SPANDIDOS PUBLICATIONS

Strong expression of chemokine receptor CXCR4 by pancreatic cancer correlates with advanced disease

THOMAS WEHLER[1*], FELIX WOLFERT[2*], CARL C. SCHIMANSKI[2,3], INES GOCKEL[3,4], WOLFGANG HERR[1], STEFAN BIESTERFELD[6], JOACHIM K. SEIFERT[4], HASSAN ADWAN[5], MARTIN R. BERGER[5], THEODOR JUNGINGER[4], PETER R. GALLE[2] and MARKUS MOEHLER[2]

[1]Third Department of Internal Medicine; [2]First Department of Internal Medicine; [3]The Interdisciplinary Translational Oncological Laboratorium (ITOL); [4]Institute of Surgery, Johannes Gutenberg University of Mainz, Mainz; [5]AG Toxicology and Chemotherapy, German Cancer Research Institute, Heidelberg; [6]Institute of Pathology, Johannes Gutenberg University of Mainz, Mainz, Germany

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Abstract. Certain chemokines have been proposed to distinctly contribute to tumor growth, dissemination and local immune escape. Expression of the chemokine receptor CXCR4 has been linked to tumor progression in diverse tumor entities. The aim of this study was to evaluate if the expression of CXCR4 influences progression of human pancreatic cancer. CXCR4 expression of pancreatic cancer was retrospectively assessed by immunohistochemistry in 103 patients with pancreatic cancer. Intensity of CXCR4 expression was correlated with both tumor and patient characteristics. Human pancreatic cancer revealed variable intensities of CXCR4 expression. Strong CXCR4 expression was significantly associated with advanced UICC stages (P=0.03) and revealed a trend for hematogenous metastasis (P=0.09) and progressed local tumor stages (P=0.15). In summary, strong expression of CXCR4 was significantly associated with advanced pancreatic cancer.

Introduction

Adenocarcinoma of the pancreas is the fourth to fifth leading cause of cancer-related deaths in the Western world (1,2). The peak incidence is in the 65-75-year age group (3), and most patients present with advanced disease, resulting in low resection rates between 2.6-9% (4-6). The late presentation is responsible for the overall median survival of <6 months and 5-year survival rate of 0.4-5% (5,6).

Ductal adenocarcinoma of the pancreas is the most common epithelial exocrine pancreatic tumor and accounts for >85% of all malignant pancreatic tumors. Tumors (80-90%) are located in the head of the gland (7). Pancreatic carcinoma often metastasizes to multiple lymph nodes and to more than one lymph node group. The most common sites for distant intra-abdominal metastases are liver and peritoneum, and the most common extraperitoneal site for metastasis is the lung.

The majority of patients develop disease recurrence within 2 years after resection. Liver metastases frequently develop earlier (after 5-11 months), indicating the presence of micrometastases at the time of surgery, whereas local/lymphatic recurrences tend to appear a little later (after 13 months).

In vivo and *in vitro* results from different tumor entities suggest, that chemokine receptors direct lymphatic and hematogenous spread and furthermore influence sites of metastatic growth (8).

Chemokines and their respective G-protein-coupled receptors were initially described to mediate different pro- and anti-inflammatory responses (9). In particular, CXCR4 was reported to regulate homing of lymphocytes in inflammatory tissues (10). Its ligand, stromal cell derived factor 1, is being expressed by endothelial cells, biliary epithelial cells and lymph nodes and attracts lymphocytes into those organs by chemotaxis (11-13).

Recently, CXCR4 has shifted into focus as it might play an important role in tumor spread of colorectal, breast and oral squamous cell carcinoma (14-16). Further data from *in vitro* and murine *in vivo* tumor models underlined the key role of CXCR4 for tumor cell malignancy, as activation of CXCR4 by SDF-1α induced migration, invasion and angiogenesis of cancer cells (17,18).

At present, no data correlated CXCR4 expression and pancreatic cancer progression, although the relevance of CXCR4 activation for pancreatic tumor cell proliferation, adhesion, migration and invasion has been described *in vitro* (17,19). Therefore, we evaluated the expression of CXCR4 in

Correspondence to: Dr Carl C. Schimanski, First Department of Internal Medicine, Johannes Gutenberg University of Mainz, Langenbeckstrasse 1, 55101 Mainz, Germany
E-mail: dr_schimanski@yahoo.de

*Contributed equally

Abbreviations: CXCR4, chemokine receptor 4; SDF-1α, stem cell derived factor 1α; VCAM, vascular cellular adhesion molecule

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pancreatic cancer cell lines and specimens of ductal adenocarcinoma and correlated these results with the patients' clinicopathological parameters and survival.

