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Lymphangiogenesis and cancer metastasis

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Abstract

Metastasis is a characteristic trait of most tumour types and the cause for the majority of cancer deaths. Many tumour types, including melanoma and breast and prostate cancers, first metastasize *via* lymphatic vessels to their regional lymph nodes. Although the connection between lymph node metastases and shorter survival times of patients was made decades ago, the active involvement of the lymphatic system in cancer metastasis has been unravelled only recently, after molecular markers of lymphatic vessels were identified. A growing body of evidence indicates that tumour-induced lymphangiogenesis is a predictive indicator of metastasis to lymph nodes and might also be a target for prevention of metastasis. This article reviews the current understanding of lymphangiogenesis in cancer, anti-lymphangiogenic strategies for prevention and therapy of metastatic disease, quantification of lymphangiogenesis for the prognosis and diagnosis of metastasis and *in vivo* imaging technologies for the assessment of lymphatic vessels, drainage and lymph nodes.

Keywords: lymphangiogenesis • cancer metastasis • cancer metastasis prediction and prevention • imaging of lymphatic vessels

Anatomy and function of the lymphatic system

The lymphatic system consists of lymphatic vessels and the lymphoid organs, which include lymph nodes, tonsils, spleen, Peyer's patches and the thymus. This system regulates tissue pressure by draining extravasated fluid back into the blood circulation. Almost all tissues are penetrated by lymphatic capillaries except some avascular tissues such as the epidermis, cartilage, cornea, retina, hair and nails and some vascularized organs including the brain and the retina [1]. Lymphatic capillaries consist of a single layer of overlapping leave-like shaped lymphatic endothelial cells (LECs) and their ends open into the tissue periphery. They are attached to the extracellular matrix by filaments, and it is assumed that through these connections, lymphatic vessels are forced to open when interstitial tissue pressure rises upon fluid extravasation from the blood stream [2]. Interstitial fluid, molecules and cells enter the lymphatic capillaries between discontinuous button like cell junctions [3]. The drained fluid, also known as lymph, is moved through lymphatic capillaries by skeletal muscle action and respiratory movements into larger collecting vessels that are surrounded by a basement membrane and flow-promoting smooth muscle cells. Eventually

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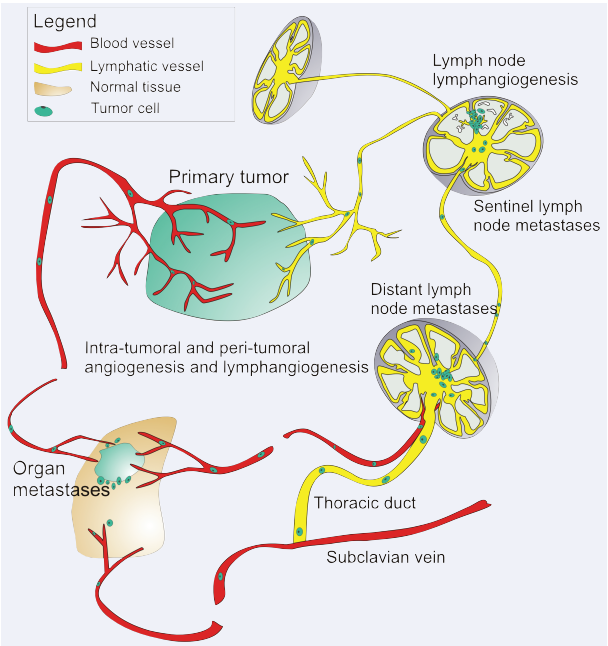

Fig. 1 Tumour-induced lymphangiogenesis at the tumour site and in tumour draining lymph nodes promotes metastasis to lymph nodes and beyond.

the lymph is released into the blood stream *via* the thoracic duct, at the junction of the jugular and subclavian veins. On the way from the peripheral tissues to the blood stream, the lymph fluid passes a cascade of lymph nodes.

In addition to its function in tissue pressure regulation, the lymphatic system is an important component of the immune system. Soluble antigens and antigen-presenting cells are transported *via* lymphatic vessels into lymphoid organs including the lymph nodes, tonsils, spleen and Peyer's patches, where immune responses are initiated. In addition, specialized lymphatic vessels, the lacteals, are involved in the absorption of dietary fat and fat soluble vitamins [1].

