CXCR6 upregulation contributes to a pro-inflammatory tumor microenvironment that drives metastasis and poor patient outcomes in hepatocellular carcinoma

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Abstract

CXC chemokines and their cognate receptors have been implicated widely in cancer pathogenesis. In this study, we report a critical causal relationship between CXCR6 expression and tumorigenesis in the setting of human hepatocellular carcinoma (HCC). Among the CXC chemokine receptors, only CXCR6 was detected in all the hepatoma cell lines studied. Moreover, in HCC tissue CXCR6 expression was significantly higher than in noncancerous liver tissues. Reduction of CXCR6 or its ligand CXCL16 in cancer cells reduced cell invasion in vitro and tumor growth, angiogenesis, and metastases in vivo. Importantly, loss of CXCR6 led to reduced Gr-1+ neutrophil infiltration and decreased neoangiogenesis in hepatoma xenografts via inhibition of pro-inflammatory cytokine production. Clinically, high expression of CXCR6 was an independent predictor of increased recurrence and poor survival in HCC. Human HCC samples expressing high levels of CXCR6 also contained an increased number of CD66b+ neutrophils and microvessels, and the combination of CXCR6 and neutrophils was a superior predictor of recurrence and survival than either marker used alone. Together, our findings suggest that elevated expression of CXCR6 promotes HCC invasiveness and a pro-tumor inflammatory environment and is associated with poor patient outcome. These results support the concept that inhibition of the CXCR6-CXCL16 pathway may improve prognosis after HCC treatment.
**Introduction**

Hepatocellular carcinoma (HCC) ranks fifth in frequency worldwide among all malignancies and is the third leading cause of cancer mortality (1). Although hepatectomy represents the most effective treatment to obtain long-term survival, over 70% of patients relapse within 5 years, and the overall survival for HCC patients remains poor (2). As an orphan tumor in regard to translational research and therapeutic options, it is important to get a better understanding of the underlying mechanisms leading to tumor invasion and metastasis, and to develop prognostic and therapeutic strategies in this disease.

Diverse roles have been reported for chemokines and their receptors in tumor biology, including direct effects on cancer cells, such as proliferation and invasion, and indirect effects on regulating inflammation and immunity (3, 4). The chemokines can be subdivided into four classes, the C-C, C-X-C, C, and C-X3-C chemokines, in which the C-X-C chemokine network is a unique group of cytokines and receptors known for their disparate manner in regulating inflammation and cancer (5-7). Studies have indicated that only a few genes, among them chemokine-receptor genes, had a significant role in determining the metastatic destination of different tumors (8). Different tumor types have distinct chemokine-receptor expression profiles, with CXCR4 and CCR7 extensively investigated (3). Notably, CXCR4 is widely expressed in at least 23 different cancer types including HCC, and associated with more aggressive disease and poorer patient prognosis (3, 9, 10). The CXCL12–CXCR4 axis is therefore of particular importance in cancer metastasis, and currently represent the
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top target for therapeutic intervention. Likewise, CCR7 is the second frequently expressed chemokine-receptor in tumor cells, where the CCL21–CCR7 axis is primarily responsible for lymph node and brain metastases (3, 11, 12).

In HCC, various chemokines and their receptors may not play the same role as they do in other tumors. After scrutinizing ten C-C chemokine-receptors including CCR1 to 10, we and others have identified that CCR1 was the only one expressed constitutively in all the human hepatoma cell lines examined (13, 14). RNA interference and animal HCC model further confirmed the contribution of CCL3/CCL5-CCR1 axis to HCC metastasis and progression (13, 14). Apart form the C-C chemokine-receptors, other chemokine-receptors may also play a role during the malignant progression of this fetal disease. However, except for CXCR4, the expression patterns of C-X-C, C and C-X3-C chemokine-receptors on HCC cells and the associated biological and clinical significance remains largely unknown.

Herein, we showed that CXCR6 and its ligand CXCL16 were consistently expressed in all the 8 hepatoma cell lines. We further identified CXCR6 as an independent predictor for increased recurrence and poor survival in HCC patients. Down-regulation of CXCR6 resulted in reduced invasive activities, tumor formation and metastasis of hepatoma cells. Moreover, CXCR6 could lead to a pro-tumor microenvironment in vivo via producing pro-inflammatory cytokines. In addition, the combination of CXCR6 and neutrophils was a superior predictor for recurrence and survival to either marker used alone.
Materials and Methods

Human HCC Samples. Archived tissues for tissue microarray (TMA) construction were obtained from a cohort of 240 patients who received curative resection of HCC between 2002 and 2006, as previously described (15). TMA construction and patient follow-up were included in the Supplementary Materials.