Materials and methods

Cell culture. The human pancreatic cancer cell lines BxPc-3, Colo-357, T3M4, MiaPaca, AsPc-1, Capan-1, SU-8686 and Panc-1 were cultured in DMEM (Invitrogen, Germany) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin (Cambrex, Germany) and 1 mM L-glutamine (Invitrogen, Germany).

Western blot analysis. Tumor cells were washed with PBS and lysed in 0.5% NP-40 solution. Protein (100 μg) was loaded on a 10% SDS-PAGE gel. The gel was transferred onto a PVDF membrane following seperation. The respective proteins were detected with anti-CXCR-4 (1:500, CIO115, Capralogics, USA; 1:1000 donkey anti-goat IgG 2nd antibody SC-2020 by Santa Cruz) and anti-actin (1:1000, A2066, Sigma, Germany; 1:1000 goat anti-rabbit IgG 2nd antibody 170-6515 by Biorad, USA) and were visualized by ECL Western blot analysis system (Amersham Biosciences, USA).

Tissue samples. For retrospective analysis of CXCR4 expression in ductal adenocarcinoma, paraffin-embedded routine samples from the local Institute of Pathology were used, which had been intra-operatively obtained from 90 patients with pancreatic cancer who underwent Whipple surgery at the Department of General and Abdominal Surgery of the University of Mainz and from 13 patients with metastases who underwent endosonographic biopsy of their primary cancer at the First Department of Internal Medicine for routine diagnosis. The morphological classification of the carcinomas was conducted according to the World Health Organization (WHO) specifications. Patients were followed up on a regular basis depending on the procedure performed.

Immunohistochemical staining. The LSAB+ system from DakoCytomation (K0690; DakoCytomation Inc., CA, USA) was used to detect the protein CXCR-4 (anti-CXCR-4, CIO115, dilution 1:300; Capralogics). In brief, samples were exposed to 70˚C for 1 h in a humified oven and hereafter deparaffinised. After pre-incubation with hydrogen peroxide (3%) for 5 min and consecutive incubation with human fresh frozen plasma for 1 h, the primary antibodies were applied for 1.5 h at room temperature. After incubation with the secondary antibody (pooled swine anti-goat, anti-mouse, anti-rabbit-antibody; LSAB+ kit) for 30 min, the samples were exposed to streptavidin peroxidase for another 30 min and chromogen-solution (LSAB+ solution) for 15 min (LSAB+ kit, respectively). Counterstaining was performed with haematoxylin (Sigma, Germany). For negative controls only the secondary antibody was used. A negative control was performed for every pancreatic cancer sample (N=103). For positive controls formalin-fixed and paraffin-embedded tissue samples of the human spleen were applied.

Evaluation of immunostaining. Immunostaining was evaluated by three authors independently (F.W., W.S., S.B.), blinded to patient outcome and all clinicopathological findings. The immunohistochemical staining was analyzed according to a scoring method as previously validated and described (14): the tumors were classified into four groups based on the homogeneous staining intensity: 0, absent; 1, weak; 2, intermediate; 3, strong staining. In the case of heterogeneous staining within the same sample, the respective, 0.5 points higher score was chosen, if >50% of cells revealed the higher staining intensity. If evaluations did not agree, specimens were re-evaluated and re-classified according to the assessment given most frequently by the observers.

Table I. Patient and tumor characteristics.

	Patient characteristics
Total number	103
Median age (years)	65
Gender	
Female	46 (47%)
Male	57 (53%)
T-Status	
1	5 (5%)
2	22 (21%)
3	67 (65%)
4	9 (9%)
N-Status	
0	30 (29%)
1	70 (68%)
2	1 (1%)
Unknown	2 (2%)
M-Status	
0	91 (88%)
1	12 (12%)
Grading	
1	5 (5%)
2	56 (54%)
3	37 (36%)
4	2 (2%)
Unknown	3 (3%)
5-year survival	16%

Statistics. The correlation of CXCR4 staining intensity with clinicopathological patterns was assessed with the χ^2 test and with the unpaired Student's t-test, when appropriate. Survival rates were visualized applying Kaplan-Meier curves, and P-values were determined by log-rank test. $P<0.05$ was considered significant and $P<0.001$ highly significant in all statistical analyses.

Results

CXCR4 expression in pancreatic cancer cell lines. CXCR4 expression of pancreatic cancer cell lines revealed varying expression intensities as depicted in Fig. 1A.

