was approved by the Institutional Ethical Review Board of the hospital.

2.2. Cell lines and cell culture

Huh-7 and HepG2 cell lines were purchased from the American Type Culture Collection (ATCC, VA, USA) and cultured in a humidified 5% CO_2 incubator at 37 °C. Both cell lines were incubated in DMEM (Thermo Fisher Scientific, MA, USA) supplemented with 10% foetal bovine serum (FBS, Thermo Fisher Scientific) and 1% penicillin/streptomycin (KeyGENBioTECH, Jiangsu, China).

2.3. Reagents

Recombinant human IL-8 (rIL-8) was purchased from Peprotech (NJ, USA). The Stattic STAT3 inhibitor and U0126 ERK1/2 inhibitor were purchased from Selleckchem (Houston, TX) and Abcam (Cambridge, UK), respectively. Primary antibodies, including SNAI1 (#3879), E-cadherin (#5296), STAT3 (#4904), phospho-STAT3 (#9145), Akt (#4691), phospho-Akt (#4060), MAPK Family Antibody Sampler Kit (#9926), Phospho-MAPK Family Antibody Sampler Kit (#9910), and β-Tubulin (#2128), were obtained from Cell Signaling Technology (MA, USA). The primary antibody TWIST1 (ab175430) was obtained from Abcam. The secondary antibodies HRP-goat anti-mouse (#7076) and HRP-goat anti-rabbit (#7074) were purchased from Cell Signaling Technology.

2.4. The cancer Genome Atlas analysis

The gene expression data and relevant clinical features were obtained from the TCGA public website (https://cancergenome.nih.gov/). Finally, the data of 343 hepatocarcinoma samples were retained for further analysis.

2.5. Migration and invasion assays

In order to evaluate the wound healing ability of Huh-7 and HepG2 cells, a straight scratch was made using a 200 μL pipette tip to simulate a wound, which was observed and measured immediately and 24 h after making the scratch. Cell invasion was examined using 24-well transwell chambers coated with Matrigel (Corning Costar, Cambridge, MA, USA). Chambers have upper and lower culture compartments that are separated by polycarbonate membranes with 8-μm pores (Corning Costar). The bottom chamber was filled with 10% FBS as a chemoattractant. Cells in serum-free medium were seeded at 5×10^4 per well in the top chamber and incubated for 40 h. Cells that migrated to the underside of the membrane were stained with 0.1% crystal violet, imaged, and counted with a microscope (Zeiss, Gottingen, Germany). All experiments were performed in triplicate.

2.6. IL-8 stimulation and pathway inhibition assays

Huh-7 and HepG2 cells were incubated at a density of 2×10^5 cells per well in 6-well plates for 24 h. After incubation in serum-free medium for an additional 12 h, rIL-8 was added at a final concentration of 100 ng/ml. Then, 20 μM Stattic or 10 μM U0126 was added to the culture medium 2 h prior to IL-8 co-culture. Cells were harvested for further analysis at 12, 24, and 36 h after treatment.

2.7. RNA extraction and quantitative real-time PCR

Total RNA was extracted from 53 paraffin-embedded HCC specimens with a HiPure FFPE RNA Plus Kit (Magen, Guangzhou, China) and 34 frozen HCC tissues with RNAiso Plus (TaKaRa, Kyoto, Japan). Then, 0.5 μg of total RNA was reverse-transcribed to cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). Qualitative real-time PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa) according to the manufacturer's instructions. The relative mRNA expression was quantitated using the comparative Ct method (ΔCt) and calculated as $2^{-\Delta Ct}$, which was normalized to the housekeeping gene β-actin. The definition of high- or low-relative mRNA expression was based on the median value, which was set as the boundary value [15].

The primer sequences are as follows:

IL-8: forward: 5′GGGTGGAAAGGTTTGGAGTAT3′;
reverse 5′TAGGACAAGAGCCAGGAAGAA3′;
β-actin: forward 5′CATGTACGTTGCTATCCAGGC3′;
reverse 5′CTCCTTAATGTCACGCACGAT3′.

2.8. Western blot assay

The concentrations of protein were determined using the BCA protein assay kit (Beyotime, Shanghai, China). An equal amount of 20 μg protein in each sample was subjected to SDS-PAGE separation, followed by blotting onto a polyvinylidene difluoride membrane (Millipore, MA, USA). The membrane was probed overnight at 4 °C using specific primary antibodies including SNAI1, TWIST, E-cadherin, STAT3, phospho-

STAT3, Akt, phospho-Akt, MAPK Family Antibody Sampler Kit, Phospho-MAPK Family Antibody Sampler Kit and β-Tubulin, followed by incubation with a horseradish peroxidase–conjugated secondary antibody, HRP-goat anti-mouse or HRP-goat anti-rabbit, for 1 h at room temperature. The signal was visualized with an enhanced chemiluminescence detection reagent (Tanon, Shanghai, China) using a Chemiluminescent Imaging System (Tanon).

