CCL18 promotes epithelial-mesenchymal transition, invasion and migration of pancreatic cancer cells in pancreatic ductal adenocarcinoma

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Abstract. CCL18 is a chemokine that is primarily expressed in monocytes, macrophages and immature dendritic cells and plays a crucial role in immune and inflammation responses. Recently, CCL18 was found to play pivotal roles in the development of several kinds of cancers, but its expression status and role during the tumorigenesis of pancreatic cancer remain unknown. In this study, we performed immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) to evaluate the expression of CCL18 in human pancreatic ductal adenocarcinoma (PDAC) tissues and preoperative serum, respectively. The results showed that both cancer epithelial cells and mesenchymal macrophages in PDAC tissues positively expressed CCL18. Serum CCL18 levels were significantly higher in patients with PDAC in comparison to healthy controls. The expression of CCL18 in both cancer epithelial cells and mesenchymal cells was correlated with lymph node metastasis, histopathological grading and overall survival in 62 PDAC patients. *In vitro* assays showed that the gene and protein expression of CCL18 from U937 and THP-1 cell- derived macrophages were significantly higher than that from unstimulated U937 cells and THP-1 cells. In contrast, pancreatic cancer cell lines showed little to no CCL18 expression even after IL4 stimulation. Intriguingly, pancreatic cancer cell lines expressed the potential CCL18 receptors PITPNM3, CCR6 and GPR3. Furthermore, treatment with recombinant human CCL18 promoted the migration and invasion of pancreatic cancer cells, but had no effect on cell proliferation. Consistent with these results, CCL18 induced the expression of the epithelial-mesenchymal transition (EMT) related gene SNAIL1. Our findings suggest that the serum level of CCL18 is a potential biomarker for the diagnosis and prognosis of PDAC, and that the combined functions of CCL18 in mesenchymal and cancer cells might accelerate the progression of PDAC by promoting the epithelial-mesenchymal transition, invasion and migration of pancreatic cancer cells.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal solid tumors and is the fourth leading cause of cancer-related death in the United States. (1) The poor prognosis of pancreatic cancer mainly results from its rapid growth, invasion and early metastasis (2,3). Considerable effort has been made to treat pancreatic cancer, however, no satisfactory progress has been made during the past several years. Therefore, increased understanding of the cancer biology of pancreatic cancer and identification of novel therapeutic strategies are both urgently needed.

In addition to the extreme malignancy of the cancer epithelial cells, another hallmark of PDAC is a dense stroma surrounding the cancer cells. The stroma of PDAC is mainly composed of collagen fibers, extracellular matrix proteins, fibroblasts and inflammatory cells. An abundant extracellular matrix defends the cancer cells and is a leading cause of the enhanced malignant potential (4). It is now widely believed that the tumor-microenvironment plays key roles during both tumorigenesis and the anticancer drug response. To identify novel therapeutic pancreatic cancer targets, it is essential to investigate the interaction between the tumor and its microenvironment during the tumorigenesis of pancreatic cancer.

Among the factors associated with tumor-microenvironment interaction that are potential targets of anticancer therapies, members of the chemokine superfamily are promising candidates (5,6). Chemokines are a superfamily of chemotactic cytokines with a crucial role in the immune and inflammation responses. Chemokines are classified into four groups based on the spacing of their first two cysteine residues:

CXC, CC, C and CX3C chemokines (7,8). The most important function of chemokines is to regulate the chemotactic migration of leukocytes (9,10). Recent studies have also revealed the pivotal role of chemokines in tumor progression (11,12).

CCL18, a member of CC chemokine family, was recently found to play a pivotal role in the progression of malignant tumors. CCL18 is mainly expressed in monocytes, macrophages and immature dendritic cells (13-15). CCL18 protein attracts lymphocytes and immature dendritic cells and induces collagen deposition by fibroblasts (16). CCL18 is one of the most abundant factors that were expressed by tumor-associated macrophages (TAMs). Stimulators, such as IL4, can polarize macrophages to a phenotype of M2 and upregulate the expression of CCL18 (17). Enhanced CCL18 production has been demonstrated in tumor tissue, peripheral blood and dropsy of the serous cavity associated with several malignancies. Highly expressed CCL18 protein can suppress the maturation and recruitment of killer cells, including lymphocytes and dendritic cells, and destroy their immunocompetence (16). CCL18 can also promote the migration and invasion of cancer cells by binding them directly (6,18). Recently, in pathway analysis of a genome-wide association study, Li *et al* (19) reported that CCL18 might be a susceptibility factor for the progression of pancreatic cancer through the Th1/Th2 immune response. Therefore, we hypothesized that CCL18 may play key roles during the progression of pancreatic cancer.

In this study, we evaluated the expression of CCL18 in human PDAC tissues by immunohistochemistry and preoperative serum by ELISA, and analyzed the correlation between CCL18 expression and the clinicopathological factors of 62 PDAC patients. Furthermore, we assessed the effects of CCL18 on the proliferation, migration and invasion of *in vitro* cultured pancreatic cancer cells.

Materials and methods

Patients and samples. Sixty-two patients (46 males and 16 females, median age 59 years, range 35-75), who underwent resection of PDAC at our institution between January 2007 and December 2011, were included in this study. Clinical data was collected from a pathography of the patients. The pathological classification of these cases was based on the UICC (Union for International Cancer Control)-TNM classification of malignant tumors (20). Preoperative serum was collected from 24 PDAC patients and the control group consisted of eight age- and gender-matched healthy volunteers. The use of the clinical samples was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University, and written informed consent was obtained from each patient.

Cell lines and treatment. Human pancreatic cancer cell lines PANC-1, BxPC-3, CAPAN-2 and SW1990, and human monocyte cell lines U937 and THP-1 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and were incubated at 37˚C in a 5% CO_2 atmosphere. For the collection of pancreatic cancer cells for quantitative real-time PCR (qRT-PCR) or cell culture supernatants of these cells for enzyme-linked immunosorbent assay (ELISA), the four pancreatic cancer cell lines were cultured at a density of $5x10^5$/ml. After incubation for one day, the medium was replaced by fresh medium with or without IL-4. After incubation for additional 72 h, the supernatants and cells were collected. For the collection of macrophages for qRT-PCR or cell culture supernatant for ELISA, U937 and THP-1 cells were incubated at a density of $5x10^5$/ml and stimulated for two days by 10 ng/ml (U937) or 100 ng/ml (THP-1) of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), respectively. Non-adherent cells were removed by washing and new medium with or without 45 ng/ml IL4 was added. After incubation for three days, the cells and supernatants were collected. The two monocyte cell lines were incubated at a density of $5x10^5$/ml for seven days to collect cells and the supernatant.