Tumour-induced lymphangiogenesis promotes metastasis

The lymphatic system gained much attention when tumour-associated lymphangiogenesis was correlated with metastasis to draining lymph nodes in several mouse models; in these models, tumours expressed the lymphangiogenic growth factors vascular endothelial growth factor (VEGF)-A, VEGF-C or VEGF-D [4–8]. Since then, VEGF-A, -C and -D have been detected in a range of human tumour types, such as melanoma and breast, cervical, non-small-cell lung, prostate, colorectal and gastric cancers. Furthermore, tumour-associated lymphatic vessel density was correlated with metastasis to the draining lymph nodes, distant metastases and poor prognosis [9–12]. These studies suggested that expression of lymphangiogenic growth factors by tumour cells induces lymphangiogenesis, which promotes metastasis (*via* lymphatic vessels) to draining lymph nodes and beyond. Furthermore, the initiation of lymphatic metastasis correlated with tumour lymphangiogenesis in a human lung cancer xenograft model in mice (Fig. 1) [13].

In addition to increasing the number of lymphatic vessels, tumours also induce lymphatic vessel enlargement, which has been associated with increased passage of tumour cell clusters to sentinel lymph nodes in mouse and human tumours [13–19]. Lymphatic vessel enlargement is at least partly mediated by VEGF-C, produced by tumour cells, which was demonstrated in mouse models of melanoma and lung cancer xenografts [13, 20].

In contrast to angiogenesis, lymphangiogenesis in tumours is likely to be almost exclusively due to the proliferation and sprouting of pre-existing vessels, rather than incorporation of circulating endothelial progenitor cells. In mice that were sub-lethally irradiated and then given grafts of green fluorescent protein (GFP)-expressing bone marrow cells, the GFP-expressing cells did not incorporate into the newly formed lymphatic vessels induced by Lewis lung carcinoma xenotransplants or by VEGF-C delivery [21]. However, studies in inflamed mouse corneas or those exposed to fibroblast growth factor-2 (FGF-2) and human kidney transplants indicate that a minor number of bone-marrow derived cells, proposed to be macrophages, might contribute to the growth of lymphatic vessels [22–24].

Tumour-induced lymph node lymphangiogenesis precedes cancer metastasis to lymph nodes

In mouse models of skin carcinogenesis in which VEGF-A or VEGF-C was overexpressed in the skin, lymphangiogenesis was found to occur not only at the tumour site but also in the tumour-draining lymph nodes and to correlate with metastasis to lymph nodes and beyond [7, 8]. Interestingly, the expansion of the lymphatic vasculature in the sentinel lymph node was initiated before cancer cells arrived at these sites, indicating that VEGF-A and VEGF-C from the tumour sites drained to the lymph nodes and induced lymphangiogenesis there. Once metastatic cells reached the lymph nodes, lymphangiogenesis increased (Fig. 1) [7, 8]. Expansion of the lymphatic vasculature in pre-metastatic lymph nodes has been confirmed in mouse models of malignant melanoma, xenographic nasopharyngeal and breast carcinoma [25, 26], indicating that lymphangiogenesis in the pre-metastatic lymph node fosters tumour dissemination by creating a favourable environment for cancer cells. Tumour-induced lymph node lymphangiogenesis also occurs in cancer patients. Prominent hot spots of lymphangiogenesis were detected in metastasized lymph nodes of human melanoma patients and lymphangiogenesis in metastatic auxiliary lymph nodes of breast

cancer patients was correlated with metastasis to non-sentinel auxiliary lymph nodes [27, 28].

Tumour cell invasion into lymphatic vessels

Tumour-induced lymphangiogenesis is likely to promote tumour cell dissemination by increasing the number of entry sites into the lymphatic system. Cancer cells seem to exploit a mechanism that immune cells use to gain access to lymphatic vessels: lymphatic entry is induced in activated dendritic cells by interaction of the receptor C-C chemokine receptor type 7 (CCR7) with the chemokine C-C motif chemokine 21 (CCL21), which is constitutively expressed by the lymphatic endothelium and the lymph nodes [29]. CCR7 is as well highly expressed by several breast cancer and malignant melanoma cell lines; metastatic melanoma cell lines that express CCR7 chemotax towards CCL21 *in vitro* and towards CCL21-secreting LECs *in vivo* [30, 31]. Importantly, overexpression of CCR7 in murine B16 melanoma cells promoted metastasis to draining lymph nodes [32].