2.9. Statistical analysis

All statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). The relationship between clinical characteristics and IL-8 was analysed with the chi-square test. The correlation between EMT-TFs mRNA levels and IL-8 mRNA levels in the original data for human HCC tissues published in the TCGA database was analysed by Pearson's correlation coefficient. The association between IL and 8 mRNA levels and overall patient survival was analysed using the Kaplan-Meier method. Multiple group differences were analysed by One-way analysis of variance (ANOVA). A two-tailed P-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. IL-8 is a prognostic indicator in HCC patients

IL-1β, IL-6, IL-8, and IL-10 are the most studied inflammatory cytokines that have been identified as protumourigenic. We assessed the prognostic value of IL-1β, IL-6, IL-8, and IL-10 using the TCGA database in HCC (Fig. 1). Among the candidate inflammatory cytokines, a high IL-8 expression level was significantly associated with poor OS in HCC patients (Fig. 1A), whereas IL-1β, IL-6, or IL-10 did not show an apparent correlation with the patient survival rate (Fig. 1B, C, D).

3.2. The correlation between IL-8 expression and clinicopathological features in HCC tissues

Clinicopathological characteristics of 87 HCC patients who underwent radical surgery were collected (Table 1). Chi-square test outcomes indicated that IL-8 expression was associated with lymph node metastasis, recurrence and ALT levels. The expression level of intratumoural IL-8 was significantly upregulated in the HCC tumour site compared with the adjacent non-tumour site (Fig. 2A). Furthermore, in patients without lymphatic metastasis, the expression of IL-8 was significantly higher than the group with lymphatic metastasis

Table 1
Association between IL-8 expression and clinicopathological features in 87 cases of HCC patients[a].

Factors		IL-8		*P*
		Low expression	High expression	
Age	< Y60	32	30	0.520
	≥Y60	11	14	
Sex	Male	38	36	0.391
	Female	5	8	
HBsAg	Positive	29	25	0.307
	Negative	14	19	
TNM	I	24	27	0.599
	II-IV	19	17	
Tumour size	≤5 cm	23	26	0.598
	> 5 cm	20	18	
Lymph node metastasis	Positive	7	16	**0.034***
	Negative	36	28	
Vascular invasion	Positive	12	20	0.09
	Negative	31	24	
Recurrence	Yes	17	30	**0.007***
	No	26	14	
ALT	≤80	39	32	**0.031***
	> 80	4	12	
Serum bilirubin	≤1.0	20	29	0.632
	> 1.0	13	15	
Albumin	≥35	31	25	0.137
	< 35	12	19	
AFP	≤20	18	18	0.928
	> 20	25	26	

* $P < 0.05$.
[a] Chi-square test and *P*-values are indicated.

