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Mechanism of lymph node metastasis in prostate cancer

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Abstract

Detection of lymph node metastases indicates poor prognosis for prostate cancer patients. Therefore, elucidation of the mechanism(s) of lymph node metastasis is important to understand the progression of prostate cancer and also to develop therapeutic interventions. In this article, the known mechanisms for lymph node metastasis are discussed and the involvement of lymphatic vessels in prostate cancer lymph node metastasis is comprehensively summarized. In addition, contradictory findings regarding the importance of lymphangiogenesis in facilitating lymph node metastasis in prostate cancer are pointed out and reconciliation is attempted.

Keywords

lymphangiogenesis; lymph node metastasis; prostate cancer; VEGF-C

Most deaths from cancer occurs as a result of metastasis [1]. Lymph nodes adjacent to the primary tumor are often the first site of metastases [2–4]. Detection of lymph node metastasis is of major prognostic significance for many cancers, although lymph node metastases are themselves rarely life threatening [2,4–6]. This observation also holds true for prostate cancer where patients with lymph node metastases exhibit a poor prognosis with significantly decreased disease-specific and biochemical recurrence-free survival rates [7]. Regional lymph node metastasis has been observed in 5–12% of prostate cancer patients with clinically organ-confined cancer [7]. A study at the Mayo Clinic of 3463 prostate cancer patients who underwent radical prostatectomy and pelvic lymph node dissection demonstrated that the 10-year cancer-specific survival was significantly worse in those with positive lymph nodes [8]. Therapeutic modalities of curative intent, such as radical prostatectomy and radiation, have a low probability of success in prostate cancer patients with lymph node metastases [7,9–12]. The therapeutic importance of lymph node metastasis, especially its role in predicting disease progression and determining future post-operative therapies, underscores the necessity of understanding the process of lymph node metastasis.

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Mechanisms of lymph node metastasis

The lymphatic system is comprised of lymphatic vessels and the lymphoid organs, which include lymph nodes, tonsils, spleen, Peyer's patches and the thymus. The lymphatic capillaries initially collect the extravasated tissue fluid, plasma proteins and cells (lymph) from the peripheral tissues and drain them to larger collecting lymphatic vessels through precollector vessels. The collecting vessels carry the lymphoid fluid to regional lymph nodes. The lymphoid fluid travels through the sinuses of the lymph node from the afferent to the efferent lymph vessels (Figure 1A). The sinuses are covered with endothelium and reticulum cells. Presentation of antigens and the induction of an effective adaptive immune response to pathogens is also a physiologic function of lymph nodes. Accordingly, lymph nodes are separated into the B-lymphocyte zone (follicles) and the T-lymphocyte zone, both of which are important for the induction of the immune response (Figure 1A). Interestingly, the immune function of the lymph node is severely impaired in cancer even before tumor cells disseminate. It has been demonstrated that tumor cells induce changes in the architecture of the lymph nodes adjacent to the malignancy, even before colonizing the lymph node, by reducing the density of antigen-presenting dendritic cells and the paracortical areas of the lymph nodes with the T-lymphocytes [13,14]. The draining lymph nodes are connected to lymphatic trunks, which, in turn, are connected to the venous circulation through the thoracic duct and left subclavian vein [2–4,15]. The structure of a lymphatic vessel is different from a blood vessel – in a lymphatic vessel, there is an absence of tight interendothelial junctions. Lymphatic endothelial cells are connected to the extracellular membrane through specialized anchoring filaments, which help the vessels open and function during increased tissue pressure [2,3]. Thus, lymphatic endothelial cells become permeable to tissue fluids and cells. Tumor cells and tumor-derived growth factors, cytokines and other molecules can enter into the lymphatic tracts through the hyper-permeable lymphatic endothelial cells [3,6,15]. They subsequently traffic to the regional lymph nodes, distant lymph nodes and finally to the blood circulation, where they can metastasize to distant organs (Figure 1B) [2,6]. Therefore, tumor-associated lymphatic vessels form an easy passage of tumor cells to lymph nodes and distant organs.

