Lymphatic Vessel Density and Vascular Endothelial Growth Factor-C Expression Correlate with Malignant Behavior in Human Pancreatic Endocrine Tumors

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ABSTRACT

Metastatic dissemination of tumor cells to regional lymph nodes is a common feature of many human cancers including pancreatic adenocarcinoma. In contrast, lymph node metastasis is more variably observed in pancreatic endocrine tumors. The objective of this study was to assess the lymphatic system of human pancreatic endocrine tumors and correlate this to clinical behavior. Immunohistochemistry was performed using antibodies to two recently identified markers of lymphatic endothelium, namely, LYVE-1 and podoplanin, and to the lymphangiogenic factor vascular endothelial growth factor (VEGF)-C. As has been reported previously, we observed that in the normal pancreas, islets of Langerhans are devoid of intra-islet lymphatics, but that lymphatics are present in connective tissue in association with ducts and blood vessels. We found that both benign and malignant pancreatic endocrine tumors contain intratumoral lymphatic vessels. Lymphatic vessel density was related to the size of the tumor in benign tumors and to the presence of liver metastasis but not to lymph node metastasis in malignant tumors. VEGF-C was expressed in tumor cells: 4 of 19 (21%) benign tumors were positive, whereas 6 of 9 (67%) borderline tumors and 9 of 11 (82%) carcinomas were positive. These findings strongly suggest that lymphangiogenesis occurs in pancreatic endocrine tumors and that lymphatic invasion and the development of metastases are associated with VEGF-C expression.

INTRODUCTION

Tumor cell dissemination is mediated by several mechanisms, including local invasion, lymphatic or hematogenous spread, and direct seeding of body cavities or surfaces. It is an important indicator of prognosis in human cancers. With regard to lymphatic spread, it is still unclear whether lymph node metastasis is dependent on lymphangiogenesis or invasion of preexisting lymphatic capillaries (1). Recent studies have demonstrated a causal role for vascular endothelial growth factor (VEGF)-C–induced lymphangiogenesis in mediating tumor dissemination and the formation of lymph node metastases in an experimental pancreatic insulinoma model (2). However, little is known about the lymphatic system in human pancreatic endocrine neoplasms (PENs) and the relationship of the lymphatic system to nodal metastasis and prognosis.

A solid, trabecular or glandular arrangement of welldifferentiated cells characterizes the histologic pattern of most well-differentiated PENs (3). In most cases, these features are sufficiently distinct to permit recognition of the endocrine nature of the tumor. In other cases, immunohistochemical stains for general neuroendocrine markers (e.g., chromogranin, synaptophysin or neuron-specific enolase) are needed for tumor identification. In addition, immunohistochemical stains for hormones are necessary to characterize tumor cells and their specific hormonal products, which may cause a syndrome of endocrine hyperfunction. These tumors are challenging because of the difficulty in predicting biological behavior when using classical histopathological malignancy criteria (size, cellular atypia, necrosis, mitotic activity, and angioinvasion). The basis for distinguishing low-grade endocrine tumors from other well-differentiated endocrine carcinomas is usually the presence of metastases. The type of endocrine hypersecretion also correlates with biological behavior of the tumor. Current consensus classification is the World Health Organization (WHO) international histologic classification (3).

Well-differentiated PENs are also characterized by an abundant vascularity. Little is known, however, about the lymphatic system in these tumors. One of the main obstacles to studying tumor-associated lymphatics and lymphangiogenesis has been the lack of specific markers for lymphatic endothelium. We applied antibodies to two recently described lymphatic markers to PENs, namely, LYVE-1, a hyaluronan receptor (4), and podoplanin, a podocyte membrane glycoprotein (5), to investigate lymphatic vessels and lymphangiogenesis and their relationship to clinicopathological characteristics of these tumors. Expression of VEGF-C, a potent lymphangiogenic factor (6, 7), was also evaluated.
**MATERIALS AND METHODS**

**Patient Material.** Archival paraffin-embedded tissue from 39 cases of human PENS was retrieved from the files of the Service of Clinical Pathology, University Hospital, Geneva, Switzerland (n = 22) and from the Service d’Anatomie Pathologique, Hôpital Cochin, Paris, France (n = 17). Our study was performed in accordance with ethical standards of the Helsinki Declaration of the World Medical Association. The group included 18 male and 21 female patients. The mean age was 51 years (range, 15–81 years). Specimens of surgical duodenopancreatic resections were selected so that both normal pancreas and tumor tissue were present on the same slide. The diagnosis was based on hematoxylin and eosin morphologic characteristics as well as immunohistochemical positivity for endocrine markers such as chromogranin and synaptophysin. Compatible clinical syndromes associated with elevated serum levels of the corresponding hormones were used for the diagnosis of functional tumors. Nonfunctional tumors were not associated with any clinical syndrome, irrespective of the hormone detected in the tumor.

