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The IL-8/CXCR1 axis is associated with cancer stem cell-like properties and correlates with clinical prognosis in human pancreatic cancer cases

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CXCR1, a receptor for CXCL8/IL-8, has recently been demonstrated to be associated with cancer stem cell (CSC) populations in certain types of human cancers. However, the effect of CXCR1 on CSC and its prognostic value in human pancreatic cancer remain unknown. In this study, we evaluated the expression of CXCR1 in human pancreatic duct adenocarcinoma (PDAC) and found that positive CXCR1 expression correlated with lymph node metastasis ($P = 0.017$) and a poor survival rate (HR, 3.748; 95% CI, 1.822 to 7.712; $P < 0.001$) in patients with PDAC. In addition, we identified significant positive correlations between CXCR1 and CD44 ($P = 0.002$) and CD133 ($P = 0.017$). Further functional studies confirmed that IL-8 addition increased sphere formation, CSC populations, and cell invasion of pancreatic cancer cells and that these effects could be reversed by antagonizing CXCR1 with a CXCR1-specific antibody. Therefore, our study demonstrated that the IL-8/CXCR1 axis is associated with the CSC-like properties of pancratic cancer cells and prognosis in human pancreatic cancer. This suggested a way of targeting pancreatic CSCs by disrupting IL-8/CXCR1 axis.

Pancreatic cancer is a highly lethal malignant disease, ranking 10th in annual incidence among the different cancers, and is the 4th leading cause of cancer related death[1]. Its aggressive growth and high metastatic rate during its early stage are responsible for the high lethality[2]. While surgery is a curative treatment, only approximately 10–20% of pancreatic cancer is resectable at the time of diagnosis[3]. Therefore, chemotherapy is the only option for the rest of patients[4]. Thus, pancreatic cancer remains a dreadful disease and requires further study to reveal the molecular mechanisms of cell invasion and metastasis.

Cancer stem cells (CSCs) are defined as a subgroup of cells within a tumor that initiate and sustain the formation and growth of cancer due to their capacity to self-renew and differentiate[5]. CSCs have been isolated and identified in a growing number of tumors, such as those of colon, liver, and breast as well as pancreatic cancer[6–8]. Furthermore, pancreatic CSCs may have a role in cell invasion potential and treatment resistance[9–12]. Therefore, pancreatic CSCs could be a potential therapeutic target. Further investigation of pancreatic CSCs may lead to new therapies that prolong survival in pancreatic cancer patients.

Growing evidence suggests that CXCL8/IL-8 is associated with cell proliferation, migration and invasion in cancer[13–16]. Recent studies also show that the expression of IL-8 induces CSC activity in human breast and colon cancers. It is suggested that IL-8 could activate Stat3/NF-κB, and MAPK pathways in both tumor and stromal cells. Activation of these pathways stimulates further IL8-dependent positive feedback loops that, in turn, drive CSC self-renewal[17,18].

The role of the IL-8/CXCR1 axis in pancreatic cancer, including its effects on CSC population and prognostic value, remains unknown. Therefore, in this study we evaluated the expression of CXCR1 in PDAC patients for the first time and found that positive CXCR1 expression correlates with lymph node metastasis and a poor survival rate. In addition, we identified positive correlations between CXCR1 and CSC marker CD44 and CD133 in patients with PDAC. Our functional studies also confirmed that the IL-8/CXCR1 axis is associated with cancer stem cell-like properties in pancreatic cancer. Therefore, our study suggests a novel way of targeting pancreatic CSCs by disrupting the IL-8/CXCR1 axis.

Table 1 | The correlation between the CXCR1 expression and clinicopathological factors in resectable PDAC (n = 65)

		CXCR1 expression		
	No. of patients (%)	Positive expression n = 40 (61.5%)	Negative expression n = 25 (38.5%)	*P*-value
Age (years)				
Mean ± SD	65	56.8 ± 12.9	58.3 ± 11.0	0.634*
Gender				
Male	44 (67.7)	26 (40.0)	18 (27.7)	0.258
Female	21 (32.3)	14 (21.5)	7 (10.8)	
Histopathological grade				
G1	18 (27.7)	9 (13.8)	9 (13.8)	0.237
G2, 3	47 (72.3)	31 (47.7)	16 (24.6)	
Depth of invasion				
T1, 2	41 (63.1)	27 (41.5)	14 (21.5)	0.350
T3, 4	24 (36.9)	13 (20.0)	11 (16.9)	
Lymph node metastasis				
Negative	34 (52.3)	16 (24.6)	18 (27.7)	0.012
Positive	31 (47.7)	24 (36.9)	7 (10.8)	
Pathologic stage				
I, II	59 (90.8)	36 (55.4)	23 (35.4)	0.786**
III, IV	6 (9.2)	4 (6.2)	2 (3.1)	