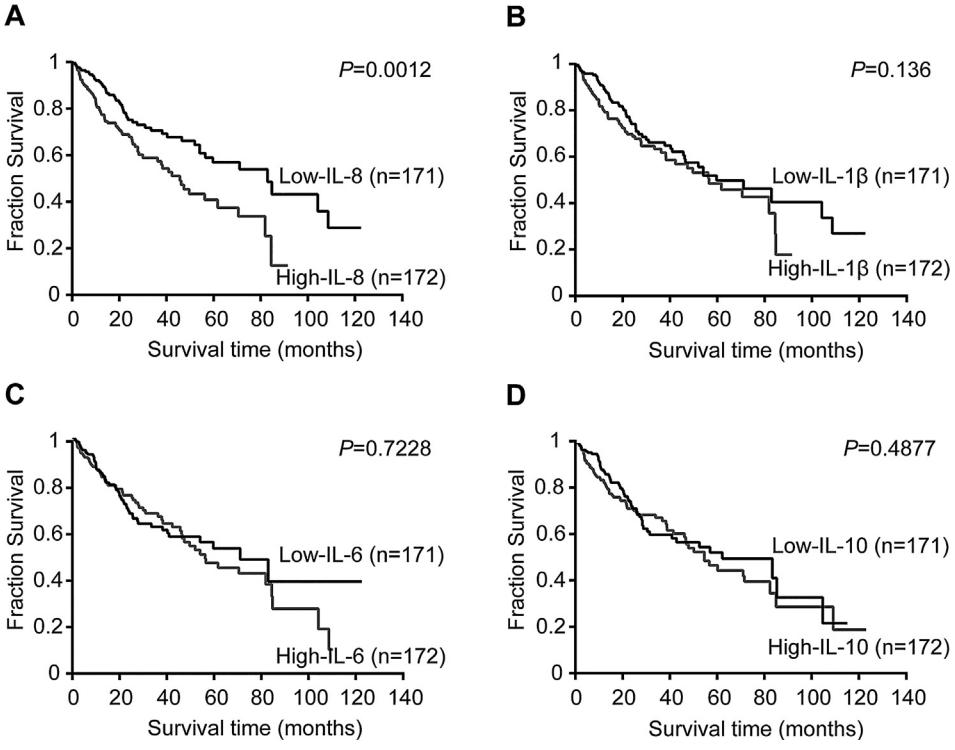

Fig. 1. Intratumoural IL-8 mRNA levels are associated with hepatocellular carcinoma (HCC) patient outcomes in the TCGA cohort. (A) HCC patients with low IL-8 expression had better OS (mean = 32.33 months) than those with high IL-8 expression (mean = 25.30 months). (B-D) There were no significant correlations between IL-1β, IL-6, or IL-10 expression and HCC patient OS. The definition of high- or low-relative mRNA expression was based on the median value, which was set as the boundary value.

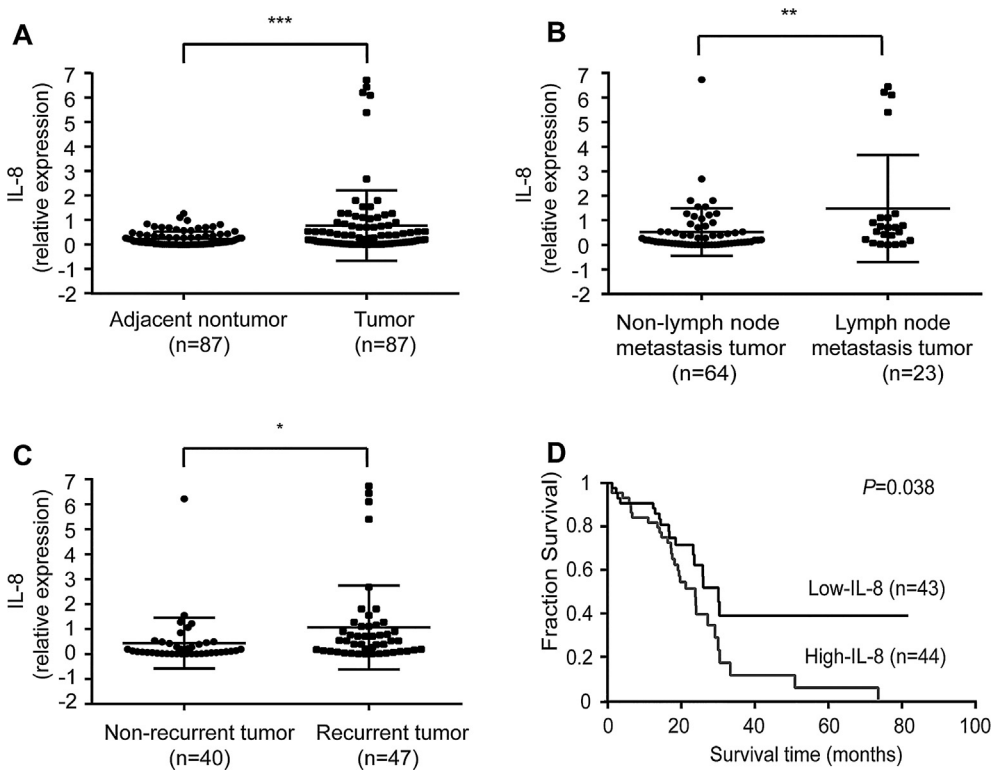

Fig. 2. The clinical significance of IL-8 mRNA levels in HCC patients. (A) The expression of IL-8 mRNA in HCC tumour tissues was higher than in adjacent non-tumour tissues (P = 0.0008). (B) Patients with lymph node metastatic tumours had higher IL-8 expression than patients with non-lymph node metastatic tumours (P = 0.0058). (C) The recurrent group had a higher IL-8 mRNA level compared to the non-recurrent tumour group (P = 0.0412). (D) Low-IL-8 expression patients exhibited longer OS times (mean = 21.06 months) compared to high-IL-8 expression patients (mean = 19.79 months). The expression levels of IL-8 mRNA were determined by qRT-PCR and normalized to an endogenous control (β-actin). *P < 0.05, **P < 0.01, and ***P < 0.001.

(Fig. 2B). Moreover, patients with recurrent cancer had remarkably higher IL-8 expression levels in tumour site tissues than non-relapsing patients (Fig. 2C). The K-M analysis demonstrated that patients with low-IL8 levels exhibited longer OS times than the patients with high-IL8 expression, which was consistent with the TCGA analysis (Fig. 2D). These results indicated that IL-8 expression at the tumour site may enhance HCC metastasis to a certain extent.

