CXCL5 contributes to tumor metastasis and recurrence of intrahepatic cholangiocarcinoma by recruiting infiltrative intratumoral neutrophils

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CXCL5 is a member of the CXC-type chemokine family that may play a role in carcinogenesis and cancer progression. This study investigates the biological function and clinical significance of CXCL5 in intrahepatic cholangiocarcinoma (ICC). We demonstrated that CXCL5 was overexpressed in ICC cell lines and tumor samples compared with paired normal tissues. CXCL5 had a direct chemoattractant effect on neutrophils *in vitro* through PI3K-Akt and extracellular signal-regulated kinase 1/2 signaling pathways. In animal studies, CXCL5 promoted tumor growth and metastasis without altering *in vitro* proliferative and invasive ability of ICC cells, and this effect was mediated by the recruitment of intratumoral infiltrative neutrophils by tumor-derived CXCL5. Immunohistochemical analysis of ICC samples showed that overexpression of CXCL5 correlated strongly with intratumoral neutrophil infiltration, shorter overall survival and high tumor recurrence. Multivariate analysis revealed that CXCL5 overexpression alone, or combined with the presence of intratumoral neutrophils, was an independent prognostic indicator for ICC. In conclusion, our data showed that CXCL5 promotes ICC growth and metastasis by recruiting intratumoral neutrophils. CXCL5 alone or combined with intratumoral neutrophils is a novel prognostic predictor for ICC patients and a potential therapeutic target.

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary hepatic malignancy after hepatocellular carcinoma (HCC), with an increasing incidence worldwide (1,2). Early detection of ICC through advances in diagnostic modalities and clinical screening has made it possible to perform curative resection. However, the long-term survival of patients with ICC remains unsatisfactory because of high recurrence rates and early metastasis (3,4). A better understanding of the molecular mechanisms associated with progression of ICC would be beneficial for the development of effective therapeutic schemes.

Inflammation has emerged as the seventh hallmark of cancer (5). Over the last decade, it has been established that cancer-related inflammation is involved in many aspects of malignancy, and particularly enhances tumor cell survival, proliferation and metastasis (6,7). Although most ICCs arise in the absence of apparent risk factors, chronic inflammation of the biliary epithelium plays a critical role in their development (8). Primary sclerosing cholangitis is the most common predisposing condition for cholangiocarcinogenesis (9,10). And other risk factors are also associated with chronic inflammation, including chronic choledocholithiasis, liver fluke infestation, hepatolithiasis, Caroli's disease and hepatitis C viral infection (9,10), suggesting possible crosstalk between inflammation and ICC development (11). Studies on the mechanisms of inflammation-associated progression and prognosis in ICC are therefore urgently needed.

Chemokines and their receptors have been identified as mediators of chronic inflammation (12). Components of the chemokine system affect multiple pathways of tumor progression including leukocyte recruitment, neo-angiogenesis, and tumor cell proliferation, survival, invasion and metastasis (13). Recently, CXCL5 (epithelial neutrophil-activating peptide-78) has been the focus of studies examining the role(s) of chemokines in carcinogenesis and tumor progression. Like other chemokines that recognize and bind the G-protein coupled receptor CXCR2, CXCL5 is a pro-angiogenic CXC-type chemokine that is an inflammatory mediator and a powerful attractant for neutrophils (14,15). CXCL5 is overexpressed in gastric (16), prostate (17,18), endometrial (19), squamous cell (20) and pancreatic cancer (21), and increased expression of CXCL5 is associated with advanced tumor stages, local invasion and metastatic potential. In our last study, we showed that CXCL5 is overexpressed in HCCs and promotes HCC growth, metastasis and intratumoral neutrophil infiltration. We also showed that CXCL5 overexpression, alone or combined with the presence of intratumoral neutrophils, can predict the prognosis of HCC patients after resection (22). However, the role of CXCL5 and its relationship with cancer-related inflammation in ICC is largely unknown.

In this study, we investigated the expression of CXCL5 in ICC cell lines and tumor samples. We explored how invasive and metastatic ability was related to changes in CXCL5 expression and investigated how changes in CXCL5 expression influenced neutrophil infiltration. Using tissue microarrays (TMAs) in ICC samples, we determined the relationship between CXCL5 expression and neutrophil infiltration and evaluated its prognostic significance.

Materials and methods

Cell lines and animals

One normal human intrahepatic biliary epithelial cell line, HIBEpiC (purchased from ScienCell Research Laboratories, Carlsbad, CA), and three human ICC cell lines, HCCC-9810, RBE (purchased from the Chinese Academy of Sciences Shanghai Branch Cell Bank, Shanghai, China) and HuH-28 (23), were used in this study. These cell lines were routinely maintained. Male non-obese diabetic-severe combined immunodeficiency (SCID) mice (4- to 6-weeks-old; Shanghai Institute of Material Medicine, Chinese Academy of Science) were housed in specific pathogen-free conditions. Animal care and experimental protocols were in accordance with guidelines established by the Shanghai Medical Experimental Animal Care Commission.

Patients and follow-up

Two independent cohorts of ICC patients were enrolled in this study. For the first cohort (snap-frozen tissues), 60 ICC samples and matched non-tumorous tissues used for quantitative real-time–PCR (qRT–PCR) and western blot analysis were consecutively collected from patients undergoing curative resection between February 2007 and December 2008 at the Liver Surgery Department, Zhongshan Hospital, Fudan University. The second cohort (paraffin-embedded tissues) used for TMA analysis consisted of 140 consecutive patients with ICC who underwent curative resection between February 1999 and November 2006 at the same department. Curative resection was defined as complete resection of tumor nodules, with the tumor margins rendered free of cancer on histologic examination, and resection of regional lymph nodes, including the hilar, hepatoduodenal ligament and caval lymph nodes, with no cancerous thrombus in the portal vein (main trunk or two major branches), hepatic veins or bile duct

Abbreviations: CM, conditioned medium; ELISA, enzyme-linked immunosorbent assay; ERK 1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HR, hazard ratio; ICC, intrahepatic cholangiocarcinoma; MAPK, mitogen-activated protein kinase; NCM, neutrophil-conditioned medium; OS, overall survival; qRT–PCR, quantitative real-time–PCR; SCID, severe combined immunodeficiency; shRNA, short hairpin RNA; TMA, tissue microarray.

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(24). Patients with ICC who had lymph node involvement beyond these lymph nodes were categorized as distant metastasis and excluded from the study (3). The histopathological diagnosis was based on the World Health Organization criteria. The histological grade of tumor differentiation was determined according to the classification proposed by Edmondson and Steiner. Liver function was assessed by the Child-–ugh scoring system. Tumor stage was determined according to the 2010 International Union against Cancer tumor-node-metastasis classification system. Ethical approval for the use of human subjects was obtained from the research ethics committee of Zhongshan Hospital, and informed consent was obtained from each patient. Patients and their detailed clinicopathologic characteristics are listed in Supplementary Table 1, available at *Carcinogenesis* Online. Follow-up data were summarized as of February 2009, and the median follow-up was 15.4 months (range 4–120 months). Follow-up procedures were described in detail in our previous report (25).

