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Analysis of tumor-induced lymphangiogenesis and lymphatic vessel invasion of pancreatic carcinoma in the peripheral nerve plexus

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Cancer cells can metastasize throughout the body by various mechanisms, including the lymphatic system, resulting in tumor-induced lymphangiogenesis that can profoundly affect patient survival. The aim of the present study was to examine the role of lymphangiogenesis in the metastasis of pancreatic cancer to the peripheral nerve plexus. Immunohistochemistry was performed to analyze specimens obtained from 70 ductal adenocarcinoma patients. The markers used included lymphangiogenic factor vascular endothelial growth factor (VEGF)-C, the lymphatic-specific marker D2-40, and cytokeratin 19, an independent prognostic factor for pancreatic tumors. The relationship between survival rate and invasion of both the lymphatic vessels and peripancreatic nerve plexus (PNP) was evaluated, with clearly elevated lymphatic vessel density (LVD) in tissues adjacent to the cancer tissues. In fact, LVD levels were higher in adjacent tissues than in localized cancer tissues, and lymphatic vessel invasion into tissues adjacent to the tumor was significantly correlated with both PNP invasion ($P = 0.005$) and lymph node metastasis ($P = 0.010$). Correspondingly, LVD in tissues adjacent to the tumor was correlated with both invasion of lymphatic vessels surrounding the tumor ($P = 0.024$) and VEGF-C expression ($P = 0.031$); in addition, VEGF-C expression was correlated with invasion of lymphatic vessels around the tumor ($P = 0.004$). Survival rates were significantly lower in patients in whom there was peritumor lymphatic vessel invasion ($P < 0.001$), extrapancreatic nerve plexus invasion ($P = 0.001$), and/or lymph node metastasis ($P < 0.001$). Based on these results, lymphatic invasion associated with adjacent tumor growth likely contributes to the development of metastatic tumors that invade the PNP. (*Cancer Sci* 2012; 103: 1756–1763)

Tumor cells can spread throughout the body by direct invasion of local tissues or via the circulatory system, either through the cardiovascular system (hematogenous metastasis) or through the lymphatic system (lymphatic metastasis).[1,2] Given its clinical importance, metastasis via the cardiovascular system has been investigated extensively in an attempt to gain a better understanding the mechanisms involved.[3] Because vascular endothelial growth factors (VEGFs), previously referred to as VEGF, are the most potent regulators of angiogenesis, they have emerged recently as critical therapeutic targets.[4–6] It has been found that VEGF-C is an important regulator of lymph vessel growth (lymphangiogenesis) and lymphatic metastasis.[7,8] Despite numerous studies indicating that the lymphatic vessels are the primary pathway for the metastasis of tumor cells,[9–11] and therefore the potential clinical significance of this pathway, relatively little is known about metastasis via the lymphatic system.

The lack of detailed information regarding the mechanisms underlying lymphatic metastasis may be attributed to a lack of viable markers for the identification of lymphatic formations. However, the emergence of VEGF-C as a dominant regulator of lymphangiogenesis has provided a new and more effective method for distinguishing between lymphatic and blood vessels related to tumor cell movement.[12,13] Increased VEGF-C expression in the primary tumor has been shown to be closely correlated with increased dissemination of the tumor to regional lymph nodes.[14] Coupled with the recent emergence of additional markers of lymphangiogenesis, including the D2-40 protein specifically expressed in the lymphatic endothelium,[15] lymphatic vessels may now be effectively marked without directly staining the vascular endothelium. Markers that specifically denote lymphangiogenesis are crucial to determine the clinical usefulness of the extent of lymph vessel involvement in tumor development as a prognostic factor.

Neurotropic growth has often been used as an indicator of poor prognosis in pancreatic cancer.[16] Although invasion of the nervous system is relatively common in patients with pancreatic cancer, the extent of cancer metastasis to the nerves via the lymphatic vessels remains unclear. In a previous study investigating the mechanism of peripancreatic nerve plexus (PNP) invasion, the distribution of lymphatic vessels in the nerve plexus surrounding the superior mesenteric artery was analyzed using D2-40. The results showed that pancreatic cancer induced lymphangiogenesis, and that lymphatic vessel density (LVD) was highly correlated with tumor differentiation, lymph node metastasis, and PNP invasion.[16–18] Together, these studies indicate that newly formed lymphatic vessels are likely to be the major pathway for the spread of tumors to the PNP.

In the present study, we used cytokeratin 19 (CK19),[19] a commonly used prognostic marker, to evaluate lymphatic invasion. It has been reported that CK19 is highly expressed in lymph nodes exhibiting disseminated tumor cells,[20] and significant levels of CK19 have been found in the peripheral blood of patients with other malignancies, including liver, breast, and pancreatic cancers.[21,22] Thus, using CK19 as a potential marker for lymphatic invasion of the PNP may both clarify the significance of lymphangiogenesis as a major route for the spread of tumor cells to the nervous system and provide mechanistic information regarding this method of tumor metastasis for further research and clinical application.