3.3. Intratumoural IL-8 mRNA expression positively correlates with epithelial-mesenchymal transition transcription factors mRNA expression

Epithelial-mesenchymal transition (EMT) is an important feature of metastatic cancer cells. During the process of EMT, SNAI1/2/3 and TWIST1/2 are important EMT transition transcription factors (EMT-TFs) that inhibit expression of the epithelial gene E-cadherin directly and/or indirectly [16]. We screened the TCGA database to evaluate correlations between IL-8 and SNAI1/2/3 and between IL-8 and TWIST1/2 mRNAs expression. Among SNAI1/2/3, the most apparent positive correlation was found between IL-8 and SNAI1 (r = 0.437, P < 0.0001, Fig. 3A), and there was a moderate positive correlation between IL-8 and TWIST1 (r = 0.281, P < 0.0001), whereas no significant correlation was indicated between IL-8 and TWIST2 (Fig. 3B).

3.4. IL-8 induces EMT and promotes migration and invasion of hepatic cancer cells

To explore the impact of IL-8 on EMT, we utilized rIL-8 to stimulate Huh-7 and HepG2 cells and detected the protein expression of SNAI1, TWIST1, and E-cadherin. Our results show that co-culture with rIL8 results in apparent increases in SNAI1 and TWIST1 expression in Huh-7 and HepG2 cells (Fig. 4A). Furthermore, after IL-8 stimulation, Huh-7 and HepG2 cells exhibited enhanced migration (2.5- and 2.3-fold) and invasion (2.2- and 2.3-fold) abilities (Fig. 4B). These data suggest that IL-8 induces EMT and promotes hepatic cancer cell migration and invasion.

3.5. The IL-8/ERK1/2/SNAI1 and IL-8/STAT3/TWIST1 pathways are involved in IL-8-induced EMT in human hepatic cancer cell lines

Previous studies have shown that constitutive activation of IL-8 in cancer cells leads to persistent downstream signalling through the PI3K/Akt [17], ERK-MAPK [18], p38 MAPK [19], and PKC [20] pathways, transducing growth signals to tumour cells and promoting tumour progression and dissemination. In order to identify the molecular basis by which IL-8 regulates SNAI1 and TWIST1 expression, we performed a series of western-blotting experiments at different time points after IL-8 stimulation. Activation of the MAPK pathway in Huh-7 and HepG2 cells was initially studied, and phosphorylation of the Thr^{202} and Thr^{204} residues of ERK was detected, whereas p38 or SAPK/JNK phosphorylation was not found. We also detected apparent phosphorylation of the Tyr^{705} residue of STAT3 in response to rIL-8 stimulation, which reached peak levels at 12 h after treatment. In the current study, we did not detect activation of AKT induced by rIL-8 (Fig. 5A).

Then, pathway inhibitors were used to further identify potential signalling pathways involved in IL-8-dependent SNAI1 and TWIST1 expression. Huh-7 and HepG2 cells were treated with a panel of pharmacological pathway inhibitors at biologically relevant concentrations. U0126 abrogated the IL-8-mediated upregulation of SNAI1 but did not influence the expression of TWIST1. Stattic significantly reversed TWIST1 protein levels but did not influence the expression of SNAI1 (Fig. 5B). Our results indicate that two different signalling pathways, IL-8/ERK1/2/SNAI1 and IL-8/ STAT3 /TWIST1, are involved in IL-8-induced EMT in liver cancer cells.

4. Discussion

Hepatocarcinogenesis constitutes multiple, complex steps, and growing evidence indicates that inflammation plays an important role in hepatocarcinogenesis and immune escape [21,22]. Inflammasome cytokines normally activate the host response when facing cellular stress and cellular damage, while they stimulate several stages of cancer formation and progression when an excessive host response provokes

