Interleukin-8, a promising predictor for prognosis of pancreatic cancer

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AIM: To investigate the value of interleukin-8 (IL-8), a pro-inflammatory chemokine, in predicting the prognosis of pancreatic cancer.

METHODS: Expression of IL-8 and its receptor CXCR1 was assessed by immunohistochemistry in pancreatic cancer and chronic pancreatitis samples. Enzyme-linked immunosorbent assay was used to detect the serum IL-8 levels in pancreatic cancer patients. Human pancreatic cancer tissues were heterotopically transplanted to the immune-deficiency mice to evaluate the effect of serum IL-8 on the tumorigenesis of the cancer samples.

RESULTS: IL-8 and CXCR1 proteins were both over-expressed in pancreatic adenocarcinoma samples (55.6% and 65.4%, respectively) compared with the matched para-cancer tissues (25.9% and 12.3%, P < 0.01), or chronic pancreatitis (0% and 25%, P < 0.05). Serum IL-8 levels in pancreatic cancer patients (271.1 ± 187.7 ng/mL) were higher than in other digestive system tumors, such as gastric cancer (41.77 ± 9.11 ng/mL, P = 0.025), colorectal carcinoma (78.72 ± 80.60 ng/mL, P = 0.032) and hepatocellular carcinoma (59.60 ± 19.80 ng/mL, P = 0.016). In vivo tumorigenesis analysis further proved that tumor tissues from patients with higher serum IL-8 levels grew faster than those with lower IL-8 levels.

CONCLUSION: IL-8 can be a fine serum marker for predicting the prognosis pancreatic cancer.

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Key words: Pancreatic cancer; Interleukin-8; CXCR1; Immunohistochemistry; Tumor implantation; Enzyme-linked immunosorbent assay

INTRODUCTION

Interleukin-8 (IL-8) is a pro-inflammatory factor, belonging to CXC chemokine family. It was initially named neutrophil-activating peptide-1 for its potent chemotactic activity on granulocytes in inflammatory and immune
diseases\cite{1,2}. Recently, it has been shown that IL-8 plays a critical role in cancer invasion, angiogenesis and metastasis\cite{3,4} and is considered as an important component of tumor microenvironment\cite{5,6}. The significance of tumor microenvironment, which can be described as the “soil” of cancer cells, has been emphasized, especially the cancer-stroma interaction, which has become critical determinants of cancer behavior\cite{7}. Stromal cells can produce IL-8 to influence the ability of invasion or metastasis of cancer cells, and the cancer cells themselves can also secrete IL-8 in an autocrine or paracrine manner, such as in breast cancer\cite{8}, gastric cancer\cite{9}, colon cancer\cite{10}, cervical cancer\cite{11}, pancreatic cancer\cite{12} and leukemia\cite{13}.

Pancreatic cancer is one of the most aggressive human malignancies and often with a dismal prognosis. The overall 5-year survival rate is only 5% or even less. Patients with pancreatic cancer have already been at advanced stage in their first visit to hospital. Around 15%-20% of the patients have the chance for tumor resection and the rest can only receive adjuvant therapies\cite{14}. Although new drugs and techniques have been developed in treating pancreatic cancer, their therapeutic effects varied. Even the first-line anticancer drug, gemcitabine, still has not achieved satisfactory results in improving patients’ outcome. The overall survival of pancreatic cancer patients is quite low, it is therefore, very important to predict the prognosis of the patients so as to enable more active treatment to prolong their lives.

Many studies\cite{8} have revealed that pancreatic cancer highly produces IL-8, which can promote angiogenesis and invasion of tumors. Serum IL-8 levels were elevated in pancreatic cancer patients\cite{15}, suggesting the feasibility of IL-8 to be a fine marker in predicting the outcome of the patients. The main aim of the present study is to investigate the prognostic value of IL-8 in pancreatic cancer patients. We examined the expression and secretion levels of IL-8 in both tumor tissue and human blood. Furthermore, we implanted the cancer tissues from patients with various serum IL-8 levels subcutaneously to the nude mice and observed the growth of each xenograft. Based on these in vitro and in vivo analyses, we aim to more precisely define the role of IL-8 in predicting the prognosis of pancreatic cancer patients.