Cell transfection

pGCSIL-GFP-short hairpin RNA (shRNA)-CXCL5 and pGC-FU-GFP-CXCL5 lentiviral vectors were purchased from Shanghai GeneChem Co. and the target shRNA sequences are listed in the Supplementary Materials and Methods, available at *Carcinogenesis* Online. pGC-FU-GFP-CXCL5 was transfected into ICC cells with lower CXCL5 expression (HCCC-9810) and pGCSIL-GFP-shRNA-CXCL5 was transfected into ICC cells that highly express CXCL5 (RBE). pGCSIL-GFP and pGC-FU-GFP lentiviral vectors were used as controls. CXCL5 expression in stably transfected clones was validated by qRT–PCR, immunoblotting and enzyme-linked immunosorbent assay (ELISA).

Isolation and preparation of human neutrophils

Human neutrophils were isolated as described previously (26). Briefly, blood was collected from ICC patients and the erythrocytes were removed using dextran sedimentation (6% dextran, 0.9% NaCl) followed by one round of hypotonic lysis using double-distilled water (ddH$_2$O). Neutrophils were purified using Ficoll-Histopaque 1077 density centrifugation and resuspended in RPMI 1640 containing 2% fetal bovine serum (FBS). Neutrophil-conditioned medium (NCM) was obtained by culturing neutrophils (3×10^6 cells/ml) in RPMI 1640 containing 2% FBS for 2 h.

Neutrophil chemotaxis assay

Neutrophil chemotaxis was assayed in a Transwell system (Corning) using 3 µm polycarbonate membranes as described previously (26). Briefly, human CXCL5 (Sigma) in RPMI 1640 containing 2% FBS or conditioned medium (CM) from ICC cells with or without CXCL5 neutralizing antibody (5 µg/ml, clone 33160; R&D Systems) was added to the lower wells. Neutrophils suspended in RPMI 1640 containing 2% FBS (2×10^5 cells/100 µl) were added to the upper wells and incubated for 30 min at 37°C, 5% CO$_2$. Neutrophils that migrated to the lower chamber were collected and counted in Neubauer chambers. Neutrophil migration toward RPMI 1640 alone was used as the negative control. To evaluate the involvement of AKT and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways in CXCL5-induced neutrophil chemotaxis, the cells were pretreated with selective inhibitors at 37°C and 5% CO$_2$ for 1 h. The chemotactic index was calculated as the ratio of the number of neutrophils that migrated to CXCL5-containing wells divided by the number of neutrophils that migrated to RPMI 1640 alone (26).

In vivo *assays for tumor growth and metastasis*

HCCC-9810-CXCL5, HCCC-9810-Mock, RBE-shRNA-CXCL5 and RBE-Mock cells (5×10^6) were suspended in 100 µl serum-free RPMI 1640 and Matrigel (BD Biosciences; 1:1) and injected subcutaneously into the upper left flank region of non-obese diabetic SCID mice. The mice were observed over 5 weeks for tumor formation. Neutrophil depletion was performed by intratumoral injections of 30 µg anti-Ly6G antibody 1A8 (BD Biosciences) twice a week from day 14 after inoculation, when tumors were palpable (27). Neutrophil depletion was confirmed at the end of each experiment using immunohistochemistry. The tumor volume was measured twice weekly with a caliper and calculated in cm^3 as follows: V = ab^2/2 [where a and b represent the largest and smallest tumor diameters measured at necropsy (22)]. Upon killing, the tumors were recovered and the volume of each tumor was determined. Lungs were removed and embedded in paraffin and the total number of lung metastases was counted under the microscope as described previously (22). Tumor tissue sections were prepared and immunoreactivity was analyzed as described using antibodies against Ly6G (1:100, 1A8; BD Biosciences) and CXCL5 (1:50, clone 33160; R&D Systems).

Cell proliferation assay, cell migration and matrigel invasion assays

Cell proliferation assay, cell migration and matrigel invasion assays are included in the Supplementary Materials and Methods, available at *Carcinogenesis* Online.

RNA isolation and qRT–PCR

RNA isolation and qRT–PCR were performed as described previously (28). The detailed information and procedures are described in the Supplementary Materials and Methods, available at *Carcinogenesis* Online.

Western blot, immunofluorescence and phospho-kinase array analysis

Western blot, immunofluorescence and phospho-kinase array analysis are included in the Supplementary Materials and Methods, available at *Carcinogenesis* Online.

Enzyme-linked immunosorbent assay

The level of CXCL5 in cell culture supernatants was determined using the Quantikine Human CXCL5 ELISA Kit (Abcam Systems) according to the manufacturer's instructions and is described in the Supplementary Materials and Methods, available at *Carcinogenesis* Online.

Immunohistochemistry and evaluation of immunohistochemical variables

Immunohistochemical staining and evaluation were performed as described previously (22) and are described in the Supplementary Materials and Methods, available at *Carcinogenesis* Online.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 for Windows. Quantitative data between groups were compared using the Student's *t*-test. Categorical data were analyzed by the chi-square test or Fisher exact test. Correlation analysis was performed between CXCL5 and CD66b. Overall survival (OS) and cumulative recurrence rates were calculated by the Kaplan–Meier method and differences were analyzed by the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

Results

Expression of CXCL5 is upregulated in human ICCs

To explore the role of CXCL5 in ICC development, we first evaluated the expression of CXCL5 in various human ICC cell lines. As shown in Figure 1A and Supplementary Figure 1A, available at *Carcinogenesis* Online, elevated expression of CXCL5 was observed in ICC cell lines compared with a normal human intrahepatic biliary epithelial cell line. In addition, CXCL5 transcript levels were significantly increased in ICC samples relative to paired non-cancerous tissues from 60 patients (Figure 1B), which was further confirmed at the protein level by western blot analysis (Figure 1C). Immunohistochemical analysis in TMA also showed upregulation of CXCL5 in tumor samples of the ICC patients (Supplementary Figure 1B, available at *Carcinogenesis* Online).

CXCL5 does not affect proliferation, migration and invasion of ICC cells *in vitro*

Stable up- or down-regulation of CXCL5 expression in transfected ICC cell lines was confirmed by qRT–PCR and western blotting (Figure 1D and Supplementary Figure 2, available at *Carcinogenesis* Online). The results were consistent with the levels of CXCL5 in cell culture supernatants determined by ELISA (Figure 1D). Downregulation of CXCL5 in RBE cells by shRNA had no significant effect on cell proliferation at any of the indicated times ($P > 0.05$). Similarly, proliferation of HCCC-9810-CXCL5 was not changed compared with HCCC-9810-Mock cells ($P > 0.05$; Supplementary Figure 3A, available at *Carcinogenesis* Online). CXCL5 had no effect on migration and invasive abilities of ICC cells *in vitro* (Supplementary Figure 3B and C, available at *Carcinogenesis* Online).