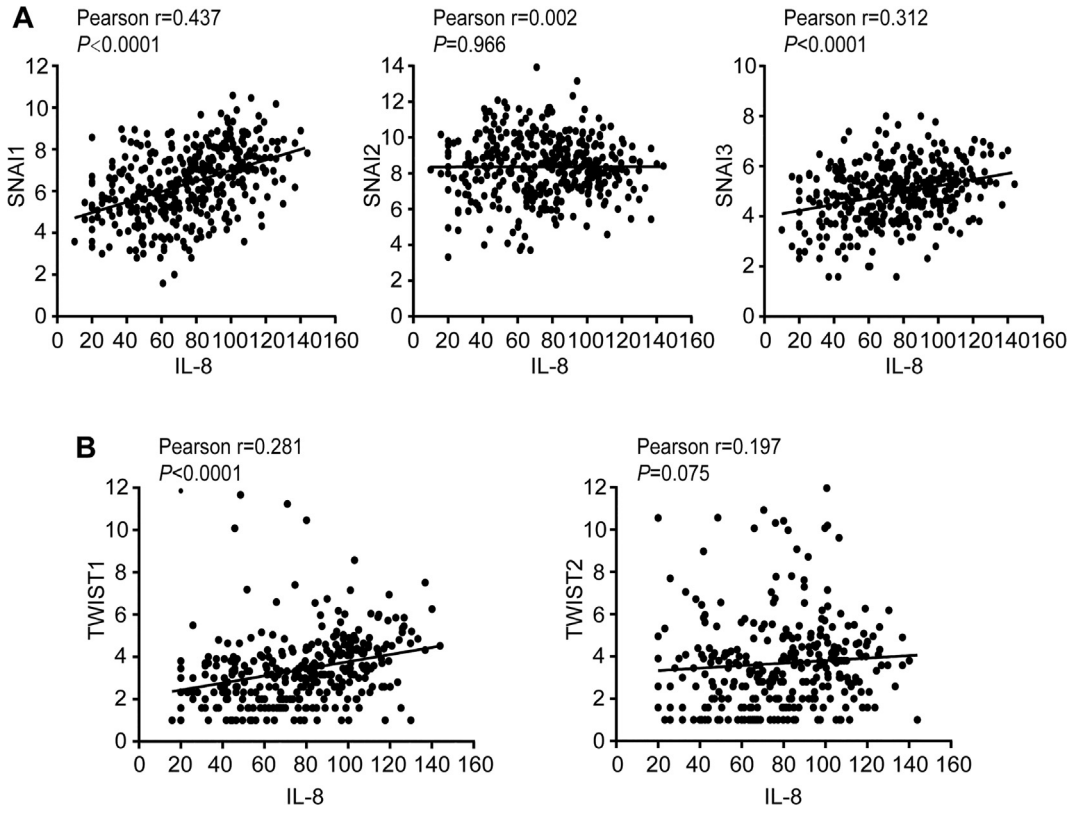

Fig. 3. Intratumoural IL-8 mRNA expression is positively correlated with epithelial-mesenchymal transition transcription factors mRNA expression. Pearson's correlation test shows the correlation between IL-8 levels and SNAI1/2/3 and TWIST1/2 expression. (A) IL-8 expression was positively correlated with SNAI1 ($r = 0.437$, $P < 0.0001$) and SNAI3 ($r = 0.311$, $P < 0.0001$). (B) There was a positive correlation between IL-8 and TWIST1 ($r = 0.281$, $P < 0.0001$), whereas there was no apparent correlation between IL-8 and TWIST2.

persistent cytokine production [23]. The tumour microenvironment is considered one of the hallmarks of cancer and is the main source of inflammasome activity [24]; the tumour microenvironment is currently being researched for the prevention, diagnosis and treatment of tumours. Immune cells, as well as tumour cells, produce pro-inflammatory cytokines to influence carcinogenesis in various stages of liver cancer [22]. If primary tumours could offer more prognostic clues, the disease outcome might be predicted at the time of diagnosis and provide guidance for personalized therapy as early as possible.

We screened the TCGA database to obtain general information, and then measured cytokines considered to be protumoural and pro-inflammatory in HCC. Interestingly, our assessment of intratumoural cytokine mRNA expression levels revealed the robust prognostic value of IL-8 for HCC patient survival. A previous study showed that preoperative high serum IL-8 levels in HCC patients correlate with large tumour size (> 5 cm), the absence of a tumour capsule, the presence of venous invasion, tumour stage, poorer disease-free survival and overall survival [25]. In addition, identification of low serum IL-8 levels pretreatment represents a promising biomarker for a longer OS in TACE-treated HCC patients [26].

IL-8, alternatively known as CXC motif ligand 8 (CXCL-8), is a multifunctional pro-inflammatory CXC chemokine [27]. Two cell surface G protein-coupled receptors, CXC motif receptor 1 (CXCR1) and CXCR2, are capable of binding to CXCL-8 and then exerting biological effects [28]. The protumoural activity of IL-8 has not been fully elucidated, however, IL-8 is considered to be an important angiogenic factor. In hepatocarcinoma cells, IL-8 upregulated vascular endothelial growth factor (VEGF)-A expression and promoted angiogenesis [29]. Similarly, a recent study showed that CXCL8 secreted by metastatic colorectal liver tumour cells at the invasion site was able to upregulate VEGF-A and promote migration through angiogenesis [30].