Immunohistochemistry and immunofluorescence. Patient PDAC tissues were formalin-fixed and paraffin-embedded. The tissue sections (3-4 μm) were rehydrated and treated with 3% hydrogen peroxide in methanol, followed by antigen retrieval. After being blocked with Dako® Protein Block, the sections were incubated with primary antibodies at 4˚C overnight. This was followed by incubation with a secondary antibody for 30 min at room temperature. Slides were then treated with streptavidin-peroxidase reagent at 37˚C for 15 min. For immunohistochemistry, Dako® DAB Chromogen was used for the color-reaction followed by nucleus counterstaining with hematoxylin. For immunofluorescence, the nucleus was counterstained by DAPI and imaged using a Nikon Eclipse E600 microscope with DP Manager Version 1.2.1.107 software. The following antibodies were used: primary antibodies: rabbit anti-CCL18 (1:200, Abcam); mouse anti-CD68 (1:100, Dako); mouse anti-CD163 (1:100, Novocastra); mouse anti-Ki67 (1:50, Abcam); mouse anti-p53 (1:200, Abcam), mouse anti-CEA (1:400, Abcam); mouse anti-CA19-9 (1:100, Abcam); mouse anti-CD34 (1:100, Abcam); secondary antibodies: anti-rabbit or anti-mouse IgG (1:200, Vector Laboratories); Alexa Fluor 594 donkey anti-rabbit or 488 goat anti-mouse IgG (H+L) (1:200, Life Technologies).

qRT-PCR. Total RNA was extracted from pancreatic cancer cells or monocyte/macrophages using TRIzol reagent according to the instructions of the manufacturer (Takara Bio). CDNA was synthesized using GoScript™ Reverse Transcription system according to the manufacturer's instructions (Promega). The relative levels of target gene mRNA to control GAPDH were determined by qRT-PCR in a 7900 HT Fast Real-Time PCR system (Applied Biosystems) using the GoTaq® qPCR Master Mix (Promega). The data were analyzed by the $2^{-\Delta\Delta Ct}$ method. The sequences are listed in Table I.

ELISA. CCL18 levels in cell culture supernatants and the serum of healthy volunteers or PDAC patients were determined quantitatively using a human PARC (CCL18) ELISA kit (Raybiotech) as described by the manufacturer.

Cell proliferation assay (MTT assay). Cells (BxPC-3, PANC-1) were seeded in 96-well plates at a density of $5x10^4$/ ml in 100 μl of complete medium and grown for 24 h. Then the medium was replaced with serum-free medium containing different concentrations of recombinant human CCL18 (rh-CCL18,

Table I. The sequences of PCR primers used in this study.

Gene	Forward primer	Reverse primer	Amplicon (bp)
CCL18	5'-CTCTGCTGCCTCGTCTATACCT-3'	5'-CTTGGTTAGGAGGATGACACCT-3'	108
PITPNM3	5'-GATGCCAGAGGAGAAGGGAC-3'	5'-TCGCTGTCTTCGTGGATCTC-3'	134
CCR6	5'-GCTCAAGTGTTCACAACCTGGAAG-3'	5'-TCCTAATGGCCCACTACAACCTG-3'	118
GPR3	5'-TCCTCTCTCTAGCCCTGCTC-3'	5'-CTCTCTGGGTACCTGGGTTG-3'	148
SNAIL1	5'-CATCCTTCTCACTGCCATGGA-3'	5'-AGGCAGAGGACACAGAACCAGA-3'	107
VEGF	5'-ATGACGAGGGCCTGGAGTGTG-3'	5'-CCTATGTGCTGGCCTTGGTGAG-3'	91
IL8	5'-AAACCACCGGAAGGAACCAT-3'	5'-CCTTCACACAGAGCTGCAGAAA-3'	101
E-cadherin	5'-AGTGCCAACTGGACCATTCA-3'	5'-TCTTTGACCACCGCTCTCCT-3'	314
CD44	5'-TGCCGCTTTGCAGGTGTAT-3'	5'-GGCCTCCGTCCGAGAGA-3'	66
CXCR4	5'-GCCTTATCCTGCCTGGTATTGTC-3'	5'-GCGAAGAAAGCCAGGATGAGGAT-3'	130
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'	87

Peprotech). After incubation for 24-72 h, cell Titer 96 AQueous One Solution Cell Proliferation assay (Promega) was added (20 μl/well) and incubated for 90 min. Finally, the optical densities (OD) were measured at 492 nm.

Transwell chamber assay. Transwell chamber migration assay was performed using Nunc 24-well 8.0-μm-pore transwell plates (Thermo Fisher Scientific) according to the manufacturer's instructions. Pancreatic cancer cells were plated into the upper chambers at $5x10^4$/ml. The lower chambers contained 5-25 ng/ml rh-CCL18 or macrophages prepared beforehand. After incubation for 24 h, non-invading cells were removed from the upper surface of the membrane using a cotton-tipped swab. Then the invading cells were fixed in methanol for 10 min and stained with 0.1% crystal violet hydrate (Sigma) for 30 min. The invading cells were counted as cells per field at 10x magnification. The invasion assay was performed in a similar fashion except the 8.0-μm pore size membrane inserts were coated with matrigel (BD Biosciences) that was diluted at 1:6 with serum-free media.

Western blotting. Total proteins were extracted from cells with RIPA cell lysis buffer (Cell Signaling) on ice for 30 min. Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 2% fat-free milk in PBS at room temperature for 1 h and probed with primary anti-SNAIL1 antibody (1:500, Abcam), E-cadherin (1:500, Abcam) or β-actin (1:1,000, Peproteck) antibodies at 4˚C overnight. Membranes were incubated with peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5,000, Beyotime) at room temperature for 45 min. Immunoreactive protein bands were visualized with an ECL detection kit (Thermol Biotech). The experiment was repeated three times.

Statistical analysis. Statistical comparisons of means were performed by Student's t-test, whereas χ^2 test was applied to analyze the relationship between CCL18 expression status and clinicopathological factors. Cutoff value of CCL18-positive macrophage counts was calculated by performing non-parametric receiver operating characteristics (ROC) using Dr. SPSS II for windows. The cutoff value was defined according to the best predictive values calculated by ROC analysis (cutoff value: 19.5 cells/40x magnification). Statistic significance was defined as $P<0.05$.

Results

CCL18 is expressed in PDAC tissue. To evaluate the expression status of CCL18 in human PDAC tissues, we performed immunohistochemical analysis of CCL18 in PDAC tissues from 62 patients. Compared with normal pancreas, there was dramatically increased expression of CCL18 in PDAC tissues (Fig. 1A-E). Among all the cases tested, 61.29% (38/62) stained positively for CCL18 in cancer cells and 70.97% (44/62) were positive in mesenchymal cells, while 45.16% (28/62) stained positively in both cancer and mesenchymal cells. Thus, 85.48% (53/62) showed a positive expression of CCL18 in cancer and/or mesenchymal cells. Notably, in cases with positive staining in both cancer and mesenchymal cells, the staining of CCL18 in cancer epithelial cells was weaker than that in mesenchymal cells (Fig. 1D). These results demonstrated that both the cancer epithelial and mesenchymal cells of PDAC tissue positively express CCL18.

