RESEARCH ARTICLE

Study on the Relationship Between CXCR4 Expression and Perineural Invasion in Pancreatic Cancer

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Abstract

Background: Recent reports have shown that C-X-C chemokine receptor 4 (CXCR4) plays an important role in metastasis. Despite a clear understanding of the protein's structure and properties, its functional role remains elusive. We conducted the present study to evaluate the expressions of CXCR4 in pancreatic cancer, and to investigate its relationship with clinicopathological parameters, especially perineural invasion(PNI). Materials and Methods: The association between CXCR4 expression and perineural invasion was determined by immunohistochemistry in pancreatic cancer patients (n=51). Results: CXCR4 expression was correlated with the existence of PNI and the type of PNI (p=0.042, p=0.040). TIMP-2 expression was also correlated with the existence, the pathway and degree of PNI (p=0.000, p=0.006, p=0.000). Conclusions: Our results suggest an association between PNI and expression of CXCR4 and TIMP-2 in pancreatic cancer. CXCR4 may promote the occurrence of PNI in pancreatic cancer cells by decreasing the inhibition of TIMPs on MMP.

Keywords: Pancreatic cancer - Perineural invasion - CXCR4 - TIMP-2

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Introduction

Pancreatic cancer is a fatal disease with an annual incidence that approaches its mortality rate (7.28/100,000, 6.61/100,000) (Chen et al., 2013). The majority of patients develop disease recurrence within 2 years after resection (Lowenfels et al., 2004). Large detailed pathohistologic studies have shown that one of the most persistent characteristics of pancreatic cancer is perineural invasion (PNI) (Pour et al., 2003). PNI was an independent and poor prognostic factor in pancreatic cancer patients. Moreover, intrapancreatic PNI status may be associated with tumor recurrence (Zhang et al., 2013). Some studies have shown that the mechanism of PNI involves the microenvironment around the nerve that promotes tumor cell infiltration and diffusion along nerve fibers (Ayala et al., 2001).

Many tumors, including lung cancer, breast cancer, melanoma, glioblastoma, pancreatic cancer, cholangiocarcinoma and basal cell carcinoma, have reported the important role of CXCR4 in mediating tumorigenicity, progression, proliferation, and metastasis (Richard et al., 2008; Tang et al., 2008). The invasion of tumor cells is a complex, multistage process. During the process of pancreatic ductal cancer PNI, MMP and TIMP may also play an important role in degrading the matrix around the tumor and the nerve tissue.

To elucidate PNI underlying mechanism in human pancreatic ductal cancer, we investigated relations between PNI and expression of CXCR4, MMP-2, MMP-9, TIMP-1and TIMP-2.

Materials and Methods

Patients and specimens

Formalin-fixed, paraffin-embedded complete tumor specimens from 51 patients (range, 35 to 68 years) with pancreatic ductal adenocarcinoma from 1985 and 2005 were collected from Tianjin Cancer Hospital, China. These cases all had undergone a partial duodenopancreatectomy; had complete prognosis data, had main tumor lesions and normal-appearing portions on the same slide. Informed consents were obtained from all the patients.

For these 51 ductal adenocarcinoma, tumor stage and histopathological grading were recorded according to the classification of World Health Organization Classification of Tumors (Miyazaki et al., 1997). There were 8 Stage I, 6 Stage II, 24 Stage III and 13 Stage IV.

Immunohistochemical analysis

Seven primary antibodies neuron-specific enolase (NSE), CXCR4, MMP-2, MMP-9, TIMP-1 and TIMP-2 were purchased from ZhongShan Company (Guangzhou Province, P. R. China). (MMP-9, 1:100 diluted polyclonal from Lab Vision Corp and CXCR4, 1:100 diluted polyclonal from Lab Vision Corp)

A standard streptavidin peroxides technique was used for the immunohistochemical detection. The major procedures were described as the following: deparaffinizing, antigen retrieval, serum blocking, primary antibody, washing, blocking, biotinylated secondary antibody, washing, blocking, chromogen (DAB),

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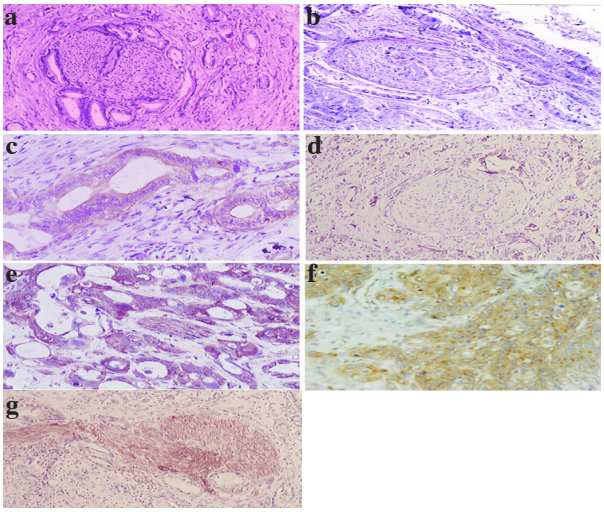