Local extension of tumor cells from the primary tumor into the surrounding lymphatics through a process called permeation is one means through which tumor cells can enter into the lymphatic vessels [3]. In addition, tumor cells can be stimulated by cytokines produced by the lymphatic vessels, which promote chemotactic diffusion of tumor cells into the lymphatics [3,4]. Finally, many tumors have the ability to secrete growth factors that induce the growth of new lymphatic vessels from a precursor, a process called lymphangiogenesis [2,4–6,16–18].

Lymphangiogenesis & lymph node metastasis

During embryogenesis lymphatic vessels develop from blood vessels [15,19]. Therefore, lymphangiogenesis and angiogenesis are stimulated by the same family of growth factor proteins. VEGF-A/VPF is the most potent growth factor for angiogenesis [20]. Other VEGF family members, such as VEGF-C and VEGF-D, are potent lymphangiogenic factors [2,5,6, 21–25]. Flt-4, also known as VEGF receptor-3 (VEGFR-3), is the tyrosine kinase receptor for VEGF-C or -D in lymphatic endothelial cells. Upon activation, this receptor triggers signaling events to initiate the proliferation and migration of lymphatic endothelial cells [2,6,17,23,26–29]. Neuropilin-2, a nontyrosine kinase receptor, is also expressed in lymphatic endothelial cells and acts as a coreceptor for VEGF-C during lymphangiogenesis [30,31]. Both VEGF-C and -D are expressed by tumor cells and, therefore, promote lymphangiogenesis from the tumor-associated surrounding lymphatics [2,6,17,23]. Importantly, cancer cells have also been shown to express neuropilin-2 and/or VEGFR-3, thus suggesting autocrine regulation of lymphangiogenic growth factors [32]. Our laboratory has reported one such autocrine regulation of VEGF-C and its receptor neuropilin-2 in prostate cancer – this autocrine function promotes

the survival of prostate cancer cells during oxidative stress and, thereby, is important for metastatic progression [33].

Although not as potent as VEGF-C or -D, VEGF-A also stimulates lymphangiogenesis upon binding with another VEGF receptor, VEGFR-2 (KDR/Flk-1), in an experimental mouse model of lymphangiogenesis [34,35]. The contribution of the VEGF-A/VEGFR-2 axis in inducing lymphangiogenesis in human tumor remains to be determined. Furthermore, both VEGF-C and -D induce angiogenesis because of their ability to bind VEGFR-2 in vascular endothelium cells [26,36–38]. Owing to the functional overlap of angiogenic and lymphangiogenic growth factors, angiogenesis and lymphangiogenesis are often coupled and can be stimulated by a single growth factor [39,40]. Apart from VEGF-C/-D and their cognate receptors, other growth factors are capable of inducing lymphangiogenesis to varying degree. These include angiopoietin-2 and -1 and its receptor Tie-2 [41,42], PDGF [43], IGF-1 and -2 [44], FGF family members [45], HGF [46,47], integrins (which crosstalk with VEGFR-3 signaling) [48,49], lymphotoxin β receptor [50] and EphrinB2 [15]. Although VEGF-C and -D and their receptor, VEGFR-3, have been implicated in tumor lymphangiogenesis, the contribution of these other factors in tumor lymphangiogenesis remains to be elucidated.

