Podoplanin expression in advanced-stage gastric carcinoma and prognostic value of lymphatic microvessel density

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In gastric cancer, lymph node metastasis is a major prognostic factor. Tumor lymphangiogenesis promotes metastasis in experimental models, but in human tumors data about the presence and clinical significance of lymphatic vessels in the tumor area are controversial. We investigated 70 patients with advanced-stage gastric carcinoma and the pathological examination showed 40 cases with intestinal subtype and 30 cases with diffuse subtype. Forty three from 70 cases had regional lymph node metastasis. Additional slides were stained with an antibody against podoplanin, and lymphatic microvessel density (LMVD) was evaluated in the tumoral and peritumoral areas. Lymphatic vessels were identified in tumor area in all cases and LMVD was higher in the peritumoral than in the tumor area. Podoplanin-positive vessels in tumor area were usually small, with narrow lumen. A significant correlation was found between LMVD and stage of the tumor ($p<0.002$) and lymph node metastasis ($p<0.031$), but not with the pathological subtype and grade of the tumor. We found tumor cells in the lumen of lymphatic vessels in 11 cases, whereas tumor cells expressing podoplanin were found in 4 cases of less differentiated diffuse subtype gastric carcinoma. In conclusion, our results suggest that LMVD predicts tumor stage and lymph node metastasis, and podoplanin-positive tumor cells select a subgroup of tumors with high potential of invasion and metastasis.

Key words: gastric cancer, podoplanin, lymphatic microvessel density (LMVD), lymphangiogenesis, prognosis.

The management of patients with gastric cancer still represents a problem in terms of therapy, mainly because the large majority is admitted with advanced stage tumors. For these reasons, despite all efforts in the field of surgical treatment and chemotherapy, the prognosis is poor, with a very low rate of survival at 5 years. One of the best clinical criteria to estimate prognosis in patients with gastric cancer is the existence or absence of metastasis in regional lymph nodes, essential to establish the therapeutic protocols and to predict the outcome [1].

Invasion of lymphatic vessels by the primary tumor is a well known parameter of unfavorable prognosis that significantly increases the risk for lymph node and systemic metastasis [2]. Despite the clinical importance of this finding, studies on lymphangiogenesis in gastric tumors are scarce in the literature, and usually restricted to microvessel counts. This is probably due to the reduced number of specific markers for the lymphatic endothelium. On the other hand, in last years some markers with higher sensitivity for lymphatic endothelium, such as VEGFR3, Lyve-1, and podoplanin (D2-40), have been introduced [3].

Podoplanin was originally detected on the surface of rat podocytes, as a 38 kDa membrane mucoprotein linked to the flattening of foot processes in puromycin-induced nephrosis [4], a rat model of human minimal change nephropathy, with extensive flattening of podocyte foot processes and severe proteinuria, associated with a significant decrease of podoplanin.

Podoplanin belongs to the family of type-1 transmembrane sialomucin-like glycoproteins. A C-type lectin-like receptor-2 (CLEC-2) was identified as an endogenous receptor of podoplanin on platelets [5] and this interaction may regulate tumor invasion and metastasis. Podoplanin is a sialoprotein located on the surface of podocytes, playing an important role in preventing cellular adhesions and the membrane pattern of

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a) b)