Abbreviations: PDAC, pancreatic duct adenocarcinoma.
*This comparison was performed using Student's t-test. The other *P*-values were obtained with Pearson's χ^2 test.
**This comparison was performed with Fisher's exact test.

Results

Positive CXCR1 expression correlates with poor prognosis in PDAC. We first determined the expression status in the formalin-fixed paraffin-embedded sample of 65 PDAC. Forty of the 65 patients (61.5%) exhibited positive CXCR1 expression in cancer tissue. The median percentage of cells with CXCR1 expression in the positive cases was 14.7% (range 0.9–66.2%). CXCR1 expression was significantly associated with lymph node metastasis (P = 0.012, Table 1). However, there were no significant correlations between CXCR1 expression and age, gender, histopathological grade, depth of

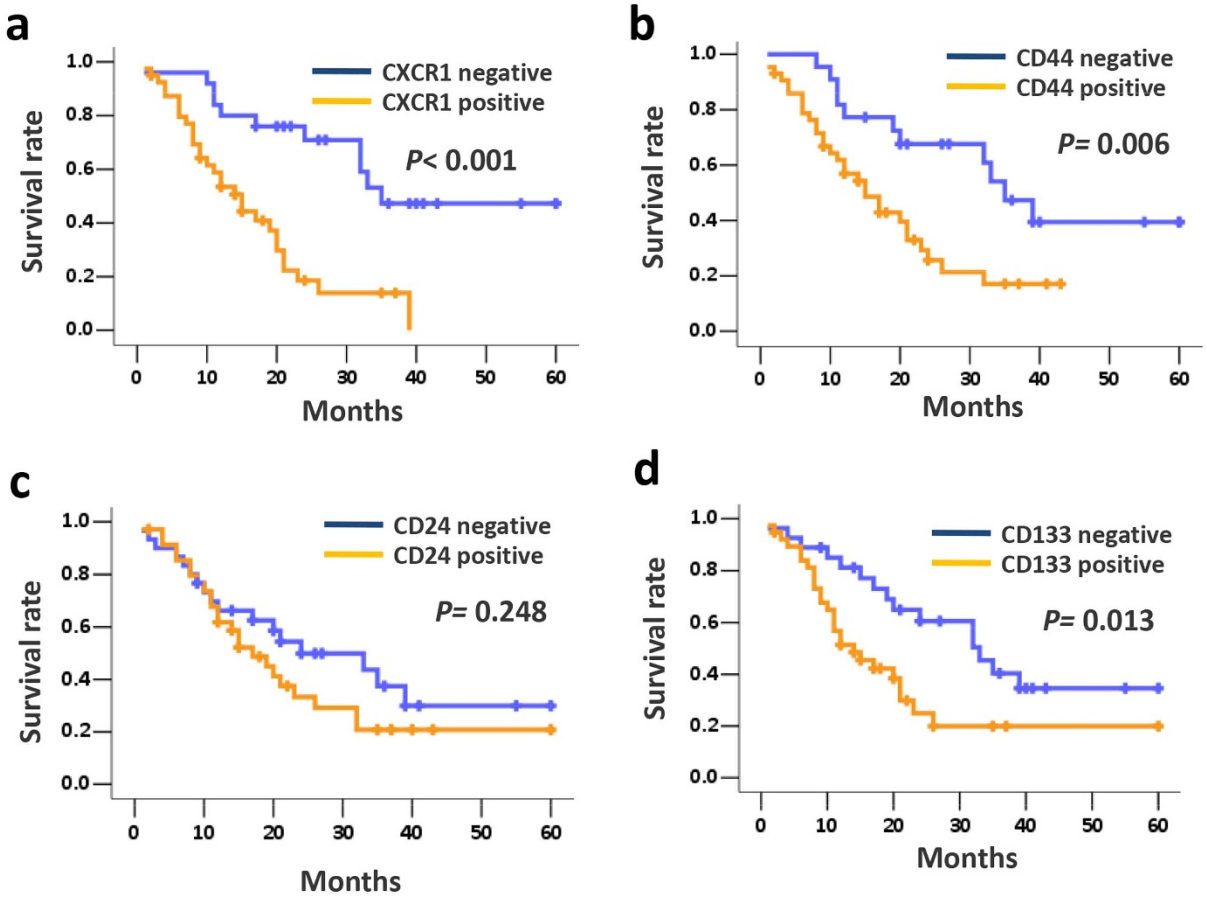

Figure 1 | Kaplan–Meier survival curves for patients with CXCR1 and CSC marker expression in PDAC patients: CXCR1 (a), CD44 (b), CD24 (c), and CD133 (d). P-values were calculated by the log-rank test.

Table 2 | Univariate and multivariate Cox regression analyses of CXCR1 expression for OS of patients with PDAC (n = 65)

Variables	Case number	Hazard ratio (95% CI)	*P*-value
Univariate analysis			
Age (<65 years vs. ≥65 years)	44/21	0.923 (0.483 to 1.765)	0.809
Gender (male vs. female)	43/22	0.583 (0.292 to 1.165)	0.127
pT (1/2 vs. 3/4)	41/24	1.694 (0.913 to 3.143)	0.095
pN (negative vs. positive)	34/31	3.118 (1.619 to 6.003)	0.004
pStage (I/II vs. III/IV)	59/6	6.320 (2.347 to 17.020)	<0.001
Histologic grade (G1 vs. G2/G3)	18/47	2.138 (1.004 to 4.553)	0.049
CXCR1 expression (negative vs. positive)	33/32	3.748 (1.822 to 7.712)	<0.001
Multivariate analysis			
pT (1/2 vs. 3/4)	41/24	1.667 (0.853 to 3.259)	0.135
pN (negative vs. positive)	34/31	2.091 (0.917 to 4.765)	0.079
pStage (I/II vs. III/IV)	59/6	5.955 (1.779 to 19.931)	0.004