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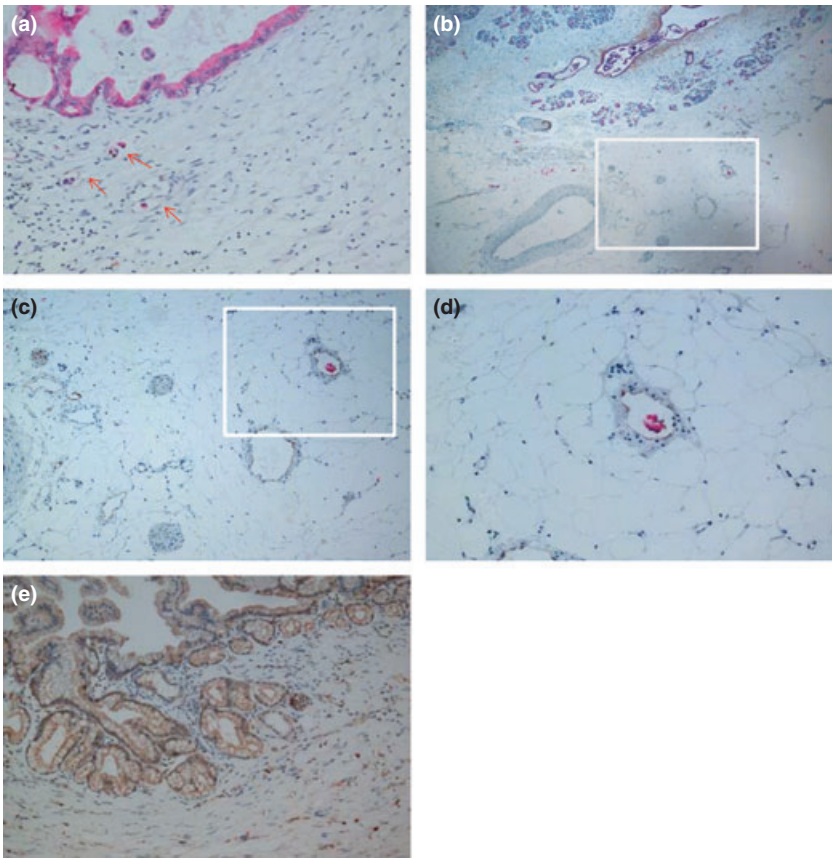

Fig. 1. Cytokeratin 19 (CK19) and D2-40 dual immunohistochemistry revealing lymphatic vessel (LV) invasion, peripancreatic plexus invasion, and lymph node metastasis. (A) High-density area of LV in peripancreatic tissue. Most lymphatic vessels exhibited lumen-like structures with tumor cells inside. Red arrows indicate lymphatic endothelial cells stained by D2-40 and pancreatic cancer cells stained by CK19 (original magnification ×200). (B) Lymphatic vessels, blood vessels and nerve bundles showing CK19-stained tumor cells inside the LV (original magnification ×40). (C) Staining of pancreatic tumor cells for CK19 (red) and peripancreatic LV for D2-40 (brown-yellow; original magnification ×100). Panel C shows the boxed area in (b) and panel D shows the boxed area in (c). (D) Tumor cells staining positive for CK19 (red) were found inside the peripancreatic LV (brown-yellow; original magnification ×200). (E) Vascular endothelial growth factor-C-stained regions found in the plasma of tumor cells (brown-yellow yellow; original magnification ×200).

Materials and Methods

Patients and specimens. Tissue samples were collected from 70 patients who underwent surgery for pancreatic cancer between September 2003 and December 2004. The patient group consisted of 45 men and 25 women ranging in age from 37 to 82 years (mean age 59 years). For inclusion in the study, patients must not have received chemotherapy, radiotherapy, or any other treatment and were required to be free of any metastases, as determined using imaging or intraoperative examination prior to surgery. Patients with distant organ metastases, as determined by imaging and surgical exploration, were excluded from the study. All patients underwent superior mesenteric artery nerve plexus resection and regional lymph node dissection. Clinical classifications were based on the *Classification of Pancreatic Carcinoma*.[23]

The present study was approved by the Ethics Committee of the Second Military Medical University of Shanghai, China. Specimens obtained from the patients included both tumor and tumor-adjacent tissues ($\leq$2.0 mm). Samples were collected from tumors of the nerve plexus in the pancreatic head and the nerve plexus-containing tissue around the hepatic artery and hepatoduodenal ligament. Samples of cancerous tissues from the nerve plexus surrounding the celiac artery in the pancreatic body and tail, superior mesenteric arterial plexus tissue (left), and splenic plexus tissue were also obtained. Furthermore, samples of peripancreatic lymph node and adipose tissue were collected.

