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Original article

Expression of vascular endothelial growth factors-C and -D correlate with evidence of lymphangiogenesis and angiogenesis in pancreatic adenocarcinoma

Bo Zhang MD, Wen-Hua Zhao MM[1], Wu-Yuan Zhou MM, Wen-Sheng Yu MD, Jin-Ming Yu MD*, Sheng Li MD**

Shandong Tumor Hospital, Jinan 250117, Shandong Province, China

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Abstract

Background: We analyzed the intratumoral and peritumoral microvessel density (MVD) and microlymphatic vessel density (MLVD) in pancreatic adenocarcinoma (PAC) and recorded the expression of vascular endothelial growth factor (VEGF)-C and -D. These data were tested for their significance for tumor progression. *Methods*: The tissue samples were obtained from 30 patients with PAC. The expression of VEGF-C and -D, MLVD, MVD was assayed by immunohistochemical staining. The expression of VEGF-A and -C, and -D mRNA was detected by semi-quantitative RT-PCR. *Results*: Immunohistochemical analysis revealed the presence of VEGF-C and -D immunoreactivity in 73% (22/30) and 57% (17/30). The positive rates of VEGF-C and -D protein in central portion of tumors (30% and 16.7%) were significantly lower than those in marginal portion (73.3% and 56.7%). The group with high expression of VEGF-C and -D in marginal portion had higher incidence of lymph node metastasis, lymphatic invasion and venous invasion. The MLVD in both of the VEGF-C and -D positive groups was higher than that in the negative groups, and the lymph node metastasis increased. MVD in the VEGF-C positive group was higher than that in the negative group. *Conclusions*: The expression of VEGF-C and -D in the marginal portion of tumor significantly associated with lymphatic metastasis and prognosis in patients with PAC, and may induced lymphangiogenesis. VEGF-C was important in the regulation of angiogenesis and lymphangiogenesis in PAC.

Keywords: Vascular endothelial growth factor-C; Vascular endothelial growth factor-D; Pancreatic adenocarcinoma; Lymphangiogenesis; Angiogenesis; Lymph node metastasis; Intratumoral and peritumoral vessel density; Lymphatic vessel endothelial hyaluronan receptor-1; Microlymphatic vessel density

1. Introduction

Pancreatic adenocarcinoma (PAC) is an aggressive tumor associated with high mortality. Despite efforts in the past 50 years, conventional treatment approaches, such as surgery, radiation, chemotherapy, or combinations of these, have had little effect on the course of this aggressive neoplasm and 5-year survival rates remain below 5% [1]. Furthermore, the 5-year survival rate after resection is 12–20% in larger series [2,3]. Lymph node metastasis and blood vessel invasion after resection are important factors affecting the survival and prognosis of PAC patients. Most tumor spread is dependent on both the angiogenic and lymphangiogenic systems. Lymphangiogenesis and angiogenesis are important in the growth, progression, and metastasis of human carcinomas. The mechanisms of tumor invasion, dissemination and lymph node metastasis are complex, and some studies confirmed the importance of the lymphatic system by the discovery of several lymphatic endothelium markers, including lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), Prox-1, Podoplanin. In addition, vascular endothelial growth factor (VEGF)-C and -D, members of the

* Corresponding author.
** Corresponding author. Tel.: +86 13705311949; fax: +86 531 87984079.
E-mail address: drlisheng@sohu.com (S. Li).
[1] Co-first author.

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VEGF family of growth factor, have been identified as potent factors in stimulating lymphoangiogenesis through their receptor VEGFR-3 [4].

Some studies confirm that VEGF-C and -D are major factors associated with the growth of lymphatic endothelial cells [5,6], and several investigators found that inhibition of VEGF-C and -D activity reduced the level of lymphangiogenesis and lymph node metastasis [7,8]. These findings suggest that VEGF-C and -D are important regulators of lymphangiogenesis in a variety of cancers.

Since PAC metastasizes early, we hypothesize that this feature is related to the tumor's capability to induce and stimulate tumor-associated lymphangiogenesis and angiogenesis. We therefore analyzed the intratumoral and peritumoral microvessel density (MVD) and microlymphatic vessel density (MLVD) in PAC and recorded the expression of VEGF-C and -D. These data were then tested for their significance for tumor progression.