### MATERIALS AND METHODS

#### Sample collection

Eighty-one pancreatic ductal adenocarcinoma (PDC) specimens with matched para-cancerous pancreas were collected during resection in the surgically treated patients. Five cases of chronic pancreatitis (CP) served as controls. All patients with pancreatic cancer had been followed up for survival and outcome until October 2008. Except for four patients who were alive till the end of follow-up, the rest all died.

The clinical information of the patients is presented in Table 1. Besides the patients with PDC, patients with chronic and acute pancreatitis (AP) were also included.

| Groups | Sex | Age (yr) |
|--------|-----|----------|
|        | Male | Female | Range | Median |
| PDC    | 13   | 14     | 45-80 | 58     |
| CP     | 13   | 2      | 9-58  | 40     |
| AP     | 3    | 5      | 37-73 | 51     |
| DA     | 3    | 5      | 47-65 | 58     |
| GC     | 1    | 2      | 47-55 | 53     |
| CRC    | 3    | 1      | 48-68 | 63     |
| HCC    | 1    | 1      | 55-72 | 63.5   |

PDC: Pancreatic ductal carcinoma; CP: Chronic pancreatitis; AP: Acute pancreatitis; DA: Duodenal adenocarcinoma; GC: Gastric carcinoma; CRC: Colorectal carcinoma; HCC: Hepatocellular carcinoma.

#### Immunohistochemical analysis

Unstained 3-μm sections were cut from the paraffin blocks and deparaffinized by routine procedures. Envision solution (K4065, DAKO, Denmark) was added to detect the primary antibody and 3,3’-diaminobenzidine was applied as chemicon. Sections were counterstained with hematoxylin. Primary antibodies against IL-8 (AHC0881) and CXCR1 (AHR1522Z) were purchased from BioSource (California, United States). In immunohistochemical (IHC) analysis, all the focal cases were considered negative and cases showing diffused expression were considered positive. Only the positive signals in epithelial cells were taken into account.

### Ethics

This study was approved by the Ethics Committee of the Second Military Medical University (Shanghai, China). Informed consent was obtained from each patient before tissue specimen and blood samples were collected.
Enzyme-linked immunosorbent assay
The concentration of serum IL-8 in the patients was determined quantitatively with human IL-8 enzyme-linked immunosorbent assay (ELISA) detection kit provided by BioSource following the manufacturer's instructions. The up-limit of the test was 500 ng/mL.

Animal models and heterotopic implantation of human tumor tissues
Eleven severe combined immunodeficiency (SCID) nude mice aged 6-7 wk were housed under specific pathogen-free conditions. Animals were inoculated with subcutaneously (sc) transplanted fresh tissue cubes (1 mL) from pancreatic cancer patients with a detected serum IL-8 level. Animals were monitored daily by general clinical observation throughout the study period. Tumor volumes were calculated based on the following formula: tumor volume = (length × width^2)/2. Animals were euthanized when their tumors reached an appropriate size (0.3 cm^3) and the latent period was recorded. All experimental manipulations were undertaken in accordance with the NIH Guide for the Care and Use of Laboratory Animals, with the approval of the Biomedical Ethics Committee of the Second Military Medical University (Shanghai, China).

Histological analysis of implanted tumor tissues
After implanted with human tumor tissues heterotopically, animals with growing tumor were sacrificed by craniocervical dislocation. The mice were then weighed, and the sc tumor tissues were excised. Part of the tumor tissues underwent routine histological examination and the rest was stored in liquid nitrogen for future use. Histology of the corresponding human tumor tissues was also reviewed.