Tissue samples were approximately 2.0 × 1.5 × 0.3 cm in size. Specimens were fixed in 10% paraformaldehyde for 24 h, embedded in paraffin wax and then sliced (3 μm). Sections were dried at 37°C and stained using H&E, D2-40, and CK19 dual immunohistochemistry (IHC) or VEGF-C IHC stains.

Immunohistochemistry. The procedures for D2-40 and CK19 dual IHC and VEGF-C IHC were as follows. The paraffin wax was removed from sections using xylene, followed by gradual hydration with ethanol (from 100% to 70%). After tissues had been processed in 3% H_2O_2 methanol buffer, they were incubated at 4°C overnight in the presence of primary antibodies, namely D2-40 mouse mAb (1:100 dilution; Dako, Carpinteria, CA, USA), CK19 rabbit mAb (1:100 dilution; Epitomics, Burlingame, CA, USA), and VEGF-C mAb (1:100 dilution; Invitrogen, Camarillo, CA, USA). The following day, tissues were incubated at room temperature for 30 min in the presence of secondary antibodies, namely horseradish peroxidase-conjugated IgG (Polylink DS kit; Zhong Shan Goldbridge Biotechnology) and alkaline phosphatase (AP)-conjugated IgG (or streptavidin peroxidase [SP] secondary antibody and peroxidase-labeled streptavidin in the case of VEGF-C). Samples were stained with diaminobenzidine and AP-Red, followed by hematoxylin staining for 1 min. Slides were subsequently examined under a microscope. As a negative control, the primary antibodies were replaced with PBS.

Histopathological analysis. Lymphatic vessels were observed in areas where D2-40-positive staining was present as brown-yellow granules in the lymphatic endothelial membrane and cytoplasm. In addition, D2-40 staining was used to observe lymphatic vessel morphology and distribution. According to the method of Ohno *et al.*,[24] D2-40 staining was used to determine LVD in intratumor tissues, tumor-adjacent tissues, and PNP tissues. Briefly, D2-40 positivity was determined by light microscopy at a magnification of ×40, with the number of positive lymphatic capillaries counted at a magnification of ×200 (0.739 mm^2/field for three fields). Mean values were then calculated to obtain LVD. Scoring of CK19-positive cells was as described previously.[25]

The VEGF-C-positive cells appeared as brown-yellow granules in the cytoplasm of pancreatic cancer cells. Each section

was examined a minimum of 10 times at a magnification of ×400 to count both the number of positive cells and the total number of cells. Samples in which >30% cells (be area) were stained were considered to be VEGF-C positive.

Data analysis. Data were analyzed using SPSS 13.0 (SPSS, Chicago, IL, USA), with differences between groups evaluated by the Chi-squared test. Differences in the LVD of tumors, tumor-adjacent tissues, and peripancreatic plexus tissues were compared using either *t*-test or one-way ANOVA with *post hoc* Dunnett's test. Survival rates were evaluated using the Kaplan–Meier estimator and Cox's proportional hazards model. Survival rates were compared using the log-rank test. $P < 0.05$ was considered significant.

Results

Clinical data. Analysis of samples tissues revealed that most tumors were located in the head of the pancreas or in the lesser pancreas (71.4%). Of the tumors sampled, 68.6% demonstrated high levels of differentiation. A large proportion of tumors was observed to be associated with positive peripancreatic nerve invasion (67.1%) or negative large vascular invasion (72.9%). Finally, high levels of VEGF-C expression were detected in 61.4% of samples. These data support a relationship between pancreatic cancer progression and peripancreatic nerve invasion, with lymphangiogenesis as a primary means for the spread of tumor cells to the nervous system.

Lymphatic vessel and peripancreatic plexus invasion in non-cancerous tissues associated with lymph node metastasis. To explore the role of lymphangiogenesis in the metastatic process, 47 patients (67.1%) with peripancreatic plexus invasion were analyzed (Fig. 1). Using IHC analysis revealed that both lymphatic endothelial cells and pancreatic cells were positive for D2-40, as evidenced by brown-yellow staining, with approximately half of all sampled tissues exhibiting positive staining (Table 1). In contrast, CK19 staining (red cytoplasmic staining on microscopic slides) was detected in pancreatic cancer cells only. Red areas corresponding to CK19 staining indicate high levels of tumor invasion by lymphatic vessels into non-cancerous tissues, including adjacent tissues and the peripancreatic nerves (Fig. 2). The VEGF-C-positive cells were detected primarily in the cytoplasm of cancerous cells, although there was some limited staining for VEGF-C in the stroma (data not shown). In all, tissue samples from 41 patients (58.6%) were found to be positive for peripancreatic lymphatic vessel invasion by tumor cells. Of these patients, 13 (31.7%) showed evidence of tumor cell invasion into the peripancreatic nerve adjacent to the lymphatic structures. The lymphatic vessels in the remaining 29 patients (41.4%) were unaffected, wherein the peripancreatic nerve surrounding the lymphatic vessel exhibited no signs of tumor cell invasion. Based on Chi-squared analysis, invasion of tumor cells from lymphatic vessels into adjacent tissues was significantly associated with PNP metastasis ($P = 0.005$), lymph node metastasis ($P = 0.010$), and VEGF-C expression ($P = 0.004$; Table 2). Notably, there was no correlation between the invasion of tumor cells from lymphatic vessels and gender ($P = 0.492$), tumor size ($P = 0.441$), tumor differentiation ($P = 0.104$), or tumor location ($P = 0.701$). There was no significant correlation between invasion of the PNP and lymph node metastasis ($P = 0.108$).