Tumour neovasculature not only provides nutrients for the growth of tumour cells but also creates paths for metastasis. In addition, IL-8/CXCR2 promotes vascular mimicry (VM) of tumour cells. VM is the ability of tumour cells to generate vascular-like structures without the presence of blood vascular endothelial cells [14,31]. In HCC, VM plays an important role in tumour cell portal vein invasion, which might be mediated by high expression levels of matrix metalloproteinases (MMP) 2 and -9 [32]. Notably, IL-8 exhibited positive regulation of MMP-1, -2, and -9 [33]; in addition, colorectal tumour CXCL8 enhanced invasion via the AKT/GSK3β/β-catenin/MMP7 pathway by upregulating BCL-2 [30]. These mechanisms may contribute to IL8-induced HCC metastasis.

Another important factor for hepatocellular carcinoma metastasis is EMT. EMT occurs in response to complicated signalling pathways that activate the expression of specific transcription factors (TFs) called EMT-TFs, which cause the transition from well-differentiated adenoma to invasive carcinoma [16]. Features of EMT cells are decreased expression of E-cadherin, occludins, and cytokeratins, which are known to be molecules involved in the epithelial phenotypes, as well as increased expression of molecules for the mesenchymal transition, such as N-cadherin and vimentin [34]. The SNAI and TWIST families are the most well-studies EMT-TFs and are thought to be major transcription factors frequently involved in EMT in a variety of cancers. However, other genes, such as ZEB1 and ZEB2, are also EMT-TFs [35]. Although we selected SNAI1 and TWIST1 as representative EMT-TFs for the evaluation of the IL-8-induced EMT effect on HCC cells in this study, the relationship between IL-8 and other EMT-TFs needs to be further elucidated.

Several studies have reported different signalling pathways underlying IL-8-induced EMT. For example, IL-8 alone or with IL-6 induced the appearance of cells with EMT and stemness characteristics among breast cancer cells [36]. Serglycin promotes breast cancer cell

