Lymphangiogenesis and Lymphatic Metastasis of Tumor

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Abstract There has been data demonstrating that there are lymphatic vessels in tumors and that tumor-induced lymphangiogenesis can contribute to metastatic spread. Identification of lymphangiogenic factors, as well as suitable markers that distinguish blood from lymphatic vascular endothelium. Recently discovered and certificated markers are VEGFR-3, Podoplanin, Prox-1, LYVE-1, etc. Studies approved that VEGF-C and VEGF-D bind to VEGFR-3, inducing and regulating lymphangiogenesis. In several human cancers, increased expression of VEGF-C is correlated with regional lymph node metastasis, and often considered as having prognostic value. VEGF-D stimulates lymphangiogenesis and metastatic spread of tumor cells, the high expression may be related to poor prognosis. So hopefully, every process of VEGFR-3 signal pathway may serve as an ideal target for controlling tumor growth and metastasis.

Key words Lymphangiogenesis; VEGF-C; VEGF-D; Lymphatic Metastasis

The metastatic spread of tumor is responsible for the majority of cancer deaths, and with few exceptions, all cancers can metastasize. Tumor metastasis may occur through a number of pathways: (a)local tissue invasion; (b)direct seeding of body cavities or surfaces; (c)hematogenous metastasis; (d)lymphatic metastasis.

The lymphatic system serves to collect and transport interstitial fluid (lymph) within tissues, and plays an important role in the immune response. The lymphatic system also constitutes one of most important pathways of tumor dissemination. The lymphatic microvasculature is uniquely adapted for the continuous removal of interstitial fluid and proteins and is an important entry point for leukocytes and tumor cells. Specialized functions of lymphatic vessels suggested the differences in the molecular composition between the lymphatic and blood vascular endothelium. However, the extent to which the two type cells differ is still unclear, and few molecules that are truly specific to lymphatic endothelial cells have been identified to date, with which it becomes more clear and easier to pay attention to tumor lymphangiogenesis and lymphatic metastasis.

Distinct markers for lymphatic endothelium

Vascular endothelial growth factor receptor 3 (VEGFR-3), also known as flt-4, the earliest cloned lymphatic vessel marker, could be found in human fetal and adult tissues, the consistent expression of which in lymphatic endothelia and its absence from endothelia of all large blood vessels has been confirmed, but was also found in specific subsets of capillary endothelia [1]. Bronislaw *et al.*[2] also proved VEGFR-3 is specifically expressed on lymphatic endothelium and may play a role in lymphangiogenesis.

Podoplanin, as well as on lymphatic endothelia, has been localized to podocytes, parietal epithelial cells of Bowman´s capsule, lung, choroid plexus, leptomeninges, osteocytes, and osteoblasts[3]. Podoplanin and VEGFR-3 antigens were found to overlap in lymphatic endothelium, benign vascular tumors, and angiosarcomas [4]. The study of Wang Yan *et al.* suggested that podoplanin is a more specific lymphatic vessel marker in NSCLC tissues [5].

Prox-1 on endothelium is detectable only on lymphatic endothelium of human fetal and adult tissues, but on non-endothelium at least during development. Prox-1 is also expressed in the lens, heart, liver, pancreas, and the central nervous system[6]. In embryos, the homeobox gene Prox-1 is expressed in a subpopulation of endothelial cells that, after budding from veins, gives rise to the mammalian lymphatic system. In Prox1-/- embryos, this budding becomes arrested at around embryonic day(E) 11.5, resulting in embryos without lym-

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phatic vasculature[7].

LYVE-1 (lymphatic vessel endothelial HA receptor): the discovery of which, a HA receptor, expressed predominantly in lymphatic vessels, highlights another aspect of HA biology: its continuous transit through the lymphatic system and its potential involvement in lymph node homing by CD44+ leukocytes and tumor cells. Its application as a marker to study tumor lymphangiogenesis is recognized[8]. However, the study of Mouta-Carreira C *et al.* showed that LYVE-1 is not exclusive to the lymph vessels, but is also present in normal hepatic blood sinusoidal endothelial cells in mice and humans. So it is a good idea to demonstrate lymphatic by combining LYVE-1 and Prox 1[9].

Other lymphatic markers such as 5′-Nucleotidase, desmoplakin, Lyp-1 etc., due to lack of comparative study, tire application as distinct markers for lymphatic endothelium still needs verification.