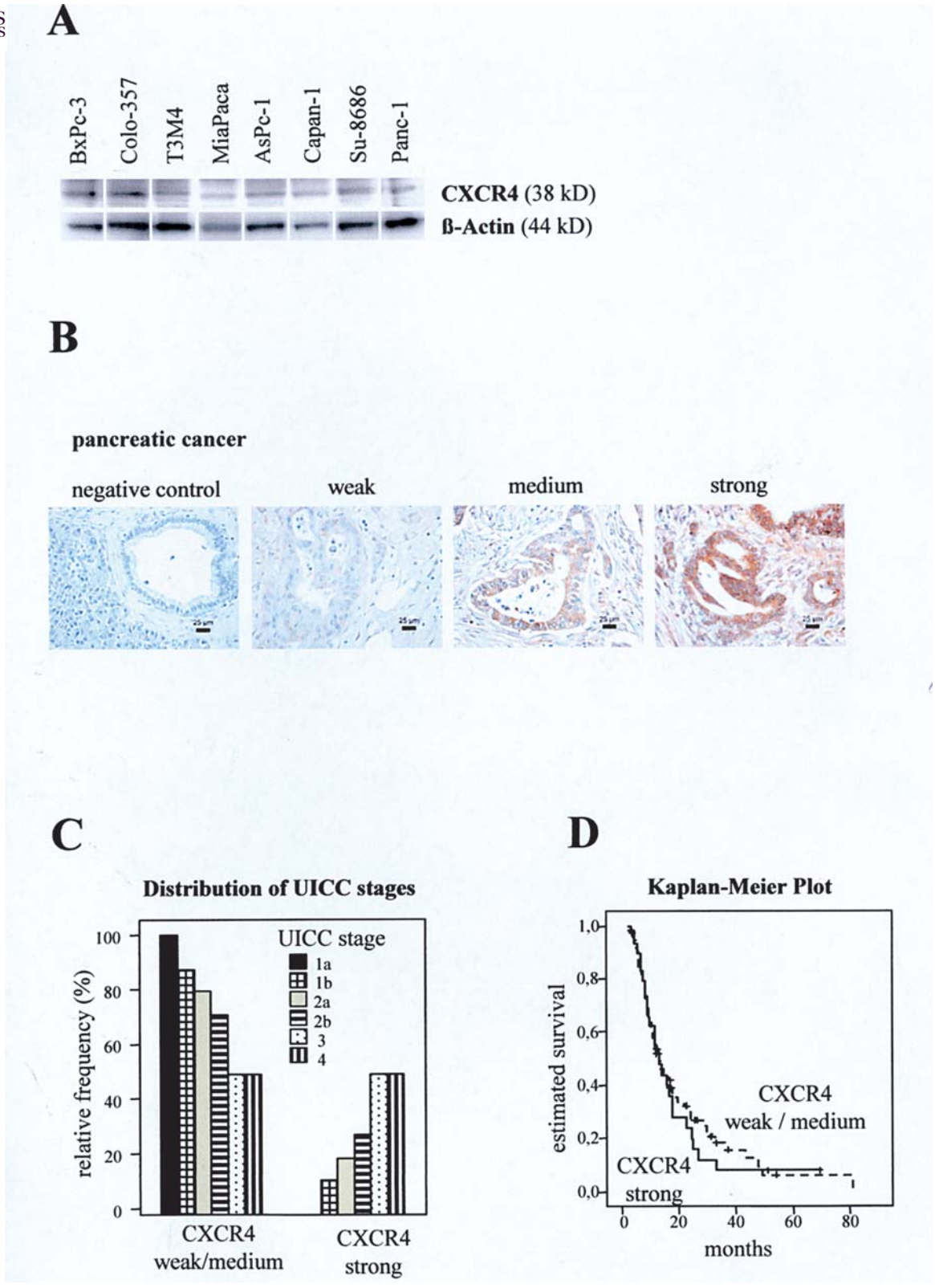

Figure 1. (A) Varying expression intensities of CXCR4 in diverse human pancreatic cell lines. (B) Depicts CXCR4 expression in healthy pancreas as well as the respective cytoplasmatic expression grades of CXCR4 (weak, medium and strong) in pancreatic cancer. (C) Distribution of UICC grades dependent on CXCR4 expression status. (D) The probability of survival of pancreatic cancer patients dependent on CXCR4 expression intensity is given in relation to time after histological confirmation.

Tumor characteristics and patient profiles. The selected group of patients represents the typical characteristics of pancreatic cancer in industrialized countries, except for a lower percentage of cases with distant metastases as shown in Table I.

Immunohistochemical staining of CXCR4 in pancreatic cancer. The staining of normal human pancreas for CXCR4 revealed a weak cytoplasmatic, and in only few specimens an additional weak membranous location of CXCR4 (Fig. 1B). A nuclear

Table II. Patient and tumor characteristics dependent on intensity of CXCR4 expression.