Peri- and intra-tumoral lymphangiogenesis

Lymphangiogenesis occurs at the tumour periphery and in the inner-tumour cell mass. Although it is obvious that peri-tumoral lymphatic vessels are involved in metastasis, it is not clear if, and to what extent, intra-tumoral lymphatic vessels are involved in this process, because they are often collapsed or might be occluded by tumour cells [12]. Ferritin injected into tumours was not efficiently drained into intra-tumoral lymphatic vessels [33] and specific inhibition of intra-tumoral lymphatic vessels did not prevent metastasis to lymph nodes in mouse models of prostate cancer. Also, intra-tumoral lymphatic vessel density did not correlate with lymph node metastasis in oral cavity or laryngeal carcinomas [34, 35]. In contrast, in human malignant melanomas, oropharyngeal carcinomas and head and neck squamous cell carcinomas, intra-tumoral lymphatic vessel density was correlated with lymph node metastasis [27, 35, 36].

Lymphatic vessel markers and lymphangiogenic growth factors

The discovery of novel markers to distinguish blood and lymphatic vessels enabled the investigation of lymphatic vessels and lymphangiogenesis in health and disease. The first markers identified were the homeobox transcription factor Prox1 [37, 38], the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) [39, 40], podoplanin [41] and vascular endothelial growth factor receptor-3 (VEGFR-3) [42]. Prox1, a transcription factor, is activated in a subset of venous endothelial cells during embryogenesis and commits these cells to the lymphatic lineage [37, 43–45]. LYVE-1 is a cell membrane protein of unknown function and is the earliest marker of LECs in development [46, 47] (Fig. 2). Podoplanin, another cell membrane protein, is involved in proper lymphatic vessel development and might have a role in carcinoma formation [48, 49]. VEGFR-3 is a cell membrane receptor for VEGF-C and VEGF-D and is essential for vascular development [42, 50]. These markers are discussed in more detail in a recent publication [51].

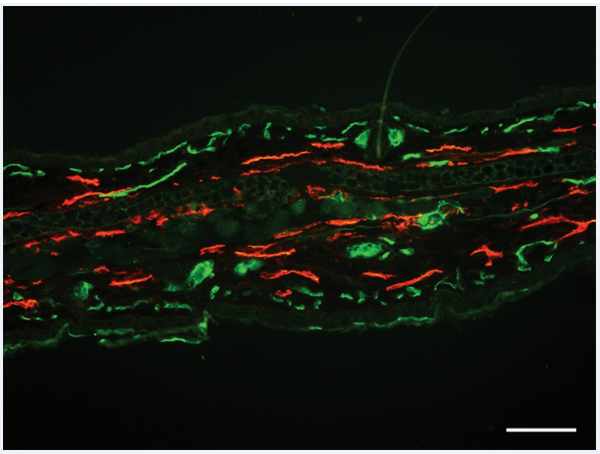

Fig. 2 Immunofluorescence staining for markers of lymphatic and blood vessels on a mouse ear section. Lymphatic vessels are stained with an antibody to LYVE-1 (red) and blood vessels are stained with Meca-32 (green). Scale bar = 100 μm

These markers have been used to isolate LECs from human skin. Cultured LECs have been analysed by transcriptional and proteomic profiling to identify additional markers [52–55]. A recent study reported the isolation of mouse LECs from normal skin and from metastatic fibrosarcoma. After *in vitro* cultivation, transcriptional profiling of these cells revealed markers that were up-regulated on tumour-associated lymphatic vessels, compared to markers of normal vessels such as endothelial-specific adhesion molecule, endoglin and CD200 [55].