Twenty-four pairs of fresh-frozen human HCC and matched nontumor liver tissues, and 6 normal liver tissue samples from patients with hepatic hemangioma were obtained for Western blotting assay. Ethical approval from the Zhongshan Hospital Research Ethics Committee and patient written informed consent were obtained.

Cell Lines and Treatments. Eight human hepatoma cell lines Hep3B, HepG2, Huh7, PLC/PRF/5, SK-HEP-1, MHCC97L, MHCC97H and HCCLM3, and an immortalized human liver cell line L-02 were used as described in the Supplementary Materials.

Transfection of in vitro growing cell lines with Lentiviral Delivered short hairpin RNA (shRNA; universal negative control shRNA [shCtl] and CXCR6 or CXCL16-targeting shRNA [shR6, shL16]), and treatment with specific inhibitors or stimulators were performed as described in the Supplementary Materials.

Immunohistochemistry and Western Blotting Analyses. Immunohistochemistry and Western blotting analyses were performed as described in the Supplementary
Materials. The primary antibodies used were listed in Supplementary Table 1.

**Cell Proliferation, Cell Cycle, and Invasion Assays.** Cell proliferation, cell cycle, and invasion assays were performed as described in the Supplementary Materials.

**Tumor Models.** Mice were manipulated and housed according to the protocols approved by the Shanghai Medical Experimental Animal Care Commission. $1 \times 10^7$ shR6/shL16-SK-HEP-1 and $2 \times 10^6$ shR6/shL16-HCCLM3 cells or shCtl cells were subcutaneously injected into the flank of each mouse respectively (6 in each group, male BALB/c-nu/nu, 6-8 weeks), as described in the Supplementary Materials.

**Real-time Reverse Transcription PCR (RT-PCR).** Chemokine-receptor expression was analyzed using a Chemokines and Receptors PCR Array (Cat. No. PAHS-022A; SABioscience, Fredrick, MD), according to the manufacturer’s instructions. Real-time RT-PCR was performed as described in the Supplementary Materials. Primers were listed in Supplementary Table 2.

**Enzyme-Linked Immunosorbent Assays (ELISA).** Antibody sandwich ELISAs were used to evaluate IL-6 and IL-8 levels in the hepatoma cell conditioned medium according to the manufacturer’s instructions (R&D System, Minneapolis, MN).
**Statistics.** Statistical analyses were conducted using SPSS 15.0 software. Data are expressed as mean ± SE. Univariate survival analysis was calculated by the Kaplan-Meier method, compared by the log-rank test. Variables showed $P < 0.1$ in univariate analyses were adopted as covariates in multivariate analysis. Multivariate survival analysis was evaluated by Cox's proportional hazards model in a forward stepwise manner with the log-likelihood ratio significance test. The Pearson chi-square test was used to compare qualitative variables; and quantitative variables were analyzed by the Student’s $t$ test or Spearman’s rho correlation test. $P < 0.05$ (two-tailed) was considered significant.

**RESULTS**

**High Expression of CXCR6 Correlates with Poor Prognosis of HCC Patients.**

We first examined the mRNA expression of 8 chemokine-receptors in 8 hepatoma cell lines with varying metastatic capability. Only CXCR6 was detected in all hepatoma cell lines, averaging 1.2% of HPRT1 (range, 0.4–2.1%) (Supplementary Table 3). CXCR4 expression was also detected in some but not all hepatoma cell lines; however, other chemokine-receptors showed minimal or no expression. Also, CXCL16 was expressed in all hepatoma cell lines, averaging 107.4% of HPRT1 (range, 25.7–228.2%). Western blotting showed that highly metastatic cells (MHCC97H, HCCLM3 and SK-HEP-1) showed higher levels of CXCR6 and CXCL16 proteins, whereas low-metastatic Huh7, Hep3B cells and normal liver cell line L-02 showed lower levels of CXCR6 and CXCL16, suggesting involvement of
CXCR6-CXCL16 axis in HCC aggressiveness (Fig. 1A).

By analyzing 24 pairs of HCC samples (Fig. 1B; Supplementary Fig. 1), we found that CXCR6 protein levels were low in peritumor liver tissue, relatively higher in corresponding HCC by 1.8-fold ($P < 0.0001$, vs. peritumor liver), and particularly higher in HCC with vascular invasion by 2.2-fold ($P = 0.032$, vs. tumors without vascular invasion). In contrast, no significant difference in CXCL16 protein levels was detected between HCC and paired peritumor liver tissue (Supplementary Fig. 1).