Figure 1. (a, b): The types of PNI in Pancreatic Cancer, H&E Staining: (a). Surrounding Pathway of PNI, × 10; (b). Intra Pathway of PNI, ×10. Figure 1 (c, d, e, f, g) Immunohistochemical staining of cancer cells in pancreatic cancer: (c).CXCR4 expressing in the cytoplasm and cell membrane, ×20; (d). MMP-9 expressing in the cytoplasm, no immunostaining in nerve tissue, ×10; (e).TIMP-1 expressing in the membrane and cytoplasm in a diffuse pattern, nerve tissue expressing TIMP-1, ×40; (f) TIMP-2 expressing in the cytoplasm, ×40. (g). NSE expressing in nerve tissue, ×40

washing, dehydrating, mounting and observation. All series included negative controls (the primary antibody absent) and positive controls (breast cancer tissues) gave satisfactory results.

When stained for NSE, CXCR4, MMP-2, MMP-9, TIMP-1 and TIMP-2 , tumor cells with brown cytoplasm were considered positive. We observed 10 fields per section at 400× magnification, and positive cell numbers were counted in 100 random cancer cells in every field. Semiquantitative classes were chosen by two pathologists, as described (Ma et al., 2008), after consensus discussion and careful revision of all slides.

A mean percentage of positive tumor cells were determined in at least five areas at a magnification of 400 x. The percentage was assigned to one of the four following categories: 0, <5%; 1, 5-25%; 2, 25-50%; 3, >50%. The grading scale ranged from no detectable signal (0) to strong signal (3) seen at low power. The immunohistochemical staining score for each sample was calculated as intensity x the percentage of positive cells. The weighted score was recorded as follows:0 score; +, 1~3 scores; ++, 4~6 scores; +++, >6 scores.

Analysis of perineural invasion

The presence of PNI was assessed in all pancreatic cancer specimens by two independent observers blinded to patient status. We also identified the existence of nerve tissues by examining NSE expression. PNI was defined as positive if cancer cells infiltrate into the perineurium or neural fascicules as reported previously (Ma et al., 2008).

On the basis of the number of PNI found microscopically at 100 magnifications in one histological section. The degree of PNI was divided into four stages, as previous described: ne0, none of the peripheral nerve is invaded; ne1, PNI was difficult to find, with one to five occurrences per slide; ne2, PNI was easy to find, with six to ten occurrences per slide; and ne3, PNI was very easy to find, with more than ten occurrences per slide. The types of PNI: surrounding pathway of PNI: tumor cells can be observed between epineurium and innerneurium (Figure 1a). intra pathway of PNI: tumor cells can be observed inside innerneurium (Figure 1b) (Ma et al., 2008)

Table 1. Correlation between Clinocopathologic Parameters and Univariate Analysis of Survival in Pancreatic Cancer

parameters		number	live	dead	*p* value
age	<50	15	6	9	0.109[a]
	50-70	33	5	28	
	>70	3	1	2	
sex	Male	30	8	22	
	Female	21	5	16	
Tumor diameter					0.310[a]
	<2cm	1	0	1	
	2-5cm	19	3	16	
	>5cm	31	10	21	
Clinical stages					0.011[b]
	I+II	13	7	6	
	III+IV	38	6	32	
Involvement of surgical margins					1.000[b]
	Negative	18	5	14	
	Positive	33	8	25	
Invasion around pancreas					0.419[b]
	Negative	10	1	9	
	Positive	41	12	29	
Lymph nodes metastasis					1.000[b]
	Negative	27	7	20	
	Positive	24	6	18	
PNI					0.006[b]
	Negative	18	9	9	
	Positive	33	4	29	
Distant metastasis					0.474[b]
	Negative	39	9	30	
	Positive	12	4	8	

Statistical analyses: [a]Pearson chi-Square ; [b]Fisher's Exact Test

Statistical analysis

Statistical analyses were performed using the SPSS software package 11.0 (SPSS, Inc. Chicago, IL, USA). The correlations among CXCR4, MMP-2, MMP-9, TIMP-1 and TIMP-2 expression and clinicopathologic variables were analyzed using Pearson Chi-square analysis respectively. Kaplan-Meier curves were constructed for univariate and multivariate analyses using a Cox proportional hazards model to examine the potential prognostic variables (clinocopathologic factors). All of the statistical tests were two-sided. P values of less than 0.05 were considered statistically significant.