Lymphangiogenesis

Lymphangiogenic factor

(1) **VEGF-C** VEGF-C, the VEGFR-3 ligand, were first purified from human prostatic carcinoma cells by Joukov *et al.* in 1996, which stimulated the migration of bovine capillary endothelial cells in collagen gel, and also suggested that VEGF-C is regulator of endothelia, whose effects may extend beyond the lymphatic system, where Flt-4 is expressed, acting in a paracrine manner [10]. Simona *et al*[11], via a collagen gel " sandwich" assay, proved that VEGF-C selectively induced tube formation of lymphatic vessels but not blood vessels, and played a role in the regulation of lymphatic vessel survival and/or formation. But via isolating primary lymphatic and blood microvascular endothelial cells by immunoselection with the lymphatic marker LYVE-1, it was suggested that VEGF-C promoted survival and tube formation of both cell types, enhanced the enlargement of primary lymphatic vessels, and induced vascular anastomosis of blood and lymphatic vessels. In agreement with its function, VEGF-C binds VEGFR-3 may induces tyrosine autophosphorylation of VEGFR-3 and VEGFR-2, while both blood and lymphatic endothial cells lineages expressed VEGFR-2, whereas VEGFR-3 was predominantly expressed in lymphatic endothial cells.

(2) **VEGF-D** VEGF-D, also known as c-fos-induced growth factor, is closely related in structure with VEGF-C, they are secreted homodimeric glycoproteins. But unlike VEGF-C, VEGF-D activates only VEGFR-3, which suggests that it may be lymphangiogenic but not angiogenic [12]. Stacker *et al.* analyzed the role of VEGF-D by inducing VEGF-D-293 tumorsin SCID/NOD mice, taining of tumor sections showed that LYVE-1^+cells were restricted to the outer connective tissue capsule surrounding the tumor in control 293 tumors and did not form vessel structures, even they did, on rare occasions, they were never observed in the tumor mass. In contrast, LYVE-1^+cells in VEGF-D-293 tumors frequently formed into large vessel structures, devoid of erythrocytes, within the tumor mass in addition to some positive cells in the capsule region. This show that VEGF-D can induce both tumor lymphangiogenesis, promotes lymphatic spread of tumors[13]. But due to the study of Megan E *et al*[12] in VEGF-D-deficient mice the lymphatic vessels and lymph nodes appeared to develop and function normally, showing that inactivation of the VEGF-D gene alone does not significantly perturb lymphatic development.

(3) **Mechanism of action** It is considered that the increase in interstitial pressure and hypoxia, etc. during the enlargement of the tumors give raise to the increase in secretion of VEGF-C and/or VEGF-D, which bind there receptors and induce tyrosine autophosphorylation of them, inducing lymphangiogenesis. VEGFR-3 has an essential role in the development of embryonic blood vessels; however, after midgestation its expression becomes restricted mainly to the developing lymphatic vessels. Veikkola T *et al.* [14] created transgenic mice overexpressing a VEGFR-3-specific mutant of VEGF-C (VEGF-C156S) or VEGF-D in epidermal keratinocytes under the keratin 14 promoter, both transgenes induced the growth of lymphatic vessels in the skin, whereas the blood vessel architecture was not affected. These results demonstrated that stimulation of the VEGFR-3 signal transduction pathway is sufficient to induce specifically lymphangiogenesis in vivo. Bronislaw *et al.* [2] proved that blocking VEGFR-3 completely and specifically prevented both physiologically

normal and tumor VEGF−C−enhanced lymphangiogenesis in the adult mouse but had no effect on either blood angiogenesis or the survival or function of existing lymphatic vessels. So we can conclude that the activation of VEGFR−3 by its ligands VEGF−C and −D is necessary for lymphangiogenesis.

Features of distribution of lymphatic vessels of tumor and lymphatic metastasis

There has been data establishing that lymphatic vessels can form in solid tumors and that tumor−induced lymphangiogenesis can contribute to metastatic spread [12]. But recently, Schneider M *et al* found in a histomorphological analysis of lymphatic vessels in pancreatic cancer resection specimens that both intratumoral and peritumoral tissue were devoid of active lymphangiogenesis. Intratumoral lymphatic vessels were frequently collapsed and non −functional, whereas peritumoral lymphatic vessels were enlarged, and numerous lymphatic vessels were seen in metastases [15]. Many scholars give the hypothesis that the phenomena have been due to the consistent elevated interstitial pressure, or the invasiness of tumor cell, destroying the lymphatic net, leaving only the endothelial residues. It is also pointed by Huang JH *et al.* that lymphangiogenesis induced by VEGF−C predominantly takes place in the tumor stroma tissue, and mature lymphatic vessels are not found in cancer nests [16]. While some recent studies showed that high lymphatic vessel density may be a significant unfavorable prognostic factor for long −term survival in breast cancer [17]. Lymphangiogenesis predominantly influenced metastasis−free survival, which is regulated by VEGF−C and VEGF−D, but it is not an independent prognostic factor [18, 19].