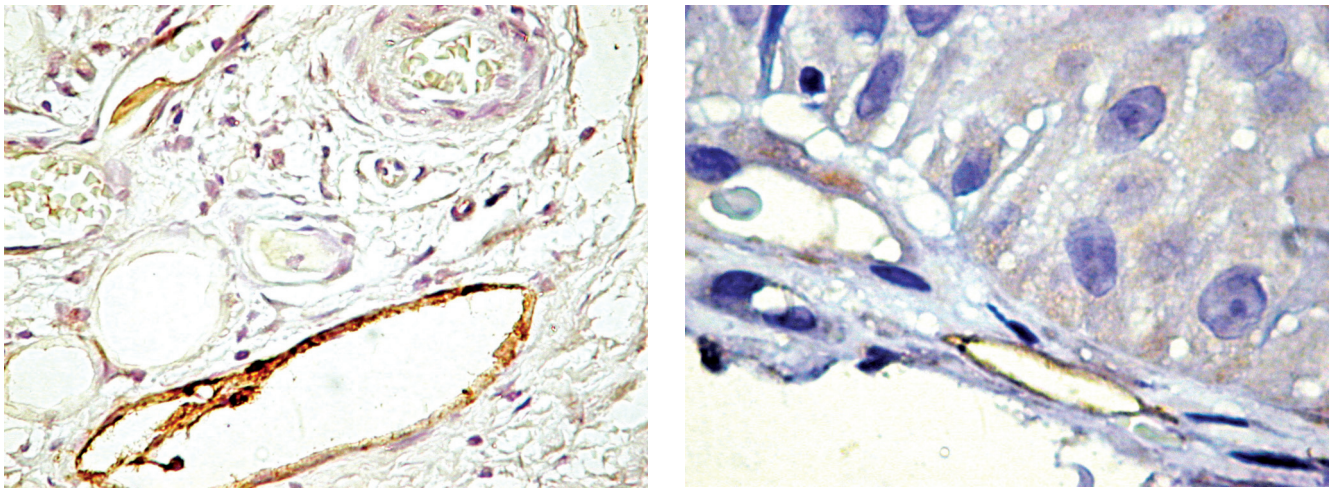


Figure 1. Control cases. Podoplanin is expressed by the lymphatic endothelium but not by the blood vessels' endothelium in the gastric submucosa (a) and stroma of the oral squamous cell carcinoma (b). x400.

podoplanin immunohistochemical reaction supports this function. Podoplanin is crucially involved in lymphatic vessels formation [6], but the mechanism by which podoplanin regulates this process remains unknown.

Data about the relationship between lymphatic microvessel density (LMVD) and conventional factors of prognosis are controversial. The mechanism(s) by which tumor cells enter the lymphatic vessels is largely unknown. Two hypotheses were proposed: invasion of preexisting vessels and formation of new lymphatics (true lymphangiogenesis). It was not yet demonstrated the origin of newborn lymphatics and if these vessels have proliferative potential.

In spite of the major importance in gastric cancer, lymphangiogenesis has more unknown aspects than angiogenesis. Here, we investigated the expression of the lymphatic endothelium marker podoplanin in gastric cancer and the prognostic value of LMVD.

Patients and methods

Seventy patients admitted with gastric carcinoma T2-T4 (16, 42, and respectively 12) were investigated. All patients have been subjected to gastrectomy and regional lymph node dissection. Specimens removed during the surgical procedure were processed using the standard histological technique. Step sections, 5µm thick, were stained with haematoxylin-eosin for the pathological diagnosis and grading (G). There were 18 G1 cases, 34 G2 cases and 18 G3 cases. The pathologic diagnosis was of intestinal-type carcinoma in 40 cases and diffuse type in 30 cases.

Additional slides were stained with anti-podoplanin antibody, clone 18H5, dilution 1:500, treated by microwave for antigen retrieval in buffer phosphate pH6 (10 minutes), using the technique previously described [7]. The working system was LSAB+, diaminobenzidine was used as chromogen, and nuclei were stained with Lillie's modified haematoxylin. All reagent used in this study were from DakoCytomation (Glostrup, Denmark), excepting for podoplanin (Abcam, Cambridge, United Kingdom). To validate the specificity of the immunohistochemical method for podoplanin, we tested the reaction on sections from 5 cases with gastric ulcer and 5 with oral squamous cell carcinoma. Slides from these control cases were submitted to the same immunohistochemical procedure. Microscopic evaluation was performed with Nikon Eclipse i80 and images were acquired with digital camera as JPEG format. LMVD was calculated at x 200 magnification based on the "hot spots" system previously proposed by Weidner [8], and adopted by the first international consensus conference on the methodology of lymphangiogenesis quantification [9]. Paired *t* Student test was used for statistical analysis, and values <0.05 were considered as significant.

Results

Podoplanin-positive lymphatic vessels were found in all control cases and the positive reaction was restricted to the lymphatic endothelium and no staining was found in the blood vessels endothelium in both gastric ulcers *(fig.1a)* and squamous cell carcinoma *(fig.1b)*. This result showed that podoplanin-positive vessels are lymphatics, and assures reproducibility of lymphatic microvessel count. No epithelial cells of the gastric mucosa stained for podoplanin were found in patients with gastric ulcer.

In gastric tumors, lymphatic vessels identified with podoplanin were different in size and shape, with thin wall, without perivascular cells, and were found in both tumor and peritumoral areas. In normal tissue at distance from the tumor, lymphatics had regular borders, without content in the

lumen *(fig.2).* Occasionally, we found positive reaction in scattered macrophages of the stroma, an aspect that is reported in the literature, and it can be used as internal positive control. The pattern of the immunohistochemical reaction was cytoplasmic diffuse, without membrane enhancement.