Peripancreatic nerve plexus invasion and analysis of lymphatic vessel distribution in the pancreas, peripancreatic region, and adjacent lymph nodes. Lymphatic vessels, blood vessels, and

Table 1. Patient characteristics

Characteristics	
Total no. patients	70
Age (years)	
Mean	59.0
Range	37–82
Gender	
No. men	45 (64.3)
No. women	25 (35.7)
Tumor size	
Diameter ≥4.0 cm	40 (57.1)
Diameter <4.0 cm	30 (42.9)
Tumor location	
Head of pancreas/lesser pancreas	50 (71.4)
Body and tail of pancreas	20 (28.6)
Tumor differentiation	
High	48 (68.6)
Low	22 (31.4)
Large vascular invasion	
Positive	19 (27.1)
Negative	51 (72.9)
Lymph node metastasis	
Positive	37 (52.9)
Negative	33 (47.1)
Peripancreatic nerve invasion	
Positive	47 (67.1)
Negative	23 (32.9)
Peritumoral LVI	
Positive	41 (58.6)
Negative	29 (41.4)
VEGF-C expression	
Positive	43 (61.4)
Negative	27 (38.6)

Unless indicated otherwise, data show the number of patients in each group, with percentages given in parentheses. LVI, lymphatic vessel invasion; VEGF-C, vascular endothelial growth factor C.

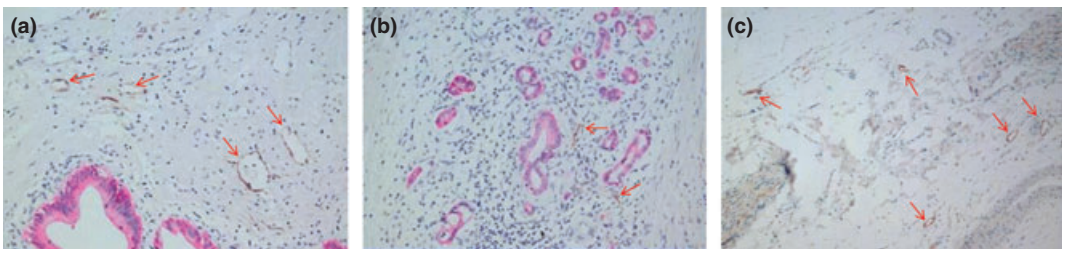

Fig. 2. Cytokeratin 19 (CK19) and D2-40 dual immunohistochemical analysis of lymphatic vessel density (LVD). (a) Peripancreatic tissue showing areas of high LVD. Most lymphatic vessels maintained lumen-like structures (original magnification ×200). (b) Within pancreatic cancer tissues, low LVD was observed. The lymphatic vessels lost their structure due to the pressure created by the tumor (CK19 [red]-stained pancreatic cancer cells in D2-40 [brown-yellow]-stained lymphatic endothelial cells; original magnification ×200). (c) The peripancreatic nerve plexus region showing areas of high LVD. Most lymphatic vessels presented with enlarged lumen-like structures. Arrows indicate lymphatic endothelial cells stained with D2-40. (Original magnification ×100.)

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Table 2. Invasion of lymphatic vessels into adjacent tissues and clinicopathological features

Clinicopathological features	*n*	Invasion of the lymphatic vessels into adjacent tissues		*P*-value
		Positive (*n*)	Negative (*n*)	
Gender				
Male	45	25	20	0.492
Female	25	16	9	
Tumor location				
Head of pancreas/lesser pancreas	50	30	20	0.701
Body and tail of pancreas	20	11	9	
Tumor size				
Diameter $\geq$4.0 cm	40	25	15	0.441
Diameter <4.0 cm	30	16	14	
Tumor differentiation				
High	48	25	23	0.104
Low	22	16	6	
Lymph node metastasis				
Positive	37	27	10	0.010
Negative	33	14	19	
PNP invasion				
Positive	47	33	14	0.005
Negative	23	8	15	
VEGF-C expression				
Positive	43	31	12	0.004
Negative	27	10	17	

PNP, peripancreatic nerve plexus; VEGF-C, vascular endothelial growth factor C.

nerve bundles were all located in both tumor-adjacent tissues and peripancreatic tissue. Lymphatic vessels specifically demonstrated non-uniform distribution by staining, indicating a collapse of the lymphatic vessels around the tumor that resulted in partial or complete loss of their whole lumen-like structure (Fig. 2b). Conversely, lymphatic vessels of the peripancreatic tissue, particularly those of the plexus, maintained their lumen structure (data not shown). In all, 15 patients (21.4%) exhibited peripancreatic lymphatic vessels that remained in close contact with, or even penetrated, the nerve bundle (Fig. 3).