Histologic grade (G1 vs. G2/G3)	18/47	1.444 (0.623 to 3.343)	0.391
CXCR1 expression (negative vs. positive)	33/32	2.830 (1.271 to 6.297)	0.011

Abbreviations: OS, overall survival; PDAC, pancreatic duct adenocarcinoma; pT, pathologic T stage; pN, pathologic lymph node metastasis; pStage, pathological stage; CI, confidence interval.

invasion, or TNM stage. Kaplan-Meier analysis revealed that the median survival time of the patients with negative CXCR1 expression was 35.4 months whereas that of patients with positive CXCR1 expression was 15.6 months (log-rank = 14.779, $P < 0.001$, Fig. 1a and Table 2). To exclude confounding effects, we performed Cox proportional hazards regression analysis. Multivariate analysis confirmed that positive CXCR1 expression (hazard ratio, 3.748; 95% confidence interval, 1.822 to 7.712; $P < 0.001$) was significantly associated with decreased overall survival (Table 2).

CXCR1 expression positively correlates with the expression of CSC markers in PDAC. We have hypothesized that CXCR1 was associated with CSC-like properties in pancreatic cancer. We next investigated the association between CXCR1 expression and reported pancreatic CSC marker, incluing CD44, CD24 and CD133, in human pancreatic cancer specimens. We could identify significant positive correlations between CXCR1 and CD44 ($P = 0.002$) and between CXCR1 and CD133 ($P = 0.017$) (Fig. 2 and Table 3). We also observed a borderline correlation between CXCR1 and CD24 although the difference was not significant (Table 3). In addition, we also evaluated the correlation between the expression of pancreatic CSC markers and patient survival time in 65 PDAC patients. We could confirm that overexpression of CD44 (log-rank = 7.492, $P = 0.006$) and CD133 (log-rank = 6.205, $P = 0.013$) was associated with decreased survival time (Fig. 1b–d). Therefore, our data suggests that CXCR1 was associated with CSC-like properties in human pancreatic cancer.