Metastasis to the regional lymph nodes through the lymphatic vessels is a common step in the progression of cancer and an important prognostic factor in many types of cancer. Tumor lymphangiogenesis could conceivably promote the spread of tumors to local lymph nodes. Massi D *et al.* concluded that the intratumorous lymphatic vessel area is the most significant factor predicting sentinel lymph node metastasis [20]. Nakamura Y *et al.* consider that increased podoplanin expression is significantly associated with lymph nodes metastasis [21]. Recent evidence suggests that VEGF −C and VEGF −D promote lymphangiogenesis, and that tumor lymphangiogenesis in turn promotes lymphatic metastasis. Many clinical researches about carcinoma such as papillary thyroid carcinoma, esophageal squamous cell carcinoma, pancreatic cancer, etc. also revealed that lymph nodes metastasis, lymphatic inflitration are obviously related to VEGF−C or VEGF−D [22]. But Schneider M *et al.* [15] advanced that active lymphangiogenesis was not required for lymphovascular spread of cancer. VEGF −C may activate pre −existing lymphatic endothelium and thus promote local tumor growth and support the entry of cancer cells into peritumoral lymphatics. Sipos B *et al.*[23] results consents: increased lymphangiogenic activity is not required for and does not significantly affect the lymphatic spread of pancreatic ductal adenocarcinoma. Wobser M *et al* [24] series VEGFR−3/CD31 immunohistochemical staining of primary melanoma does not serve as a valid marker to predict lymph node involvement.

(1) **Function of VEGF−C**

In several human cancers, increased expression of VEGF−C in primary tumors is correlated with regional lymph node metastasis. The role of VEGF−C in human tumor metastasis is therefore likely to involve lymphangiogenesis as well as its capacity to induce activation of pre−existing lymphatic endothelium. Kawai *et al.*[25] suggested that active lymphangiogenesis promoted by up-regulation of VEGF121 −induced elevated VEGF −C, was ongoing within sentinel lymph nodes from NSCLC patients, even before metastasis. Some recent studies approve that, VEGF −C secreted by cancer cells plays an important role in LEC migration in pancreatic cancer and NSCLC lymphangiogenesis [26, 27]. Li YS *et al.*[28] suggested that VEGF−C promotes the proliferation of peritumoral lymphatic vessels and that lymphatic invasion and metastasis to lymph nodes are frequently induced in invasive micropapillary carcinoma of breast. Takizawa H *et al.*[29] demonstrated in their study that VEGF−C promotes lymph node metastasis while being influenced by the strength of the VEGF −C autocrine loop, and the VEGF−C/VEGFR−3 ratio can be a useful predictor of lymph node metastasis in NSCLC. Mylona E *et al.* approved that VEGF−C was found to be an independent

indicator of patient´s poor prognosis and the simultaneous expression of tumor VEGF−C and stromal VEGFR−3 yielded additional prognostic information[30].

However, there are also researches disaffirm such pertinence: Arinaga *et al* [31] study found no relationship between over−expression of VEGF−C and lymph node metastasis. Even more, after analyzing 122 patients with breast cancer, Al−Mowallad A *et al* [32] study failed to show any prognostic value for plasma VEGF−C level in patients with breast cancer. Also, Gisterek Ⅰ *et al.*[33] did not reveal any prognostic value of VEGF−C.

Moreover, some scholars suggest that VEGF−C expression influence lymphatic size rather than being involved in the increase of lymphatic vessel number [34]. It was found in Mŏbius C *et al*[35] study that the expression of VEGF−C was significantly different between the two histological types of esophageal tumors. Patients with squamous cell carcinoma and lymph node metastases had a significantly higher VEGF−C expression ($P < 0.01$),and in patients with adenocarcinoma of the esophagus there was no correlation between VEGF−C expression and clinicopathological parameters, which suggested that the malignant change of tumors and their lymphatic spread are complex, multi−step processes, several different genes might be involved, which might be determined by specificity of the tumors themselves.