Lymphangiogenesis in tumors bears similarities with lymphangiogenesis in normal tissues. In order to study lymphatic vasculature in the tumor microenvironment, several lymphatic endothelium-specific markers are useful [6]. VEGFR-3 is one such marker and is also expressed by vascular endothelial cells in the tumor microenvironment [51]. Lymphatic vessel endothelial hyaluronan-receptor (LYVE)-1, a homolog of CD44, is another marker that is present in normal kidney, pancreas, adrenal and thyroid epithelia, as well as in hepatic blood sinusoidal endothelial cells [52]. Podoplanin, which is a glomerular podocyte membrane mucoprotein, is widely expressed in small lymphatic vessels [53] of vascular tumors, as well as in osteoblasts, kidney podocytes, peritoneal mesothelial cells, glandular myoepithelial cells, ependymal cells and lung alveolar type I cells. The homeobox transcription factor Prox-1 is also a marker for lymphatic vessels [54]. Since no single marker can specifically stain tumor lymphatic endothelium, these markers are often used in tandem to visualize lymphatic vessels [55]. Studies of these markers along with lymphangiogenic growth factors have revealed important aspects of tumor lymphangiogenesis and its correlation with lymph node and distant metastasis for many cancers. Increased lymphatic vessel density (LVD) was observed in many primary tumors expressing high levels of VEGF-C/-D, which were also correlated with regional lymph node metastasis and patient survival [2,21–23,25,56,57]. Interestingly, there are examples where such correlations do not exist [58,59].

Lymphangiogenesis, both intratumorally and peritumorally (Figure 1B), has been documented in human tumor specimens and also in tumor xenografts [60]. New enlarged lymphatic vessels at the periphery of VEGF-C/-D-expressing primary tumors (peritumoral lymphangiogenesis) have been observed in many cancer types [55,60]. Tumor cells, along with interstitial fluid containing several secreted factors from the tumor, enter into the lymphatics through the enlarged, highly permeable junctions of lymphatic endothelium and drain into the lymph nodes (Figure 1B).

The importance of intratumoral lymphangiogenesis for lymph node metastasis remains controversial. Functional lymphangiography studies with intravital microscopy in experimental animal tumor models suggest that intratumoral lymphatic vessels (ILVs) are compressed by the growing tumor mass and become nonfunctional [61]. On the other hand, ILVs carrying tumor emboli have been reported in tumor xenografts overexpressing VEGF-C [62] as well as head-and-neck squamous cell carcinomas [63] and oral/oropharyngeal cancer [64]. Another aspect of lymphangiogenesis in cancer is the detection of lymphangiogenesis in the sentinel lymph nodes (the first lymph node[s] to which cancer is likely to spread from the

primary tumor) [65]. Interestingly, lymphangiogenesis in those nodes occurs prior to the onset of metastasis. Both VEGF-C- and VEGF-A-expressing tumors exhibit lymph node lymphangiogenesis [66,67]. Thus, angiogenic growth factors secreted from the primary tumor appear to arrive in the sentinel lymph nodes prior to the metastatic tumor cells and alter the lymph node structure by inducing lymphangiogenesis [66,67]. Therefore, this phenomenon might explain tumor-reactive lymphadenopathy, where enlargement of draining lymph nodes occurs without the presence of detectable metastatic tumor cells. Thus, lymph node lymphangiogenesis may facilitate tumor cell transport to the lymph nodes, thereby establishing a microenvironment where tumor cells can reside and metastasize [65].

Prostate cancer lymph node metastasis & its dependence on lymphangiogenic growth factors

Correlative studies linking lymph node metastasis with the lymphangiogenic axis in human prostate cancer

A number of clinical studies have examined the relationship between lymphangiogenesis and prostate cancer lymph node metastasis (Table 1). These studies produced conflicting results. Although some detected lymphangiogenesis in prostate cancer tissues, which were correlated with lymph node metastasis, others failed to observe such a correlation. Interestingly, most of these studies have observed increased expression of VEGF-C in tumor tissues of prostate cancer patients with lymph node metastasis. The major point of dispute has been whether the increased expression of lymphangiogenic growth factors and their receptors, such as VEGFR-3, in prostate cancer tissues induced lymphangiogenesis for lymph node metastasis, or whether they might facilitate increased invasion of tumor cells into lymphatic vessels. Thus, we will discuss the major findings of each study and attempt to reconcile these conflicting results.