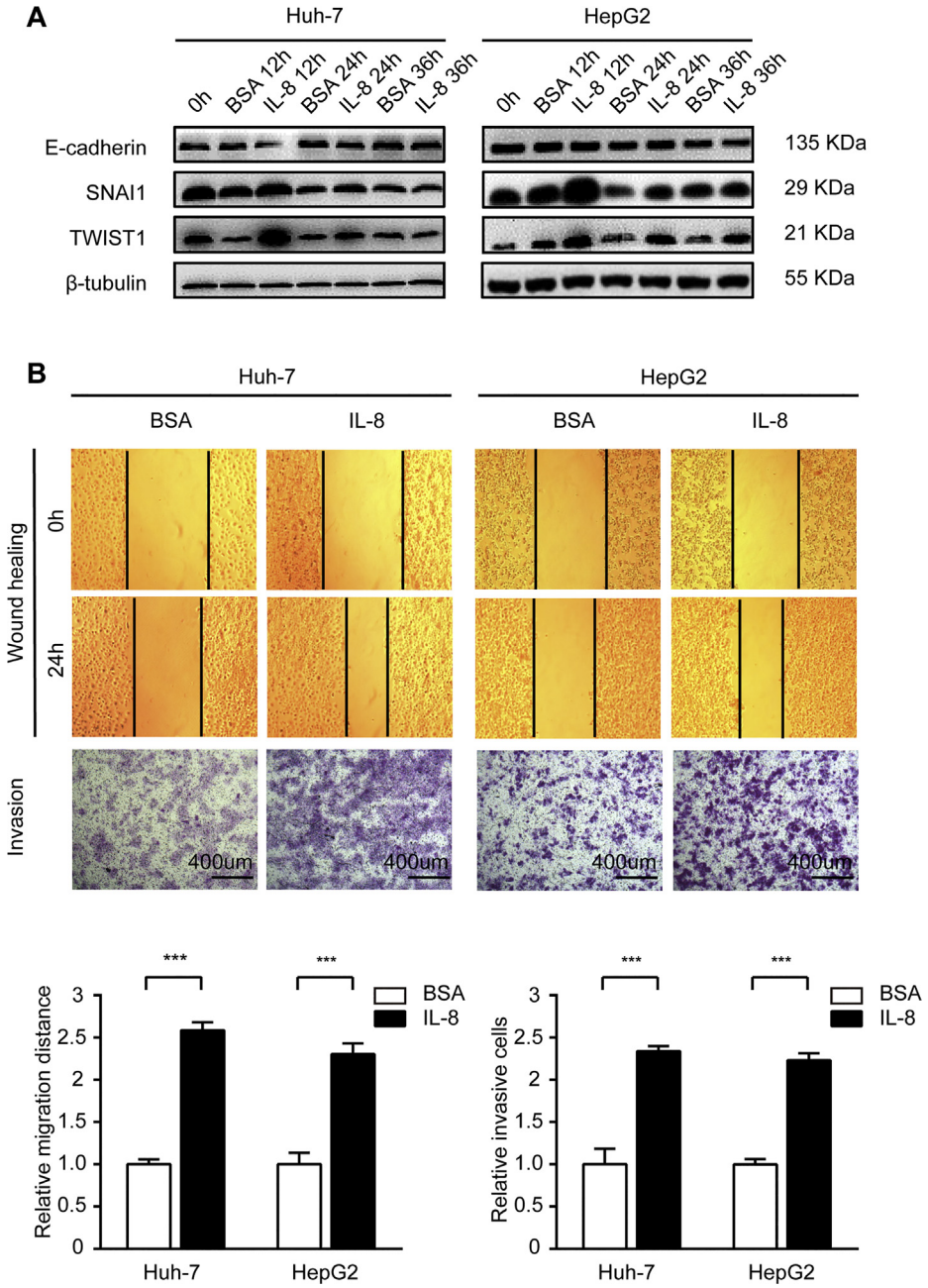

Fig. 4. IL-8 induces EMT and promotes migration and invasion of hepatic cancer cells. (A) Stimulation of rIL-8 increased SNAI1 and TWIST1 and reduced E-cadherin expression. Western blotting showed a transient time-dependent increase in SNAI1 and TWIST1 expression in HCC cells after rIL-8 treatment. (B) Wound healing assays and transwell invasion assays indicated that after IL-8 stimulation, Huh-7 and HepG2 cells exhibited enhanced migration and invasion abilities. Values are expressed as the mean ± SD, *P < 0.05, **P < 0.01, and ***P < 0.001. Cells treated with BSA were used as a negative control.

aggressiveness via autocrine activation of the IL-8/CXCR2 signalling axis [37]. IL-8/CXCR2 could also induce glioblastoma (GBM) cells into an intermediate stem cell–like state [14,28,38]. However, studies on the effect of IL-8 on HCC EMT are still very limited. It was shown that neurotensin (NTS)-induced IL-8 activated the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) pathways and then stimulated a pro-oncogenic inflammatory microenvironment by inducing M2-type TAMs, which dramatically promoted EMT of HCC cells [39]. Macrophage-secreted IL-8 promoted HCC cell migration and invasion by inducing EMT through the JAK2/STAT3/SNAI1 signalling pathway [40]. Our results clearly showed that IL-8 upregulated SNAI1 and TWIST1 expression and that there are two different signalling pathways contributing to IL-8-induced EMT in HCC cells. Although transforming growth factor-beta (TGF-β) is considered a strong EMT inducer [41], the intratumoural expression of TGF-β does not show an apparent effect on the overall survival of HCC patients based on the TCGA database (not shown). These results indicate that the effects of the inflammasome on HCC are complicated and may rely on the combined effect of multiple factors.

Occasionally, a prognostic marker, such as HER2 expression in breast cancer, also serves as a therapeutic target [42]. Translational medicine now views the well-studied IL-6 family cytokines as major therapeutic targets for clinical intervention in inflammatory diseases and cancers [43–45]. Many studies have begun to consider targeting IL-8 in cancer therapy. According to our results, IL-8 mRNA expression levels in primary tumours are apparently correlated with OS in HCC patients. Therefore, we need to identify the biological functions of tumour cell-intrinsic and tumour cell-extrinsic IL-8. In our future studies,