To determine the association of CXCR6 expression in HCC with disease outcome, TMAs from 240 HCC patients were examined by immunostaining (Fig. 1C). We found that CXCR6 expression in peritumor liver tissue was weak or minimal. In HCC, CXCR6 expression was strong in 73, moderate in 97, weak in 52 and negative in 18 cases, and located diffusely in the cytoplasm and cell membrane. For further analysis, patients were dichotomized into CXCR6 -low (negative and weak; $n = 70$) or -high (moderate and strong; $n = 170$) groups. Statistically, CXCR6 expression was significantly higher in HCC with vascular invasion ($P = 0.042$) and in advanced tumor stage ($P = 0.045$) and positively correlated with CXCL16 expression ($P = 0.002$) (Supplementary Table 4). There was a striking inverse association between CXCR6 intensity and recurrence-free survival (RFS) ($P = 0.007$), and to a less extent overall survival (OS) ($P = 0.064$) (Fig. 1D). The median OS and RFS time for CXCR6$^{\text{high}}$ patients were 35.5 and 20.5 months, as compared with 61.0 and 84.0 months for CXCR6$^{\text{low}}$ patients, respectively. Multivariate analysis revealed that CXCR6 intensity in tumors was an independent prognosticator for both OS and RFS.
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(Table 1; Supplementary Tables 5, 6). Thus, CXCR6 expression is a valuable predictor for recurrence and survival in HCC patients.

In peritumor liver, CXL16 constitutively showed moderate or strong intensity in both cytoplasm and nuclei, and in HCC, CXCL16 was positive in either cytoplasm or nuclei or both (Supplementary Fig. 1A). Based on the combined cytoplasm and nuclear intensities, patients were categorized into CXCL6 -low (n = 114) or -high (n = 126) groups. CXCL16\textsuperscript{high} patients had worse OS (median months: 45.0 vs. 60.0, \( P = 0.46 \)) and RFS (median months: 24.0 vs. 34.0, \( P = 0.65 \)) than CXCL16\textsuperscript{low} patients, however the difference did not reach statistical significance (Supplementary Fig. 1B; Supplementary Tables 5, 6).

**CXCR6 Knockdown Inhibits HCC Cell Invasion in vitro.**

Given CXCR6’s clinical significance in HCC, we wondered whether silenced endogenous CXCR6 expression could exhibit biological effects on hepatoma cells. Two cell lines with the most abundant level of CXCR6 protein, i.e., SK-HEP-1 and HCCLM3, were introduced CXCR6-knockdown via specific shRNA carried by Lenti-virus system. Transwell assays demonstrated that CXCR6-knockdown substantially inhibited invasive activity of hepatoma cells \textit{in vitro} (Fig. 2A-B), but had no impacts on cell proliferation and cell cycle distribution (data not shown). Because CXCR6 is the only known chemokine-receptor of CXCL16 (16), we then investigated whether CXCL16 impact hepatoma cell invasion. The addition of recombinant CXCL16, as well as CXCL16-knockdown, did not impact hepatoma cell proliferation
and cell cycle either (data not shown). Intriguingly, CXCL16, in its soluble form or trans-membrane full-length form, could prominently promote control SK-HEP-1 and HCCLM3 cell invasion, but not CXCR6-knockdown cells (Fig. 2A-B). Likewise, down-regulation of CXCL16 markedly inhibited invasive activity of hepatoma cells in vitro (Supplementary Fig. 3A). These results indicate that the contribution of CXCR6 on HCC cell invasion may be due to CXCR6-CXCL16 signaling and an autocrine loop of CXCL16 production.

**CXCR6 Knockdown Promotes Membrane Translocation of Caveolin-1 and β-Catenin, with p38 and GSK3β Pathways Involved.**

To elucidate the mechanistic roles of CXCR6, the effect of CXCR6 disruption on expression of adhesion/invasion-associated proteins was examined. We showed that caveolin-1 and β-catenin were significantly up-regulated in CXCR6-knockdown cells compared to control cells, while CXCL16 stimulation substantially decreased caveolin-1 and β-catenin expression in control cells but not CXCR6-knockdown cells (Fig. 2C). Meanwhile, E-cadherin, N-cadherin, vimentin and plakoglobin were detected, but no significant change consequent on CXCR6-knockdown was shown (Supplementary Fig. 4A).

Further effort was taken to the effect of CXCR6-knockdown on the distribution of caveolin-1 and β-catenin. Stable cell lines grown at high density were fractionated into membrane and nucleus. As shown in Fig. 2D, the membrane-localization of caveolin-1 and β-catenin was obviously enhanced in CXCR6-knockdown cells, while
the amount of caveolin-1 and β-catenin in nuclei had no evident difference compared to control cells. These data indicate that CXCR6-CXCL16 signaling may affect hepatoma cell invasion though regulating the functional translocation of caveolin-1 and β-catenin.