A range of soluble factors induce lymphangiogenesis (Table 1). The first lymphangiogenic growth factors identified were VEGF-C and VEGF-D, which bind to VEGFR-3 on LECs and induce receptor phosphorylation and downstream signalling [56, 57]. VEGF-A was shown to induce lymphangiogenesis by binding to VEGFR-2 on LECs [7, 58]. Additional lymphangiogenic growth factors include hepatocyte growth factor, which binds to the c-met receptor [59], angiopoietin-1 (Ang-1) binding to its receptor Tie2 [60, 61], as well as FGF-1 and -2 [62, 63], insulin-like growth factors 1 and 2 (IGF-1 and -2) [64], platelet derived growth factors (PDGF) [65],

Table 1 Lymphangiogenic growth factors

Growth factor	Receptors on lymphatic vessels	References
VEGF-A	VEGFR-2	[58]
VEGF-C	VEGFR-2, VEGFR-3	[56]
VEGF-D	VEGFR-2, VEGFR-3	[57]
Hepatocyte growth factor	c-met	[59]
Ang-1	Tie-2	[60, 61]
FGF	FGFR-3 and possibly others	[63]
PDGF-BB	PDGFR-α and -β	[65]
GH	Growth hormone receptor	[67]
AM	Calcitonin receptor-like receptor (calcrl) associated with receptor activity-modifying protein 2	[148]
ET-1	Endothelin B receptor	[68]

adrenomedullin (AM) [66], growth hormone (GH) [67] and endothelin-1 (ET-1) [68]. Recently, transforming growth factor-β_1 (TGF-β_1) has been identified to be a negative regulator of lymphangiogenesis in wound healing [69]. The relative contribution of these different factors towards lymphangiogenesis and their interplay remains to be elucidated.

Lymphangiogenic growth factors are secreted not only by tumour cells but also by tumour-associated stromal cells, in particular tumour-associated macrophages, which has been reported for example in mice bearing human gastric carcinoma xenotransplants [70] and in human melanoma [27]. In human cervical cancer, VEGF-C and VEGF-D expression by macrophages correlated with lymphatic vessel density and frequency of lymph node metastasis [71]. Immunization with the model antigen keyhole limpet haemocyanin in complete Freund's adjuvant was observed to induce B cells to produce VEGF-A [72] whereas dendritic cells, macrophages and neutrophils express VEGF-C in mice with airway inflammation [73].

Preventive and therapeutic potential of anti-lymphangiogenic treatment

There have been several important studies performed to determine whether anti-lymphangiogenic agents can prevent metastasis to lymph nodes. Similar to angiogenesis, lymphangiogenesis does not occur in adults, except in the endometrium during pregnancy and in pathological settings such as chronic inflammation, tissue repair, and tumour growth [1, 74, 75]. Therefore, anti-lymphangiogenic strategies are expected not to interfere with normal lymphatic vessel function. Several studies in experimental cancer models showed that inhibition of lymphangiogenesis reduced the incidence of metastasis to lymph nodes; in most of these studies, the VEGF-C/VEGF-D/VEGFR-3 axis was inhibited before the onset of metastasis. Tumour lymphangiogenesis and lymph node metastasis were reduced by VEGFR-3 blocking antibodies in a mouse model of breast carcinoma and an orthotopic model of gastric cancer [76, 77], by a VEGF-D neutralizing antibody in a tumour model of EBNA 293 (cells expressed VEGF-D) [6], by a soluble VEGFR-3 fusion protein (a 'VEGF-C/D trap') in models of melanoma, gastric, prostate, breast and lung cancer [78–80], and by VEGF-C small interfering (si)RNA in a breast cancer model and in gastric cancer xenografts [81, 82]. Recently, an anti-neuropilin-2 antibody was shown to inhibit tumour-induced lymphangiogenesis in experimental mammary and brain tumour models and to delay metastasis to sentinel lymph nodes [83]. Neuropilin-2 is a cell-surface transmembrane protein expressed on tumour-associated but not normal lymphatic vessels [83]. It functions as a co-receptor for VEGF-C and VEGF-A [84] and is not required for developmental lymphangiogenesis [85].

Inhibition of the VEGF-C/-D/VEGFR-3 pathway in adult mice did not affect normal lymphatic vessels [86] and was not reported to induce oedema formation, so this appears to be a relatively safe therapeutic strategy. However, although targeting the VEGF-C/VEGF-D/VEGFR-3 axis efficiently blocks lymphangiogenesis and reduces metastasis to the lymph nodes, it does not completely inhibit formation of metastases in lymph nodes. Furthermore, long-term expression of VEGFR-3-IgG inhibited macrometastasis but not micrometastasis [13]. This indicates that complete blockage of lymphatic metastasis could require targeting of additional lymphangiogenic pathways and of the mechanism of lymphatic vessel invasion by tumour cells. Recently etodolac, an inhibitor of cyclooxygenase-2, inhibited lymphangiogenesis and lymph node metastasis in a mouse model of gastric cancer. Etodolac decreased VEGF-C and – D levels in tumour-associated macrophages, suggesting that reducing macrophage-mediated tumour lymphangiogenesis might help prevent lymph node metastasis [70].