Patients were grouped according to tumor location and classification, and the relationship between LVD and peripancreatic neural invasion analyzed. Patients were first divided into two groups, one consisting of 50 patients with tumors located in the head of the pancreas/lesser pancreas and the second consisting of 20 patients with tumors in the body and tail of the pancreas. The LVD was determined for each tissue individually (Table 3), with the results indicating that the average LVD was higher in tumor-adjacent tissues than in tumor tissues (11.1 ± 4.8 *vs* 3.7 ± 2.1/field, respectively; $P < 0.001$; Fig. 2). The LVD in the nerve plexus region near the head of the pancreas was significantly higher than that in tissues adjacent to the hepatic artery and the hepatoduodenal ligament plexus ($P = 0.026$). In addition, LVD in both the celiac plexus tissues and splenic plexus tissues was significantly higher than that in the nerve plexus tissue surrounding the superior mesenteric artery ($P < 0.001$). The rate of PNP invasion in tissues from the pancreatic head plexus and celiac plexus (left) was 60.0% and 65.0%, respectively. The rate of PNP invasion was higher in both these tissues compared with that in other tissues of the pancreatic plexus (Table 3), indicating the likelihood of a relationship between tumor location and PNP invasion.

The relationship between adjacent lymphatic vessels and pancreatic tumors was, in turn, related to clinicopathological factors. As indicated in Table 4, there was no significant difference in LVD in tumor-adjacent tissues positive for lymph node invasion ($P = 0.549$). However, there was evidence of significantly greater PNP invasion in groups positive for lymphatic vessel invasion in adjacent tissue compared with the group negative group for lymphatic vessel invasions ($P = 0.042$). Similarly, significantly higher PNP invasion was seen in tumor-adjacent tissues that were VEGF-C positive compared with those that were VEGF-C negative ($P = 0.031$). Finally, LVD in tumor-adjacent tissues was higher in tissues positive for lymphatic vessel invasion ($P = 0.024$). Together, these results suggest that invasion of lymphatic vessels into tumor-adjacent tissue is likely to be related to pancreatic cancer prognosis. Furthermore, there may be a relationship between prognosis and lymphatic vessel invasion, tumor differentiation, PNP invasion, and lymph node metastasis.

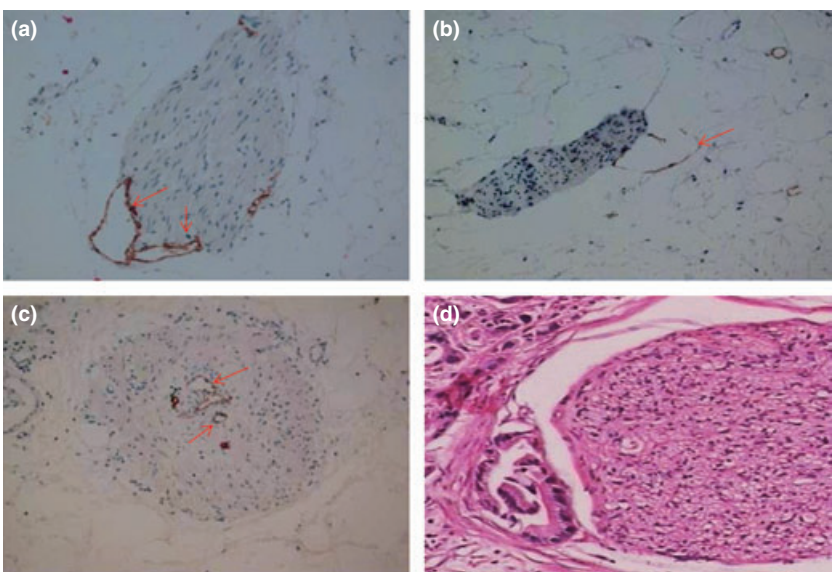

Fig. 3. (a–c) Cytokeratin 19 (CK19) and D2-40 dual immunohistochemical analysis of the distribution of lymphatic vessels (LV) in the peripancreatic plexus. (a) Peripancreatic LV accompanied by the peripancreatic plexus, showing close contact or invasion into the epineurium (original magnification $\times$200). (b) Peripancreatic LV in close contact or invading the epineurium. The LV are seen as semi-open structures (original magnification $\times$200). (c) Peripancreatic LV directly entering the peripancreatic nerve bundle (original magnification $\times$200). (d) Invasion of the peripancreatic plexus showing infiltration of the epineurium and partial perineurium (H&E staining; original magnification $\times$100). Arrows indicate lymphatic endothelial cells stained by D2-40.