Because p38 MAPK and GSK3β have well-established role in modulating caveolin-1 and β-catenin expression (17, 18), we assumed that they may operate in CXCR6-CXCL16 signaling. We found that phosphorylation of p38 was markedly increased, while inactivation (i.e., phosphorylation) of GSK3β was obvious, in CXCR6-knockdown cells (Fig. 3A). Notably, CXCL16 stimulation repressed phosphorylation of p38 and GSK3β in control cells, whereas no obvious changes were seen in CXCR6-knockdown cells (Fig. 3A), showing a parallel expression pattern with caveolin-1/β-catenin and indicating that these pathways contributed to CXCR6-knockdown mediated inhibition of hepatoma cell invasion. Indeed, a p38 inhibitor SB202190 augmented, while a GSK3β inhibitor TDZD8 repressed, in vitro invasion of hepatoma cells (Fig. 3B). There were no significant differences in the invasive activities between CXCR6-knockdown and control hepatoma cells, in the presence of SB202190 or TDZD8 (Fig. 3B), further supporting that p38 and GSK3β have a crucial role in CXCR6 mediated tumor promotion. Consistent with the results of in vitro invasion assay, Western blotting showed that SB202190 further amplified, while TDZD8 partly recovered, the down-regulation of caveolin-1 and β-catenin induced by CXCL16 stimulation in hepatoma cells (Fig. 3C). In contrast, Rac1, Rho1, Cdc42, ERK and Akt signaling showed no significant change after
CXCR6-knockdown (Supplementary Fig. 4B).

**CXCR6 Knockdown Inhibits Tumorigenicity, Neutrophil Recruitment, Angiogenesis, and Metastasis of Hepatoma Cells in vivo.**

We next explored the effects of CXCR6-knockdown in vivo. CXCR6-knockdown hepatoma cells were transplanted into nude mice subcutaneously in the left flank, with control cells in the right flank (n = 6). We found that the weights of shR6-SK-HEP-1 (0.13 ± 0.04g) and shR6-HCCLM3 (0.49 ± 0.14g) -derived xenografts were significantly lighter than those of shCtl-SK-HEP-1 (0.41 ± 0.09g; P = 0.016) and shCtl-HCCLM3 (2.02 ± 0.41g; P = 0.013) (Fig. 4A-B). Similar results were seen when comparing tumor xenografts from shL16 and shCtl hepatoma cells (Supplementary Fig. 3B-C). In line with the in vitro findings that CXCR6-knockdown didn’t impact cell proliferation, there were no significant differences in Ki-67 expression between the shR6 and shCtl -derived xenografts (data not shown). Remarkably, the densities of infiltrating Gr-1+ neutrophils in shR6-SK-HEP-1 (112.0 ± 11.3/×200 high power field [HPF]) and shR6-HCCLM3 (162.6 ± 14.9/HPF) -derived xenografts were significantly lower than those of shCtl-SK-HEP-1 (266.4 ± 30.5/HPF; P = 0.032) and shCtl-HCCLM3 (373.2 ± 42.1/HPF; P = 0.019), respectively (Fig. 4C-D). We also compared the densities of infiltrating F4/80+ macrophages between the two groups, but the difference was not statistically significant (data not shown). Due to that neutrophils play a central role in tumor angiogenesis (19), we further investigated activities of neoangiogenesis between the
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two groups. As expected, CD31+ microvessel densities in shR6-SK-HEP-1 (17.8 ± 2.1/HPF) and shR6-HCCLM3 (24.4 ± 3.5/HPF) -derived xenografts significantly decreased compared to those of shCtl-SK-HEP-1 (36.6 ± 3.8/HPF; \( P = 0.021 \)) and shCtl-HCCLM3 (58.6 ± 6.2/HPF; \( P = 0.011 \)), respectively (Fig. 4C-D). These data indicate that CXCR6 mediated tumor promoting effect may partly be attributed to enlarged inflammatory and angiogenic responses.

Then, to investigate the impact of CXCR6-knockdown on lung metastasis of hepatoma cells, shR6-HCCLM3 and control cells were implanted subcutaneously into the flanks of different nude mice (n = 6 each group). Serial sections confirmed that the pulmonary metastatic rates and metastatic tumor clusters per mouse were 100% (6/6) and 128.3 ± 12.3 in shCtl-HCCLM3 group, but were 33% (2/6) (\( P = 0.014 \)) and 39.5 ± 7.5 (\( P = 0.001 \)) in shR6-HCCLM3 group, respectively (Fig. 4E-F). Importantly, the metastatic foci of shR6-HCCLM3 group were mainly grades I-II, whereas most of metastatic foci in the controls were grades III-IV (Fig. 4E-F).