We found lymphatic vessels in tumor area, located between nests and cords of malignant cells. Their size and shape was variable from one case to another and even in the same microscopic field from the same case. Many podoplanin-positive vessels were small, with narrow lumen and located in the close vicinity of tumor cells *(fig.3a).* Lymphatics with these morphological aspects were most frequently recognized in the tumor area, representing 54,24% of the cases. Large vessels from the tumor area were often irregular in shape with one or more small branches *(fig.3b).* In the majority of the cases, tumor cells were closely attached to the outer side of the wall.

The presence of tumor cells in the lumen of podoplanin-positive vessels was found in 11 from 70 cases. In these cases, tumor cells were negative to podoplanin, while endothelium was intensely stained for podoplanin. The outer side of tumor cells was coated with a thin rim of podoplanin-positive material *(fig.4),* that signals out the potential of podoplanin to stimulate invasion and metastases.

In the next step we evaluated the LMVD in both tumoral and peritumoral areas. Results were correlated with the stage of the tumor, pathological subtype, grade, and lymph node metastasis. We found no significant correlation between the number of lymphatics and pathological subtype and grade of the tumor ($p<0.1$, and $p<0.23$, respectively). A significant correlation was found with the stage of the tumor ($p<0.002$) and presence of metastasis in regional lymph nodes ($p<0.031$). Actually, in all cases with lymph node metastasis a high LMVD of podoplanin-positive vessels was found mainly in the tumor area, but a significant increase was also found in peritumoral tissue. Data concerning LMVD in tumoral and peritumoral areas are shown in Table 1.

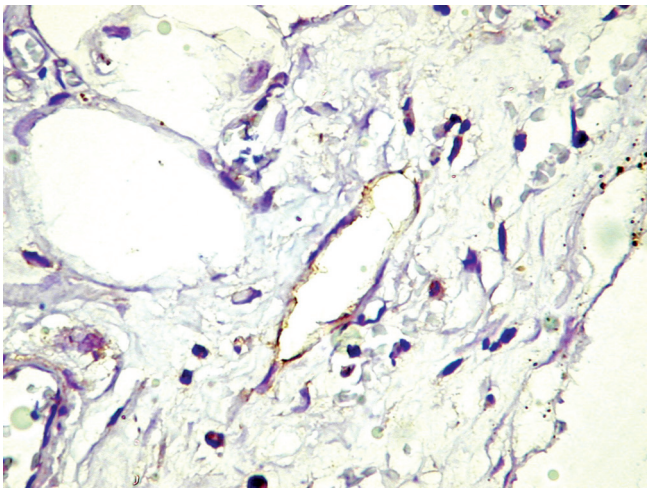


Figure 2. Lymphatic vessel with endothelium positive for podoplanin. Close to the vessel can be noticed some positive macrophages. All other components of the connective tissue are negative. Anti-podoplanin, x400.

Table 1. Lymphatic microvessel density (LMVD) evaluated as podoplanin-positive microvessels related to the tumor stage in tumor and in peritumoral areas

Tumor stage (T)	LMVD in tumor area	LMVD in peritumoral area	Total LMVD
T2 (n=7)	4.3	5.8	10.1
T3 (n=36)	7.2	9.6	16.8
T4 (n=27)	14.22	18.2	32.4
Total (n=70)	29.29	40.7	70.6

a)

b)

