CXCR6 Upregulation Contributes to a Proinflammatory Tumor Microenvironment That Drives Metastasis and Poor Patient Outcomes in Hepatocellular Carcinoma

Qiang Gao1, Ying-Jun Zhao4, Xiao-Ying Wang1, Shuang-Jian Qiu1, Ying-Hong Shi1, Jian Sun1, Yong Yi1, Jie-Yi Shi1, Guo-Ming Shi1, Zhen-Bin Ding1, Yong-Sheng Xiao1, Zhong-Hua Zhao2, Jian Zhou1,3, Xiang-Huo He4, and Jia Fan1,3

Abstract

CXCR6 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 proinflammatory cytokine production. Clinically, high expression of CXCR6 was an independent predictor of increased recurrence and poor survival in HCCs. 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 protumor 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. Cancer Res; 72(14); 1–11. ©2012 AACR.

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, more than 70% of patients relapse within 5 years, and the overall survival for patients with HCCs 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 4 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 HCCs and is 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 represents the 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 HCCs, various chemokines and their receptors may not play the same role as they do in other tumors. After scrutinizing
10 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 fatal 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 biologic and clinical significance remain 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 patients with HCCs. Downregulation of CXCR6 resulted in reduced invasive activities, tumor formation, and metastasis of hepatoma cells. Moreover, CXCR6 could lead to a protumor microenvironment in vivo via producing proinflammatory 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 HCCs 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 HCCs and matched nontumor liver tissues and 6 normal liver tissue samples from patients with hepatic hemangioma were obtained for Western blotting. Ethical approval from the Zhongshan Hospital (Shanghai, PR China) 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 shL16] and CXCR6- or CXCL16-targeting shRNA (shR6, shL16)] and treatment with specific inhibitors or stimulators were conducted as described in the Supplementary Materials.

Immunohistochemistry and Western blotting

Immunohistochemistry and Western blotting were conducted as described in the Supplementary Materials. The primary antibodies used are listed in Supplementary Table S1.

Cell proliferation, cell-cycle, and invasion assays

Cell proliferation, cell-cycle, and invasion assays were conducted 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. A total of 1 × 10⁶ shR6/shL16-SK-HEP-1 and 2 × 10⁶ 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

Chemokine receptor expression was analyzed using a Chemokines and Receptors PCR Array (Cat. No. PAHS-022A; SA Bioscience), according to the manufacturer’s instructions. Real-time reverse transcription PCR (RT-PCR) was carried out as described in the Supplementary Materials. Primers are listed in Supplementary Table S2.

ELISA

Antibody sandwich ELISAs were used to evaluate interleukin (IL)-6 and IL-8 levels in the hepatoma cell conditioned medium according to the manufacturer’s instructions (R&D System).

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 and compared by the log-rank test. Variables showing P < 0.1 in univariate analyses were adopted as covariates in multivariate analysis. Multivariate survival analysis was evaluated by Cox proportional hazards model in a forward stepwise manner with the log likelihood ratio significance test. The Pearson χ² test was used to compare qualitative variables; and quantitative variables were analyzed by the Student t test or Spearman correlation test. P < 0.05 (2-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 S3). 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. S1), we found that CXCR6 protein levels were low in peritumor liver tissue, relatively higher in corresponding HCCs by 1.8-fold (P < 0.0001, vs. peritumor liver), and particularly higher in HCCs 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 HCCs and paired peritumor liver tissue (Supplementary Fig. S1).

To determine the association of CXCR6 expression in HCCs with disease outcome, TMAs from 240 patients with HCCs were examined by immunostaining (Fig. 1C). We found that CXCR6 expression in peritumor liver tissue was weak or minimal. In HCCs, 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 HCCs 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 S4). There was a striking inverse association between CXCR6 intensity and recurrence-free survival \(\text{RFS}; P=0.007\) and to a less extent overall survival \(\text{OS}; P=0.064\); Fig. 1D). The median OS and RFS time for CXCR6\textsuperscript{high} patients were 35.5 and 20.5 months, as compared with 61.0 and 84.0 months for CXCR6\textsuperscript{low} patients, respectively. Multivariate analysis revealed that CXCR6 intensity in tumors was an independent prognosticator for both OS and RFS (Table 1; Supplementary Tables S5 and S6). Thus, CXCR6 expression is a valuable predictor for recurrence and survival in patients with HCCs.