The concentration of CCL18 in the peripheral blood serum of patients with PDAC is significantly higher than that of healthy controls. Currently, several serum markers, such as CEA and CA19-9, are commonly used to detect the origin, recurrence and metastasis of pancreatic cancer (21,22). Since CCL18 is a soluble protein and is upregulated in PDAC tissues, we hypothesized that serum levels of CCL18 might serve as a diagnostic or follow-up marker in PDAC. We measured the concentration of soluble CCL18 in serum samples of patients with PDAC (n=24) and in healthy donors (n=8) by ELISA. The serum level of CCL18 in PDAC patients was 8,913.60 pg/ml to 270,117.30 pg/ml (65,337.19±63,287.63 pg/ml) while that of healthy donors was 3,721.57 pg/ml to 25,046.21 pg/ml (17,510.83±8,717.47 pg/ml) (P=0.039) (Fig. 1F). Moreover, 75% (18/24) of the PDAC samples demonstrated higher CCL18

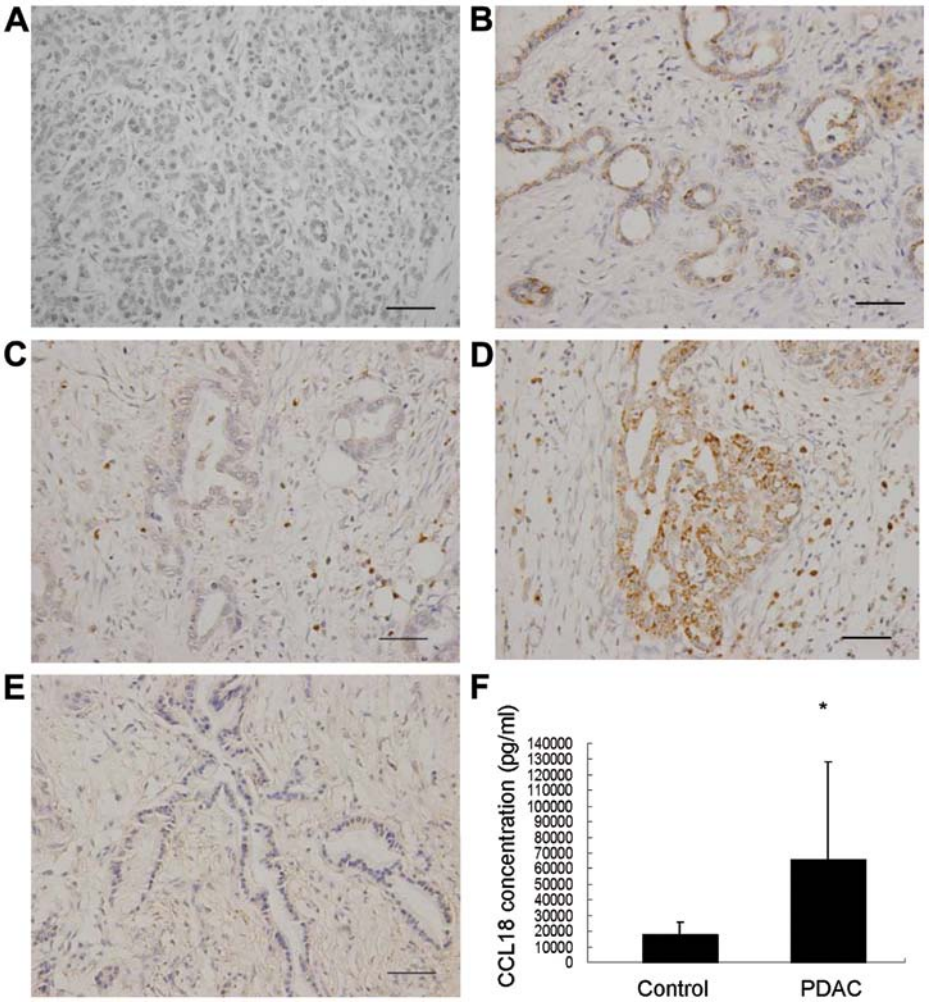

Figure 1. CCL18 is positively expressed in PDAC tissue. (A-E) Expression and localization of CCL18 in pancreatic tissues. (A) Normal pancreas; (B-E) PDAC [(B) cancer cells +/mesenchymal cells -, (C) cancer cells -/mesenchymal cells +, (D) cancer cells +/mesenchymal cells +, (E) cancer cells -/mesenchymal cells -]. (F) Concentration of CCL18 in the serum of healthy controls and patients with PDAC (*P<0.05 versus control). (A-E) Scale bar, 50 μm.

levels than the highest value measured in the control group. Therefore, the serum CCL18 levels were significantly higher in patients with PDAC in comparison to healthy controls, suggesting that serum CCL18 level is a potentially useful biomarker for the diagnosis and prognosis of PDAC.

CCL18 expression in both cancer and mesenchymal cells correlates with PDAC tumor progression and a worse survival rate for PDAC patients. We next analyzed the correlation between the expression of CCL18 and the clinicopathological factors of 62 PDAC patients. The clinical data are summarized in Tables II and III. The results revealed that CCL18 expression in both cancer and mesenchymal cells was significantly correlated with lymph node metastasis (P=0.015) and UICC stage (P=0.037) (Table II). Moreover, the number of CCL18-expressing mesenchymal cells was significantly associated with tissue CA19-9 expression level (P=0.030) (Table III).

We further examined the correlation between the expression of CCL18 and the overall survival of these 62 PDAC patients by Kaplan-Meier analysis. CCL18 expression in cancer cells (Fig. 2A, P=0.311), mesenchymal cells (Fig. 2B, P=0.358), and the count of mesenchymal positive cells (Fig. 2C, P=0.057) was not statistically associated with survival of PDAC patients. Interestingly, PDAC patients with CCL18 expression in both cancer and mesenchymal cells had a significantly worse overall survival rate than patients without CCL18 expression in either cell type (Fig. 2D, $\chi^2 = 6.165$, P=0.013).

CCL18-positive cells in the mesenchyme of PDAC tissues are M2-polarized macrophages. Previous reports showed that CCL18 is mainly expressed in the monocyte-macrophage system and is highly expressed in tumor-associated macrophages (TAMs) (17). It is thus possible that the CCL18-positive mesenchymal cells in our tested PDAC tissues are macrophages. To test this hypothesis, we performed immunofluorescence staining of both CCL18 and the macrophage marker CD68. CCL18-expressing cells co-localized with CD68 positive staining (Fig. 3A-C), indicating that CCL18-expressing cells are macrophages. Moreover, in agreement with a previous report (6), additional double staining of CD163 and CCL18 indicated that CCL18-expressing macrophages were a subset of CD163-positive M2-polarized macrophages (Fig. 3D-F).