Results

Clinocopathologic data

In 51 patients, 33 cases (64.7%) can be observed with PNI. The follow-up data was updated on December 4, 2005 (median follow-up was 14.8 months, range, 0.5~102 months). The result of one-way survival analysis conducted to identify clinocopathologic factors associate with survival were shown in Table 1. PNI and Tumor stage

were found to be significant prognostic factors ($p<0.05$). Kaplan-Meier curve analysis suggested that the median survival time for PNI-positive patients was significantly lower than PNI-negative patients (Figure 2). PNI indicated poor prognosis of pancreatic cancer patients. However, this result was not be supported by the Cox multivariate analysis (Table 2).

IHC

Staining of the CXCR4 protein was identified in the cytoplasm and/or cell membrane of cancer cells but was not detected in the nerves, the normal acinar cells and ductal cells of noncancerous region in pancreatic cancer tissue (Figure 1c), weak staining for the CXCR4 was observed in some lymphocytes in the specimens. The immunopositive ratio of CXCR4 in the pancreatic cancer cell was 84.3%. The presence and type of PNI significantly correlated with the expression of CXCR4 ($p=0.0029$, $p=0.002$) (Table 3). There were no correlations between the expression of CXCR4 and degree of PNI. For other clinicopathologic parameters, high CXCR4 expression was found to be associated significantly with metastasis ($p<0.05$).

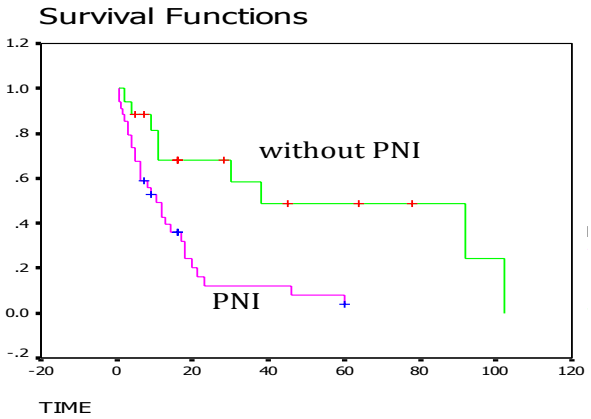

Figure 2. Kaplan-Meier Curve of Pancreatic Ductal Cancer Patients with Different PNI Status

Table 2. Multivariate Analyses for Prognostic Variables using a Cox Regression

	B	SE	Wald	df	Sig.	Exp(B)
Tumor size	0.085	0.365	0.054	1	0.816	1.089
Lymph nodes metastasis	0.125	0.353	0.126	1	0.723	1.133
Invasion around pancreas	-0.718	0.43	2.784	1	0.095	0.488
PNI	0.837	0.556	2.269	1	0.132	2.31
Distant metastasis	0.109	0.462	0.055	1	0.814	1.115
Clinical stages	0.713	0.651	1.201	1	0.273	2.041

The immunopositive ratio of different proteins in cancer cells in the pancreatic cancer tissue specimens for MMP-2, MMP-9, TIMP-1 and TIMP-2 were 62.7%, 64.7%, 88.2%, 68.6%. MMP-2 and MMP-9 were strongly expressed in the cytoplasm of cancer cells, and only a few cases observed the weak expression in stromal cells. No immunostaining was detected in nerve tissue (Figure 1d). Immunoreactivity of TIMP-1 was intensely present in the membrane and cytoplasm of cancer cells in a diffuse pattern (Figure 1e). In addition to the weak expression of stromal cells was observed, middle-intense expression of TIMP-1 was observed in the nerve tissue. Positive staining of TIMP-2 protein was identified in the cytoplasm of cancer cells (Figure 1f). The expression level of TIMP-2 was varied scattered among different parts in the same sample. TIMP-2-positive cells were found at the invasive margin of tumor and adenoductal structures. NSE was highly expressed in nerve tissue, and the cancer cells were negative (Figure 1g). For PNI, there was a significant correlation between TIMP-2 expression and PNI factors, including the existence of PNI, degree of PNI, and types of PNI ($p<0.001$), Pancreatic cancer cells with high expression of TIMP-1 easily invaded nerve with an intra pathway of PNI ($p<0.05$). Although there was no positive correlation between PNI and the expression of MMP-9, and there was a positive correlation between expression of TIMP-2 and MMP-9 ($p<0.001$). For other clinocopathologic factors, TIMP-2 expressions were correlated with lymph node metastasis, metastasis distance, and tumor diameter in pancreatic cancer patients ($p<0.001$, $p<0.05$, $p<0.001$, respectively). The patients with high MMP-9 expression were easy to invade the tissues around pancreas ($p<0.001$). There was no positive correlation between the expression of TIMP-1 and clinicopathologic factors.