PENS were divided into three groups according to the WHO international histologic classification (3), which was used to establish clinicopathological correlations (Table 1). The first group consisted of 19 benign PENS characterized by a well-differentiated tumor limited to the pancreas with a size of <2 cm, a mitotic count always less than 2 per high-power field (HPF), and no evidence of angioinvasion or metastases. The second group was composed of nine PENs with criteria of uncertain behavior. They were all confined to the pancreas. The diagnosis was based on hematoxylin and eosin morphologic characteristics as well as immunohistochemical positivity for endocrine markers such as chromogranin and synaptophysin. Compatible clinical syndromes associated with elevated serum levels of the corresponding hormones were used for the diagnosis of functional tumors. Nonfunctional tumors were not associated with any clinical syndrome, irrespective of the hormone detected in the tumor.

**Immunostaining.** Immunohistochemical stainings were performed on 10% neutral buffered formalin-fixed, paraffin-embedded specimens. Briefly, serial 3- to 5-μm sections were cut, mounted on silane-coated glass slides and air dried overnight. Sections were deparaffinized, rehydrated, and pretreated with H2O2/methanol to block endogenous peroxidase activity. The following primary antibodies were used: mouse monoclonal antihuman chromogranin A (1:400; Immunotech, Switzerland), mouse monoclonal antihuman insulin (1:500; Biogenex, Basel, Switzerland), rabbit polyclonal antihuman glucagon (1:1,000; DakoCytomation, Basel, Switzerland), mouse monoclonal antihuman serotonin (1:20; DakoCytomation), rabbit polyclonal antihuman glucagon (1:1,000; DakoCytomation), rabbit polyclonal antihuman VIP (1:10; DakoCytomation), rabbit polyclonal antihuman LYVE-1 antibody (ref. 4; 1:100), rabbit polyclonal antihuman podoplanin (ref. 5; 1:1,500), mouse monoclonal antihuman CD31 (1:100; Dako), mouse monoclonal antihuman CD34 antibody (1:10; Serotec, Dottikon, Switzerland), and polyclonal goat antihuman VEGF-C (1:80; R&D Systems, Bad Zurzach, Germany). For anti-chromogranin, anti-VIP, anti-CD31, anti-CD34, anti-LYVE-1 and anti-podoplanin antibodies, microwave treatment was used. Sections were incubated for 1 hour at room temperature with the diluted primary antibodies, which were then revealed by the avidin-biotin-peroxidase complex method (Vectastain; Vector Laboratories, Burlingame, CA). Peroxidase activity was revealed with 3% 3,3′-diaminobenzidine as the chromogen diluted in PBS containing 0.015% H2O2. Sections were weakly counterstained with Mayer’s hematoxylin and mounted in Eukitt. Negative controls included omission of the fist antibodies. For LYVE-1 and VEGF-C immunostaining, antigen-antibody complexes were revealed by means of the rabbit/horseradish peroxidase Envision system (DakoCytomation).

LYVE-1 and podoplanin were considered to be positive when staining was intense and diffuse along endothelium. LYVE-1– and podoplanin-positive vessels with erythrocytes in the lumen were evaluated separately. Lymphatic vessel density (LVD) was determined by counting the number of LYVE-1– or podoplanin-positive vessels per HPF (>400) in the tumor. This included both intratumoral and stromal vessels; however, the vast majority of vessels were stromal. Vessels were counted in 10 randomly selected fields per section by two independent observers. VEGF-C was considered positive when staining was cytoplasmic. Staining was graded semiquantitatively as follows: 0, absent; 1, focal positive isolated tumor cells; 2, 20% to 50% of tumor cells positive; and 3, >50% of tumor cells positive (8).