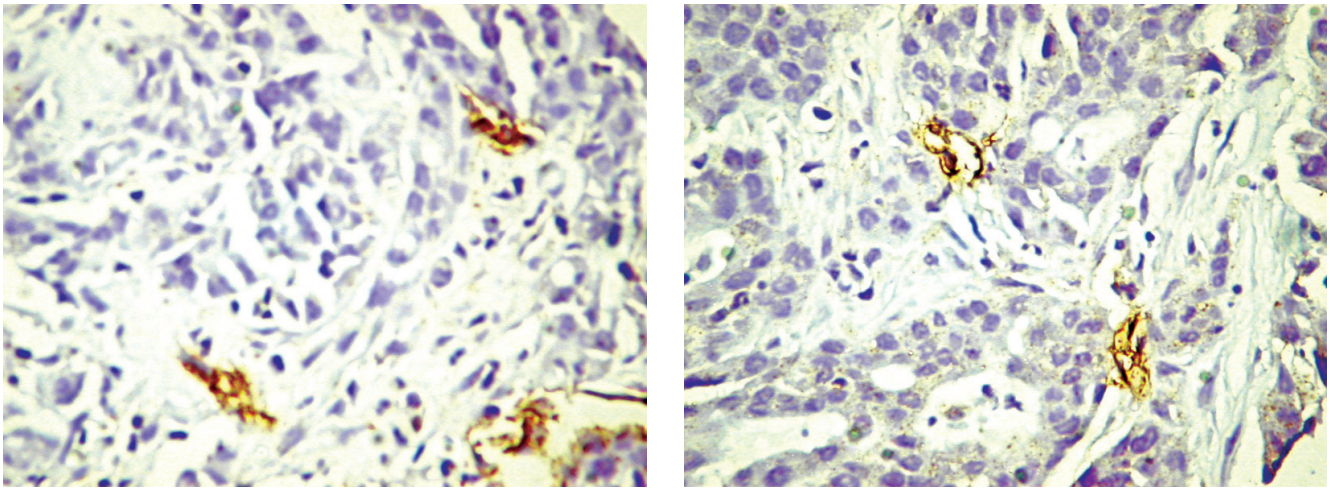


Figure 3. Podoplanin positive vessels in tumor area. Small size lymphatics with narrow lumen (a), Lymphatics in contact with tumor cells (b).

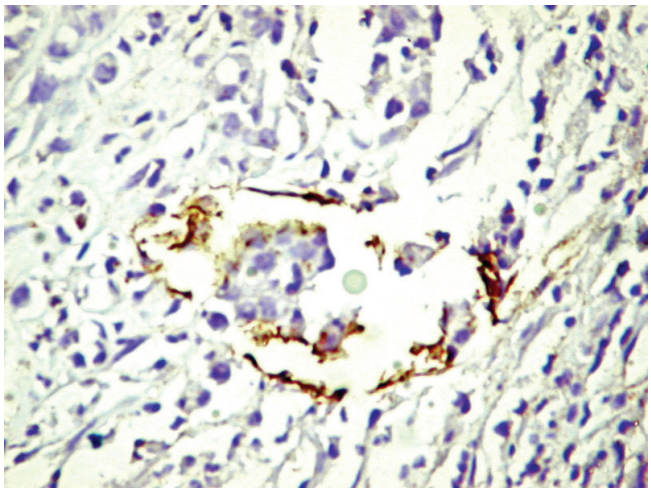


Figure 4. Tumor cells in the lumen of a podoplanin-positive vessel. Tumor cells show a thin layer of positive material. Note the discontinuous layer of endothelial cells.

We found a significant increase in LMVD in both tumoral and peritumoral areas parallel to the stage of the tumor. On the other hand, it must be mentioned that the number of podoplanin-positive vessels is higher in the peritumoral than in the tumor area. Based on these data, we suggest that LMVD might have a predictive value for the stage of the tumor. 43 of the cases included in the study had regional lymph node metastases. Data concerning the relation between LMVD and the lymph node status are shown in Table 2. It can be noticed the close correlation between the number of lymphatics in tumoral and peritumoral areas and presence of lymph node metastasis.

Table 2. Relationship between LMVD and lymph node status in tumor and peritumoral areas

Lymph node status	LMVD in tumor area	LMVD in peritumoral area
N+ (n=43)	12.4	17.8
N- (n=27)	4.1	6.8

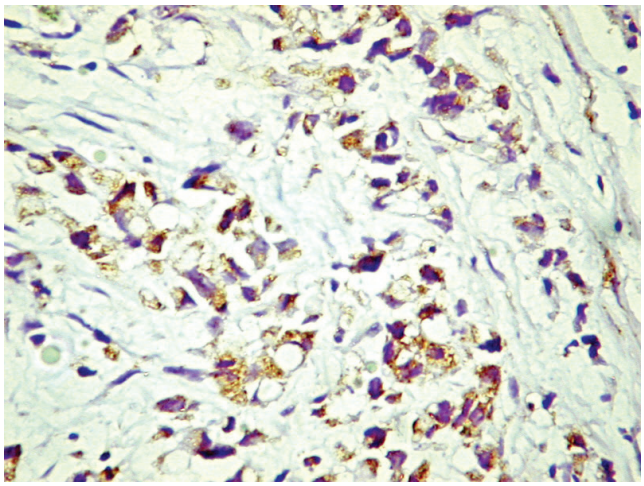
One particular aspect, not found in the literature, was noticed in 4 less differentiated G3 tumors, in which tumor cells expressed podoplanin with cytoplasmic granular pattern. The immunoreaction was heterogeneous, moderate or strong, and more evident at the border with the tumor stroma *(fig.5a).* The reaction was intensely positive in the majority of tumor cells in two cases of diffuse subtype *(fig.5b).*