Statistical analysis
The IHC results were analyzed by χ² test. And the ELISA assay results were expressed as arithmetic mean ± SD. The comparisons between groups of patients were made using analysis of variance, and Fisher's Least Significant Difference test was used for animal models. The difference was considered significant at \( P < 0.05 \). SPSS 10.0 was applied as a statistical tool. Survival rates were calculated by the Kaplan-Meier method.

RESULTS
IL-8 and CXCR1 expression in PDC
IL-8 was expressed in 55.6% (45/81) of pancreatic cancer specimens, whereas in 25.9% (21/81) of non-cancer tissues \((P < 0.01, \chi^2 = 14.727)\) (Table 3). IL-8 immunoreactivity was absent in CP (0%, 0/5) \((P = 0.016, \chi^2 = 5.827)\) (Table 3). The positive signal was localized in the cytoplasm of cancerous or normal ductal cells (Figure 1). Focal stromal cells could express both IL-8 and CXCR1. The relationship between the expression of IL-8 or CXCR1 and the histological grading of pancreatic cancer is shown in Table 3. The CXCR1 expression level was significantly higher in pancreatic cancer samples (65.4%, Table 3).

| IL-8 | Matched para-cancer tissue | Chronic pancreatitis |
|------|---------------------------|---------------------|
| +    | 5                         | 21                  |
| -    | 11                        | 60                  |

| CXCR1 | Matched para-cancer tissue | Chronic pancreatitis |
|-------|---------------------------|---------------------|
| +     | 10                        | 1                   |
| -     | 6                         | 71                  |

Table 3 Interleukin-8 and CXCR1 expression in pancreatic cancer, matched para-cancer tissue and chronic pancreatitis.

Figure 1 Interleukin-8 and CXCR1 expression in pancreatic cancer. A: interleukin-8 (IL-8) expression in pancreatic cancer (× 200); B: CXCR1 expression in pancreatic cancer (× 200); The positive signals of IL-8 and CXCR1 were mainly located in the cytoplasm of cancer cells. For IL-8, focal positivity was found in the fibroblasts; C: Survival analysis of pancreatic cancer patients. “Censored” means patients were still alive till the end of follow-up study. The patients without IL-8 expression survived longer than those IL-8 positive.
53/81) than in samples from the adjacent non-cancerous pancreas (12.3%, 10/81) ($P < 0.001, \chi^2 = 48.026$). No statistical difference was observed between histological grading and IL-8 or CXCR1 expression.

Overall survival analysis was performed in the patients. Most people died within 3 years after surgery and only four were alive till the end of follow-up. The median survival was 5 mo (range, 1-36 mo) in the IL-8 positive group and 24 mo (range, 1-39 mo) in the IL-8 negative group. Although IL-8 positive patients seemed to live shorter than IL-8 negative ones, no significant difference was observed by Kaplan-Meier method ($P = 0.245$).

### Serum IL-8 levels in pancreatic cancer patients and pancreatic cancer cell lines

Serum IL-8 level was measured in patients with PDC ($n = 27$), AP ($n = 8$), CP ($n = 15$) and other kinds of cancer from digestive system, such as duodenal adenocarcinoma (DA, $n = 4$), gastric carcinoma (GC, $n = 3$), rectal colonic carcinoma (CRC, $n = 4$), and hepatocellular carcinoma (HCC, $n = 2$). Blood was collected before surgery in the cancer patients. The serum IL-8 levels were significantly higher in PDC ($271.1 \pm 187.7$ ng/mL) than in CP ($97.02 \pm 130$ ng/mL, $P = 0.002$), AP ($133.6 \pm 162.9$ ng/mL, $P = 0.041$), GC ($41.77 \pm 9.11$ ng/mL, $P = 0.025$), CRC ($78.72 \pm 80.6$ ng/mL, $P = 0.032$) or HCC ($59.6 \pm 19.8$ ng/mL, $P = 0.016$). No significant difference was observed between PDC and DA ($168.7 \pm 212.4$ ng/mL, $P = 0.247$). Two cancer cell lines originated from metastatic site (SW1990 and Patu8988s) showed a much high level of IL-8 secretion (Table 4).