U937 and THP-1 cell derived macrophages secrete high levels of CCL18 while cultured pancreatic cancer cells

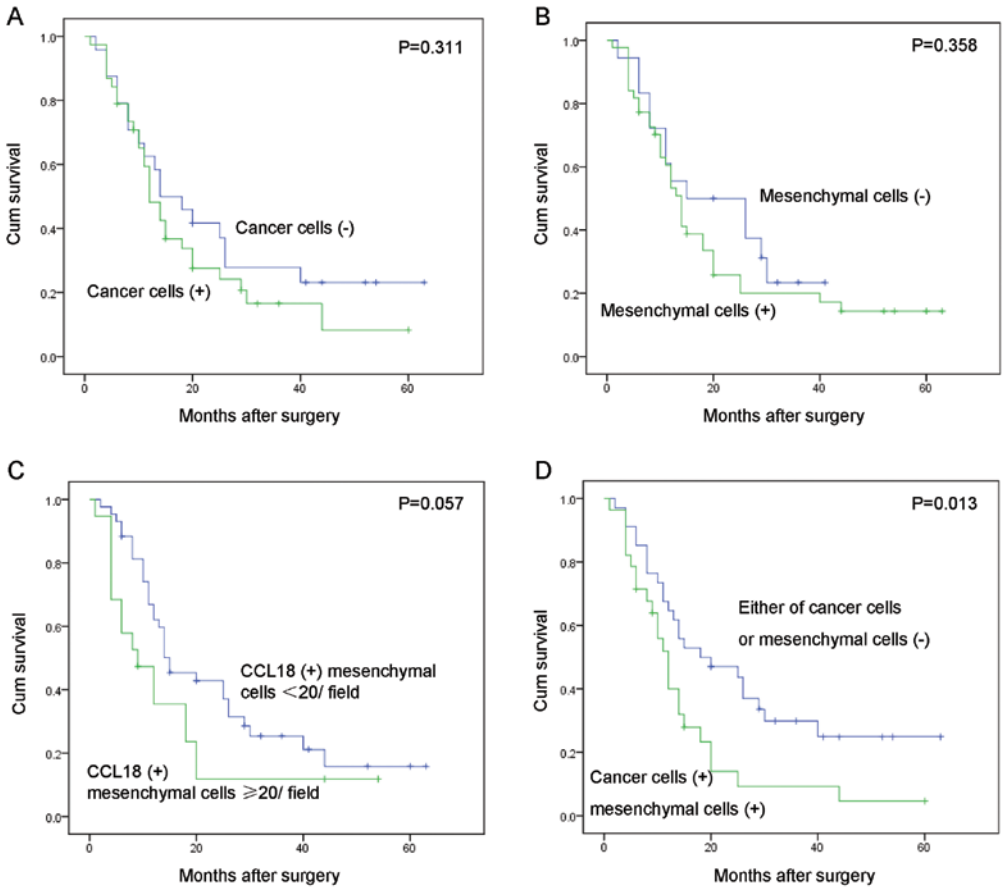

Figure 2. Kaplan-Meier analysis of overall survival of PDAC patients. Expression of CCL18 in 62 PDAC patients was plotted against the time of overall survival. (A) Cancer cells positive versus cancer cells negative expression of CCL18. (B) Mesenchymal cells positive versus mesenchymal cells negative espression of CCL18. (C) Lower (<20 per view of field) CCL18-positive cell counts versus higher CCL18-positive cell counts (≥20 per view of field). (D) CCL18-positive expression in both cancer and mesenchymal cells versus CCL18-negative expression in either cell types.

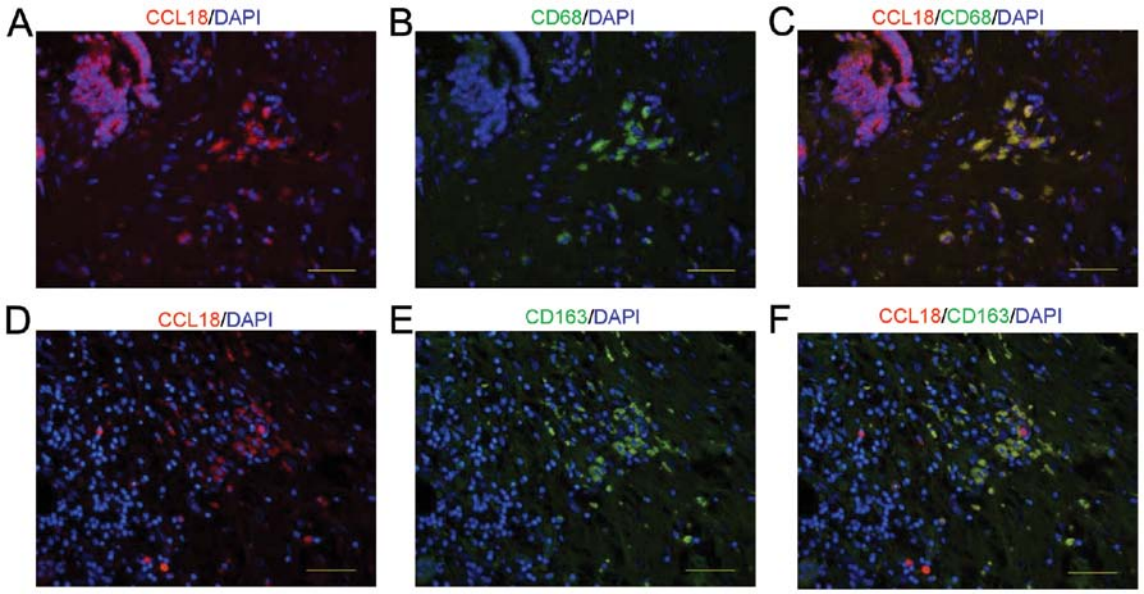

Figure 3. CCL18-positive cells in the mesenchyme of pancreatic cancer tissue are macrophages. Double staining of CCL18 and macrophage specific markers CD68 and CD163 by immunofluorescence. (A-C) Double staining of CCL18 (red) and CD68 (green). (A) CCL18, (B) CD68, (C) Merged image. (D-F) Double staining of CCL18 (red) and CD163 (green). (D) CCL18, (E) CD163, (F) Merged image. Nuclear counterstain with DAPI is blue. Double staining in yellow shows co-localization. Scale bar, 50 μm.

express limited levels of CCL18. U937 and THP-1 cell lines are representative monocyte/macrophage cells and widely used as human monocyte cells. Macrophage differentiation can be induced in both cell lines by stimulation with PMA

Table II. Correlation of CCL18 expression with clinical data from PDAC patients.