**CXCR6 Knockdown Shifts Cytokine Secretion Pattern of HCC Cells, and IL-6 and IL-8 Induction Contribute to CXCR6-Mediated HCC metastasis.**

Given that CXCR6-knockdown resulted in reduced inflammatory leukocyte recruitment and angiogenesis, we hypothesized that CXCR6-knockdown may alter cytokine production of hepatoma cells. As illustrated in Fig. 5A, mRNA expression of IL-17F, IL-6 and IL-8 were significantly inhibited in shR6-SK-HEP-1 cells compared to control cells (72%, 51% and 40% inhibition respectively), which were markedly
up-regulated by CXCL16 stimulation in control cells (9.2, 2.8 and 1.9-folds respectively) but not shR6-SK-HEP-1 cells (data not shown). Likewise, in shR6-HCCLM3 cells, mRNA expression of IL-8, IL-6 and IL-1β represented the top three cytokines decreased compared to control cells (72%, 67% and 57% inhibition respectively), or up-regulated under rCXCL16 stimulation (1.8, 1.8 and 2.4-folds respectively). IL-1β, IL-6, and IL-8 from tumor cells have been linked to the recruitment of immature myeloid cells and neutrophils (19, 20), thus, substantiated our findings in xenograft tumor model. We then chose IL-6 and IL-8 for further analysis. ELISA assays confirmed that IL-6 and IL-8 concentrations were much lower in shR6-SK-HEP-1 (36% and 61% inhibition respectively) and shR6-HCCLM3 (42% and 37% inhibition respectively) cells than their relative control cells (Fig. 5B). Similarly, we observed a significant decrease of IL-6 and IL-8 levels in shL16 compared to shCtl hepatoma cells (Supplementary Fig. 3D).

To characterize functional properties of IL-6 and IL-8, specific stimulating or blocking experiments were done. The addition of exogenous IL-6 and IL-8 significantly rescued the inhibitory effects of CXCR6-knockdown on hepatoma cell invasion (Fig. 5C). Consistently, blocking IL-6R or IL-8RA or IL-8RB activities and neutralizing IL-6 or IL-8 actions with specific antibodies markedly reversed the invasion of hepatoma cells enhanced by CXCL16 (Fig. 5D). Interestingly, similar to the effect of CXCL16, IL-6 or IL-8 administration reduced the membrane accumulation of caveolin-1 and β-catenin via p38 and GSK3β pathways (Fig. 5E), while cell proliferation and cell cycle distribution were not affected (data not shown).
Altogether, these pro-inflammatory cytokines, via modulating both tumor microenvironment and tumor cells, significantly contributed to the tumor promoting role of CXCR6.

**Combination of CXCR6 and Neutrophil Levels Has Better Prognostic Value for HCC.**

We analyzed the densities of tumor-infiltrating CD66b+ neutrophils, CD68+ macrophages, CD8+ T-cells and CD34+ microvessels in clinical HCC samples, to address whether the *in vitro* findings and mice models mirror human HCC. TMA analysis of 240 patient specimens revealed that high expression of CXCR6 was associated with increased tumor-infiltrating CD66b+ neutrophils (17.4 ± 2.9 vs. 30.0 ± 4.2 cells/1-mm core, *P* = 0.031; Fig. 6A), but not CD68+ macrophages nor CD8+ T-cells (data not shown). Furthermore, there existed a positive correlation of microvessel density with tumor-infiltrating CD66b+ neutrophils (r = 0.242, *P* = 0.0001; Fig. 6B). Representative immunostaining images of cases with concordantly high or low levels of CXCR6, CD66b and CD34 were shown in Fig. 6C.

Patients whose tumors expressed above-median levels of neutrophils (> 12 cells/1-mm core) also exhibited significantly decreased trend in both RFS (median months: 23.0 vs. 40.0 for neutrophil\textsuperscript{high} vs. neutrophil\textsuperscript{low} groups, *P* = 0.039) and OS (median months: 33.0 vs. 63.0 for neutrophil\textsuperscript{high} vs. neutrophil\textsuperscript{low} groups, *P* = 0.026). Multivariate analyses revealed intratumoral neutrophil density as an independent index for RFS but not OS (Table 1; Supplementary Tables 5, 6). Using the
combination of the two parameters increased the prognostic value, as compared to CXCR6 or neutrophil alone (Fig. 6D and Table 1). Patients with simultaneously high levels of CXCR6 expression and neutrophil infiltration were 2.60 (HR, 2.60, 95% CI, 1.51-4.47; \( P = 0.0006 \)) and 1.96 (HR, 1.96, 95% CI, 1.17-3.30; \( P = 0.011 \)) times more likely to suffer from recurrence and death than cases with CXCR6\textsuperscript{low}/neutrophil\textsuperscript{low}, respectively (Table 1; Supplementary Tables 5, 6). Therefore, evaluation of both CXCR6 expression and neutrophil infiltration is a powerful predictor of prognosis, further supporting the findings of altered inflammatory infiltration in mice hepatoma model.