Discussion

Specific markers of the endothelial cells of lymphatic vessels have multiple functions in normal and pathological conditions, which are useful to investigate changes in the tumor tissue correlated with lymphangiogenesis, and to identify new therapeutic strategies. Some questions related to tumor lymphangiogenesis are still unanswered, and these include mechanism of migration of tumor cells into lymphatics that is crucial in development of metastases, and differences between preexisting and newly formed lymphatics. Available data about the proliferative character of lymphatics are poor and the significance of their presence in tumor area is still controversial. The results of this study show podoplanin-positive vessels in tumor area in all cases of gastric carcinoma examined and we found a significant correlation with tumor stage and lymph node metastases.

Our results showed that podoplanin is expressed by the lymphatic endothelium but not by the blood vessels' endot-

a)



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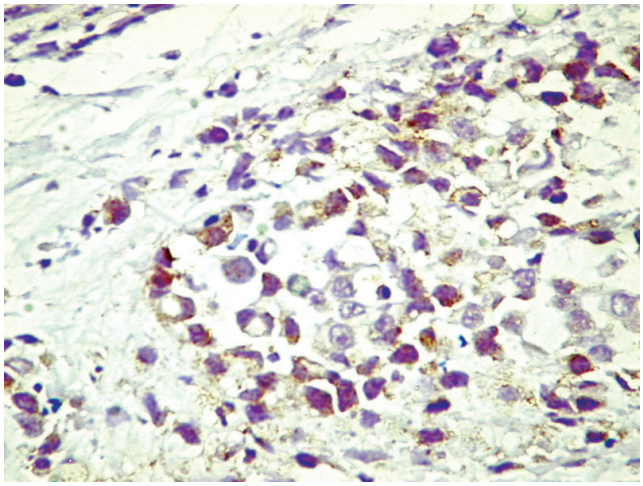

Figure 5. Tumor cells positive for podoplanin, with cytoplasmic granular pattern (a). Numerous podoplanin-positive cells in diffuse gastric carcinoma (b).

helium. Unless entirely specific, podoplanin expression can discriminate between blood and lymphatic vessels. The immunohistochemical identification of podoplanin was performed in order to evaluate the relationship between LMVD and clinical prognostic factors and to demonstrate the presence of tumor cells within lymphatics and we found these findings in 11 cases. Other authors used podoplanin in the immunophenotyping of some vascular tumors or to evaluate lymphangiogenesis in different types of carcinoma [10, 11, 12, 13, 14].

Podoplanin consists of an extracellular domain, a small transmembrane portion and a cytoplasmic tail, which is the active site for protein kinase C. Kato et al [15] showed that EDxxVTPG segment of the extracellular domain is important for the activity of podoplanin, and threonin residues are crucial for platelet aggregation. The interaction between the endogenous receptor CLEC-2 and podoplanin seems to contribute to invasion and metastases [5, 16]. It was demonstrated that podoplanin mediates remodeling of the actin cytoskeleton of tumor cells, and some authors hypothesized that this change could be involved in tumor progression [17, 18]. These data are supported by our observations, which showed tumor cells within lymphatic vessels coated by a thin rim of podoplanin-positive material. Podoplanin expressed on the surface of tumor cell within the lymphatic lumen could induce platelet aggregation by interaction with CLEC-2. Kato et al [16] showed that CLEC-2-Fc inhibits platelet aggregation induced by podoplanin. Based on these data, both podoplanin and its specific receptor can be potential targets for metastases treatment.