### Table 4 Interleukin-8 concentration in the supernatant of pancreatic cancer cell lines

| Cell line | IL-8 concentration (ng/mL) |
|-----------|-----------------------------|
| BxPC3     | 81.38                       |
| CFPAC-1   | 3.906                       |
| SW1990    | > 500                       |
| Patu 8988s| 498.9                       |

SW1990 and Patu8988s originated from metastatic sites showed much higher levels of interleukin-8 (IL-8) than BxPC3 and CFPAC-1.

Histology of human tumor tissues implanted into SCID mice

Tumors subcutaneously implanted into the SCID mice were mostly composed of strands of cells, and glandular architectures were not very obvious (Figure 2). Intracellular vacuoles could be observed in some regions as immature or primary glands, indicating their adeno-epithelial origin. Unlike cancers growing in human pancreas, the desmoplastic reaction was not so remarkable and only several fibroblastoid spindle cells appeared in the implanted cancer tissues. Nerve and vessel invasion by can-

![Figure 2](image-url)
cancer cells was easily observed in human cancer tissues, but none was detected in transplanted cancer tissues.

**In vivo growth of heterotopically transplanted tumor tissues and its correlation with serum IL-8 levels**

Among all the 11 cases of human tumor tissues implanted into SCID mice, seven tumors grew subcutaneously in animals. We set the terminal goal of growth at 0.3 cm³ and recorded the latent period. The slowest growing period was 93 d and the fastest was 44 d. Seven animals with tumors were divided into three groups based on the median latent period of 61 d for analysis: group 1, failed to form tumor, n = 4; group 2, latent period < 61 d, n = 3; and group 3, latent period ≥ 61 d, n = 4. Serum IL-8 level in group 2 (140.1 ± 33.4 ng/mL) was much higher than in group 1 (23.4 ± 17.4 ng/mL) and group 3 (23.2 ± 16.1 ng/mL) (P < 0.001). There was no significant difference between group 1 and group 3 (P = 0.998).

**DISCUSSION**

In this study, the expression level of IL-8 and its receptor CXCR1 was elevated in pancreatic cancer and serum IL-8 level was significantly higher in patients with pancreatic cancer than in those with pancreatitis and also higher than in the patients with other kinds of tumors from digestive system. *In vitro* tumor tissues from pancreatic cancer patients with a higher serum IL-8 level grew faster and behaved more aggressively than those with low serum IL-8 levels. Follow-up data showed that patients with high serum IL-8 levels had a relatively lower survival than those with low serum IL-8 levels. Due to the insufficient number of patients involved in the follow-up analysis, no statistical difference was achieved in the two groups.

IL-8 is a member of the CXC chemokine family and is a chemotactic factor for T cells, neutrophils, and basophils. Besides its pro-inflammatory role, IL-8 has been evaluated as a pro-oncogenic effector in various types of human cancers, including leukemia, astrocytoma, melanoma, breast cancer, ovarian cancer, lung cancer, prostate cancer, colon cancer, renal cell carcinoma, gastric cancer and pancreatic cancer[18]. The most critical effect of IL-8 on cancer cells is its strong angiogenic potential and ability of promoting invasion and metastasis[19-21]. *In vitro* IL-8 can enhance the proliferation and survival of endothelial cells and through its receptor CXCR1 can upregulate the expression of matrix protein, MMP-2 and MMP-9[22]. IL-8 can mimic the role of vascular endothelial growth factor (VEGF), transactivate VEGFR2 and promote angiogenesis[23]. Various signals or pathways can induce IL-8 expression in cancers[24-26] and the whole IL-8-involved network is very complicated. It has been confirmed that nuclear factors, NF-κB and AP-1 are the upstream regulators of transcription of IL-8 mRNA, both of which may cooperate with each other in the production of IL-8. In our study, a higher expression level of IL-8 and CXCR1 was detected in pancreatic cancer tissues than in para-cancerous pancreas. Although IL-8 could be elevated in colon and gastric cancers, the serum IL-8 level in pancreatic cancer was remarkably higher than in colon and gastric cancers. Moreover, IL-8 level was even higher in acute pancreatitis, in which IL-8 is considered to be a reliable indicator in evaluating the severity of inflammation and necrosis[27]. These data suggest that pancreatic cancer cells have a higher capability to produce chemokines than inflammatory cells, and function in an autocrine manner, which fulfills the composition of tumor microenvironment.