CXCL5 promotes ICC growth and metastasis in tumor xenograft models

After injection into non-obese diabetic SCID mice, all cell lines successfully formed tumors. The tumor size of RBE-Mock-derived xenografts was $2.02 \pm 0.37 \, \text{cm}^3$, significantly larger than that of xenografts derived from RBE-shRNA-CXCL5 cells ($0.82 \pm 0.26 \, \text{cm}^3$; $P < 0.01$). Similarly, the tumor size of HCCC-9810-CXCL5-derived xenografts was $1.19 \pm 0.79 \, \text{cm}^3$, markedly larger than HCCC-9810-Mock-derived tumors ($0.25 \pm 0.09 \, \text{cm}^3$; $P < 0.05$;
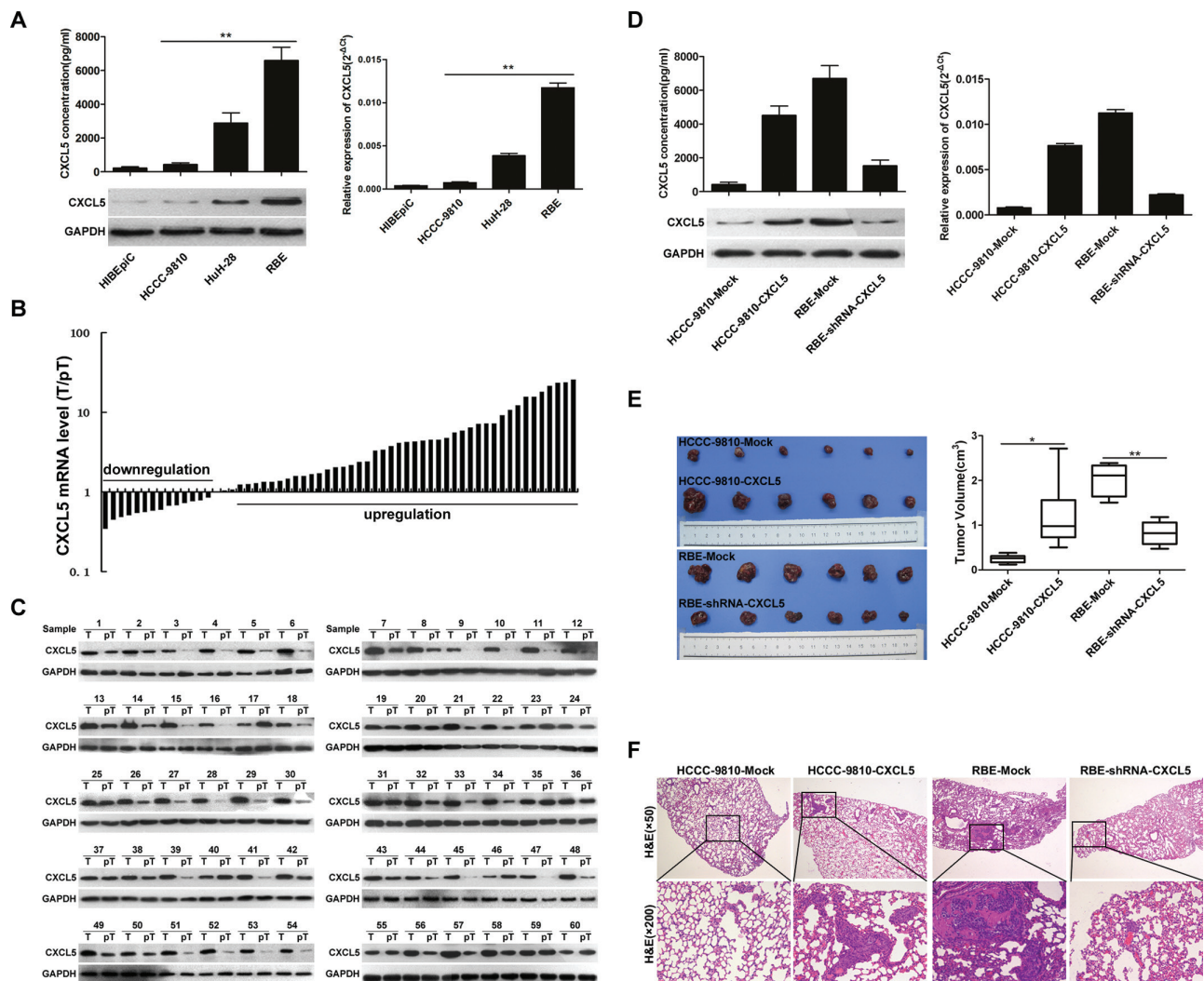
Fig. 1. CXCL5 is upregulated in human ICCs and promotes ICC growth and metastasis *in vivo*. (**A**) Expression of CXCL5 in one human normal intrahepatic biliary epithelial cell line (HIBEpiC) and three ICC cell lines (HCCC-9810, HuH-28 and RBE) was examined by real-time PCR, western blot and ELISA. (**B**) CXCL5 expression in 60 paired samples of ICC and surrounding tissues was measured by real-time PCR. (**C**) Western blot showing the expression of CXCL5 protein in tumor tissue (T) and paired non-cancerous tissues (N) from these ICC patients. (**D**) qRT–PCR, western blot and ELISA confirmed CXCL5 expression levels in stably transfected and parent ICC cells. (**E**) CXCL5 promotes ICC growth in a tumor xenograft model. (**F**) CXCL5 promotes pulmonary metastasis of ICC in a tumor xenograft model. (*$P < 0.05$; **$P < 0.01$).

Figure 1E). Pulmonary metastasis occurred in 83.3% (5/6) of the RBE-Mock mice, a higher rate than observed in the RBE-shRNA-CXCL5 mice (1/6). The number of metastatic nodules of each grade in the lung was also greater in the RBE-Mock mice (Figure 1F). Pulmonary metastasis occurred in 75% (4/6) of HCCC-9810-CXCL5 mice, compared with 16.7% (1/6) of HCCC-9810-Mock mice (Figure 1F).

CXCL5 induces neutrophil migration in vitro *and mediates intratumoral neutrophil infiltration* in vivo
CXCL5 induced neutrophil cell migration within the range of 0.01–100 nM, with a peak effect at 10 nM (Figure 2A). Accordingly, 10 nM CXCL5 was used for all following experiments. The chemotactic effect of ICC cells was evaluated in the presence of ICC cell CM obtained with increasing incubation times (24–72 h). When compared with control conditions, CM from ICC cells with high expression levels of CXCL5 (RBE or HCCC-9810-CXCL5) induced a significant increase in neutrophil migration, whereas CM from cells with low levels of secreted CXCL5 (RBE-shRNA-CXCL5 or HCCC-9810) induced only a slight increase in neutrophil migration (Figure 2B). The chemotactic effect of ICC cells on neutrophils was abolished by

CXCL5 neutralizing antibody (Supplementary Figure 4, available at *Carcinogenesis* Online).

We next tested the effect of CXCL5 on neutrophil infiltration *in vivo*. Notably, intratumor Ly6G+ neutrophil infiltration was significantly higher in the RBE-Mock-derived xenografts than in RBE-shRNA-CXCL5-derived xenografts and significantly lower in the HCCC-9810-Mock-derived xenografts than in HCCC-9810-CXCL5-derived xenografts (Figure 2C). However, we did not observe any difference in the number of other intratumor infiltrative stromal cells including F4/80+ macrophages, αSMA+ fibroblasts and tryptase+ mast cells in the presence of CXCL5 (Supplementary Figure 5, available at *Carcinogenesis* Online).