Parameters	No. of patients	Cancer cells		P-value	Mesenchymal		P-value	Mesenchymal positive cell counts		P-value	Positive mesenchymal and cancer cells		P-value
		-	+		-	+		< 20	≥20		No	Yes	
Cases	62	24	38		18	44		43	19		34	28	
Age													
≤60	35	15	20	0.309	12	23	0.226	25	10	0.448	21	14	0.251
>60	27	9	18		6	21		18	9		13	14	
Gender													
Male	46	18	28	0.576	13	33	0.528	30	16	0.19	26	20	0.435
Female	16	6	10		5	11		13	3		8	8	
Tumor location													
Head	56	22	34	0.572	16	40	0.567	38	18	0.397	3	26	0.434
Body/tail	6	2	4		2	4		5	1		4	2	
Tumor size													
≤2.5 cm	16	4	12	0.157	6	10	0.288	11	5	0.592	8	8	0.435
>2.5 cm	46	20	26		12	34		32	14		26	20	
Differentiation													
Well	20	7	13	0.449	7	13	0.335	16	4	0.169	11	9	0.602
Moderate/poor	42	17	25		11	31		27	15		23	19	
T stage													
T1+T2	22	12	10	0.052	9	13	0.109	16	6	0.449	15	7	0.096
T3+T4	40	12	28		9	31		27	13		19	21	
Lymph node metastasis													
Negative	41	19	22	0.072	14	27	0.173	31	10	0.115	27	14	0.015
Positive	21	5	16		4	17		12	9		7	14	
TNM stage													
I+IIA	31	15	16	0.096	11	20	0.201	24	7	0.135	21	10	0.037
IIB+III	31	9	22		7	24		19	12		13	18	
Perineural invasion													
Absent	52	20	32	0.596	16	36	0.394	34	18	0.117	30	22	0.247
Present	10	4	6		2	8		9	1		4	6	
Vascular permeation													
Absent	43	17	26	0.536	12	31	0.497	32	11	0.158	23	20	0.484
Present	19	7	12		6	13		11	8		11	8	
Pre-therapeutic CA19-9 level													
<37 U/ml	19	7	12	0.536	8	11	0.115	13	6	0.57	11	8	0.484
≥37 U/ml	43	17	26		10	33		30	13		20	23	

(23,24). Previous reports showed that IL4 could stimulate the polarization of peripheral blood monocytes, causing the monocytes to adopt an M2 phenotype and release of high levels of CCL18 (6). We used these two cell lines as monocyte models to verify the expression of CCL18 in monocytes and macrophages. In agreement with previous reports, our results

Table III. Correlation of CCL18 expression with Ki67, P53, CEA, CA19-9 and CD34 expression in 62 cases of PDAC.

		Cancer cells			Mesenchymal			Mesenchymal positive cell counts			Positive mesenchymal and cancer cells		
	No. of patients	-	+	P-value	-	+	P-value	< 20	≥20	P-value	No	Yes	P-value
Cases	62	24	38		18	44		43	19		34	28	
Ki67													
Negative	25	13	12	0.067	6	19	0.336	19	6	0.259	15	10	0.341
Positive	37	11	26		12	25		24	13		19	18	
P53													
Negative	28	12	16	0.364	7	21	0.363	22	6	0.124	16	12	0.471
Positive	34	12	22		11	23		21	13		18	16	
CEA													
Negative	15	7	8	0.333	2	13	0.110	8	7	0.112	8	7	0.563
Positive	47	17	30		16	31		35	12		26	21	
CA19-9													
Negative	29	11	18	0.557	8	21	0.519	24	5	0.030	14	15	0.237
Positive	33	13	20		10	23		19	14		20	13	
CD34													
Negative	48	19	29	0.525	13	35	0.377	31	17	0.117	27	21	0.455
Positive	14	5	9		5	9		12	2		7	7	

showed that CCL18 mRNA level (Fig. 4A) and secreted CCL18 protein (Fig. 4E) were low in untreated U937 cells, while both were significantly upregulated after conversion of U937 cells to macrophages by PMA. Impressively, IL4 further dramatically enhanced the CCL18 mRNA level (Fig. 4B) and secretion of CCL18 protein (Fig. 4F) in PMA stimulated U937 cells. We observed no stimulation of CCL18 protein expression in THP-1 cells after PMA treatment (Fig. 4G), but PMA treatment significantly increased the levels of CCL18 mRNA (Fig. 4C). Similar to U937 cells, IL4 significantly upregulated the mRNA level (Fig. 4D) and secretion of CCL18 protein (Fig. 4H) in THP-1 cells stimulated with PMA.

To determine gene and protein expression of CCL18 in pancreatic cancer cells, various pancreatic cancer cell lines (PANC-1, BxPC-3, SW1990 and CAPAN-2) were subjected to qRT-PCR and ELISA analysis of CCL18. All four pancreatic cancer cell lines showed faint CCL18 mRNA expression levels that were similar to those of THP-1 and U937 cells (Fig. 4I). With the exception of the CAPAN-2 cells, which displayed a faintly detectable level of CCL18, the soluble CCL18 protein level in the cell culture supernatant was less than the detection limit of CCL18 ELISA kit (0.2 pg/ml) (Fig. 4J). IL4, which can upregulate the expression of CCL18 in monocyte and macrophage U937 and THP-1 cells, did not induce CCL18 mRNA expression (Fig. 4I) or the soluble CCL18 protein level in cell culture supernatant (Fig. 4J). These results showed that in cultured pancreatic cancer cells, CCL18 was either not expressed or was only expressed at a very low level.

Pancreatic cancer cell lines express the potential CCL18 receptors PITPNM3, CCR6 and GPR3. CCL18 is considered an 'orphan ligand', with its cognate receptor and the underlying pathways unidentified. Recent studies have suggested some potential receptors of CCL18. The study by Catusse *et al* revealed that CCL18 acts agonistically to and diminishes the CXCR4-mediated effects of CXCL12 via GPR30 (25). Chen *et al* demonstrated that CCL18 could promote the invasion and migration of breast cancer cells by binding PITPNM3, a membrane-associated phosphatidylinositol transfer domain-containing protein (6). Zissel *et al* (26) proposed that the chemokine receptor CCR6 is a CCL18 receptor with the ability to initiate fibroblast activity. We examined the gene expression of these three potential receptors of CCL18 in pancreatic cell lines by qRT-PCR and found that they were expressed at different levels in different cell lines. BxPC-3 cells expressed only PITPNM3, while PANC-1, CAPAN-2 and SW1990 cells expressed various levels of PITPNM3, CCR6 and GPR3 (Fig. 5). These results suggest the possibility of the existence of CCL18 receptors in pancreatic cancer cells and that CCL18 may have effects on the biological behavior of pancreatic cancer cells.