Table 3. Comparison between lymphatic vessel density and peripancreatic neural invasion

Tumor location	Specimen of pancreatic plexus	*n*	LVD	Positive pancreatic plexus invasion (%)
Head of pancreas	Pancreatic head plexus tissue	30	6.5 ± 3.2*	60.0
	Common hepatic artery tissue and hepatoduodenal ligament plexus tissue	22	5.2 ± 2.5	44.0
Body and tail of pancreas	Celiac plexus tissue (left)	13	8.0 ± 3.1†	65.0
	Splenic plexus tissue	7	6.9 ± 2.9†	25.0
	Superior mesenteric arterial plexus tissue (left)	5	3.5 ± 1.9	35.0

*$P < 0.05$ compared with common hepatic artery tissue and hepatoduodenal ligament plexus tissue. †$P < 0.001$ compared with superior mesenteric arterial plexus tissue (left). Lymphatic vessel density (LVD) data are given as the mean ± SD.

Table 4. Lymphatic vessel density distribution in adjacent tissues and clinicopathological features

Clinicopathological features	*n*	LVD	*P*-value
Gender			
Male	45	11.3 ± 4.7	0.686
Female	25	10.8 ± 5.0	
Tumor location			
Head of pancreas/lesser pancreas	50	10.9 ± 4.7	0.601
Body and tail of pancreas	20	11.6 ± 5.1	
Tumor size			
Diameter ≥4.0 cm	40	11.0 ± 4.4	0.821
Diameter <4.0 cm	30	11.2 ± 5.3	
Tumor differentiation			
High or middle	48	11.0 ± 5.0	0.800
Low	22	11.3 ± 4.3	
Lymphatic vessel invasion in adjacent tissue			
Positive	41	12.2 ± 4.8	0.024
Negative	29	9.6 ± 4.4	
Lymph node metastasis			
Positive	37	11.4 ± 4.4	0.549
Negative	33	10.8 ± 5.2	
PNP invasion			
Positive	47	11.9 ± 4.6	0.042
Negative	23	9.4 ± 4.9	
VEGF-C expression			
Positive	43	12.1 ± 4.7	0.031
Negative	27	9.6 ± 4.6	

Lymphatic vessel density (LVD) data are given as the mean ± SD. PNP, peripancreatic nerve plexus; VEGF-C, vascular endothelial growth factor C.

Survival rate analysis. *Univariate analysis.* Follow-up was initiated after radical surgery for pancreatic cancer for the 60 patients (85.7%) who underwent this procedure, resulting in a median survival of 21 months. The survival rate by the end of 1st, 2nd, 3rd, and 5th years after surgery was 84.2%, 38.7%, 18.5%, and 7.7%, respectively (Fig. 4). Kaplan–Meier estimation was used to analyze the potential effects of individual factors on survival rates, including gender, tumor location, tumor size, tumor differentiation, large vasculature (e.g. portal vein and superior mesenteric vein) invasion, invasion of lymphatic vessels into tumor-adjacent tissue, PNP invasion, and lymph node metastasis.

Survival rates between groups were compared using the log-rank test. The results indicate that the survival rate of pancreatic cancer patients is significantly correlated with vascular invasion ($P = 0.003$), lymph node metastasis into adjacent tissues ($P < 0.001$), the extent of tumor differentiation ($P = 0.030$), and PNP invasion ($P = 0.001$). These factors strongly influenced long-term survival, as indicated in Table 5.

Multivariate analysis. The factors identified by univariate analysis were subsequently examined by Cox's proportional hazard models. This analysis revealed that survival rates were independently affected by major vascular invasion ($P = 0.009$), invasion of lymphatic vessel into tumor-adjacent tissues ($P = 0.011$), PNP invasion ($P = 0.046$), and lymph node metastasis ($P = 0.002$). However, tumor differentiation had no significant effect on prognosis ($P = 0.221$). Table 6 lists the *P*-values, associated risk factors, and 95% confidential intervals for each of these factors.

Discussion

Pancreatic cancer is a devastating neoplasm commonly associated with poor prognosis, particularly in cases of metastasis through the lymphatic system. The 5-year survival rate for pancreatic adenocarcinoma[26] is <5% and the only currently available method to treat the disease is complete tumor removal. Even in the case of successful tumor removal, long-term survival may only be improved by as little as 18–24%.[27] In 64–100% of patients, pancreatic cancer is complicated by neural invasion.[28] Neural invasion is a cause of distant metastasis and retroperitoneal recurrence. Understanding the mechanisms and pathways governing nerve invasion will provide valuable insight for the development of novel therapeutic strategies.[29]

In the present study, 46 patients (59.7%) with peripancreatic neural invasion and 47 patients (61%) with regional lymph node invasion were evaluated, including dual staining for CK19 and D2-40 or standard IHC staining for VEGF-C in samples from 41 patients. The results revealed that the lymphatic vessels present in tumor-adjacent tissues were often invaded by cancerous cells. Perineural lymphatic invasion was also found in 13 patients (18.6%). Most commonly, when lymphatic vessels surrounding the tumor were unaffected, the peripancreatic nerve was not invaded.