CXCL5 induces neutrophil migration through PI3K-Akt and ERK1/2-MAPK signaling pathways
To analyze the mechanism underlying CXCL5-mediated chemotaxis of neutrophils, we performed a phospho-kinase array on human neutrophils with or without CXCL5 treatment to detect the activation of 46 kinases involved in several signaling pathways, including Akt, mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription family, Src family kinase, focal adhesion
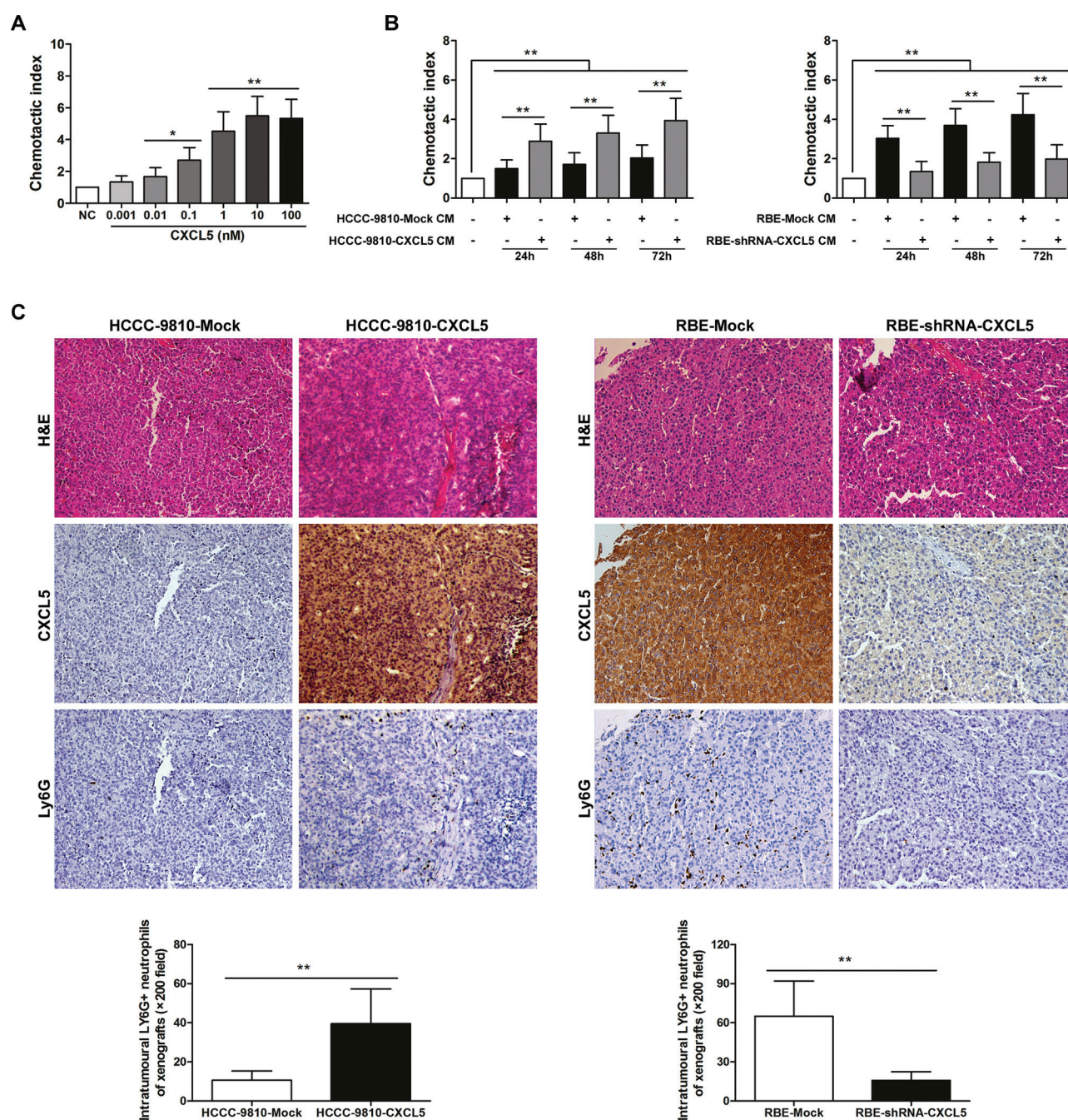
Fig. 2. CXCL5 promotes neutrophil migration *in vitro* and *in vivo*. (**A**) *In vitro* transwell assays show that CXCL5 induces concentration-dependent migration of neutrophils. (**B**) CM from ICC cells attracts neutrophils more strongly with increasing incubation time (24–72h). (**C**) CXCL5 mediates neutrophil infiltration in xenograft SCID mice models. Representative images from tumor sample serial sections stained with hematoxylin and eosin, CXCL5 and Ly6G are shown. (*$P < 0.05$; **$P < 0.01$).

kinase, cell cycle/check point proteins and transcription factors, and others (Figure 3A). Among the six phosphorylated proteins that were upregulated >100%, three were involved in the PI3K/Akt pathway, including p-AKT^{Ser473}, and three were involved in the ERK1/2-MAPK pathway (p-ERK1/2$^{T202/Y204}$, p-RSK1/2/3$^{S380/S386/S377}$ and p-RSK1/2$^{S221/S227}$; Figure 3B). To validate these data, we performed western blots to detect the phosphorylation of these proteins in neutrophils stimulated with CXCL5 and confirmed that CXCL5 activates PI3K/Akt and ERK1/2-MAPK pathways (Figure 3C).

To determine whether the PI3K/Akt and ERK1/2-MAPK pathways play a crucial role in neutrophil recruitment by CXCL5, we pretreated neutrophils with selective inhibitors of the PI3K/Akt pathway (LY29004) and ERK1/2-MAPK pathway (U0126). Blocking both pathways completely inhibited the recruitment of neutrophils toward CXCL5 (Figure 3D), suggesting that CXCL5 directly chemoattracts neutrophils through the PI3K-Akt and ERK1/2-MAPK signaling pathways in ICC.