CCL18 promotes migration and invasion of pancreatic cancer cells in vitro, but has no effect on cell proliferation. To investigate whether CCL18 could promote the progression of PDAC, we assessed the cell proliferation, migration and invasion of pancreatic cancer cells by MTT assay, transwell migration

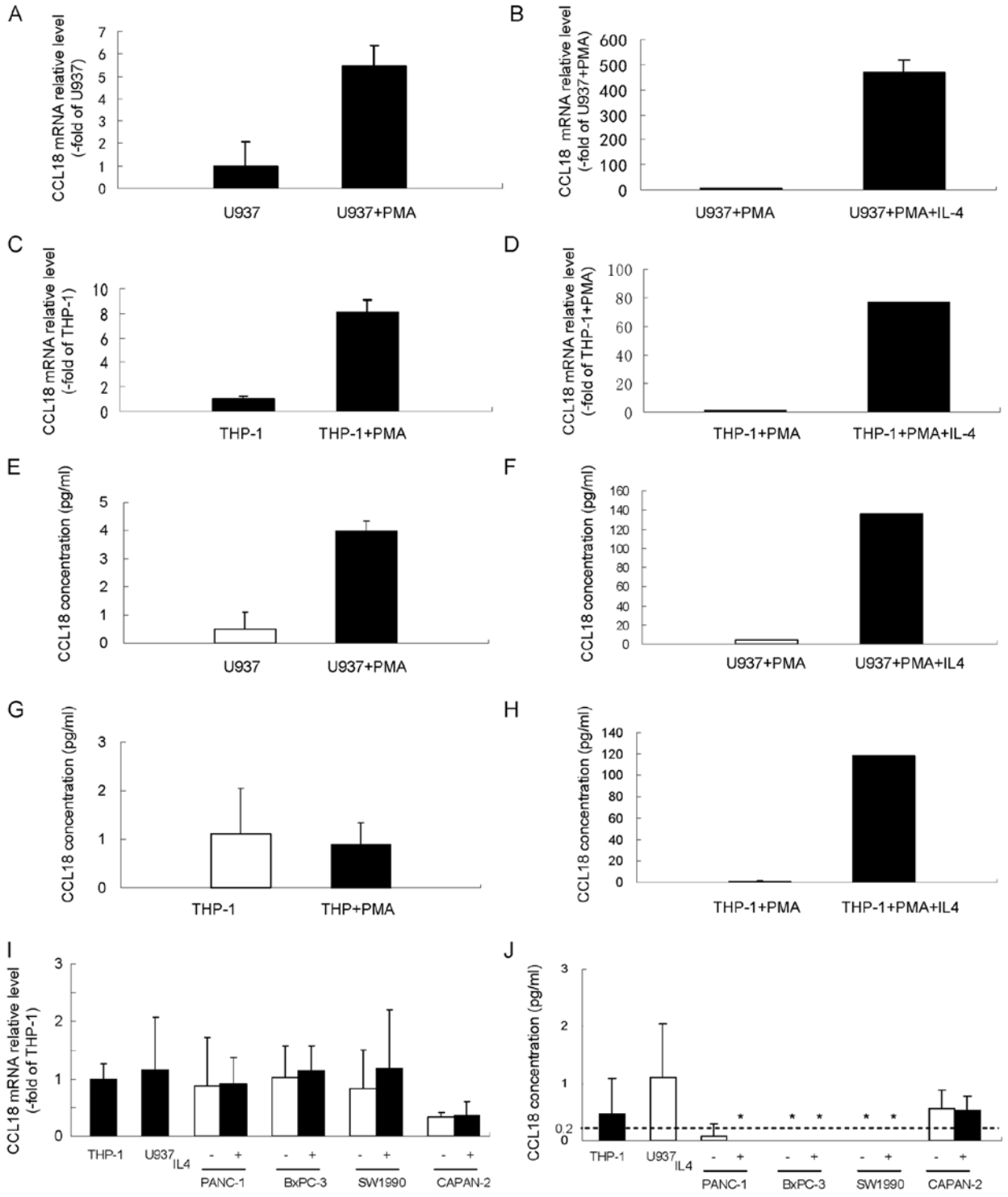

Figure 4. U937 and THP-1 cell derived macrophages secrete high levels of CCL18 while pancreatic cancer cells express limited levels of CCL18. CCL18 mRNA (A-D and I) and protein (E-H and J) expression levels in U937 or THP-1 cell derived monocyte-macrophages and pancreatic cancer cells are shown. *Undetectable.

assay and transwell invasion assay, respectively. MTT assay showed that treatment of pancreatic cancer BxPC-3 (Fig. 6A) and PANC-1 (Fig. 6B) cells with 5 ng/ml to 25 ng/ml rh-CCL18 for 24, 48 and 72 h did not noticeably alter cell proliferation. In contrast, cell migration after treatment with rh-CCL18 was significantly increased in both BxPC-3 and PANC-1 cells. Migratory capability of BxPC-3 cells was significantly higher after treatment with 10 ng/ml rh-CCL18 in both BxPC-3 cells (1,191±141 versus control 699±54 migratory cells/field, P=0.0006, Fig. 6C) and PANC-1 cells (715±59 versus control 383±66 migratory cells/ field, P<0.0001, Fig. 6D). Transwell invasion assay revealed that rh-CCL18 significantly increased the number of invading cancer cells compared to serum-free medium in both BxPC-3 (Fig. 6E, P<0.0001) and PANC-1

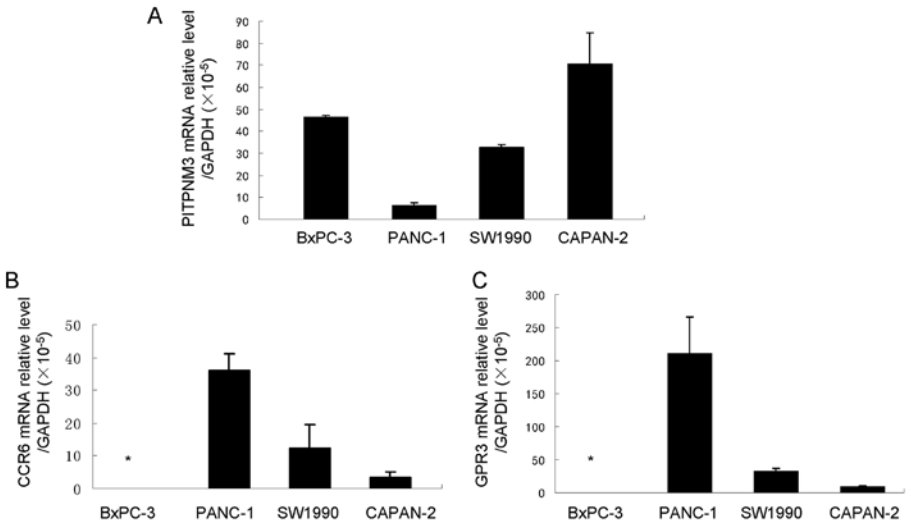

Figure 5. Potential CCL18 receptors are expressed at varying levels in different pancreatic cancer cell lines. The mRNA expression of PITPNM3, CCR6 and GPR3 was determined by qRT-PCR in pancreatic cancer cell lines. (A) PITPNM3; (B) CCR6; (C) GPR3. The expression ratio to GAPDH was calculated. *Undetectable.