The IL-8/CXCR1 axis induces the stem-cell-like mammosphere-forming phenotype in pancreatic cancer cells. Because CXCR1 overexpression correlated with the expression of CSC markers and was associated with decreased survival time in human PDAC, we then investigated effects of the IL-8/CXCR1 axis on stem-cell-like mammosphere-forming phenotype in pancreatic cancer cells. As shown in Fig. 3a, cells that were treated with recombinant human IL-8 exhibited an enhanced capacity for inducing mammosphere formation when compared to controls. Using a human CXCR1/IL-8 RA antibody (R&D Systems Inc., Minneapolis, MN), we further showed that blockage of IL-8/CXCR1 abolished the CSC inducing activity. To confirm the effects of the IL-8/CXCR1 axis on CSC, we identified that only 0.63% of cultured monolayer Capan1 cells but 1.37% of IL-8 treated cells showed the pancreatic CSC associated phenotype of $CD44^+CD24^+$[19] (Fig. 3b). Similarly, we also observed that treatment with IL-8 antibody diminished the percentage of $CD44^+CD24^+$cells induced by IL-8 (Fig. 3b). These results indicate that the IL-8/CXCR1 axis induces the stem-cell-like mammosphere-forming phenotype in pancreatic cancer cells.

The IL-8/CXCR1 signaling axis promotes the migration and invasion of the pancreatic cancer cells. Recent evidence has suggested that pancreatic carcinomas harbor a distinct subpopulation of putative CSCs that are responsible for tumor progression and metastasis[12]. We next tested the effects of IL-8/CXCR1 signaling on cell migration and invasion using transwell chambers with or without Matrigel coating. The IL-8 treatment inhibited both the

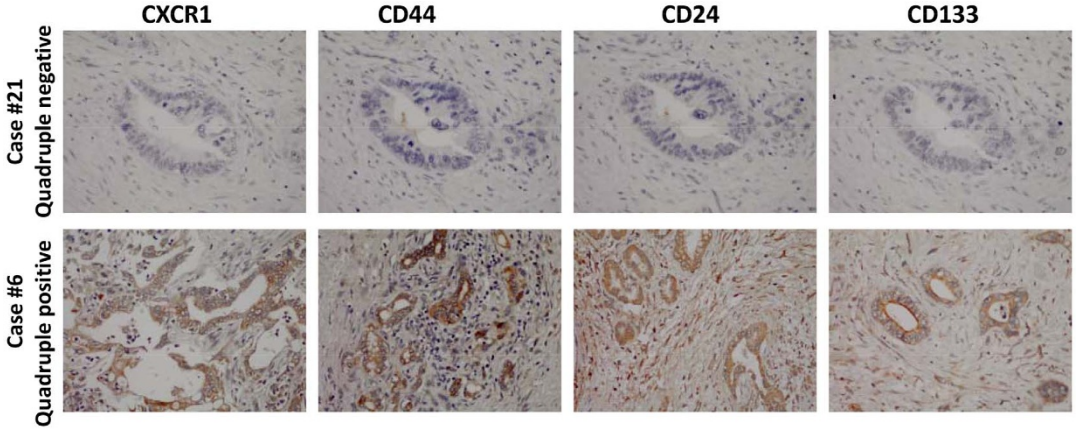

Figure 2 | Immunohistochemistry (IHC) staining of PDAC specimens with antibodies specific for CXCR1, CD44, CD24, and CD133. Original magnification, ×200.

Table 3 | Correlation between expression of CXCR1, CD44, CD24 and CD133 in resectable PDAC (n = 65)

	CXCR1 expression		
	Positive expression n = 40 (61.5%)	Negative expression n = 25 (38.5%)	*P*-value*
CD44 expression			
Negative (n = 22)	8 (12.3)	14 (21.5)	0.002
Positive (n = 43)	32 (49.2)	11 (16.9)	
CD24 expression			
Negative (n = 30)	15 (23.1)	15 (23.1)	0.079
Positive (n = 35)	25 (38.5)	10 (15.4)	
CD133 expression			
Negative (n = 27)	12 (18.5)	15 (23.1)	0.017
Positive (n = 38)	28 (43.1)	10 (15.4)	

Abbreviations: PDAC, pancreatic duct adenocarcinoma.
*P-values were obtained with Pearson's χ^2 test.

migration and invasion of Capan 1 cells in the transwell assays. Furthermore, blockade of IL-8 signaling with IL-8 antibody significantly inhibited the IL-8 induced cell migration and invasion (Fig. 4a–b).