(2)Function of VEGF−D

VEGF−D and VEGFR−3 are novel independent prognostic marker molecules aiding to identify patients with poor prognosis after curative resection of gastric adenocarcinomas[36]. In Ishikawa M *et al*[37] study on 105 cases of early gastric cancers, the lymph node metastasis was significantly related to the expression of VEGF−D in undifferentiated adenocarcinomas, and the expression increase with the depth of infiltration. The expression of VEGF−D in a mouse tumor model stimulated lymphangiogenesis in solid tumors; this process promoted the metastatic spread of tumor cells via the lymphatics but could be blocked by a neutralizing VEGF−D antibody [38]. Von Marschall Z *et al.*[39] suggested that the presence of lymphatic metastases is a strong indicator for poor prognosis in patients with ductal pancreatic cancer, in which VEGF−D plays a pivotal role in stimulating lymphangiogenesis and lymphatic metastasis. Orlandini *et al.*[40] demonstrated that β−catenin is a negative regulator of VEGF−D mRNA stability, thus VEGF−D might play effect only in β−catenin−deficiency cases in inducing lymph node metastasis. Yasuoka H *et al*[41] findings indicate that VEGF−D expression and increased lymph vessel density may have an important role for lymph node metastasis in papillary thyroid carcinoma. Hu WG *et al.*[42] pointed that, after analyzing 69 patients with pathologically confirmed colorectal carcinoma who received routine follow−up, the VEGF−D expression was significantly correlated with lymph node metastasis and long−term prognosis and could be applied as prognostic markers in colorectal carcinoma. But Gisterek Ⅰ *et al*[33] did not reveal any prognostic value of VEGF−D. George *et al.*[43] verified that VEGF−D mRNA expression was significantly lower in both polyps and colorectal cancer compared with normal mucosa, and there was no association between VEGF−D and lymphatic spread. While some recent studies demonstrated that VEGF−D induced lymphangiogenesis, promotes metastasis to lymph nodes and lungs, and yet represses hemangiogenesis and tumor outgrowth[44]. Further studies are needed to elucidate the function of VEGF−D and its relationship with VEGF−C and VEGFR−3.

Therapeutic prospect of anti− lymphangiogenesis

Tumor metastasis to sentinel lymph nodes represents the first step of tumor dissemination in most human cancers and serves as a major prognostic indicator for disease progression. A lot of studies have revealed that tumors can actively induce the formation of lymphatic vessels via expression of the lymphangiogenic factors, VEGF−C and VEGF−D, which are capable of activating VEGFR−3 signal pathway, correlates with up−regulated lymphangiogenesis and regional lymph node metastasis. VEGFR−3 activation promotes lymphatic endothelial cell proliferation, migration, and survival via the extracellular signal−regulated kinase, the phosphatidylinositol 3−kinase/AKT, and the c−Jun NH2−terminal kinase pathways. So hopefully, therapeutic targeting of the VEGFR−3 pathway will be an effective way in anti−lymphangiogenesis and in turn, anti−metastasis[45], in which every process may serve as an ideal tar-

get for controlling tumor growth and metastasis [46]. For example, inhibiting VEGF-C expression in tumor cell with small interfering RNA vectors to inhibit tumor lymphangiogenesis[47], inhibiting VEGFR-3 activity by a blocking anti-VEGFR-3 antibody [48], targeting at the proteolytic activation of VEGF-C and VEGF-D[49]; using soluble VEGFR-3 fusion protein ("VEGF-C/-D-trap") to block effect of VEGF-C [50]; with neutralizing antibodies of VEGF-D to inhibit metastasis of VEGF-D over-expressed cases [51]; and blocking generation of VEGF-D by estrogen antagonist[52]; moreover, blockade of the VEGFR-3 pathway by small molecule kinase inhibitors efficiently inhibits experimental tumor lymphangiogenesis and metastasis and might also represent a novel therapeutic avenue for the treatment of human cancers. Recently, a gene therapy approach using recombinant adeno-associated virus expressing soluble VEGFR-3 resulted in blockade of lymph node metastasis in a melanoma model in mice [53]. Together, these findings indicate that blockade of the VEGFR-3 pathway efficiently inhibits lymph node metastasis and likely also reduces the incidence of distant organ metastases. To sum up, although there are a series of problems in tumor lymphangiogenesis and lymphatic metastasis remain elusive, along with the continuous studies, anti-lymphangiogenesis will hopefully become a new pattern in biotherapy of tumor.

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