Podoplanin is constantly expressed by the lymphatic endothelium and not by the blood vessel endothelial cells, as we noticed in the present study. The high sensitivity and specificity allows us to count lymphatics in tumoral and peritumoral areas. Lymphatic vessels in the tumor area were also detected by others in pancreatic endocrine tumors and their density correlates with the expression of VEGF-C (vascular endothelial growth factor-C) [19]. To the best of our knowledge, this is the first report on the presence of lymphatics in tumor area in gastric carcinoma, and we demonstrated the increase of LMVD as the stage of the tumor increases. On this basis, at the present is not possible to establish if these vessels are preexisting, or newly formed vessels. On the other hand, an active lymphangiogenesis might be found in human tumors; in fact Shimizu et al [20] demonstrated in an experimental model the inhibitory effect of anti-VEGFR-3 on regional metastasis and reduction of LMVD. Lymphatics from the peritumoral area are significantly larger than those found in the tumor area, and their density is significantly lower in tumors of the pancreas [3]. Here, we have confirmed that peritumoral lymphatics were larger and their density was higher than in tumor area.

Podoplanin is not an entirely specific marker for lymphatic endothelium. In last years, it was shown that podoplanin is expressed not only by the lymphatic endothelium, but also by some human malignant tumors, like squamous cell carcinoma [21, 22, 23], malignant mesothelioma [24, 25], germ cell tumors [26], some brain tumors [27], and some subtypes of vascular tumors [4]. In this study, we found for the first time that podoplanin was expressed in tumor cells of four cases of gastric carcinoma, all of diffuse subtype and less differentiated, with regional lymph node metastases. Although the number of positive cases is low, our data suggest that podoplanin expression in tumor cells correlates with high grade of the tumors, invasion and lymph node metastases. Our data support recent evidence, which proposed a role for podoplanin in invasion and metastasis [28, 29], based on the strong expression of podoplanin in tumor cells of cases with lymph node metastasis and mainly in tumor cells at the interface with tumor stroma [30].

In conclusion, our immunohistochemical study of podoplanin expression in advanced-stage human gastric carcinoma showed high sensitivity of this marker for lymphatic endothelium, but its moderate specificity. Lymphatic vessels were detectable in both tumor and peritumoral areas. Evaluation of LMVD has prognostic value in gastric carcinoma and correlates with stage of the tumor and lymph node status and our results support the evaluation of LMVD in both tumoral and peritumoral areas. LMVD do not correlate nor with the grade of the tumor neither with the pathological subtype of gastric carcinoma. Finally, the immunohistochemical expression of podoplanin in tumor cells as a cytoplasmic, granular product of reaction seems to be of worst prognosis, and correlates with less differentiated tumors and might have a potential role in invasion and metastasis.

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References

[1] TOBLER N, DETMAR M . Tumor and lymph node lymphangiogenesis – impact on cancer metastasis. J Leukoc Biol, 2006, 80: 691–696.

[2] NAKAMURA Y, YASOUKA H, TSUJIMOTO M et al. Importance of lymph vessels in gastric cancer: a prognostic indicator in general and a predictor for lymph node metastasis in early stage cancer. J Clin Pathol, 2006, 59: 77–82.

[3] JI RC, ESHITA Y, KATO S. Investigation of intratumoural and peritumoral lymphatics expressed by podoplanin and LYVE-1 in the hybridoma-induced tumours. Int J Exp Path, 2007, 88: 257–270.

[4] BREITENEDER-GELEFF S, MATSUI K, SOLEIMAN A et al. Podoplanin, novel 43 kDa membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol, 1997, 151: 1141–1152.

[5] SUZUKI-INOUE K, KATO Y, INOUE O et al. Involvement of the snake toxin receptor CLEC-2 in podoplanin-mediated platelet activation by cancer cells. J Biol Chem, 2007, 282: 25993–26001.

[6] SCHACHT V, RAMIREZ MI, HONG YK et al. T1α/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. EMBO J, 2003, 22: 3546–3556.

[7] CÎMPEAN AM, RAICA M, IZVERNARIU D et al. Lymphatic vessels identified with podoplanin. Comparison of immunostaining with three different detection systems. Rom J Morphol Embryol, 2007, 48: 139–143.

[8] WEIDNER N, SEMPLE JP, WELCH WR et al. Tumor angiogenesis and metastases: correlation in invasive breast carcinoma. N Engl J Med, 1991, 324: 1–8.

[9] VAN DER AUWERA I, CAO Y, TILLE JC et al. First international consensus on the methodology of lymphangiogenesis quantification in solid human tumours. Br J Cancer, 2006, 95: 1611–1625.

[10] SINZELLE E, VAN HUYEN JP, BREITENEDER GELEFF S et al. Intrapericardial lymphangioma with podoplanin immunohistochemical characterization of lymphatic endothelial cells. Histopathology, 2000, 37: 93–94.