Neutrophils promote ICC cell proliferation, migration and invasion

To examine the effect of neutrophils on ICC cell biology, we incubated ICC cell lines (HCCC-9810, HuH-28 and RBE) with CM from neutrophils derived from ICC patients, as described in the previous study (29). Proliferation analysis demonstrated that NCM promoted ICC growth *in vitro* (Figure 4A). Moreover, compared with control medium, NCM significantly enhanced the migration and invasion abilities of HCCC-9810, HuH-28 and RBE cells (Figure 4B and C).
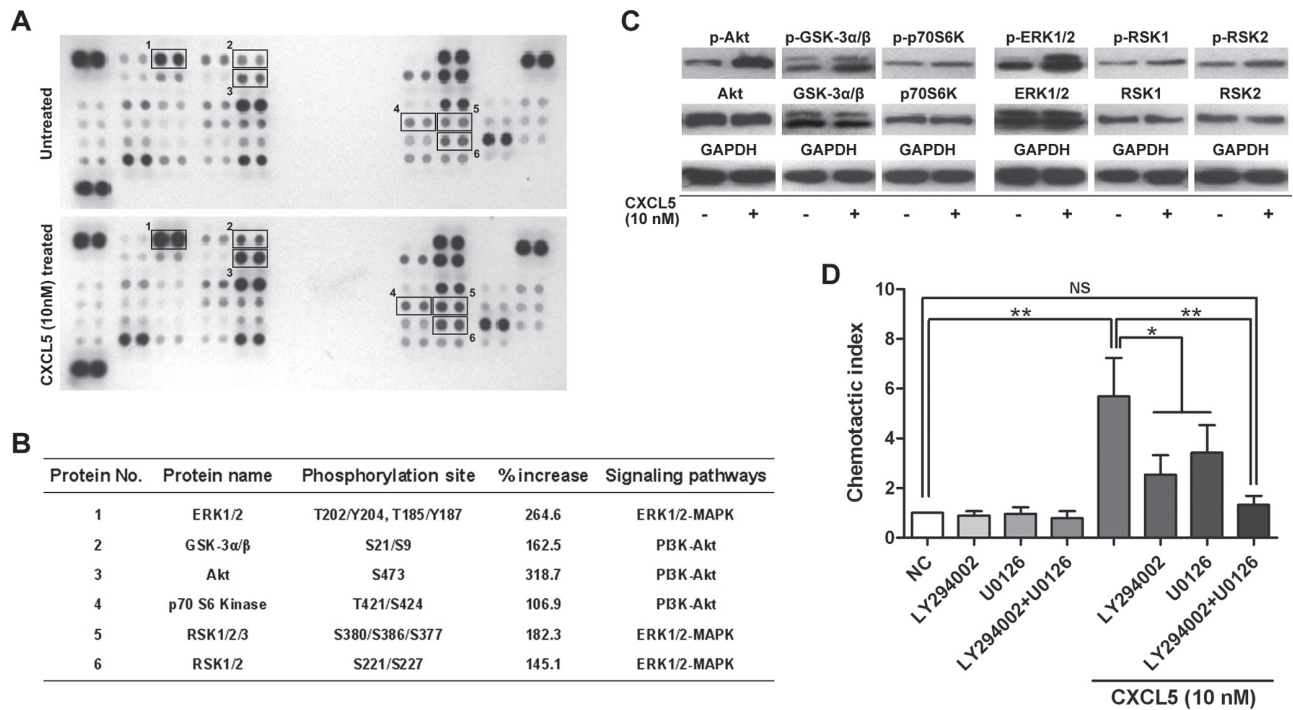
Fig. 3. CXCL5 induces neutrophil migration through PI3K-Akt and ERK1/2-MAPK signaling pathways. (**A**) Signaling pathways activated by CXCL5 in neutrophils were screened by probing a human phospho-kinase array with lysates from untreated human neutrophils or cells treated with 10 nM CXCL5. Proteins that showed increased phosphorylation upon CXCL5 treatment are indicated in boxes. (**B**) Phosphorylated proteins that were upregulated at least 1-fold in human neutrophils after CXCL5 treatment. (**C**) Validation of p-Akt^{Ser473}, p-GSK3α/β$^{Ser21/9}$, p-p70S6K$^{Thr421/Ser424}$, p-ERK1/2$^{Thr202/Tyr204}$, p-RSK1^{Ser380} and p-RSK2^{Ser227} levels in neutrophils treated with CXCL5 by western blot analysis. (**D**) Neutrophils were pretreated with 50 μM LY294002 (PI3K inhibitor) or 10 μM U0126 (ERK1/2 inhibitor) for 1 h and stimulated to migrate toward CXCL5 (10 nM) for 2 h. (*$P < 0.05$; **$P < 0.01$).

In vivo stimulation of ICC progression by CXCL5 is mediated by the recruitment of intratumoral infiltrative neutrophils

As CXCL5 mediated neutrophil infiltration and promoted ICC progression *in vivo* but not *in vitro,* and neutrophils enhanced the proliferative and invasive ability of ICC cells, we evaluated whether neutrophils play a crucial role in CXCL5-mediated progression of ICC in the SCID mice tumor xenograft model. Neutrophil depletion significantly reduced tumor growth and metastasis in RBE-Mock-derived xenografts, a similar effect to CXCL5 knockdown. Moreover, depletion of neutrophils in HCCC-9810-CXCL5-derived xenografts completely abrogated the pro-ICC effect induced by CXCL5 over-expression (Figure 5A–C). These results suggest that the effect of CXCL5 on ICC progression *in vivo* is mediated by the recruitment of infiltrative intratumoral neutrophils.

Expression of CXCL5 alone and combined with the presence of intratumoral neutrophils correlates with poor prognosis in ICC patients

We investigated the expression of CXCL5 and CD66b (a marker thought to be uniquely expressed by human neutrophils) by immuno-histochemical staining in a TMA composed of primary tumors from 140 ICC patients in cohort 2 (Figure 6A and B). CXCL5 expression was significantly correlated with tumor size ($P = 0.025$), microvascular/bile duct invasion ($P = 0.040$) and lymphatic metastasis ($P = 0.049$). Other clinical characteristics, including age, sex, preoperative serum α-fetoprotein, CA19-9 and ALT, were not directly related to CXCL5 expression (Supplementary Table 1, available at *Carcinogenesis* Online).

At the last follow-up, 108 patients had recurrent tumors and 109 patients had died, including 7 patients who died of liver failure without evidence of disease recurrence. The OS and cumulative recurrence rates for cohort 2 were 55.7% and 56.9% at 1 year, 29.1% and 75.2% at 3 years, and 21.3% and 79.5% at 5 years, respectively. Based on the

immunohistochemical data, ICC patients of this cohort were divided into two groups: high CXCL5 expression and low CXCL5 expression. The 1-, 3- and 5-year survival rates of the CXCL5^{low} patients were significantly higher than the corresponding survival rates of the CXCL5^{high} group (68.6% versus 42.9%, 39.8% versus 18.5% and 31.7% versus 16.4%, respectively; Figure 6D). Similarly, CXCL5^{high} ICC patients had the poorest prognosis at 1, 3 and 5 years, with higher cumulative recurrence rates than CXCL5^{low} patients (71.4% versus 42.0%, 85.4% versus 64.9% and 90.3% versus 69.0%, respectively; Figure 6D). The presence of intratumoral neutrophils significantly correlated with OS [$P < 0.001$, hazard ratio (HR) = 2.115] and time to recurrence ($P < 0.001$, HR = 2.266; Supplementary Table 2, available at *Carcinogenesis* Online), consistent with previously reported results (30).