Analysis of lymphatic vessels within the PNP showed differential LVD distribution according to region, with the highest levels observed in pancreatic head plexus tissues and progressively lower levels observed in tissues from the celiac plexus, splenic plexus, common hepatic artery, hepatoduodenal ligament plexus, and superior mesenteric arterial plexus (Table 3). Consequently, metastasis can be considered to occur more frequently in certain regions, such as the pancreatic head plexus.[30] The consistency between elevated LVD surrounding tissues and PNP invasion implies that the distribution of lymphatic vessels strongly influences the invasion of PNP. A reasonable explanation for this observation is that the lymphatic system represents the predominant route through which tumor cells spread to the peripancreatic nerve. A previous study has shown that tumors of the pancreatic head tend to grow towards the celiac plexus and posterior hepatic plexus, a finding that differs considerably from that of tumors of the pancreatic body and tail, which tend to spread towards the splenic plexus and celiac plexus.[31] The findings of the present study support those of previous studies, indicating the potential prognostic importance of varying levels of LVD.

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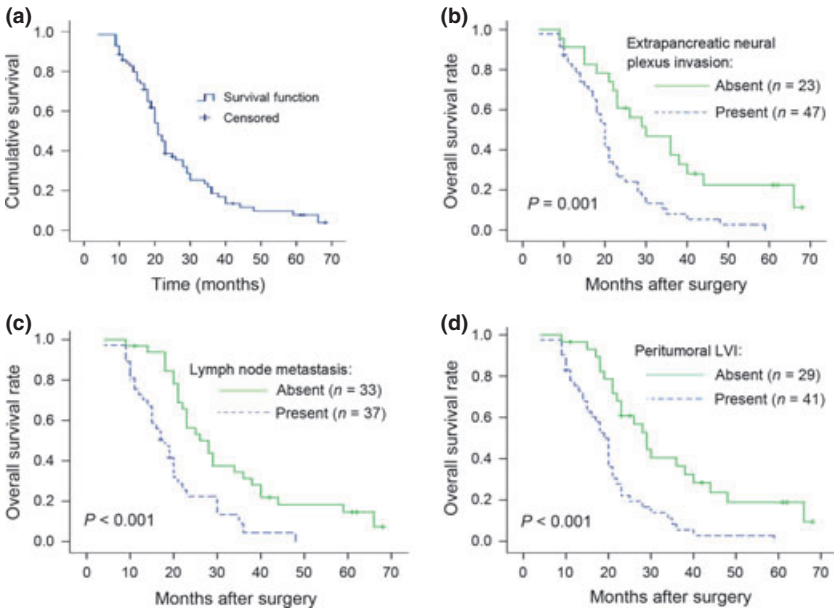

Fig. 4. Survival analysis (Kaplan–Meier method). (a) Follow-up was initiated after surgery for pancreatic cancer. A total of 60 patients (85.7%) completed follow-up interviews. The median survival time was 21 months. At the end of 1, 2, 3, and 5 years after surgery, survival rates were 84.2%, 38.7%, 18.5%, and 7.7%, respectively. (b) Peripancreatic nerve plexus (PNP) invasion was closely correlated with survival ($P = 0.001$). Survival at 5 years was significantly greater in the patient group without PNP invasion. (c) Lymphatic vessel (LV) metastasis was closely correlated with survival ($P = 0.001$). Patients with no LV metastasis had significantly greater survival at 5 years. (d) Peritumor LV invasion (LVI) was correlated with survival ($P < 0.001$). Patients with no LVI had significantly better survival at 5 years.

Table 5. Factors contributing to prognosis, as determined using univariate analysis

	No. patients	Medium survival rate (months)	2-year survival rate (%)	5-year survival rate (%)	*P*-value for 5-year survival rate
Gender					
Male	45	23	43.7	11.0	0.074
Female	25	18	29.3	0.0	
Tumor location					
Head of pancreas/lesser pancreas	50	21	38.3	6.2	0.907
Body and tail of pancreas	20	21	40.0	10.0	
Tumor size					
Diameter $\geq$4.0 cm	40	21	33.6	3.1	0.459
Diameter <4.0 cm	30	23	45.1	15.2	
Tumor differentiation					
Low	22	16	24.1	0.0	0.030
High	48	23	45.1	11.7	
Major vascular invasion					
Yes	19	15	17.4	0	0.003
No	51	23	46.4	10.1	
Lymphatic vessel invasion in adjacent tissue					
Yes	41	19	22.2	0	<0.001
No	29	29	60.8	18.9	
Lymph node metastasis					
Positive	37	18	22.3	0	<0.001
Negative	33	28	56.3	14.6	
PNP invasion					
Positive	47	20	26.7	0	0.001
Negative	23	30	60.9	22.5	

P-values were calculated by the log-rank test. PNP, peripancreatic nerve plexus.