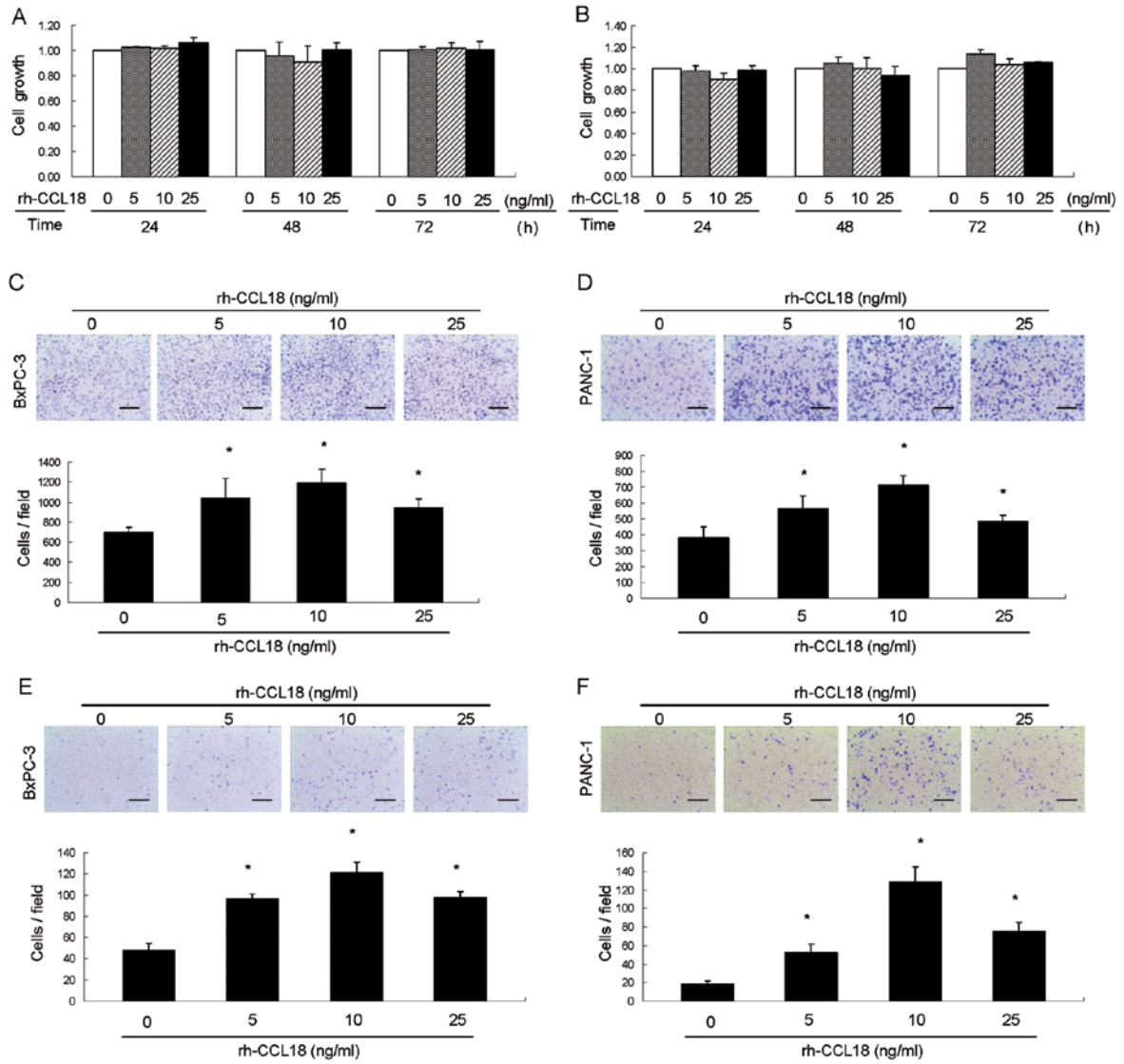

Figure 6. CCL18 promotes migration and invasion but not proliferation of pancreatic cancer cells *in vitro*. (A and B) The effect of CCL18 on proliferation of pancreatic cancer cells [(A) BxPC-3, (B) PANC-1]. (C-F) The effect of CCL18 on migration (C and D) and invasion (E and F) of pancreatic cancer cells. The migratory or invading cells were counted under a microscope at magnification, x10 in five random fields per insert assays (*P<0.05 versus control). (C-F) Scale bar, 200 μm.

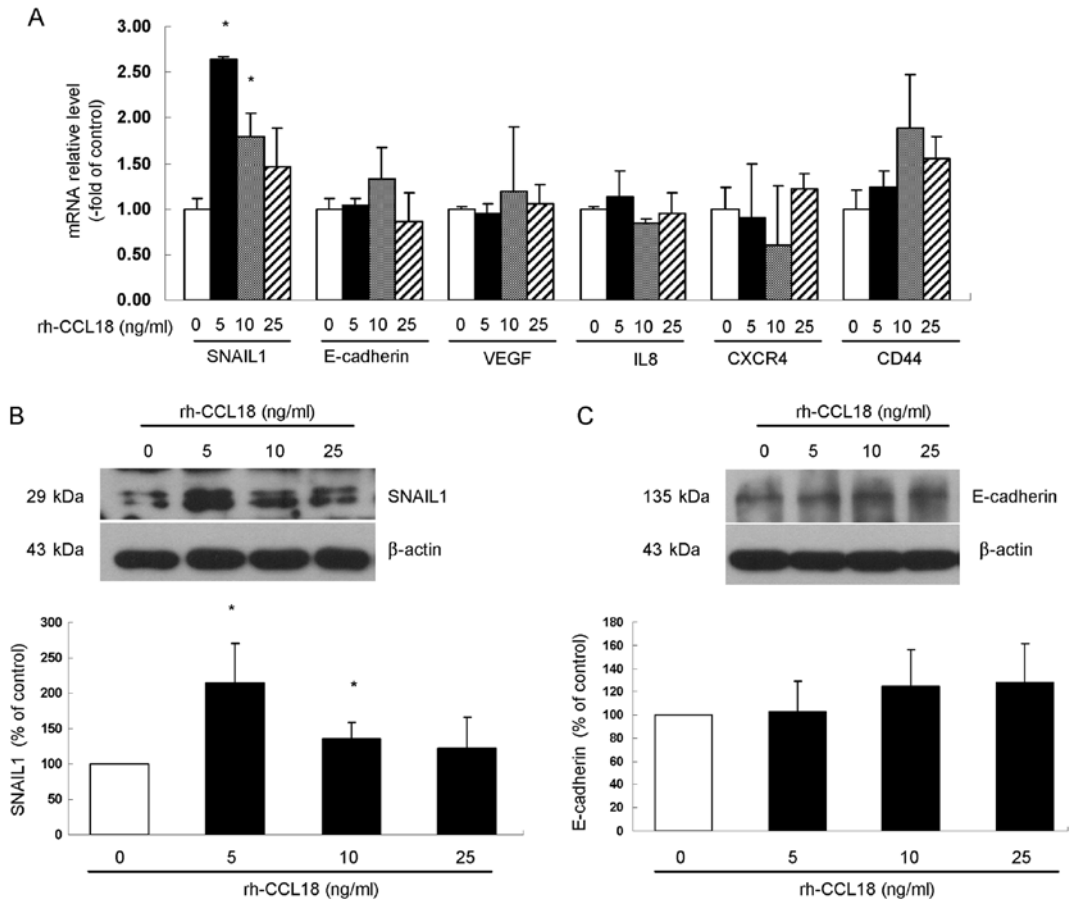

Figure 7. CCL18 promotes the expression of EMT-associated factor SNAIL1. (A) The effect of CCL18 on the mRNA expression of EMT markers, angiogenesis related markers and pancreatic cancer stem cell markers in BxPC-3 cells. (B and C) Effect of CCL18 on the protein expression of EMT related markers SNAIL1 and E-cadherin in BxPC-3 cells (*P<0.05 versus control).