Discussion

Here, we demonstrate that CXCR1 positively correlates with pancreatic CSC markers. In addition, we found that CXCR1 expression correlated with lymph node metastasis and lower survival rate in patients with pancreatic ductal adenocarcinoma. Functional studies showed that IL-8 addition increased sphere formation, CSC population, and cell invasion of pancreatic cancer cells and that these effects could be reversed by CXCR1 blockade using a CXCR1-specific blocking antibody, further supporting the correlation in patients. Our study suggests a way of targeting pancreatic CSCs by disrupting the IL-8/CXCR1 axis.

CXCR1 is a G-protein coupled receptor which binds IL-8 with high affinity. It is mainly expressed in neutrophils and was originally

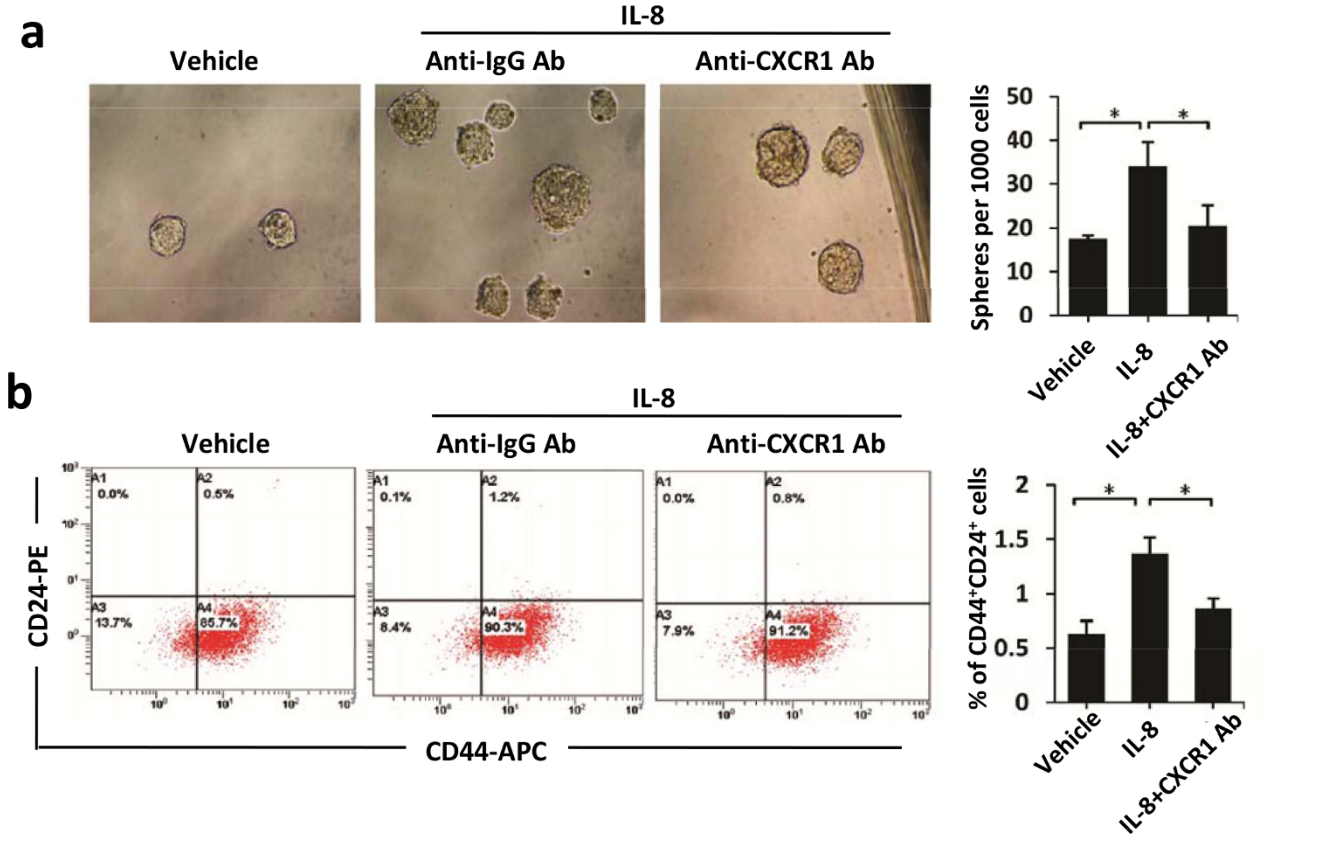

Figure 3 | The IL-8/CXCR1 axis induced the stem-cell-like mammosphere-forming phenotype in pancreatic cancer cells. (a) Capan1 cells were cultured in sphere formation media and then treated with vehicle, IL-8 (100 ng/ml), or the CXCR1 antibody (20 μg/ml) as indicated. After 15 days of treatment, spheroids larger than 50 μm were counted. Ten wells were counted in each experimental set. The results are presented as the means ± SD of values obtained in three independent