[11] BIRNER P, OBERMAIR A, SCHINDL M et al. Selective immunohistochemical staining of blood and lymphatic vessels reveals independent prognostic influence of blood and lymphatic vessel invasion in early-stage cervical cancer. Clin Cancer Res, 2001, 7: 93–97.

[12] CUNNICK GH, JIANG WG, GOMEZ KF et al. Lymphangiogenesis and breast cancer metastasis. Histol Histopathol, 2002, 17: 863–870.

[13] SEDIVY R, BECK-MANNAGETTA J, HAVERKAMPF C et al. Expression of vascular endothelial growth factor-C correlates with the lymphatic microvessel density and the nodal status in oral squamous cell cancer. J Oral Pathol Med, 2003, 32: 455–460.

[14] STRAUME O, JACKSON DG, AKSLEN LA. Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. Clin Cancer Res, 2003, 9: 250–256.

[15] KATO Y, FUJITA N, KUNITA A et al. Molecular identification of Aggrus/T1α as a platelet aggregation-inducing factor expressed in colorectal tumors. J Biol Chem, 2003, 278: 51599–51605.

[16] KATO Y, KANEKO MK, KUNITA A et al. Molecular analysis of the pathophysiological binding of the platelet aggregation-inducing factor podoplanin to the C-type lectin-like receptor CLEC-2.Cancer Sci, 2007, 3: 1–8.

[17] WICKI A, CHRISTOFORI G. The potential role of podoplanin in tumour invasion. Br J Cancer, 2007, 96: 1–5.

[18] MOUSTAKAS A, HELDIN CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci, 2007, 98: 1512–1520.

[19] RUBBIA-BRANDT L, TERRIS B, GIOSTRA E et al. Lymphatic vessel density and vascular endothelial growth factor-C expression correlate with malignant behavior in human pancreatic endocrine tumors. Clin Cancer Res, 2004, 10: 6919–6928.

[20] SHIMIZU K, KUBO H, YAMAGUCHI K et al. Supression of VEGFR-3 signalling inhibits lymph node metastasis in gastric cancer. Cancer Sci, 2004, 95: 328–333.

[21] KATO Y, KANEKO M, SATA M et al. Enhanced expression of Aggrus (T1α/podoplanin), a platelet aggregation-inducing factor in lung squamous cell carcinoma. Tumor Biol, 2005, 26: 195–200.

[22] MARTIN-VILLAR E, SCHOLL FG, GAMALLO C et al. Characterization of human PA2.26 antigen (T1α-2, podoplanin) a small membrane mucin induced in oral squamous cell carcinomas. Int J Cancer, 2005, 113: 899–910.

[23] WICKI A, LEHEMBRE F, WICK N et al. Tumor invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. Cancer Cell, 2006, 9: 261–272.

[24] KIMURA N, KIMURA I. Podoplanin as a marker for mesothelioma. Pathol Int, 2005, 55: 83–86.

[25] ORDONEZ NG. The diagnostic utility of immunohistochemistry and electron microscopy in distinguishing between peritoneal mesotheliomas and serous carcinomas: a comparative study. Mod Pathol, 2006, 19: 34–48.

[26] KATO Y, SASAGAWA I, KANEKO M et al. Aggrus: a diagnostic marker that distinguishes seminoma from embryonal carcinoma in testicular germ cell tumors. Oncogene, 2004, 23: 8552–8556.

[27] MISHIMA K, KATO Y, KANEKO MK et al. Podoplanin expression in primary central nervous system germ cell tumors: a useful histological marker for the diagnosis of germinoma. Acta Neuropathol, 2006a, 111: 563–568.

[28] MISHIMA K, KATO Y, KANEKO MK et al. Increased expression of podoplanin in malignant astrocytic tumors as a novel molecular marker of malignant progression. Acta Neuropathol, 2006b, 111: 483–488.

[29] KUNITA A, KASHIMA TG, MORISHITA Y et al. The platelet aggregation-inducing factor aggrus/podoplanin promotes pulmonary metastasis. Am J Pathol, 2007, 170: 1337–1347.

[30] ADACHI Y, NAKAMURA H, KITAMURA Y et al. Lymphatic vessel density in pulmonary adenocarcinoma immunohistochemically evaluated with anti-podoplanin or anti-D2-40 antibody is correlated with lymphatic invasion or lymph node metastases. Pathol Intern, 2007, 57: 171–177.