In models of inflammation and wound repair, several anti-lymphangiogenic strategies have been successful – these strategies might also be efficient in inhibiting tumour-induced lymphangiogenesis. A small molecule antagonist of the integrin subunit $\alpha 5$ inhibited inflammation-induced lymphangiogenesis in the mouse cornea [87], antibodies to integrin subunits α_1 or α_2 blocked VEGF-induced lymphangiogenesis in mouse skin [88], and a specific antagonist of the endothelin receptor type B decreased lymphangiogenesis in a matrigel plug assay in mice [68]. Moreover, siRNA knockdown of endothelial-specific molecule-1 blocked *in vivo* lymphangiogenesis in matrigel assays [54] and a recent study indicated that transforming growth factor (TGF)-β_1 inhibits lymphangiogenesis during wound repair [69]. Additional targets might be revealed by characterizing proteins that transcriptional and proteomic profiling studies showed were expressed on activated LEC [52–55].

The ability of anti-lymphangiogenic agents to prevent the early stages of metastasis has been established in animal models. However, the effect of anti-lymphangiogenic therapies on established

metastases is less clear. Blockade of lymphangiogenesis by adenoviral delivery of VEGFR-3-IgG after the tumour cells had already spread out did not suppress lymph node metastasis in lung cancer xenografts in mice [13]. The preventative and interventional effects of a blocking antibody against VEGFR-3 were recently investigated in a mouse xenograft model of melanoma. Although lymph node metastasis was almost completely blocked when the blocking antibody was administered at the time of tumour cell injection, the lymph node tumour burden was 47% lower in mice that were given antibody after the tumour cells had already started to spread [76]. It remains to be determined whether anti-lymphangiogenic treatment can prevent metastasis from lymph nodes to distant organs.

Tumour lymphangiogenesis as a prognostic indicator for lymph node metastasis

Malignant tumours, including melanoma and breast and prostate cancers, often metastasize first *via* lymphatic vessels to their regional lymph nodes [89]. The status of regional lymph nodes is an important parameter to determine the stage of disease progression in different tumour types, including breast cancer and melanoma. Lymph node status is used to predict patient survival times and determine their clinical management strategy [10, 90]. To determine the node status, regional lymph nodes (or only sentinel lymph nodes in the case of breast cancer and melanoma patients) are removed and sections are analysed to detect metastases. The procedure of lymph node dissection and examination is elaborate and is associated with significant morbidity and costs [91–93]. In addition, tumour-draining lymph nodes can be missed, resulting in false prognoses. Several non-invasive methods are currently available to discern normal lymph nodes from those with metastases; however, they are not sensitive enough to detect micrometastases [94]. In addition, there is no solid method for predicting disease progression in patients with small tumours without lymph node involvement. Therefore, more reliable markers that can predict lymph node metastasis in early and advanced cancers are needed for therapeutic decision-making [10]. Several novel lymphangiogenesis parameters might be more sensitive prognostic indicators of metastasis to lymph nodes and disease progression than current methods. These include lymphatic vessel density at tumour sites and in tumour draining lymph nodes, expression of lymphangiogenic growth factors, invasion of cancer cells into lymphatic vessels and altered lymph flow and volume in tumour draining lymph nodes.