experiments. Statistical significance was calculated using the ANOVA tests. *$p < 0.05$. (b) The effects of the IL-8/CXCR1 axis on CSC population. Cultured monolayer Capan1 cells were treated with vehicle, IL-8 (100 ng/ml), or the CXCR1 antibody (20 μg/ml), as indicated, for 5 days. Cells were then dissociated and stained with human CD44 antibody and CD24 antibody following the manufacturer's protocol. The percentage of $CD44^+CD24^+$ cells was calculated. The results are presented as the mean ± SD of values obtained in three independent experiments. Statistical significance was calculated using the ANOVA tests. * $p < 0.05$.

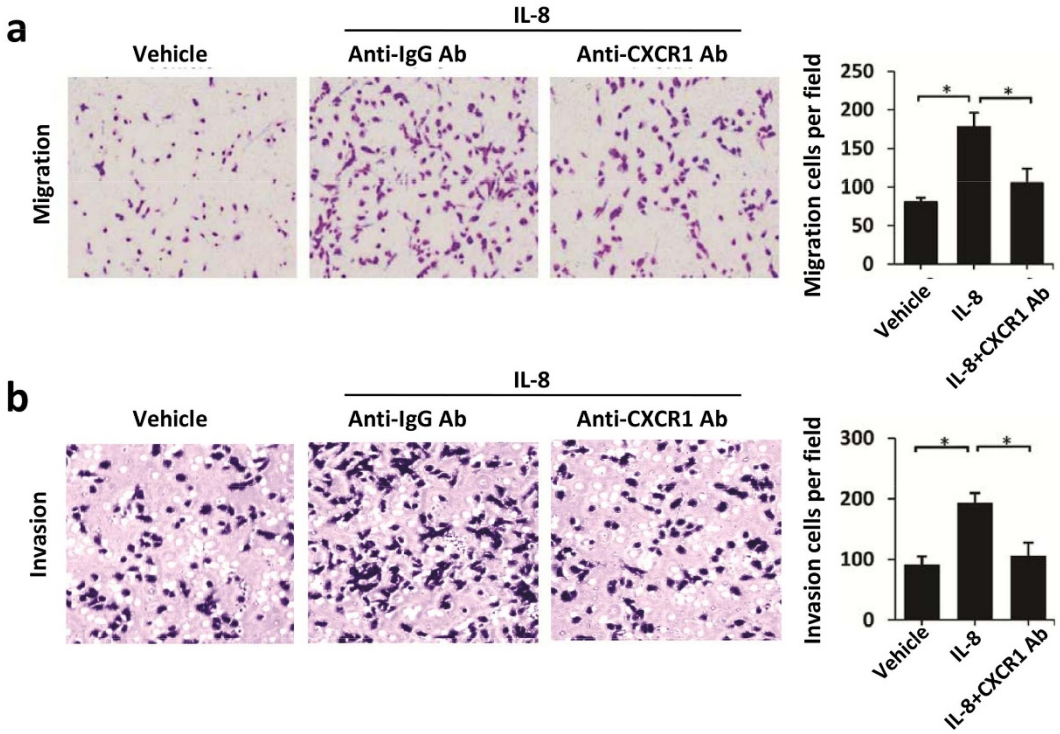

Figure 4 | The IL-8/CXCR1 signaling axis promoted the migration and invasion of the pancreatic cancer cells. Migration (a) and invasion (b) assays were performed on Capan1 cells treated with vehicle, IL-8 (100 ng/ml), or the CXCR1 antibody (20 μg/ml) using a transwell cell chamber. The number of cells that invaded through the membrane was counted in 10 fields under the ×20 objective lens. Original magnification, ×200. The results are presented as the means ± SD of values obtained in three independent experiments. Statistical significance was calculated using the Student's t-test. *$p < 0.05$.