Major vascular invasion, lymphatic vessel invasion in tumor-adjacent tissue, PNP invasion, and lymph node metastasis were shown to be the most dominant factors affecting survival rate, as determined using Kaplan–Meier analysis. Thus, both lymphatic metastasis and PNP invasion are critical factors impacting survival rates. Recent studies have shown that lymphangiogenesis may be tumor induced, thus providing a means for invasion and metastasis.[32,33] Both VEGF-C and VEGF-D have been shown to be capable of stimulating lymphatic vessel formation by activating VEGF receptor 3 (VEGFR-3). Signaling via this pathway assists lymph node metastasis of various tumors, including pancreatic cancer, whereas blocking the VEGF-C/D–VEGFR-3 signaling pathway inhibits lymphangiogenesis and lymph node metastasis.[34–36] In contrast, binding of VEGF-C to VEGFR-3 induces lymphangiogenesis in tumor tissues and causes lymphatic vessel dilation in surrounding tissues.[37,38] Although binding of VEGF-C to VEGFR-2 enhances microvascular formation, the binding of VEGF-C to VEGFR-3 promotes lymphangiogenesis.[39] Clinically, high VEGFR-3 expression has been associ-

Table 6. Cox's proportional hazards model of factors influencing survival rate

Factors that may influence prognosis	*P*-value	Risk (95% CI)
Adjacent lymphatic vessel invaded	0.011	2.099 (1.90–3.705)
Peripancreatic neural invasion	0.046	1.822 (1.010–3.289)
Regional lymphatic metastasis	0.002	2.428 (1.385–4.256)
Major vascular invasion	0.009	2.189 (1.212–3.953)
Extent of tumor differentiation	0.221	1.424 (0.809–2.506)

CI, confidence interval.

ated with lymphatic vessel metastasis, ultimately impacting long-term survival.[40]

The formation of new lymphatic vessels associated with tumor growth may be an important prognostic factor. Bjorndahl *et al.*[41] reported that insulin-like growth factor (IGF)-1 and IGF-2 were capable of inducing and promoting lymphangiogenesis and lymph node metastasis. The results of the present study demonstrate that VEGF-C/D–VEGFR-3 signaling pathways may interact with IGF to form new lymphatic vessels, a potential route for metastasis. In addition, VEGF-C has been found to play a further role in lymphatic endothelial cell proliferation as a specific factor involved in chemotaxis. The entry of cancerous cells from the pancreas into lymphatic vessels is likely facilitated by endothelial junctions with the assistance of proteinase, an inhibitor of apoptosis.[42] These cells may also invade into nerves through neural adhesion molecules.[43] The process of metastasis through the lymphatic system involves several pathways, each of which is affected by the growth of cancerous tissue in the pancreas.

The close association between pancreatic cancer and peripancreatic nerve invasion has prompted extensive research into the mechanism of pancreatic cancer cell affinity for this particular neural structure. Studies have consistently suggested that nerve growth factor is a crucial mediator of neural invasion.[44,45] The determination of other growth factors that are involved in neural invasion, such as transforming growth factor-α,[46] glial cell line-derived neurotrophic factor,[47] artemin,[48] and fractalkine,[49] has allowed for the emergence of the molecular model of neurotrophism. Furthermore, neural cells and pancreatic cancer cells share a number of similar biological properties, including similarities in growth factor receptors and cell surface adhesion molecules, which may promote their interaction.[30] The relationship between perineural invasion and the lymphatic system surrounding those nerves suggests that perineural invasion occurs not only as a result of direct infiltration, but also by way of lymphatic vessels, most commonly through newly formed lymph vessels, possibly due to cancerous growth.

Together, the results of the present study indicate that tumor cells are able to release several factors, including VEGF-C, that serve to stimulate the formation of lymphatic vessels in tissues adjacent to the cancerous growth. Furthermore, some of these secreted factors may play a role in both the creation of new lymph vessels and the promotion of tumor cell entry into these newly formed passages, thus providing a route for metastatic invasion of surrounding neural tissues. The involvement of neurotropic adhesion facilitates further spread of tumor cells, accounting for common metastasis into the nerve plexus.

The present study provides strong evidence that tumor-induced lymphatic vessel formation and PNP invasion are particularly important factors influencing patient survival. Regulation of lymphangiogenesis by VEGF-C and regulation of neurotrophism using nerve growth factors is a potential goal that may result in the development of more effective clinical treatments, allowing for medical intervention in cases of pancreatic cancer than may result in an improved prognosis for these patients. The results of the present study are the first step in a process that will require further investigations, using both animal models and advanced molecular biology techniques, before the findings can be applied to a clinical setting.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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