	CXCR4 expression			
	Weak (1)	Intermediate (2)	Strong (3)	Statistics
Total number	10 (10%)	63 (61%)	30 (29%)	
Median age (years)	66		65	NS
Gender				
Female	36		10	NS (P=0.13)
Male	37		20	
T-Status				
1+2	22		5	NS (P=0.15)
3+4	51		25	
N-Status[a]				
0	23		7	NS
+	48		23	
M-Status				
0	67		24	NS (P=0.09)
+	6		6	
UICC-Status				
1+2	64		21	P=0.03
3+4	9		9	
Grading 1				
1+2	42		19	NS
3+4	28		11	
5-year survival	18%		15%	NS

[a]N-status could not be obtained from two patients, whereas grading could not be obtained for three patients. NS, not significant.

staining of CXCR4 was not observed. Similarly, a predominantly cytoplasmatic location was observed in human pancreatic cancer. The respective expression rate for CXCR4 was 100% and varied from weak (10%), intermediate (61%) to strong (30%). Negative controls of human pancreatic cancer remained negative for all tissue samples (Fig. 1B). As positive control, splenic lymphocytes revealed a strong CXCR4 expression matching human pancreatic cancer tissue. Similarly, inflammatory infiltates in pancreatic tissue showed a strong CXCR4 expression (data not shown).

Relevance of CXCR4 expression in pancreatic cancer. Strong CXCR4 expression significantly correlated with progressed pancreatic cancer, indicated by the UICC stages III and IV (P=0.03; Fig. 1C, Table II). Furthermore, strong CXCR4 expression revealed a strong trend towards hematogenous dissemination (M-Status; P=0.09) and progressed local disease (T-status; P=0.15), whereas no correlation was seen for lymphatic dissemination (N-Status). CXCR4 expression did not impact on grading, medium age or gender. Concerning survival, a strong CXCR4 expression was not significantly correlated with decreased survival, as indicated by Kaplan-Meier and log-rank tests (Fig. 1D), neither was survival significantly influenced by CXCR4 in the subgroup of patients undergoing Whipple surgery (N=90).

Discussion

In diverse tumor entities, expression of the chemokine receptor CXCR4 has been linked to tumor dissemination and poor prognosis. Therefore, we analyzed the expression profile of CXCR4 in a large series of human pancreatic cancer cell lines and patient samples. The human pancreatic cancer cell lines and tumor samples analyzed, revealed different intensities of CXCR4 expression, as previously described for colorectal cancer (14). A cytoplasmatic staining of CXCR4 was observed in all cases, whereas fewer cases had an additional membranous localization of CXCR4. An inducible translocation of CXCR4 from the membrane to the cytoplasma has been reported previously (30).

In our pancreatic cancer patients, a strong CXCR4 expression was significantly associated with progressed pancreatic cancer as indicated by the UICC system and revealed a clear, though not significant trend towards M1-Status

tumors. Hence, our results imply a relevant influence of CXCR4 on proliferation and hematogenous dissemination of pancreatic cancer *in vivo*. Furthermore, our data strengthen the *in vitro* observations by Mori and colleagues, who described a dose-dependent SDF-1α stimulation of migration and invasion in pancreatic cancer cells (17). In addition, they confirm current murine *in vivo* studies, demonstrating that CXCR4 expression mediates metastasis of pancreatic cancer cells (20).

Our results are also in line with recent studies from our group and others, describing a similar effect of CXCR4 on disease dissemination in different tumor entities (14,15,21,22). Furthermore, CXCR4 expression was up-regulated in glioblastoma and its inhibition resulted in tumor growth arrest (23).

CXCR4 is regulated by different external factors, such as hypoxia (hif-1-pathway), as well as by internal alterations as the inactivation of tumor suppressor genes pVHL or over-expression of NFκB and DNA methylation (24-26).

Homing factors, inducing chemotaxis to target organs of dissemination, have been proposed as the major inductor of tumor cell dissemination and metastatic growth, as the filter theory does not sufficiently explain the growth of metastases in target organs (27-29). Herein, CXCR4 certainly favours the 'homing' theory, as CXCR4 expression co-mediates dissemination of primary tumors to different organs through the chemotactic factor SDF-1α (27). Moreover, SDF-1α expression is most intense in typical 'homing organs' such as lungs, bone marrow, liver and lymph nodes as compared with other non-homing tissues (11,16). Pathophysiologically, endothelial cells co-express SDF-1α and VCAM-1, thus mediating tumor-cell/endothelial cell attachment. CXCR4 activation by SDF-1α induces ß-integrin expression, binding VCAM-1 on endothelial cell (30,31). Similar pathophysiological processes must be proposed for pancreatic cancer dissemination.

In conclusion, chemokines have been connected to tumor growth, dissemination and local immune scape (32,33). Our *in vivo* results expand these data for human pancreatic cancer, as expression of CXCR4 was associated with advanced stages of this disease. Thus, inhibition of pancreatic cancer progression by CXCR4 antagonists might be a promising therapeutic option.

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