ICC patients with high CXCL5 expression had more intratumoral neutrophil infiltration than patients with low CXCL5 expression ($n = 140$, $r = 0.567$, $P < 0.001$; Figure 6C). Regarding the combined effect of CXCL5 expression and intratumoral neutrophil presence on ICC prognosis, the 1, 3 and 5 year OS rates in the CXCL5^{high}/intratumoral neutrophils^{high} patients were 36.7%, 14.3% and 7.4%, respectively, significantly lower than those in the CXCL5^{low}/intratumoral neutrophils^{low} patients (73.5%, 44.6% and 36.2%, respectively; Figure 6F). The 1, 3 and 5 year cumulative recurrence rates in the CXCL5^{high}/intratumoral neutrophils^{high} patients were 80.0%, 92.5% and 92.5%, respectively, significantly higher than those in the CXCL5^{low}/intratumoral neutrophils^{low} patients (31.2%, 59.4% and 65.2%, respectively; Figure 6F). Univariate and multivariate analyses revealed that, along with lymphatic metastasis, microvascular invasion and tumor-node-metastasis stage, CXCL5 expression and the co-index (CXCL5/intratumoral neutrophils) were independent prognostic factors for both OS ($P = 0.005$, HR = 1.762 and $P < 0.001$, HR = 2.827) and time to recurrence ($P = 0.023$, HR = 1.605 and $P < 0.001$, HR = 2.799; Supplementary Table 2, available at *Carcinogenesis* Online).
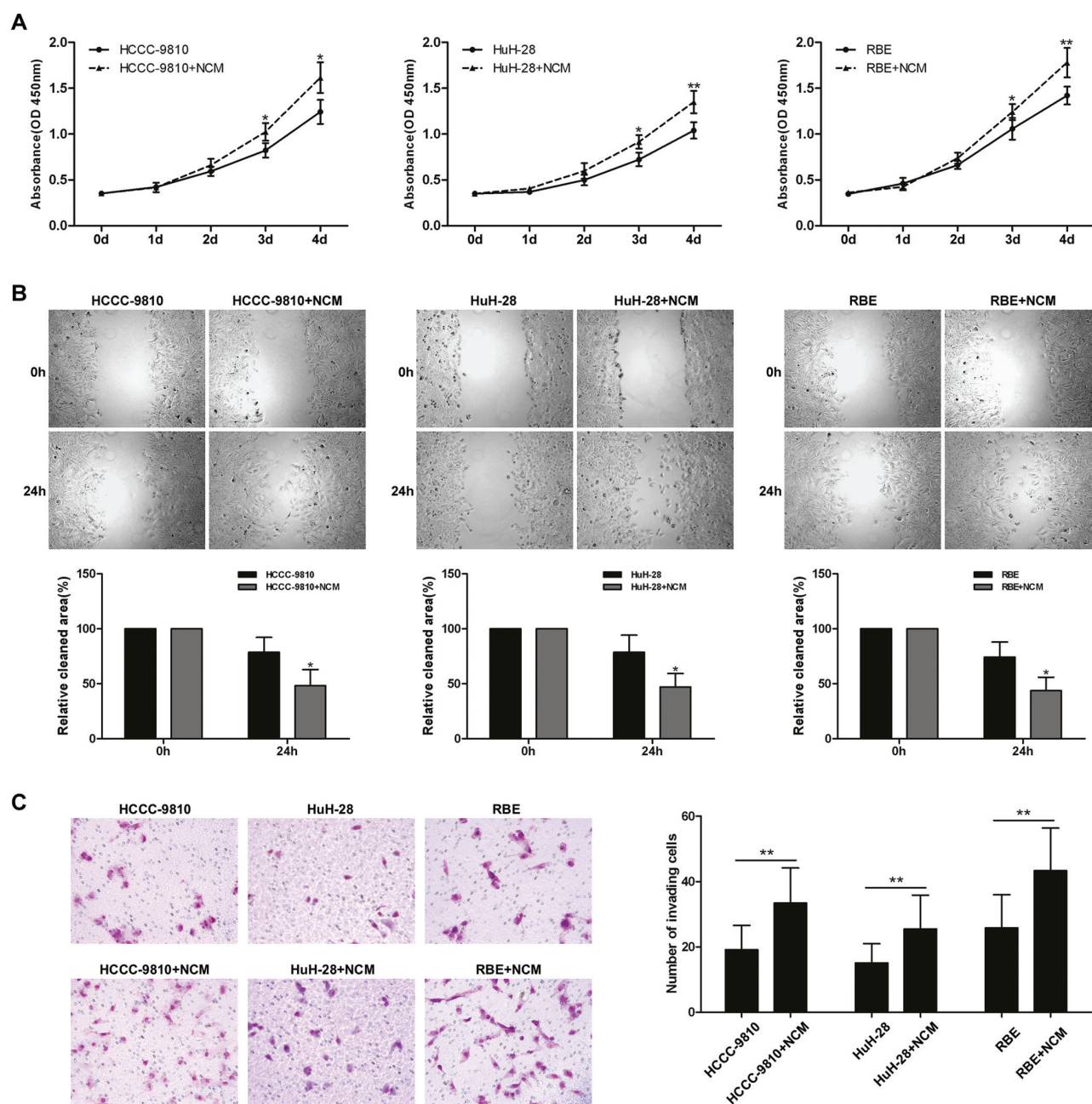
Fig. 4. Neutrophils promote proliferation, migration and invasion of ICC cells *in vitro*. (**A**) Cell proliferation measured by CCK8 assay showed that NCM significantly promoted ICC cell proliferation after 48 h. (**B**) In wound-healing migration assays, microscopic examination at 24 h postwounding revealed a significant increase in the wound closure rate of ICC cells (HCCC-9810, HuH-28 and RBE) cultured with NCM compared with the controls. (**C**) *In vitro* invasive assays showed that the number of invasive ICC cells was higher when cells were cultured with NCM than for cells cultured without NCM. (*$P < 0.05$; **$P < 0.01$).

Discussion

This study confirmed that the expression of CXCL5 was markedly increased in ICC tissues compared with adjacent non-tumorous tissues. Moreover, overexpression of CXCL5 contributed to the growth and metastasis of ICCs *in vivo* and was associated with poor prognosis of ICC patients. These results indicate that CXCL5 plays a substantial role in tumor progression. Although CXCL5 has been shown to participate in various cellular functions, including proliferation, migration and invasion of cancer cells, in several studies (17,18,20) including our recent study on HCC (22), we did not observe any direct effect of CXCL5 on ICC cells *in vitro*, indicating that the ability of CXCL5 to promote ICC progression might be dependent on interactions with host cells.