**Discussion**

In this study, we found that CXCR6 content was low in normal hepatocytes, increased in noninvasive HCC cells, and reached the highest level in invasive HCC. This progressively increased expression profile paralleled with deterioration of the disease, suggesting a role of CXCR6 in HCC progression. Then, analyzing the association of CXCR6 expression with pathological characteristics in 240 HCC patients revealed a significant correlation of CXCR6 expression with tumor vascular invasion, advanced tumor stage and recurrence/metastasis. Multivariate analysis showed that HCC patients with high CXCR6 expression in general had worse survival and increased recurrence than those with low expression. Further, the effects of CXCR6/CXCL16 interaction on tumor invasion and metastasis were directly demonstrated in our *in vitro* and *in vivo* studies. In mice subcutaneous xenografts,
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down-regulation of CXCR6 led to severe suppression of tumor growth, inflammatory infiltration, angiogenesis and lung metastasis of hepatoma. To our knowledge, this is the first report that CXCR6 expression is critical for HCC invasion and metastasis.

In line with these findings, in prostate cancer, suppressed CXCR6 expression was associated with decreased invasive activities, tumor growth, and reduced expression of proangiogenic factors (21-23). Similarly, CXCR6 expression identifies a discrete subpopulation of human melanoma cells with a highly aggressive stem cell phenotype (24). However, contrasting conclusions also existed. CXCR6 showed no clinical relevance and prognostic significance, while high expression of CXCL16 correlated with better prognosis and down-regulation of CXCL16 promoted tumor migratory behavior in renal cell carcinoma (25). Additionally, a study in colorectal cancer highlighted that high CXCL16 expression correlated with a better patient prognosis (26), while in our study HCC patients with high expression of CXCL16 were prone to suffer from increased recurrence and reduced survival. Our results also rule out that different or inverse roles of the two types of CXCL16, the trans-membrane and soluble forms, like that reported in renal cell carcinoma (25), existed in HCC. In addition, we found constitutively high CXCL16 expression in peritumor liver tissue. Given that constitutive CXCL16 expression has also been demonstrated in bone marrow and lung (23, 27), the two most common metastatic sites of HCC next to the liver, CXCR6-CXCL16 axis mediated site-specific metastasis of HCC can be envisioned.

Our results suggested that CXCR6-CXCL16 signaling may affect hepatoma cell
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Evidence has indicated that caveolin-1 is a tumor suppressor and participates in Wnt-independent regulation of transcriptional activity of β-catenin (17, 28). In particular, caveolin-1 recruits β-catenin to caveolae membranes for its link with E-cadherin and thus promoting cell adhesion (29). Thus, caveolin-1-dependent regulation of β-catenin might be the underlying mechanism for enhanced cell–cell adhesion and reduced tumor metastases resulting from CXCR6 knockdown. Moreover, the parallel dynamics of caveolin-1 expression/translocation and p38 activation prompted us to hypothesize that p38 may involve in the up-regulation of caveolin-1 expression, as previously described (17). Meanwhile, we observed the dampened GSK3β activity and concomitant up-regulation and membrane accumulation of β-catenin in CXCR6-knockdown cells, which coupled with reduced invasive activity of hepatoma cells. GSK3β is a well-established downstream molecule of p38, in which p38 inactivates GSK3β by direct phosphorylation, resulting in accumulation of β-catenin (18). Taken together, we propose that CXCR6-knockdown activates p38, while phosphorylates and inactivates GSK3β, leading to β-catenin accumulation; and simultaneously up-regulates caveolin-1, resulting in recruitment of β-catenin to the plasma membrane, whereby modulating cell adhesion and inhibiting tumor metastasis.