**Statistical Analysis.** Differences in LVD between insulinomas and other tumors and their relationship to lymph node metastasis, liver metastasis, or angioinvasion were evaluated using the Mann-Whitney test. The Spearman test was used for estimating the relationship between tumor size and LVD. The differences in LVD among the three groups (benign, borderline,
and malignant) were evaluated using the Kruskal-Wallis test. 

\[ P < 0.05 \] was considered to be significant. Tests were performed with the SPSS statistical package (SPSS Base 10.0; SPSS Inc., Chicago, IL).

RESULTS

LYVE-1 and Podoplanin Antibody Specificity. Immunostaining of normal pancreatic and liver tissues was first used to assess the specificity of LYVE-1 and podoplanin for lymphatic vessels. In the pancreas, LYVE-1–positive staining was seen in vessels devoid of red blood cells with an irregular morphology. These vessels were located mainly around negative arteries and veins in the interlobular connective tissue and were sparse in the intralobular connective tissue (Fig. 1A). These vessels were also podoplanin positive (Fig. 1C). LYVE-1– and podoplanin-positive vessels were CD31 positive (Fig. 1B) but CD34 negative. No LYVE-1– or podoplanin-positive vessels were observed within islets of Langerhans (Fig. 2A and B), whereas blood vessels were detected with CD34 (Fig. 2C) and CD31 (Fig. 2D).

In the liver, LYVE-1–positive vessels were observed in large portal tracts (Fig. 3B). Small portal tracts were usually devoid of positive vessels (Fig. 3C). Sinusoidal endothelial cells were also intensely positive, whereas portal arteries and veins and centrolobular veins were negative (Fig. 3B and C). Podoplanin-positive vessels were seen in all portal tracts, independent of their size (Fig. 3A and D). They were generally small in diameter, with several positive vessels in each portal tract, and were often localized near to the interlobular bile duct (Fig. 3D). Sinusoids, portal arteries and veins, and centrolobular veins were negative (Fig. 3A and D).

Lymphatic Vessels in Pancreatic Endocrine Tumors. The 39 PENs included in this study were classified according to their pathological characteristics (Table 1). The first group consisted of 19 benign well-differentiated PENs. Sixteen were insulinomas, one was a glucagonoma, and two were nonfunctioning tumors. The second group demonstrated criteria of uncertain behavior and consisted of nine cases. They corresponded to one insulinoma, one gastrinoma, one serotoninoma, and six nonfunctioning tumors. The third group consisted of 11 well-differentiated endocrine carcinomas. Among this group, one secreted glucagon, two secreted gastrin, and eight were nonfunctioning tumors. No significant differences were observed in sex and age between the different groups (\( P > 0.05 \)).

To characterize lymphatic vessels in pancreatic endo-

Fig. 1 Immunostaining of normal pancreas. LYVE-1–positive staining (A) and podoplanin-positive staining (C) were seen in vessels devoid of red blood cells. These vessels were located mainly around negative arteries and veins in the interlobular connective tissue. LYVE-1– and podoplanin-positive vessels were also CD31 positive (B). Magnification of \( A-C \), ×100.
crine tumors, we performed immunostaining with antibodies to LYVE-1 and podoplanin. The results are summarized in Table 2. LYVE-1- and podoplanin-positive vessels were always seen in the surrounding normal pancreas. In the PENs, positive lymphatic vessels were variably observed within the intratumoral supporting connective tissue (stroma) next to negative blood vessels (Fig. 4A) or dispersed between the tumor cells in close contact with them (Fig. 4B and C). These vessels were often similar in shape and size to normal pancreatic vessels. Occasionally, they were distinct, with a smaller, thin or ill-defined lumen (Fig. 4C). Lymphatic vessels were randomly distributed within the tumor without hot spots. Occasionally, positive vessels contained tumor cells, which is indicative of lymphatic invasion (Fig. 4D). Vessels positive for LYVE-1 or podoplanin were negative for CD34 on serial sections.