In peritumor liver, CXCL16 constitutively showed moderate or strong intensity in both cytoplasm and nuclei, and in HCCs, CXCL16 was positive in either cytoplasm or nuclei or both (Supplementary Fig. S1A). On the basis of the combined cytoplasm and nuclear intensities, patients were categorized into CXCL16-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. S1B and Supplementary Tables S5 and S6).

CXCR6 knockdown inhibits HCC cell invasion \textit{in vitro}

Given the clinical significance of CXCR6 in HCCs, we wondered whether silenced endogenous CXCR6 expression could
exhibit biologic effects on hepatoma cells. Two cell lines with the most abundant level of CXCR6 protein, that is, SK-HEP-1 and HCCLM3, were introduced CXCR6 knockdown via specific shRNA carried by Lentivirus system. Transwell assays showed that CXCR6 knockdown substantially inhibited invasive activity of hepatoma cells in vitro (Fig. 2A and B) but had no impact 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 impacts 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 transmembrane full-length form, could prominently promote control SK-HEP-1 and HCCLM3 cell invasion but not CXCR6-knockdown cells (Fig. 2A and B). Likewise, downregulation of CXCL16 markedly inhibited invasive activity of hepatoma cells in vitro (Supplementary Fig. S3A). These results indicate that the contribution of CXCR6 on HCC cell invasion may be due to CXCL6–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 upregulated in CXCR6-knockdown cells compared with control cells, whereas CXCL16 stimulation substantially decreased caveolin-1 and β-catenin expression in control cells but not in 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. S4A).

Further effort was taken for 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, whereas the amount of caveolin-1 and β-catenin in nuclei had no evident difference compared with control cells. These data indicate that CXCR6–CXCL16 signaling may affect hepatoma cell invasion, although regulating the functional translocation of caveolin-1 and β-catenin.

Because p38/mitogen-activated protein kinase (MAPK) and GSK3β have well-established roles 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, whereas 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, whereas 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.
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 carried out in triplicate (mean ± SE). *, P < 0.05; **, P < 0.01. C, expression of caveolin-1 and β-catenin in shR6 and shCtl cells was 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 with shCtl hepatoma cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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 are presented as mean ± SE from 3 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, a GSK3β inhibitor.
Consistent with the results of in vitro invasion assay, Western blotting showed that SB202190 further amplified, whereas TDZD8 partly recovered, the downregulation of caveolin-1 and β-catenin induced by CXCL16 stimulation in hepatoma cells (Fig. 3C). In contrast, Rac1, Rho1, Cdc42, extracellular signal–regulated kinase (ERK), and Akt signaling showed no significant change after CXCR6 knockdown (Supplementary Fig. S4B).