Tumor growth and metastasis requires a complex interplay between the local microenvironment and various accessory cells—most notably inflammatory cells and endothelial cells. Herein, we demonstrated that, in CXCR6-knockdown hepatoma
cells, the production of various cytokines was down-regulated leading to a disturbed tumor microenvironment, as exemplified by neutrophil infiltration and angiogenesis. In CXCR6\textsuperscript{high} tumors, significant increases in intratumor neutrophils and neoangiogenesis were observed in both mice models and human HCC samples. It is conceivable that IL-1\textbeta, IL-6, IL-8 and IL-17F secreted by CXCR6\textsuperscript{high} tumors could contribute to the recruitment of neutrophils or stimulation of angiogenesis. Of note, mounting evidence suggests that tumor-infiltrating neutrophils have a decidedly pro-tumor capacity \textit{in vivo} via promoting tumor cell proliferation, angiogenesis, and metastasis (30). As such, in CXCR6\textsuperscript{high} tumors, enhanced secretion of neutrophil chemotactic substances, massive neutrophil infiltration, and neutrophil-mediated multiple pro-tumor responses can occur sequentially. Consequently, high levels of CXCR6 or intratumoral neutrophils independently correlated with increased postoperative recurrence, and patients with simultaneously high levels of both markers had the worst clinical outcome. Additionally, IL-6/IL-6R and IL-8/IL-8R axes can directly participate in CXCR6-CXCL16 signaling mediated HCC invasion, through modulating β-catenin and caveolin-1 expression via p38 and GSK3β pathways, highly consistent with recent findings that pro-inflammatory IL-6 and IL-8 autocrine signaling loop was critical for oncogene overexpression induced tumorigenesis and epithelial-mesenchymal transition of human cancer cells (31, 32).

In conclusion, our results suggest that CXCR6 expression is an independent prognostic factor and associated with invasive growth, inflammatory recruitment and angiogenic activities of HCC. These events involve p38 and GSK3β signaling, and
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production of inflammatory cytokines from tumor cells. In particular, the accompanying tumor microenvironment is replete with pro-tumor neutrophils. Thus, strategies designed to target CXCR6 may provide a venue to ameliorate tumor progression. Given the complex nature of the tumor microenvironment, targeting of components in combination might advance the field.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Tables

**Table 1. Multivariate Analysis of Factors Associated With Survival and Recurrence**

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<th>Variables</th>
<th>Survival rate (%)</th>
<th>Multivariate</th>
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<td>1-year</td>
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<td><strong>Recurrence-free survival</strong></td>
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<td>CXCR6 (high vs. low)</td>
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<td>CD66b (high vs. low)</td>
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<td>Combination of CXCR6 and CD66b</td>
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<td><strong>Overall survival</strong></td>
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<td>III vs. I</td>
<td>71/85</td>
<td>46/69</td>
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Patients were classified according to the levels of CXCR6 and CD66b: group I, high expression of both markers; group II, high expression of either markers; and group III, low expression of both markers. For details, see Supplementary Tables 5 and 6.

HR, hazard ratio; CI, confidential interval; NS, not significant.
Figure legends

Figure 1. CXCR6 is expressed in HCC and its expression correlates with clinical outcome of HCC patients. A, Western blot revealed constitutive CXCR6 and CXCL16 expression in hepatoma cells. B, Western blot showed that CXCR6 protein level was significantly higher in HCC than paired peritumor liver tissues (n=24). In particular, CXCR6 expression in HCC with VI (n=10) was significantly up-regulated than those without VI (n=14). C, Immunostaining of CXCR6 using DAB (brown) was shown for: peritumor normal liver and HCC. Magnification, ×20. D, The cumulative recurrence and overall survival rates of 240 patients with HCC were compared between CXCR6 -low or -high groups, using Kaplan-Meier methods (log-rank test). VI, vascular invasion.

Figure 2. CXCR6/CXCL16 interaction impacts on invasive activity of hepatoma cells via modulating caveolin-1 and β-catenin expression in vitro. A, Representative images of the Transwell assays of SK-HEP-1 and HCCLM3 cell invasion. Magnification, ×40. B, Results of Transwell assays showed the relative number of invasive SK-HEP-1 and HCCLM3 cells with indicated treatments. Three independent experiments were performed in triplicate (mean ± SE). *, P < 0.05; **, P < 0.01. C, Expression of caveolin-1 and β-catenin in shR6 and shCtl cells were detected by Western blotting. Equivalent results were obtained with the addition of full-length or soluble CXCL16. D, Western blotting showed membrane translocation of caveolin-1 and β-catenin expression in shR6 compared to shCtl hepatoma cells.
Figure 3. p38 and GSK3β signaling are involved in the regulation of caveolin-1 and β-catenin expression induced by CXCR6/CXCL16 interaction. A, Expression and phosphorylation of p38 and GSK3β in shR6 and shCtl cells were detected by Western blotting. Equivalent results were obtained with the addition of full-length or soluble CXCL16. B, Results of Transwell assays showed the relative number of invasive hepatoma cells with indicated treatments. *, P < 0.05. Data were presented as mean ± SE from three independent experiments in triplicate. C, Hepatoma cells were treated with CXCL16, TDZD8 and SB202190 as indicated. Expression of indicated molecules was detected by Western blotting. SB202190, a p38 inhibitor; TDZD8, GSK3β inhibitor.