characterized by its ability to induce chemotaxis of leukocytes[20,21]. Previous studies have indicated that CXCR1 was overexpressed in many cancers and is associated with drug-resistance, invasion, and metastasis[22–26]. Previous studies reported that CXCR1 were overexpressed in pancreatic cancer samples compared with the matched para-cancer tissues[13,27], suggesting that CXCR1 may play an important role in the development of certain tumors by interacting with IL-8. Although CXCR1 has been studied in several cancer types including pancreatic cancer, the precise functional role of CXCR1 in pancreatic cancer remains unclear. In our study, we examined the level of CXCR1 protein expression in human PDAC and found a significant association between CXCR1 expression and lymph node metastasis. Further analysis indicated that CXCR1 expression is significantly correlated to the prognosis of pancreatic cancer.

CSCs are a subgroup of cells that are functionally defined by their ability to form tumors, self-renew, and differentiate[5]. Since evidence of CSCs was first found in human acute myeloid leukemia[28], CSCs have been widely identified in a number of malignancies, and the existence of CSCs in pancreatic cancer was found by Li *et al*[19]. They first identified pancreatic CSCs with surface markers CD44, CD24, and ESA. Since then, a number of CSC markers, including CD133, CXCR4, ABCG2, nestin, c-Met, and ALDH have been used to identify pancreatic CSCs[29–33]. In our study, using CD44 and CD24 markers, we identified the effects of IL-8/CXCR1 on pancreatic CSC population.

Multiple signaling pathways are involved in the maintenance or homeostasis of pancreatic CSCs, including Wnt, CXCR4, Notch, Hedgehog, Nodal/activin, FoxM1, and mTOR. More recently, a study suggested that the IL-8/CXCR1 axis may play a pivotal role in the regulation of CSCs proliferation, self-renewal, and drug-resistance, leading to tumor cell invasion and metastasis[22]. In our study, we examined the correlation between CXCR1 expression and the stemness of pancreatic CSCs. We found that CXCR1 were significantly associated with the expression of CD44 and CD133 markers. Moreover, to further confirm the role of the IL-8/CXCR1 axis in pancreatic CSCs, we found out that the IL-8/CXCR1 axis was able to enrich Capan1 sphere-forming properties and increase the proportion of CSCs in vitro. Therefore, our results suggest that the IL-8/CXCR1 axis is associated with cancer stem cell-like properties in pancreatic CSCs.

CSCs make up only a small proportion of the cancer cell population[34], but they are thought to play a crucial role in cancer progression and metastasis[35,36]. Since the dysregulation of these signaling pathways is involved in the formation of pancreatic CSCs, it is important to develop therapeutic strategies that specifically target CSCs. Previous studies have used inhibitors of the Notch signaling pathway (PF-03084014, SAHA)[37,38], Hedgehog pathway[19,39] (Sulforaphane, cyclopamine, IPI-269609), mTOR pathway (Rottlerin, rapamycin, LY294002, rapamycin)[40,41], and Nodal (rLefty, SB431542)[42]. Our study revealed that the blockade of the IL-8/CXCR1 axis would result in a significantly decreased sphere formation, CSC population, and cell invasion. Therefore, disrupting the IL-8/CXCR1 axis may be a new way to target pancreatic CSCs.

In conclusion, our study demonstrated that the IL-8/CXCR1 axis is associated with the CSC-like properties of pancratic cancer cells and prognosis in human pancratic cancer. This provides insight into additional therapeutic strategies for targeting cancer stem cells.

Methods

Patients and clinical specimens. Clinical specimens were obtained as described previously[43]. Human pancreatic cancer samples were collected from 65 patients after surgical resection at Huashan Hospital, Fudan University, Shanghai, China, between January 2003 and December 2005. Informed consent was obtained from the patients before sample collection in accordance with institutional guidelines, and the study was approved by the Committees for the Ethical Review of Research at the Fudan University Shanghai Cancer Center. All patients underwent macroscopically curative resection. Resected primary tumors and lymph nodes were histologically examined by hematoxylin and eosin staining using the tumor-node-metastasis classification

system. Histologically, all of the tumors were invasive ductal adenocarcinomas. None of the patients had received neoadjuvant radiotherapy or chemotherapy. Formalin-fixed paraffin-embedded (FFPE) samples were obtained for immunohistochemical analysis. Patient characteristics are provided in Table 1.