**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 ± 0.04 g) and shR6-HCCLM3 (0.49 ± 0.14 g)–derived xenografts were significantly lighter than those of shCtl-SK-HEP-1 (0.41 ± 0.09 g; P = 0.016) and shCtl-HCCLM3 (2.02 ± 0.41 g; P = 0.013; Fig. 4A and B). Similar results were seen when comparing tumor xenografts from shL16 and shCtl hepatoma cells (Supplementary Fig. S3B and S3C). In line with the in vitro findings that CXCR6 knockdown did not 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–derived [112.0 ± 11.3/HPF] and shR6-HCCLM3 (162.6 ± 14.9/HPF)–derived xenografts significantly decreased compared with 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 and D). We also compared the densities of infiltrating F4/80+ macrophages between the 2 groups, but the difference was not statistically significant (data not shown). Because the neutrophils play a central role in tumor angiogenesis (19), we further investigated activities of neoangiogenesis between the 2 groups. As expected, CD31+ microvessel densities in shR6-SK-HEP-1–derived (17.8 ± 2.1/HPF) and shR6-HCCLM3 (24.4 ± 3.5/HPF)–derived xenografts significantly decreased compared with those of shCtl-SK-HEP-1 (36.6 ± 3.8/HPF;
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 mouse models mirror human HCCs. 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 or 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 neutrophilhigh vs. neutrophillow groups, P = 0.039) and OS (median months: 33.0 vs. 63.0 for neutrophilhigh vs. neutrophillow groups, P = 0.026). Multivariate analyses revealed intratumoral neutrophil density as an independent index for RFS but not OS (Table 1; Supplementary Tables S5 and S6). Using the combination of the 2 parameters, increased the prognostic value as compared with 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% confidence interval (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 CXCR6low/neutrophilhigh, respectively (Table 1; Supplementary Tables S5 and S6). Therefore, evaluation of both CXCR6 expression and neutrophil infiltration is a powerful predictor of prognosis, further supporting the findings of altered inflammatory infiltration in mouse 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 HCCs. 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 pathologic characteristics in 240 patients with HCCs revealed a significant correlation of CXCR6 expression with tumor vascular invasion, advanced tumor stage, and recurrence/metastasis. Multivariate analysis showed that patients with HCCs with high CXCR6 expression in general had worse survival and increased recurrence than those with low expression. Furthermore, the effects of CXCR6/CXCL16 interaction on tumor invasion and metastasis were directly shown in our *in vitro* and *in vivo* studies. In mice subcutaneous xenografts, downregulation 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, whereas high expression of CXCL16 correlated with better prognosis, and downregulation of CXCL16 promoted tumor-migratory behavior in renal cell carcinoma (25). In addition, a study in colorectal cancer highlighted that high CXCL16 expression correlated with a better patient prognosis (26), whereas in our study, patients with HCCs 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 2 types of CXCL16, the transmembrane and soluble forms, like that reported in renal cell carcinoma (25), existed in HCCs. In addition, we found constitutively high CXCL16 expression in peritumor liver tissue. Given that constitutive CXCL16 expression has also been shown in bone marrow and lung (23, 27), the 2 most common metastatic sites of HCCs next to the liver, CXCR6–CXCL16 axis–mediated site-specific metastasis of HCCs can be envisioned.

Our results suggested that CXCR6–CXCL16 signaling may affect hepatoma cell invasion through regulating the membrane translocation of caveolin-1 and β-catenin. 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 be involved in the upregulation of caveolin-1 expression, as previously described (17). Meanwhile, we observed the dampened GSK3β activity and concomitant upregulation 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, whereas

Figure 6. Combination of CXCR6 expression and neutrophil density is a better predictor for clinical outcome. A, correlation analysis showed that CXCR6high tumors contained higher density of neutrophils than CXCR6low tumors (n = 240). B, a significant positive correlation between intratumoral CD66b+ neutrophils and CD34+ microvessels, derived from 240 patients with HCCs, was detected. C, two representative cases with simultaneously high or low levels of CXCR6, CD66b, and CD34 are 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 patients with HCCs 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.
phosphorylates and inactivates GSK3β, leading to β-catenin accumulation; and simultaneously upregulates caveolin-1, resulting in recruitment of β-catenin to the plasma membrane, thereby 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 showed 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-high tumors, significant increases in intratumor neutrophils and neoangiogenesis were observed in both mouse models and human HCC samples. It is conceivable that IL-1β, IL-6, IL-8, and IL-17F secreted by CXCR6-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 protumor capacity in vivo via promoting tumor cell proliferation, angiogenesis, and metastasis (30). As such, in CXCR6-high tumors, enhanced secretion of neutrophil chemotactic substances, massive neutrophil infiltration, and neutrophil-mediated multiple protumor 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. In addition, 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 proinflammatory 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 HCCs. These events involve p38 and GSK3β signaling and production of inflammatory cytokines from tumor cells. In particular, the accompanying tumor microenvironment is replete with protumor 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Grant Support
The study was supported by The Major Project of NSFC (No.81030038), National Key Sci-Tech Project (2008ZX10002-019), Shanghai Rising Star Program (No. 10QA140130), National Natural Science Foundation of China (No. 8107992 & No. 30091432), FANEDD (No. 2011183), Shanghai NSFC (No.10ZR140600 & No. 09ZR140620), and Shanghai “Chen Guang” project (No. 11CG02). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 14, 2011; revised April 4, 2012; accepted April 18, 2012; published OnlineFirst June 18, 2012.

References