CXCL5 was first identified as a neutrophil-activating inflammatory peptide with homology to interleukin-8 (15). CXCL5 was shown to have a strong effect on neutrophil recruitment in studies of rheumatoid arthritis (14), chronic obstructive pulmonary disease (31) and HCC (22); however, its role in ICC is largely unknown. The study by Okabe *et al.* (32) identified CXCL5 as an important mediator of the interaction between cholangiocarcinoma and cancer-associated fibroblasts. In this study, we revealed a striking correlation between CXCL5 production and neutrophil infiltration in ICC. First, we showed that CXCL5 acts as a direct chemoattractant for neutrophils *in vitro*. Next, through CXCL5 overexpression or knockdown in ICC cells, we revealed a role of CXCL5 in recruitment of neutrophils to the tumor site *in vivo*. Finally, we validated the relationship between CXCL5 and neutrophil infiltration using clinical ICC samples.
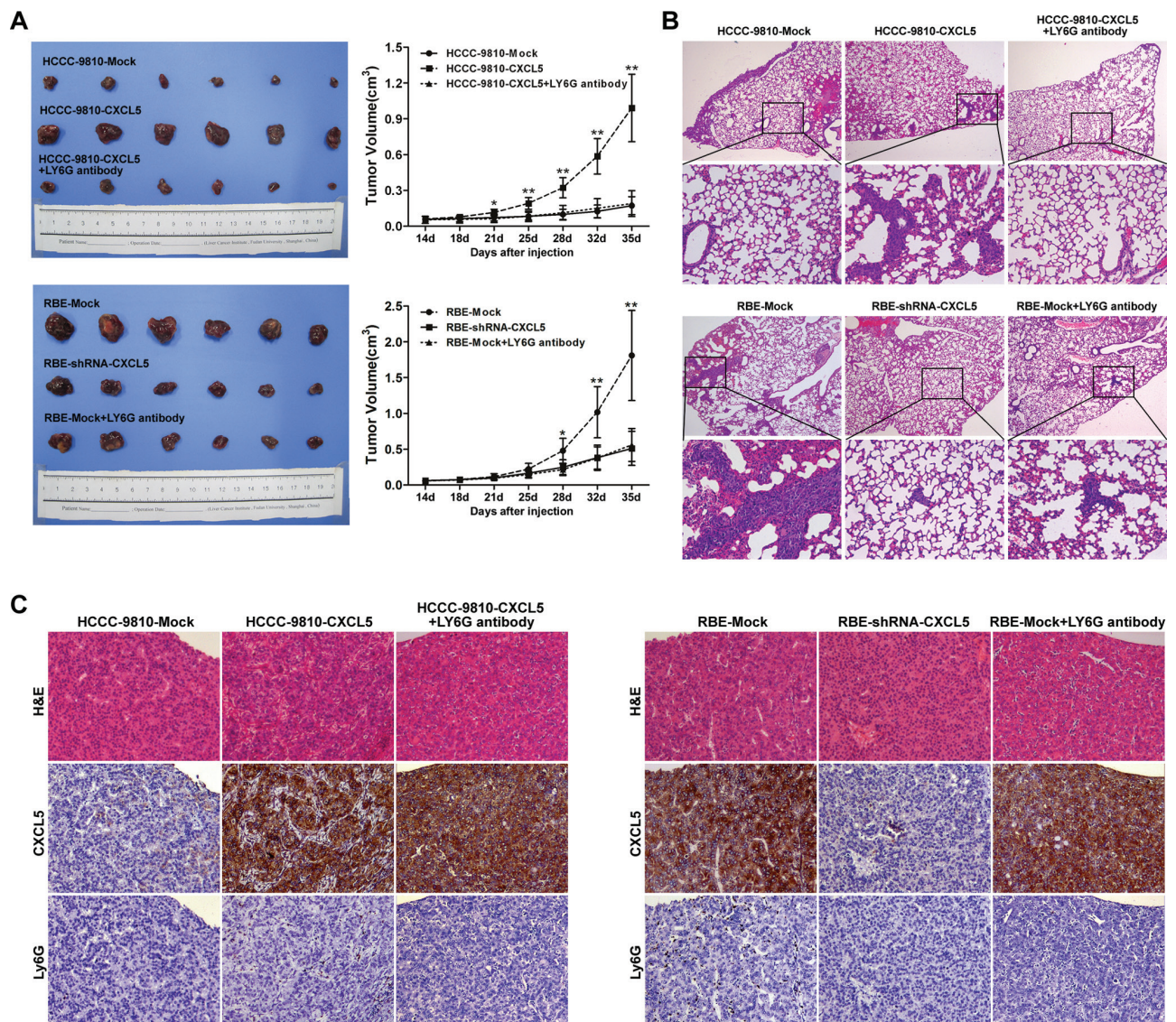
Fig. 5. Intratumoral infiltration of neutrophils recruited by CXCL5 plays a crucial role in ICC progression *in vivo*. (**A**) The effect of CXCL5 on the growth of ICC is mediated by recruitment of infiltrative intratumoral neutrophils. (**B**) The infiltration of intratumoral neutrophils mediated by tumor-derived CXCL5 plays a crucial role in the metastasis of ICC. (**C**) Representative images from tumor sample serial sections stained with hematoxylin and eosin, CXCL5 and Ly6G for each group. Magnification ×200. (*$P < 0.05$; **$P < 0.01$)

The migration of neutrophils across the tumor vasculature is mainly mediated by CXC chemokines, especially glutamic acid–leucine–arginine motif positive-CXC chemokines that bind to and activate CXCR1 and/or CXCR2 (33). Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor have been shown to be upregulated in ICC and may be responsible for the prominent neutrophilic infiltration (34). In this study, we demonstrated that CXCL5 activates several signaling pathways in human neutrophils, especially the PI3K-Akt and ERK1/2-MAPK pathways. Activation of Akt and ERK1/2 is crucial for chemotaxis and migration of neutrophils induced by chemoattractants (35,36). Furthermore, blocking the PI3K-Akt and ERK1/2-MAPK signaling pathways completely inhibited the CXCL5-induced migration of neutrophils. We also tested for the presence of other CXC chemokines in ICC and found that only CXCL1 was expressed (Supplementary Figure 6, available at *Carcinogenesis* Online). However, CXCL1 had no effect on neutrophils in the *in vitro* chemotaxis assay and showed no correlation with intratumoral neutrophils in TMA (data not shown). Thus, we suggest that CXCL5 plays a crucial role in recruiting neutrophils to the tumor site through activation of the PI3K-Akt and ERK1/2-MAPK signaling pathways in ICCs.

Neutrophils are primary inflammatory cells that are essential to protect the host during early phases of microbial infection (37). Neutrophils can potentiate cancer cell migration, invasion and dissemination by secreting immunoreactive molecules such as hepatocyte growth factor (38), oncostatin M, b2-integrins or neutrophil elastase (39). Neutrophils enhanced the proliferative and invasive ability of ICC cells *in vitro*, and observed that RBE-Mock and HCCC-9810-CXCL5-derived xenografts with high expression levels of CXCL5 had more intratumoral neutrophils, larger tumors and more pulmonary metastases *in vivo* compared with RBE-shRNA-CXCL5 and HCCC-9810-Mock-derived xenografts with low CXCL5 expression. Depletion of neutrophils in the tumors of RBE-Mock-derived and HCCC-9810-CXCL5 xenografts decreased tumor volume and pulmonary metastasis. Thus, the stimulatory effect of CXCL5 on ICC progression appears to be mediated by the recruitment of infiltrative intratumoral neutrophils.