Figure 4. Effects of down-regulation of CXCR6 on tumor growth, inflammatory recruitment, neoangiogenesis and metastasis in vivo. A, The morphologic characteristics of tumor xenografts. 1×10^7 shR6-SK-HEP-1 and 2×10^6 shR6-HCCLM3 cells were injected in the left flank of each mouse respectively (6 in each group), with control cells in the right flank of the corresponding mouse. B, Significant differences in tumor weights were revealed between shR6 group and control group. C, Representative immunostaining of Gr-1+ neutrophils and CD31+ microvessels in shR6 group and control group. Magnification, ×40. D, Substantial decrease in infiltrating neutrophil and microvessel densities were observed in shR6 group. E, Representative images of lung metastatic foci (H&E) were shown. 5×10^6 shR6-HCCLM3 cells and control cells were subcutaneously injected (6 in each group).
Magnification, ×20, ×40. F, The Lung metastasis rates and foci were significant lower and smaller in shR6 group than control group respectively.

**Figure 5.** CXCR6 activates inflammatory cytokine production and associates with HCC invasiveness. A, Real time RT-PCR results of mRNA expression of indicated inflammatory factors in CXCR6-knockdown and control hepatoma cells. shCtl Cells were treated with full-length CXCL16 (0.2μg/ml) as indicated. Equivalent results were obtained with the addition of soluble CXCL16 (not shown). B, ELISA analysis confirmed the down-regulation of IL-6 and IL-8 in CXCR6-knockdown cells. C, Results from Transwell assays showed that IL-6 or IL-8 recovered the inhibition of hepatoma cell invasion resulting from CXCR6-knockdown. D, Transwell assays showed that blocking IL-6 and IL-8 activities by indicated treatments significantly repressed the promoting effect on hepatoma cell invasion that induced by CXCL16 stimulation. E, Representative immunoblotting of indicated molecules in HCCLM3 cells treated with full-length CXCL16 or IL-6 or IL-8 or left untreated. *, P < 0.05; **, P < 0.01. Data were presented as mean ± SE from three independent experiments in triplicate.

**Figure 6.** Combination of CXCR6 expression and neutrophil density is a better predictor for clinical outcome. A, Correlation analysis showed that CXCR6\textsuperscript{high} tumors contained higher density of neutrophils than CXCR6\textsuperscript{low} tumors (n=240). B, A significant positive correlation between intratumoral CD66b\textsuperscript{+} neutrophils and CD34\textsuperscript{+}...
microvessels, derived from 240 HCC patients, was detected. C, Two representative cases with simultaneously high or low levels of CXCR6, CD66b and CD34 were shown. Adjacent sections of paraffin-embedded HCC samples stained with anti-CXCR6, anti-CD66b or anti-CD34 antibodies. Magnification, ×40. D, Kaplan-Meier analysis revealed that HCC patients in group I presented with the worst survival and the highest recurrence rate. Patients were classified according to the CXCR6 and CD66b expression: group I, high expression of both markers; group II, low expression of either markers; and group III, low expression of both markers.
Figure 5.

A

Relative mRNA expression

IL-1α  IL-6  IL-8  IL-1β  IL-6  IL-8  TNF-α

shCtL       shR8       shCtL+L16

HCCLM3

B

IL-6 (ng/ml per 10⁶ cells)

SK-HEP-1  HCCLM3

shCtL       shR8

HCCLM3

C

Relative invasion ability (the fold of shCtL)

SK-HEP-1  HCCLM3

shCtL       shR8

IL-6 (0.2 μg/ml)  IL-8 (0.2 μg/ml)

D

Relative invasion ability (the fold of shCtL)

SK-HEP-1  HCCLM3

CXCL16 (0.2 μg/ml)  IgG (1 μg/ml)

anti-IL6R (1 μg/ml)  anti-IL8RA (1 μg/ml)

anti-IL8RB (2 μg/ml)  anti-IL8 (1 μg/ml)

E

Western blot

SK-HEP-1  HCCLM3

β-catenin (90 KD)  Caveolin-1 (24 KD)

p-p38 (38 KD)  p38 (38 KD)

p-GSK3β (46 KD)  GSK3β (46 KD)

GAPDH (38 KD)  Lamin A/C (70 KD)
Figure 6.

A

CD66b+ neutrophil density

B

Microvessel density (1-mm core)

C

Case #12

Case #221

D

Probability of recurrence

Overall survival

Time (months)

I vs II P = 0.141
I vs III P = 0.003
II vs III P = 0.041

I (n = 48)
II (n = 48)
III (n = 96)

I vs II P = 0.335
I vs III P = 0.017
II vs III P = 0.056

I (n = 48)
II (n = 48)
III (n = 96)