Lymphatic vessel density

An increase in tumour-associated lymphatic vessel density correlates with lymph node metastasis and unfavourable prognosis in a number of human cancer types, including melanoma and breast, gastric, colorectal, bladder, cervical, ovarian, head and neck, and non-small cell lung cancers [9, 10, 12]. In particular, in studies of human cutaneous melanoma, lymphangiogenesis was the most sensitive prognostic indicator for lymph node metastasis – detection of lymphangiogenesis was more sensitive prognostic factor than measurement of tumour thickness [15, 27]. The density of intra- and peri-tumoral lymphatic vessels and the lymphatic vessel area correlated with shortened disease-free and overall survival times [15, 27]. Thus, quantification of tumour-associated lymphatic vessel density is a promising tool for predicting metastasis of various cancers. However, this method could vary in reproducibility; it relies on counting lymphatic vessels in 'hot spot' regions of lymphangiogenesis in tissue sections, but finding the relevant hot spots depends on the training and experience of the investigator [10]. Recently, guidelines for standardized assessment of lymphangiogenesis in human beings have been recommended [10]. By applying these guidelines, intra- and inter-observer variability might decrease and thus quantification of lymphatic vessel density might become a reliable prognostic for metastasis. However, the most sensitive and reliable method for quantifying tumour lymphangiogenesis remains to be determined.

Lymphatic invasion by tumour cells

Studies in human breast cancer revealed that tumour cell invasion into lymphatic vessels predicted lymph node involvement and was a prognostic factor for overall and disease-free survival [95, 96]. In accordance with these findings, lymphatic vessel invasion was found to be a prognostic indicator of lymph node metastasis in human melanoma [97] and of adverse outcome in several studies of gastric cancer [98–101] and node-negative carcinomas of bladder and oesophagus [102, 103]. These findings indicate that quantification of tumour cell invasion of lymphatic vessels might be used in prognosis in cancer patients with uninvolved lymph nodes.

Lymphangiogenic growth factors

Several factors are being investigated as prognostic indicators of metastasis. The most studied factor is VEGF-C. In a range of human tumours, including melanoma, breast, gastric, colorectal, cervical, endometrial, ovarian and non-small cell lung cancer, it was shown that expression of VEGF-C by tumour or tumour-associated cells correlated with lymph node metastasis and poor prognosis [9, 11, 12]. Recently, a meta-study analysed a large number of published human cancer studies that found VEGF-C expression in tumours and lymph node metastases. In 56 of these 73 studies (77%), VEGF-C expression correlated with lymph node metastasis [12]. Interestingly, VEGF-C levels change with tumour progression; this was shown in human melanomas

in which mRNA levels of VEGF-C were higher during the vertical growth phase (advanced melanoma) compared to the horizontal growth phase. The greatest levels of VEGF-C expression were found in nodal metastases, followed by regional dermal metastases and then primary tumours [104].

Fewer studies investigated tumour VEGF-A and VEGF-D expression and lymph node metastasis [12]. VEGF-D expression was correlated with lymph node metastases in 14 of 32 studies (44%) and VEGF-A was in 6 of 16 studies (38%) [12]. Further elucidation of lymphangiogenic growth factors might reveal additional suitable prognostic factors.

Several methods were applied to quantify lymphangiogenic growth factor expression in cancer patients, including detection of protein levels in serum or plasma, and mRNA or protein levels in tumour biopsies or tumour tissue sections [12].

Quantification of circulating lymphangiogenic growth factor levels in human blood would be the preferred biomarker for prognosis, because sample collection is minimally invasive and levels can be measured repeatedly. However, the validity of the method has not been established. Circulating levels of VEGF-C correlated with lymph node metastasis and differed between healthy persons and patients with oesophageal squamous cell carcinoma, malignant melanoma, cervical carcinoma, non-small cell lung carcinoma or colorectal cancer [105–109]. However, this difference was not confirmed in studies assessing circulating VEGF-C or VEGF-D levels patients with ovarian carcinoma, breast carcinoma, cervix adenocarcinoma, colorectal cancer or head and neck carcinoma [105, 110–113]. A potential reason for this discrepancy could be the fact that serum levels of lymphangiogenic growth factors vary among patients. For instance, high levels of serum VEGF-C in patients with non-metastatic cancers are in the range of those of patients with metastatic tumours [107]. Therefore, it is a challenge to interpret the levels of circulating VEGF-C for prognostic purposes without knowing each patient's pre-disease level. This issue probably also affects measurement of other lymphangiogenic factors. Furthermore, lymphangiogenic growth factors such as VEGF-C are produced during other conditions, such as inflammation [1], which could affect the reliability of this prognostic method.