Because CXCL5 acted as a strong chemotactic cytokine for neutrophils in our xenograft model and there was a significant positive correlation between CXCL5 expression and intratumoral neutrophils in clinical ICC samples, we investigated the prognostic significance
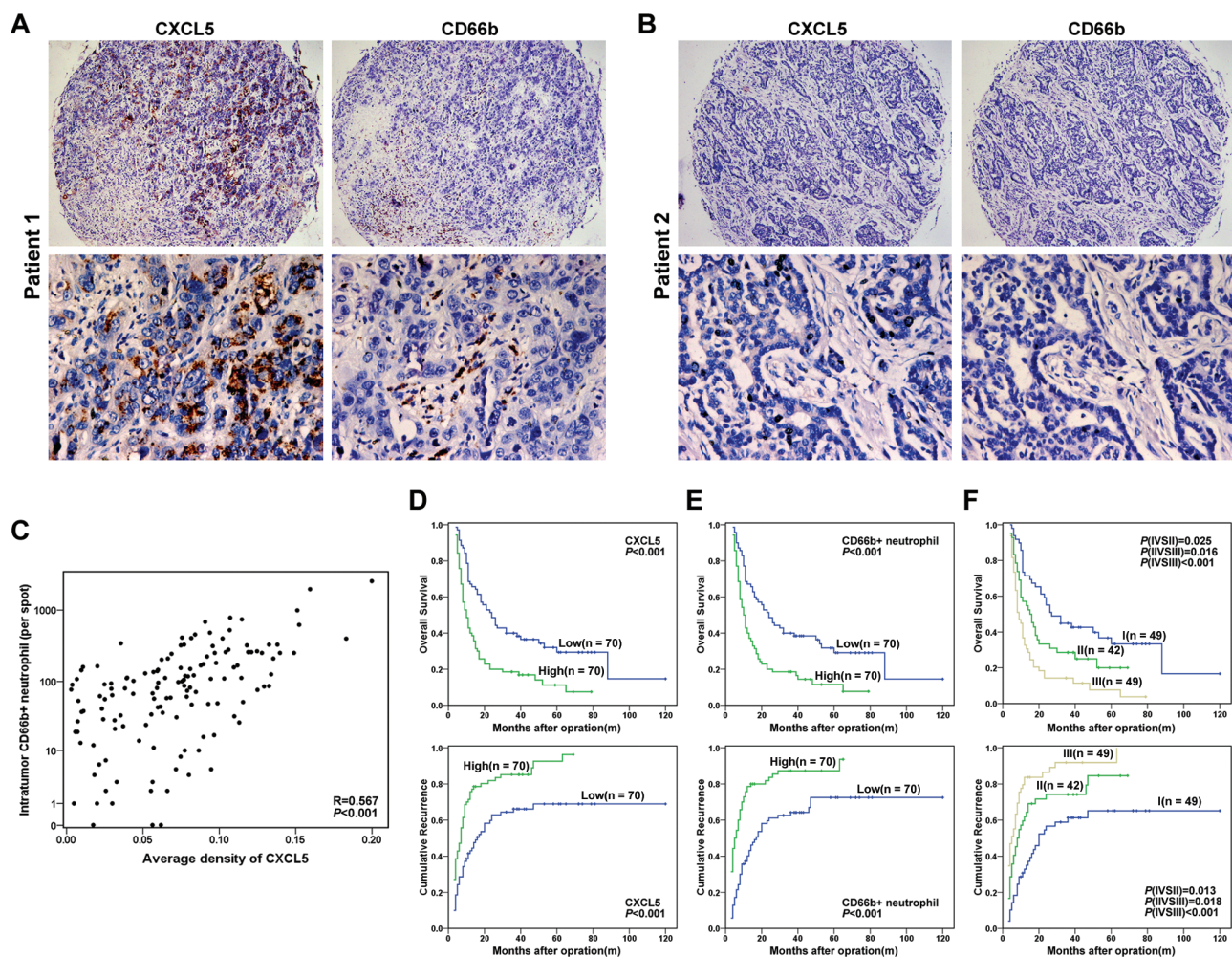
Fig. 6. Expression and prognostic value of CXCL5 and intratumoral CD66b+ neutrophils in ICC samples ($n = 140$). (**A** and **B**) Representative HCC tumor samples showing the expression of CXCL5 (brown staining in the cytoplasm of HCC cells) and intratumoral CD66b (brown staining in the cell membrane of the intratumoral neutrophils). (A) High-level expression of both CXCL5 and intratumoral CD66b. (B) Low-level expression of CXCL5 and intratumoral CD66b. (**C**) Scatter plot indicating a significant positive correlation between CXCL5 and intratumoral CD66b in cancerous tissues. (**D–F**) Prognostic values of CXCL5 and intratumoral CD66b using Kaplan–Meier analysis. I, CXCL5^{low}/intratumoral CD66b^{low}; II, CXCL5^{low}/intratumoral CD66b^{high} and CXCL5^{high}/intratumoral CD66b^{low}; III, CXCL5^{high}/intratumoral CD66b^{high}.

of intratumoral neutrophils in TMA by immunohistochemistry. The presence of intratumoral neutrophils was a poor prognostic indicator for ICC. The clinical relevance of tumor-infiltrating neutrophils has recently begun to emerge and direct associations between tumor-infiltrating neutrophils and poor clinical outcome have now been described for several types of human cancer (40), including our recent study on ICC (30). We also evaluated the prognostic value of combined CXCL5 expression and intratumor neutrophils in ICC patients by direct comparisons of prognosis among three subgroups (CXCL5^{low}/intratumor neutrophils^{low}, CXCL5^{low}/intratumor neutrophils^{high} or CXCL5^{high}/intratumor neutrophils^{low}, and CXCL5^{high}/intratumor neutrophils^{high}) in 140 ICC patients. In the TMA analysis, ICC patients who had both high CXCL5 expression and high levels of intratumor neutrophil infiltration were more likely to have poor survival following curative resection. Conversely, ICC patients who expressed low levels of CXCL5 and had low intratumor neutrophil infiltration had the best prognosis. Although the expression of CXCL5 alone was an independent predictor for OS and time to recurrence in ICC patients, the predictive range of combined CXCL5^{high}/intratumor neutrophils^{high} was more sensitive than that of CXCL5^{high} or intratumor neutrophils^{high} alone.

Emerging evidence indicates a complex and multidirectional interplay of tumor cells with components of the tumor inflammatory environment during cancer development and progression. This interplay may functionally alter and polarize the tumor microenvironment in a way that favors tumor promotion (39). The inflammatory environment includes inflammatory cells and inflammatory mediators such as chemokines, cytokines and prostaglandins (6). Chemokines are emerging as key mediators of both the homing of cancer cells to metastatic sites and the recruitment of a number of different cell types to the tumor microenvironment. Recent studies suggest that the production of chemokines by epithelial cancer cells leads to the recruitment of tumor-associated macrophages, tumor-associated neutrophils, lymphocytes, cancer-associated fibroblasts, mesenchymal stem cells and endothelial cells to the tumor microenvironment. These infiltrating cells provide a secondary source of chemokines that could affect tumor growth, cell survival, senescence, angiogenesis and metastasis (12). In this study, we confirmed that CXCL5 is a strong chemotactic cytokine for neutrophils in ICC and observed increased neutrophil infiltration in a microenvironment containing high levels of tumor-derived CXCL5, but no significant correlation with other intratumoral stromal cells, including CD68+ macrophages, αSMA+ fibroblasts, tryptase+ mast cells, or CD3+, CD4+, CD8+, CD45RO+, and Foxp3+ lymphocytes (Supplementary Table 3, available at *Carcinogenesis* Online). We also confirmed that the role of CXCL5 on ICC growth and metastasis was mediated by the recruited intratumoral neutrophils. Therefore, we suggest that ICC cells with higher expression of CXCL5 are likely to recruit more neutrophils to the tumor site,

establishing a tumor-promoting microenvironment and amplifying the inflammatory response and thus facilitating ICC metastasis and recurrence.

In conclusion, CXCL5 promotes ICC growth and metastasis by recruiting infiltrative intratumoral neutrophils. CXCL5 expression alone or in combination with intratumoral neutrophils serves as a novel prognostic indicator for ICC patients. These features of CXCL5 also make it a potential therapeutic target.

Supplementary material

Supplementary Tables 1–3, Figures 1–6 and Materials and Methods can be found at http://carcin.oxfordjournals.org/

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