With respect to LYVE-1 immunoreactivity (Table 2), only 4 insulinomas of 19 benign tumors had positively staining vessels that were localized exclusively in the tumor stroma; in these four cases, average LVD was 0.14 vessel per HPF (range, 0.1–0.2 vessel per HPF). In borderline tumors, four of nine had positive vessels in the tumor stroma; in these cases, the average LVD was 0.15 vessel per HPF (range, 0.1–0.3 vessel per HPF); no positive intratumoral vessels were seen in any of the cases. In well-differentiated carcinomas, 5 of 11 cases had positive tumor stromal vessels. Most strikingly, three of these cases contained positive intratumoral vessels. In these positive cases, the average LVD was 0.6 vessel per HPF (range, 0.1–1.2 vessel(s) per HPF).

With respect to podoplanin immunoreactivity (Table 2), all benign PENs had positive vessels in the tumor stroma. In addition, 8 of 19 benign tumors had positive intratumoral vessels. The average LVD here was 1 vessel per HPF (range, 0.3–2.4 vessel(s) per HPF). In the borderline tumors, all PENs had positive vessels in the tumor stroma, and one of nine had positive intratumoral vessels. The average LVD here was 0.7 vessel per HPF (range, 0.2–1.4 vessel(s) per HPF). In well-differentiated carcinomas, all had positive vessels in the tumor stroma, and 6 of 11 had positive intratumoral vessels. The average LVD here was 2.5 vessels per HPF (range, 0.7–3.1 vessel(s) per HPF).

Correlations between LVD and WHO Classification. The WHO classification separates PENs into different categories according to their clinical behavior. LVD evaluated by
podoplanin immunostaining was significantly higher in welldifferentiated endocrine carcinomas than in borderline or benign tumors ($P = 0.009$, Kruskal-Wallis test). No correlation was found when LVD was evaluated by LYVE-1 immunostaining ($P = 0.204$, Kruskal-Wallis test). The different histoprognostic features defining the WHO classification for PENs were then compared to LVD (Table 3). LVD evaluated by podoplanin immunostaining (Table 3) was significantly correlated to the size of the tumor in benign PENs ($r = 0.589; P = 0.008$). LVD was not correlated to size in borderline tumors ($r = 0.452; P > 0.05$) or carcinomas ($r = -0.09; P > 0.05$). No correlation was present between LVD and size in the benign group ($r = 0.191; P > 0.05$), borderline group ($r = -0.421; P > 0.05$), or carcinomas ($r = 0.05; P > 0.05$) when LYVE-1 immunostaining was considered (Table 3). There was no relationship between the image of angioinvasion and LVD with podoplanin immunostaining.

Fig. 3 Immunostaining of normal liver. LYVE-1–positive vessels (B) were observed in large portal tracts (PT). Small portal tracts were usually devoid of positive vessels (C). Sinusoidal endothelial cells in the lobules (L) were also intensely positive (B and C). Podoplanin-positive vessels were seen in all portal tracts, independent of their size (A and D). Sinusoids in the lobules were negative (A and D). In the tumor metastasis (T) seen in this image, positive vessels were identified with podoplanin (B) but not with LYVE-1 (B). Magnification: A and B, $\times 100$; C and D, $\times 400$. 
(P = 0.488) or LYVE-1 (P = 0.346) immunostaining. There was no relationship between the presence of lymph node metastasis and LVD with podoplanin (P = 0.07) or LYVE-1 (P = 0.635). There was a modest correlation between the presence of liver metastasis and LVD with podoplanin (P = 0.02) and LYVE-1 (P = 0.019).

**Inflammation/Stroma.** LYVE-1– and podoplanin-positive vessels were occasionally seen to contain red blood cells in their lumen. These positive vessels were essentially observed in areas of inflammation in peritumoral pancreatic tissue and corresponded to venules (data not shown).