(Fig. 6F, P<0.0001) cells. Invading cancer cells reached their maximum at a dose of 10 ng/ml rh-CCL18. These results indicated that CCL18 was involved in migration and invasion but not proliferation of pancreatic cancer cells.

CXCL16 increased the expression level of EMT-associated factor SNAIL1 in pancreatic cancer cells. To investigate the molecular mechanisms by which CCL18 enhances cell migration and invasion, we examined BxPC-3 cells after stimulation with rh-CCL18 and measured the mRNA expression of EMT-associated factors SNAIL1 and E-cadherin, angiogenesis-associated factors VEGF and IL8 and pancreatic cancer stem cell specific markers CD44 and CXCR4. After 24 h rh-CCL18 stimulation, gene expression of these factors was examined by qRT-PCR. The results showed that, among all six factors examined, only stimulation with rh-CCL18 significantly upregulated the gene expression of EMT-related factor SNAIL1 at the concentrations of 5 ng/ml (2.64±0.03-fold of control, P=0.020) and 10 ng/ml (1.79±0.26-fold of control, P=0.035) (Fig. 7A). Consistent with the increase in SNAIL1 mRNA after stimulation by rh-CCL18 for 24 h, the protein expression of SNAIL1 was upregulated at the concentrations of 5 ng/ml (2.14±0.56-fold of control, P=0.011) and 10 ng/ml (1.35±0.23-fold of control, P=0.046) (Fig. 7B), while different concentrations of rh-CCL18 treatment for 24 h did not change the protein level of E-cadherin (Fig. 7C).

Discussion

In this study, we found that CCL18 was positively expressed in both the epithelial and mesenchymal cells of human PDAC tissues. Moreover, serum CCL18 levels were significantly higher in patients with PDAC in comparison to those of healthy controls. Furthermore, CCL18 expression in cancer epithelial and mesenchymal cells correlated with malignant progression and shorter overall survival of the 62 PDAC patients examined. Most importantly, we observed that treatment with recombinant human CCL18 promoted the migration and invasion of *in vitro* cultured pancreatic cancer cells.

Chronic inflammation is implicated in a variety of human cancers, including pancreatic cancer (27,28). It is now becoming clear that the tumor microenvironment, which is largely coordinated by inflammatory cells including tumor-associated macrophages (TAM), tumor-associated dendritic cells (TADC) and tumor-infiltrating T cells (TIL), is an indispensable participant in the neoplastic process, proliferation, survival and migration of cancer cells (29,30). As key players in the creation of the tumor-microenvironment, chemokines, which can be produced by the tumor cells and tumor-associated inflammatory cells, may contribute directly to malignant progression (6,31).

CCL18, a vital chemokine in Th-2 immune response, was recently demonstrated to be associated with progression

of various malignant tumors, including pancreatic cancer (19,32-37). Our immunohistochemistry results revealed that CCL18 was highly expressed in human pancreatic cancer tissues. High CCL18 expression was not restricted to mesenchymal cells, 61.29% of the cancer tissues examined showed positive CCL18 staining in cancer epithelial cells, although the expression level was relatively weak in contrast to mesenchymal cells (Fig. 1). Furthermore, we demonstrated that CCL18 expression level correlated with the stage of progression and overall survival of PDAC patients (Tables II and III and Fig. 2). These data suggest that CCL18 might contribute to the tumor progression of PDAC in both cancer epithelial and mesenchymal cells.

Using immunofluorescence staining, we found that CCL18-positive mesenchymal cells were CD68 or CD163-positive macrophages (Fig. 3). In *in vitro* cell cultures, we observed that CCL18 was highly expressed in U937 and THP-1 monocytes and macrophages but very low or undetectable in pancreatic cancer PANC-1, BxPC-3, SW1990 and CAPAN-2 cells. In agreement with previous reports, (6) IL4, a stimulator of CCL18 expression, significantly upregulated the expression level of CCL18 in both PMA activated U937 and THP-1 macrophages. In sharp contrast, little to no CCL18 was detected in pancreatic cancer cells, even after stimulation with IL4 (Fig. 4). This result agrees with previous reports showing CCL18 expression under the detection limit and no CCL18 response to conventional stimulators in various carcinoma cells (32). However, the results observed in cultured pancreatic cancer cells were quite different from the immunohistochemistry results we observed using clinical PDAC samples. The discrepancy between the cell culture results and the results observed in the clinical samples is probably due to the distinct differences between the *in vivo* and *in vitro* conditions. No CCL18 homologue has yet been found in rodents (16), and the lack of an animal model makes it difficult to confirm the expression of CCL18 *in vivo*. Nevertheless, the high expression of CCL18 was mainly found in macrophages in the microenvironment of PDAC, implying that macrophages around cancer cells might promote the migration and invasion of pancreatic cancer cells and that CCL18 expression might be essential to this process.

Previous reports showed that overexpression of CCL18 by tumor tissues could promote the invasiveness of cancer cells by interacting with other tumorigenic factors (6,18). Our qRT-PCR results revealed expression of the potential CCL18 receptors PITPNM3, CCR6 and GPR3 in all four of the pancreatic cancer cell lines tested (Fig. 5). Consistently, our *in vitro* experiments demonstrated that rh-CCL18 promoted the migration and invasion activity of the pancreatic cancer cells BxPC-3 and PANC-1 (Fig. 6). These data suggest that CCL18 may affect the biological behavior of pancreatic cancer cells by binding cell surface receptors.

Finally, we investigated the molecular mechanism by which CCL18 enhanced the progression of pancreatic cancer. Of the six factors we examined, rh-CCL18 upregulated the gene expression of SNAIL1, a marker of EMT, in BxPC-3 cells (Fig. 7A). Increased SNAIL1 expression was confirmed by western blotting (Fig. 7B). Our results were consistent with previous observations that CCL18 or other chemokines are associated with EMT in various tumors (33,38-40). Taken together, these facts suggest that, at least in part, CCL18 promotes the migration and invasion of pancreatic cancer cells through SNAIL1 signaling.

In conclusion, we found that cancer epithelial cells and mesenchymal macrophages from human PDAC tissues positively expressed CCL18. The expression level of CCL18 correlated with tumor progression and the overall survival of PDAC patients. Our findings suggest that serum CCL18 level is a potential biomarker for the diagnosis and prognosis of PDAC, and that CCL18 plays an important role during the tumorigenesis of PDAC.

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