Immunohistochemistry. Immunohistochemical analysis was performed as described previously[44]. Briefly, unstained 3-μm sections were excised from paraffin blocks. The sections were stained using anti-CXCR1 (rabbit polyclonal; 1 : 300 dilution), anti-CD44 (rabbit polyclonal; 1 : 200 dilution), anti-CD24 (rabbit polyclonal; 1 : 300 dilution), and anti-CD133 (rabbit polyclonal; 1 : 300 dilution) overnight at 4°C. Staining with the secondary antibody and the avidin-biotin peroxidase complex was performed according to manufacturer's protocol (Vector Laboratories, CA). All of the procedures were performed by two independent evaluators and one pathologist, none of whom had any previous knowledge of the clinical outcomes of these cases. An immunoglobulin-negative control was used to rule out non-specific binding. Immunoreactivity for each marker was scored semi-quantitatively by evaluating the number of positive tumour cells over the total number of tumor cells as previously decribed[45].

Cell lines. The human pancreatic cancer cell line Capan1 was obtained from the American Type Culture Collection and grown in complete growth medium as recommended by the manufacturer. The cultured cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. Cell lines were regularly authenticated by verifying its morphology and testing to confirm the absence of mycoplasma contamination (MycoAlert, Lonza, Rockland, ME, USA).

Mammosphere formation assay. Cells were dissociated into single-cell suspension with 0.25% Trypsin (Gibco, Carlsbad, CA). The sphere formation media (SFM) used was DMEM-F12 (Gibco, Carlsbad, CA) supplemented with 10 ng/ml fibroblast growth factor-basic (Peprotech), 20 ng/ml epidermal growth factor (Peprotech), 2% B27 and 1% N_2. To obtain spheres from the Capan1 cell line, dissociated cells were cultured in SFM for at least 2 weeks[46]. A total of 1000 cells were suspended in SFM by limiting dilution and cultured in a well of low attached 96-well plates for 15 days. Spheroids larger than 50 μm were counted.

Flow cytometry. To identify the population of pancreatic cancer stem cells, cultured monolayer Capan1 cells were dissociated with 0.25% trypsin, washed twice with phosphate-buffered saline (PBS), and suspended in PBS containing 2% FBS at a density of 1×10^6 cells/100 μl. The dissolved cells were stained with human CD44 antibody (APC-conjugated, BD Biosciences, Franklin Lakes, NJ) and CD24 antibody (PE-conjugated, BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol. The samples were analyzed on a flow cytometer (BD LSR II, America) and the data were analyzed with the BD FACS Diva software.

Migration and invasion assay. Cell migration and invasion were determined using transwell cell migration plates (Corning, NY) and Matrigel invasion chambers (Matrigel-coated membrane, BD Biosciences). Cells (1.0×10^4) were seeded in serum-free medium into the upper chamber and allowed to invade toward the lower chamber with 10% FCS as the chemoattractant. After 12 h (for migration assays without matrigel coating) or 24 h (for invasion assays with matrigel coating), the cells that had invaded through the membrane and adhered to the underside of the membrane were counted as we described previously[43].

Statistical analyses. ANOVA and Student's t-tests were used to determine the statistical significance of differences between samples, and the results are expressed as the mean ± SD. The χ^2 and Fisher's exact test were used to analyze the association between CXCR1 expression and clinicopathological parameters. The correlations between CXCR1 expression and CSC markers expressions were determined by Pearson's χ^2 test. Overall survival (OS) was defined as the interval between the dates of surgery and death. The Kaplan–Meier method was used to compare OS among patients in different groups, and the log-rank test was used to estimate differences in survival. Univariate and multivariate analyses were based on the Cox proportional hazards regression model using SPSS 15.0 software (SPSS, Inc.). A value of $p < 0.05$ was considered to be statistically significant.

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Author contributions

P.W. conceived and designed the experiments; P.W., J.F. and H.C. carried out the experiments; and P.W., Z.M., Z.C. and L.L. analysed the data. P.W. and L.C. wrote the manuscript, and P.W., J.F., H.C., Z.M., Z.C. and L.L. discussed the results and commented on the manuscript.

Additional information

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