Lymphangiogenic growth factors have been quantified in tumour biopsy samples using RT-PCR and ELISA analyses. Increased expression of VEGF-C and VEGF-D mRNA and protein in tumour biopsies has been associated with lymph node metastasis [114–117]. However, to perform these analyses, biopsies need to be carefully collected, because intra-tumoral VEGF-C and VEGF-D levels were found to be lower than peri-tumoral levels [118–122]; biopsies derived from the tumour centre therefore might not represent VEGF-C or VEGF-D levels at the hot spots of lymphangiogenic activity.

Lymphangiogenic growth factors can also be detected on tissue sections. This method has the advantage of preserving the histological environment and allows discrimination between intra- and peri-tumoral structures and hot spots of lymphangiogenesis, as well as cell-type specific expression of lymphangiogenic growth factors [12].

Lymph node lymphangiogenesis

In mouse models of skin carcinogenesis, malignant melanoma, breast and nasopharyngeal carcinoma, the lymphatic vasculature of the lymph nodes expanded prior to tumour metastasis [7, 8, 25, 26]. Once cancer cells arrived in the lymph node, lymphangiogenesis was increased [7, 8]. Hot spots of lymphangiogenesis have been found in metastases-bearing lymph nodes in human melanoma patients [27] and lymphangiogenesis in sentinel lymph nodes of breast cancer patients was associated with metastases to non-sentinel lymph nodes [28]. These studies supported the concept that lymph node lymphangiogenesis occurs in cancer patients.

Interestingly, pre-metastatic lymphatic vessel expansion in tumour-draining lymph nodes was associated with increased lymph flow to the lymph nodes in a mouse model of metastatic melanoma [26]. Lymph flow imaging was performed *in vivo* using near infrared quantum dots or Cy5.5 labelled magnetic nanoparticles. An increased amount and rate of lymph flow through draining lymph nodes in the same mouse model was also shown by magnetic resonance imaging (MRI) after injection of a gadolinium-based contrast agent [123]. These experiments suggest that detecting increased lymph flow to sentinel lymph nodes, or increased lymph node volumes might predict metastasis to lymph nodes. Further studies on this subject are needed to confirm this hypothesis.

Diagnostic value of tumour-associated lymphangiogenesis

Although quantification of tumour-induced lymphangiogenesis is a valuable method for predicting lymph node metastasis, it is not clear whether detection of lymphangiogenesis might be used in the early detection of metastases in lymph nodes or elsewhere. Because lymphangiogenesis has been found in lymph nodes of several experimental cancer models, as well as in patients, it might be prognostic factor for metastasis, especially because it has been shown to precede cancer metastasis in mice [7, 8, 25, 26]. If pre-metastatic lymphangiogenesis is as well a feature in human cancer patients, it remains to be determined whether pre-metastatic and metastatic lymph node lymphangiogenesis can be used to develop reliable diagnostics. It may be a challenge, however, to distinguish between lymph node lymphangiogenesis evoked by cancer metastasis or by inflammation [124].

Imaging of lymphatic vessels

To analyse lymphatic vessels and their function in patients, to monitor anti-lymphangiogenic treatment, and to use lymphatic

vessel density as a prognostic or diagnostic indicator, methods are needed to image lymphatic vessel structure and their function *in vivo*. Agents to image lymphatic vessels have been administered intradermally, subcutaneously, intravenously or directly into lymphatic vessels. Dyes such as Evans Blue or colloidal carbon particles and fluorescent dyes such as fluorescein were injected intradermally or subcutaneously into the interstitial tissue of mice or human beings, to obtain indirect micro-lymphangiographies of superficial lymphatic vessels [58, 125, 126]. For example, micro-lymphangiography with fluorescin isothiocyanate (FITC)-dextran allowed non-invasive estimation of the width of lymph channels in human patients suffering from oedema [126]. Interstitial administration of the contrast agent also enabled lymphangiography of larger and deep lymphatic vessels by MRI in mice and human beings [94, 127, 128] and, less successfully, by X-ray scans [94].

Contrast agents can be delivered intravenously, *via* hyperpermeable blood vessels, through interstitial tissue, into the lymphatic vessels [94]. Particles up to several nanometres in size can extravasate from blood vessels and pool into lymphatic vessels through gap junctions between the endothelial cells. Particles up to 100 nm in diameter extravasate from the blood into the interstitial space, where they are phagocytosed by macrophages and are further transported to the lymph nodes. Particles larger than 100 nm typically remain trapped in the interstitial tissue [94].