2. Materials and methods

2.1. Patients and tumor samples

Freshly cryoconserved tumor tissue and formalin-fixed, paraffin-embedded tissue blocks from 30 PAC patients and their normal tissues of pancreas, liver, and greater omentum were obtained from resection specimens at Shandong Tumor Hospital in June 2005 and June 2006. All of the patients underwent macroscopically curative resection by Whipple procedure (18), or distal pancreatectomy (12) with lymph node dissection. There was no death due to operation. Patients included in this study had not received any preoperative chemotherapy or radiotherapy. These tissues were collected according to the protocol approved by the Ethics Committee of the Medical Faculty of the Shandong Tumor hospital. All of the resected primary tumors and normal tissues were histologically examined by hematoxylin and eosin (H&E) staining according to the tumor-node-metastasis classification system. The staging of PAC was classified by pathological examination after operation (pTNM). The duration of follow-up ranged from 8 to 18 months (median, 12 months). All of the patients were followed-up after operation as follows: plain-film radiography every 1–3 months, and computerized tomography and ultrasonography every 3–6 months. Table 1 summarizes the most important clinicopathological data on the PAC patients.

Table 1
Patients and tumor characteristics ($n = 30$)

Age	57.2 (range 35–78)
Gender	
Male	17
Female	13
Surgical intervention	
Whipple procedure	18
Distal pancreatectomy	12
pTNM stage[a]	
I–II ($pT_1pN_0pM_{0\sim}pT_3pN_0pM_0$)	12
III–IV (pT_{1-3} $pN_1pM_{0\sim}pT_{1-3}$ $pN_{0\text{-}1v}pM_1$)	18
Grade	
Well	17
Moderately/poorly	13

[a] UICC TNM classification.

2.2. Immunohistochemical staining and evaluation

Rabbit anti-human VEGF-C, VEGF-D, immunohistochemical lymphatic marker LYVE-1, CD34 polyclonal antibodies (DAKO Cytomation, Glostrup, Denmark) and S-P kits (DAKO Cytomation, Glostrup, Denmark) were used for immunohistochemical analysis. For negative controls, the primary antibodies were omitted or the primary anti-basic fibroblast growth factor antibody was incubated with specific blocking peptide in 10-fold molar excess before the staining. For the VEGF-D staining, the primary antibody was replaced with control mouse IgG2a (DAKO Cytomation, Glostrup, Denmark). Ten fields were selected for each section, and expression in 1,000 tumor cells (100 cells/fields) was evaluated with high-power (400×) microscopy. The expression of VEGF-C, and -D, was evaluated semi-quantitatively as Negative/low (<10% of tumor cells stained), high (>10% of tumor cells stained) by two independent observers blinded to the patient's status. Primary tumors were divided into two portions, which were marginal and central portions (Fig. 1) [9]. The marginal portion was within 2 mm in diameter of the invasive external edge of the tumor, whereas the central portion was with the exception of the marginal portion and necrotic area.

MLVD mainly in the marginal portion had relatively large lumens and was great in number. The number of LYVE-1 positive vessels was counted as follows: search the dense area of LYVE-1 positive vessels with low power (100×), switch to high power (400×) and select five visual fields to perform counting, and take the average value.

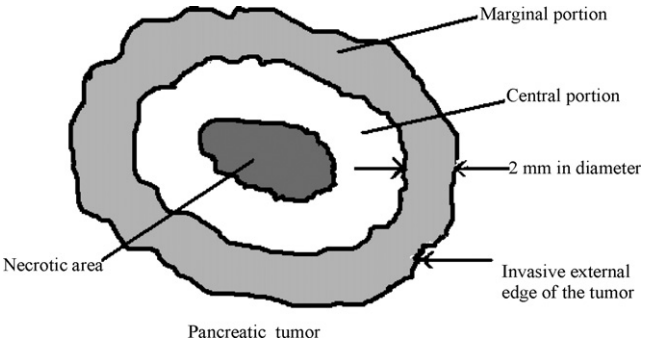

Fig. 1. Schematic illustration of the division of primary tumors. We divided primary tumors into two portions, which were marginal and central portions. The marginal portion was within 2 mm in diameter of the invasive external edge of the tumor, whereas the central portion was with the exception of the marginal portion and necrotic area. We evaluated each expression of VEGF-C and -D in the two portions of the primary tumor separately.