We have no direct experimental information on which cells are responsible for the secretion of IL-8, but according to the IHC analysis, IL-8 expression occurred mostly inside cancer cells, indicating that they might be the main source of IL-8. Pancreatic cancer cell lines have been investigated for IL-8 secretion and its mRNA expression, and quite a number of these cell lines showed high levels of IL-8 in the supernatant and mRNA expression[28]. We also analyzed IL-8 levels in the cultured supernatant of four pancreatic cancer cell lines, and found that those cell lines originated from the primary tumor site produced less IL-8 than those selected from metastatic site. Nomura et al[29] demonstrated similar results using two sublines of pancreatic cancer, both of which were sequentially selected from the parental cell line and possessed a high potential of organ metastasis, and the high IL-8 expression was closely correlated with the aggressive behavior of cancer cells. Besides cancer cells, inflammatory cells in the tumor stroma is also one of the important sources of IL-8 secretion. Neutrophils can not only promote tumor destruction[30], but also increase the growth of tumor cells[31]. Neutrophil-dependent release of VEGF-A leads to subsequent recruitment of neutrophils, resulting in angiogenesis.

Our survival analysis showed that IL-8 positive pancreatic cancer patients had a lower survival than IL-8 negative ones, whereas no significant difference was observed between the two groups possibly due to the insufficient number of cases involved. *In vivo* preclinical experiments of animal models on IL-8 have been conducted by many labs using various tumor cell lines, such as prostate cancer, lung cancer, breast cancer and pancreatic cancer[32]. In these cell lines, a high level of IL-8 is directly correlated with tumor growth, angiogenesis and metastasis in nude mice. But in ovarian cancer, IL-8 was negatively involved during tumorigenesis in animal models. Lee et al[33] demonstrated that the inverse regulation of IL-8 on xenograft growth was mainly mediated by its induction of neutrophil infiltration. In our animal model, we for the first time used the clinically resected pancreatic cancer tissues and transplanted them sc into SCID mice. The infiltration of inflammatory cells was not as evident as that observed by Lee et al[33] in ovarian cancer. The growth rate of tumors was significantly correlated with the serum level of IL-8 of the corresponding patients. The higher serum IL-8 level in the patients, the faster the tumor grew, indicating the more aggressive phenotype of this tumor, the poorer prognosis of the patients. IL-8...
level can be measured in the tumor tissues resected surgically and in the patients’ sera, it can also be detected in other kinds of clinical samples, including pancreatic juice obtained from the duodenum. Moreover, IL-8 concentration in pancreatic juice could be used to discriminate between normal pancreas and pancreatic cancer.

In summary, IL-8 was highly expressed in pancreatic cancer in both tumor tissues and blood samples. In vivo analysis showed that IL-8 would be a sensitive marker in predicting prognosis and monitoring disease progression of the pancreatic cancer patients. The patients with high serum levels of IL-8 should receive more active treatment due to the more aggressive biology of their cancer. Besides its prognostic value, IL-8 may represent a promising target for the development of adjuvant therapy for pancreatic cancer.

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