Imaging lymphatic drainage

The draining capacity of lymphatic vessels has been assessed by tracking injected radioactive colloids and fluorescent dyes. Lymphoscintigraphy is used to diagnose lymphedema, a condition characterized by non-functional lymphatic vessels. Typically, 99m-technetium (^{99m}Tc)-labelled sulphur colloid or ^{99m}Tc-human serum albumin is administered, either intradermally or subcutaneously [129]. A scintigraphic camera that detects γ emission is used to visualize the radiotracer's flow pattern. More recently, indocyan green (IC-green), an albumin-binding near-infrared dye was investigated for assessing lymphatic function and lymphedema. Using IC-green, pulsative lymph flow has been shown in pigs [130] and subcutaneous IC-green injection revealed dilated lymphatic channels and lymph flow obstruction in human patients suffering from secondary lymphedema [131]. Furthermore, lymph flow has been quantified in breast cancer patients using IC-green [132].

To identify tumour-draining lymph nodes in human cancer patients, Tc-99m-labelled sulphur colloid, albumin nanocolloid, antimony colloid or other soluble non-colloids are injected intradermally or subcutaneously around the tumour and their drainage to lymph nodes is detected by a γ scintigraphic camera [94]. A range of further radiographic contrast agents and dyes have been tested in experiments with mice and in clinical trials to track sentinel lymph nodes of tumours [133–139]. Optical methods to follow drainage patterns are increasingly considered, because of the possibility to track differently labelled dyes simultaneously. However, these methods are currently limited in imaging of nodes in deeper tissues, and in the case of quantum dots, bear toxicity issues. In mice, near-infrared dye-labelled IgGs [138], dendrimers [137], magnetic nanoparticles [26], indocyan green and different quantum dots [26, 134–137, 139] were found in lymph nodes following injection into the interstitial tissue. Lymph flow to lymph nodes could also be tracked and quantified by MRI using a gadolinium-based contrast agent [123, 140].

Non-invasive imaging of lymph node metastases

The removal of tumour-draining lymph nodes, in particular the complete dissection of auxiliary lymph node that is performed in breast cancer patients, can trigger serious adverse reactions, including lymphedema. Questions have been raised about the benefits of routinely performing auxiliary lymph node dissection [94]. Thus, non-invasive methods were developed to detect metastasis-containing lymph nodes and avoid the unnecessary dissection of metastases-free lymph nodes. Ultrasound (US) can detect changes in shape, size and echogenicity induced by metastases. Because ultrasound does not employ a contrast agent, it might be used to detect metastases in lymph nodes that obstruct the drainage of radiotracer to sentinel lymph nodes [94]. However, ultrasound cannot image deep-lying lymph nodes [94]. Conflicting results have been published regarding the sensitivity and specificity of the combination of ultrasound and fine-needle aspiration biopsy in detection of tumour cells [141, 142]. One study reported that lymph nodes could be visualized by ultrasound upon interstitial injection of microbubbles [143]. Metastasis-induced enlargement of lymph nodes can also be imaged by computed tomography (CT) or MRI [94, 129]. In addition, positron emission tomography (PET) can be used after intravenous injection of ^{18}F-fluorodeoxyglucose for direct detection of metastases in lymph nodes [94, 129]. Often PET imaging is combined with CT or X-ray analyses to combine PET data with structural information [144].

CT, MRI and US are routinely used in clinical settings to evaluate lymph nodes. However, none of these methods is sensitive enough to detect micrometastases. Micrometastases, particularly those from breast tumours, are often found in normal-sized and normal-shaped lymph nodes [145, 146]. Furthermore, lymph node enlargement is not necessarily a result of malignancy but can also be caused by hyperplasia or infections [129]. Several attempts have been made to increase the sensitivity of metastasis detection. Whereas conventional MRI is able to detect nodal metastasis as small as 8–10 mm, MRI, after injection

of lymphotropic monocrystalline iron oxide nanoparticles, detects nodal metastases as small as 2 mm [146, 147]. Further improvements in sensitivity might be achieved by the combination of different imaging modalities [129, 146]. Non-invasive imaging of lymph node lymphangiogenesis in tumour-draining lymph nodes could be more sensitive than current methods of detecting metastases.

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