Discussion

Despite recent diagnostic and management advances, pancreatic cancer remains a highly lethal disease (Siegel, et al., 2011). The intrinsic properties of neoplastic cells contribute to the rapid progression and invasiveness of this tumor. Principally malignant cells spread to the nerve tissue. There is a growing interest in preventing the recurrence of pancreatic cancer as well as understanding the fundamental nature of PNI.

Besides their well-established roles in the inflammatory reaction, physiological and pathological behaviors, more increasing evidences suggested that chemokine receptor s were closely related to tumor growth, invasion and metastasis. Among the family of chemokine receptors,

Table 3. CXCR4 IHC Result and PNI in Pancreatic Cancer (case)

Variables	PNI			Type of PNI				Degree of PNI			
	negative	positive	*p* value	S	I	S+I	*p* value	Ne1	Ne2	Ne3	*p*-value
CXCR4			0.029				0.002				0.436
-	1	7		0	5	2		4	2	0	
+	6	16		1	3	12		10	4	1	
++	9	4		0	2	2		2	0	1	
+++	4	4		2	0	2		2	0	1	

*S:surrounding pathway, I:intra pathway, S+I:both pathway

high expressions of CXCR4 were often found in tumor cells transfering to specific organs (Zhang et al. 2008). Accordingly, CXCR4 may play chemotaxis role (direct cancer cell migration) in the PNI process. However, there are few reports about chemokines recepters involving with PNI.

It has been established in the fetal rodent brain that CXCR4 modulate neuronal functions, including neurogenesis and migration (Loetscher et al., 1994; Stumm et al., 2007). It was hypothesised that in the process of mutual adaptation of cancer cells and neural, some cancer cells may get the components of peripheral nerve or the neuroectoderm. Cancer cells appeared certain new phenotype or neurogenic phenotype transformation. These changes make the interactions between cancer cells and neural tissue easy, which lead to PNI (Zhang et al., 2011).

This was supported by the result of Li et al. They co-cultured human pancreatic cancer cell line-MIA PaCa-2 and the mouse dorsal root ganglion neurons (DRGn). They found in the presence of MIA PaCa-2 cell line, the cytan size, axon length and vital impulse of DRGn were promoted. They suggested that this was possibly related with a group of growth factors secreted in the microenvironment by nerve and/or cancer cells (Li et al., 2008).

However, in this study, CXCR4 protein was only identified in the pancreatic cancer cells, which is consistent with the previous report that the CXCR4 gene is silenced postnatal in most cortical neurons (Stumm et al., 2007). Our result support that CXCR4 may only promote the migration of cancer cell has no action on the neurogenesis.

To invade the nerve, the cancer cells need to change the cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons. Matrix metalloproteinases (MMPs) have important roles in these processes because their proteolytic activities assist in degradation of ECM and basement membrane (Pytliak et al., 2012). Physiological tissue inhibitors of matrix metalloproteinases (TIMPs) inhibit the enzymes of the MMP family and preserve stromal integrity, inhibiting tumor migration. In tumor invasion and progression the balance between MMP and TIMPs is crucial. It was supported by previous research that CXCR4 can stimulate secretion of MMP-2 (Richard et al., 2008), direct invasion of lung cancer cells by up-regulation of both MMP-9 (Tang et al., 2008).

In this study, high TIMP-2 expressions are significantly negatively correlated with PNI. High expressions of TIMP-1 were also connected with an intra pathway of PNI. The correlation between the expression of TIMP-2 and MMP-9 also supported that TIMP-2, TIMP-1, MMP-9 were all involved in the course of PNI. We suspected that under the stimulation of CXCR4, an increased secretion of MMP-9 may take part in the course of PNI by increasing adhesion of cancer cells to extracellular and eventually degradation of ECM and regulating chemotactic ability and invasiving of tumor cells.

In conclusion, expressions of CXCR4 and TIMP-2 showed significant difference in different PNI status in pancreatic cancer. CXCR4 can be used as a predictor of the PNI in pancreatic cancer, and TIMP-2 can be a negative predictor. We present here a novel mechanism of PNI. CXCR4 may promot the happen of PNI in pancreatic cancer cells by decreasing the inhibition TIMPs on MMP. With the involvement of CXCR4 and TIMP-2, the cancer cell were more easily to overcome the physical barriers of nerve, form PNI, but the mechanism of PNI still need further investigation.

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