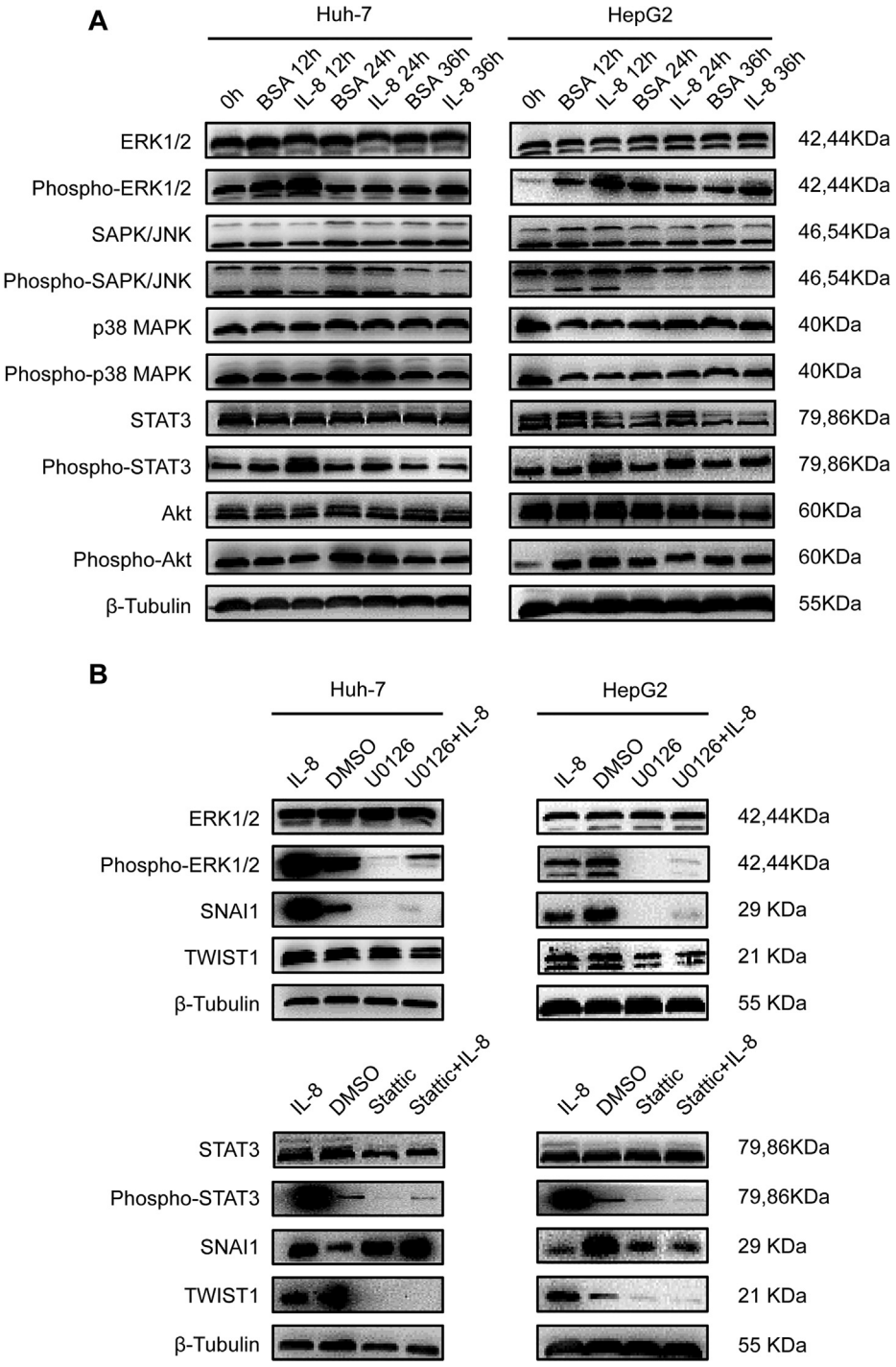

Fig. 5. The IL-8/ERK1/2/SNAI1 and IL-8/STAT3/TWIST1 signalling pathways are involved in IL-8-induced EMT in Huh-7 and HepG2 cells. (A) Protein levels of phospho- or total MAPK family proteins, STAT3 and Akt were examined in Huh-7 and HepG2 cells co-cultured with rIL-8. (B) U0126 abrogated the IL-8-mediated upregulation of SNAI1 but did not influence the expression of TWIST1. Stattic significantly reversed TWIST1 protein levels but did not influence the expression of SNAI1.

we will focus on the following aspects: (i) Whether tumour cell-intrinsic IL-8 also transcriptionally induces numerous molecular targets via CXCR1/2 activation [46] (ii) Which receptor of IL-8, CXCR1 or CXCR2, is more effective in activating the downstream signalling pathway [14]? What type of IL-8/receptor axis inhibitor (monoclonal antibody or small molecular inhibitor of CXCR1/2) is more efficient at inhibiting HCC?

In conclusion, we report the strong prognostic value of intratumoural IL-8 mRNA levels and the potential signalling pathways contributing to IL-8-induced EMT in liver cancer cells. The prognostic association of intratumoural IL-8 in primary liver tumours is intriguing and may not be affected by additional treatments, especially systemic therapies. Our results provide valuable biological information that needs to be further investigated in liver cancer target therapy research. Furthermore, the intratumoural cytokine expression of IL-8 at the mRNA level may provide insight into the prognosis of patients with hepatocarcinoma.

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Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Siqi Peng: Conceptualization, Writing - original draft. **Yutong Chen:** Data curation, Software. **Yihang Gong:** Formal analysis, Investigation. **Zizi Li:** Methodology, Visualization. **Rongzhi Xie:** Software. **Yujing Lin:** Resources. **Baojia Zou:** Data curation, Validation. **Jian Li:** Project administration, Funding acquisition. **Linjuan Zeng:** Writing - review & editing, Supervision, Funding acquisition.

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