**Table 2.** LYVE-1 and podoplanin expression in pancreatic endocrine tumors

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<th>LYVE-1–positive staining</th>
<th>Podoplanin-positive staining</th>
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<tr>
<td></td>
<td>Vessels in normal pancreas</td>
<td>Intratumoral stromal vessels</td>
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<tr>
<td></td>
<td>Vessels in normal pancreas</td>
<td>Intratumoral stromal vessels</td>
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<td>Benign tumor</td>
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**VEGF-C Expression in PENs.** Nineteen of 39 tumors demonstrated VEGF-C–positive immunolabeling in the cytoplasm of tumor cells (Fig. 5). With respect to different tumor behavior categories, 4 of 19 benign tumors had positive tumor cells (Table 4). Only 1 of the 16 insulinomas was positive, whereas the glucagonoma and 2 nonfunctioning tumors expressed VEGF-C. Expression was present in 20% to 50% of cells in one case and was diffuse in the three other cases (Fig. 5). In borderline tumors, six of nine tumors had positive VEGF-C expression in tumor cells. VEGF-C expression was limited to scattered cells in two cases (Fig. 5), present in 20% to 50% of...
cells in three cases, and diffuse in one case. In well-differentiated carcinomas, 9 of 11 cases had positive tumor cells. VEGF-C expression was limited to scattered cells in one case, present in 20% to 50% of the cells in five cases, and diffuse in three cases.

VEGF-C was more frequently expressed in carcinomas when compared with borderline or benign tumors ($P < 0.003$; Kruskal-Wallis). There was no correlation between the intensity of VEGF-C expression and clinical behavior ($P = 0.4$; Kruskal-Wallis test). There was no correlation between VEGF-C expression and LVD as evaluated by podoplanin ($P = 0.355$) and LYVE-1 ($P = 0.1$) immunostaining.

### DISCUSSION

Tumor cell spread via lymphatic capillaries is an early feature of many carcinomas, and the presence of metastases in regional lymph nodes is critical for the staging of these tumors and for determining prognosis and subsequent therapeutic modalities. Unlike pancreatic adenocarcinomas, PENs variably metastasize to regional lymph nodes and may occasionally metastasize at first to the liver. The staging of these tumors takes this into account and does not rely only on lymph node status (metastatic versus nonmetastatic). The reason for this characteristic behavior has not been established. Until recently, tumor

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<th>Correlation between LVD (as evaluated by LYVE-1 and podoplanin immunostaining) and histological data</th>
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<tr>
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<td>LYVE-1–positive staining</td>
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<td>Median size (cm)</td>
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<td>Mitotic counts &gt; 2 per HPF</td>
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<td>Lymph node metastasis</td>
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<td>Liver metastasis</td>
<td>NA</td>
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<td>Total</td>
<td>19</td>
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Abbreviation: NA, not available.

Fig. 5 In PENs, VEGF-C–positive immunolabeling was detected in the cytoplasm of tumor cells. Expression was focal (A) or diffuse (B). Magnification, ×400.
lymphatics dispersed between tumor endocrine cells. The presence of these lymphatics highlights the fact that lymphangiogenesis in human PENs is a phenomenon that already occurs in benign tumors and is insufficient to promote the development of endocrine malignancy. Furthermore, LVD was related to the clinical behavior of the tumors. In fact, our study illustrates a great variability in LVD among PENs. LVD quantitated by podoplanin (but not LYVE-1) immunostaining was significantly higher in well-differentiated endocrine carcinomas than in benign or borderline PENs. LVD as assessed by podoplanin and LYVE-1 immunostaining correlated modestly with the presence of liver metastasis. Remarkably, however, the density of lymphatic vessels was unrelated to the image of angioinvasion or lymph node metastasis. These findings strongly suggest that factors other than mere access to an increasing number of lymphatic vessels are required for some tumors to metastasize to regional lymph nodes.

Our results on PENs are in accordance with reports from others on carcinoma of head and neck (10–12), thyroid (13), and malignant melanomas (14), in which proliferating intratumoral lymphatics and LVD have been identified as a markers of poor prognosis. Our study is in contrast to results on breast cancer, in which metastatic dissemination appears to occur in the absence of lymphangiogenesis and the LVD was inversely correlated to tumor aggressiveness (15). Our present observations, combined with our previous report on murine insulinomas, point to a major role for peritumoral/stromal vessels in tumor cell dissemination. The notion that intratumoral lymphatics, which were relatively rare in human tumors and absent from murine tumors, are nonfunctional was not addressed in this study.

Few lymphangiogenic factors have been identified thus far. The best characterized are members of the VEGF family. In particular, VEGF-C has been shown to promote lymphangiogenesis both in vitro and in vivo through binding to the VEGF-C tyrosine kinase receptor, VEGFR-3 (16), which, in normal adult tissues, is expressed on lymphatic endothelium (6). The possibility that VEGF-C might promote tumor lymphangiogenesis has received much attention. Several studies on animal models have directly demonstrated the existence of tumor lymphangiogenesis (17). However, there is as yet little evidence for direct lymphangiogenesis in human tumors. Nonetheless, a number of clinical studies have reported VEGF-C expression in human tumors and illustrated a significant association between VEGF-C levels of primary tumors and lymph node metastasis (11, 18–28). This suggests that although VEGF-C may induce tumor lymphangiogenesis, it is also likely to have other functions. These include angiogenesis and the autocrine stimulation of tumor cell growth.

In addition, and of specific interest to our studies, is the hypothesis that VEGF-C could activate preexisting lymphatics, which in turn become directly involved in tumor cell chemotaxis, intralymphatic infiltration, and distant dissemination. This
would imply the existence of a reciprocal dialogue between tumor and lymphatic endothelial cells, which leads to the formation of lymph node metastases. Normal endocrine cells constitutively express VEGF (6). In PENs, VEGF expression is maintained, but at variable levels (6, 29). To evaluate the role of VEGF-C in the metastatic process, immunohistochemistry was performed. In our series, VEGF-C was variably expressed in PENs, and VEGF-C expression was correlated to malignant clinical behavior. A similar variable expression of VEGF-C in PENs has recently been reported by another group (30). Taken together with our observation that LVD is unrelated to lymph node metastasis, these data point to the requirement for a second element for the development of lymphogenous metastases, namely, activities of VEGF-C that are unrelated to lymphangiogenesis. This mechanism is illustrated in the WHO classification, in which the presence of angioinvasion determines uncertain behavior but does not imply a malignant metastatic state.

Our data on LVD contrast with the results on blood vessel intratumoral density on PENs evaluated by CD34 immunostaining, in which microvascular density was inversely correlated with behavior (29). Moreover, these authors report a lower VEGF expression in endocrine carcinomas compared with a higher expression in benign well-differentiated endocrine tumors; this is also in contrast with our observation of an increase in VEGF-C expression with malignant evolution. Taken together, these results suggest the existence of a switch from a blood vessel angiogenic to a lymphangiogenic state during the evolution of PENs toward malignancy, possibly through modification of levels of expression of members of the VEGF family. This possible angiogenic switch has been illustrated in the adenoma–carcinoma sequence during colorectal cancer progression (31). These results also suggest the existence of lymphatic drainage from the pancreas to the liver that would play a role in the development of liver metastasis.

The intratumoral localization of lymphatics in our human PEN series is in contrast to the double transgenic RipTag2 × RipVEGF-C mouse model combining pancreatic endocrine tumorigenesis and lymphangiogenesis (2), in which VEGF-C induced lymphatics surrounding the tumor nodule but did not extend into the tumor itself. However, with respect to the other variables, our results in human PENs are similar to this animal model: peritumoral lymphatics and the image of intralymphatic tumoral angioinvasion were occasionally seen in RipTag2 mice, but lymph node metastases were never seen in these animals. The VEGF-C–induced increase in peritumoral lymphatic density in double transgenic RipTag2 × RipVEGF-C mice resulted in dissemination of tumor cells to regional lymph nodes in approximately 40% of animals. It is noteworthy that this animal lymphangiogenesis model based on VEGF-C overexpression highlights, in a manner similar to our results in humans, the important role of VEGF-C in the development of a malignant phenotype via a possible two-step mechanism.

In conclusion, tumor cell metastasis to lymph nodes is a key event in disease outcome and is frequently used as a prognostic factor. Our findings strongly suggest that lymphangiogenesis occurs in human PENs and that LVD is correlated to clinical behavior, but not to lymph node status. Factors other than mere access to an increasing number of lymphatic vessels may thus be required for some tumors to metastasize to regional lymph nodes. LVD is correlated to liver metastasis, suggesting the existence of a lymphatic drainage from the pancreas to the liver. Moreover, we provide evidence for a role for VEGF-C in the development of endocrine malignancy. Our results also confirm the validity of double transgenic Rip1Tag2 × RipVEGF-C mice as a model of human PENs, in which the mechanisms of lymphangiogenesis and lymphatic tumor metastasis can be dissected and in which potential inhibitors of these processes can be tested.

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Clinical Cancer Research

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