Studies with human prostate cancer tissue specimens suggest an association of VEGF-C and VEGFR-3 expression with lymphatic dissemination of human prostate cancer (Table 1). In one report by Tsurusaki *et al.*, VEGF-C mRNA and VEGFR-3 expression were studied in needle transperineal biopsies from prostate cancer patients prior to treatment [68]. VEGF-C mRNA expression was significantly higher in cancer tissues from patients with lymph node metastases than without. VEGFR-3-positive vessels were evident in tumor stromal tissues, suggesting an increase in LVDs and this correlated with VEGF-C mRNA expression. The authors concluded that VEGF-C expression in prostatic carcinomas is an indicator of lymph node metastasis.

Similar results were obtained with prostate cancer tissues obtained by retropubic prostatectomy [69]. Whereas benign prostatic hyperplasia (BPH) tissues exhibited negative or weak expression of VEGF-C, the majority of tumor tissues exhibited increased expression with varying intensities. Tumor cells at the periphery showed stronger expression than tumor cells in the center. Patients with lymph node metastases showed more intense staining than patients without lymph node metastases. However, no significant correlation was observed between VEGF-C expression and Gleason score. Expression levels of VEGFR-3 were associated with the presence of lymph node metastases. Importantly, there was a significant correlation between VEGFR-3 levels and Gleason score. The authors concluded that the increased expression levels of VEGF-C and its receptor VEGFR-3 are associated with lymph node metastasis in prostate cancer. Interestingly no increase in LVD was suggested as a mechanism for the involvement of the VEGF-C/VEGFR-3 axis in lymph node metastasis of prostate cancer.

In line with the aforementioned studies, another study by Zeng *et.al* observed an association of VEGFR-3-positive vessels in prostate tumors with lymph node metastases [70]. They found

the expression of VEGFR-3 positive vessels in prostate tumor to be associated with Gleason grade, extracapsular extension and surgical margin. One concern of this report, which is also applicable to the previous reports, is that VEGFR-3 was also detected in the vascular endothelium of tumors and, therefore, might also represent angiogenesis [51]. Tumor angiogenesis is known to correlate with most of the clinicopathological parameters of aggressive prostate cancer [71]. VEGF-C staining was lower in BPH tissue than in adjacent carcinoma. The expression of another lymphangiogenic growth factor, VEGF-D, exhibited a different pattern, where no differences were observed between benign epithelium and carcinoma. Nonetheless a strong and consistent pattern of VEGF-D staining was observed within the fibromuscular stroma of all prostate cancer specimens. The smooth muscle cells of blood vessels surrounding the tumor also strongly expressed VEGF-D. The authors suggested that VEGF-C and VEGF-D secreted by cancer cells might activate VEGFR-3 in the endothelial cells of adjacent lymphatic vessels via a paracrine mechanism to induce lymphatic invasion, possibly by modifying vessel permeability. This function therefore favors lymph node metastasis through lymphatic vessels.

In another study by Li *et al.*, normal prostate and BPH tissues exhibited negative or weak staining for VEGFR-3, whereas a significant number of the cancer tissues exhibited high-levels of VEGFR-3 expression [72]. Again, statistically significant positive correlations were observed between VEGFR-3 expression and Gleason score, preprostate-specific antigen levels and lymph node metastasis. VEGFR-3 was also related to poor cellular differentiation and aggressive behavior. Patients with high levels of VEGFR-3 expression had a worse recurrence-free survival rate, suggesting an involvement of VEGFR-3 in the progression of prostate cancer. However, no correlation was observed between VEGFR-3 expression and extracapsular extension, surgical margins, seminal vesicle invasion or pathologic stage. Moreover, VEGFR-3 expression was not an independent prognostic factor, as determined by multivariate analysis.

At least three studies have reported conflicting results with the previous studies (Table 1). One study found no increase in LVD but rather a destruction of lymphatic vessels in malignant prostatic tissues when compared with benign prostate tissues [73]. Paraffin sections of BPH and prostate cancer tissues were stained for LYVE-1 to identify lymphatic vessels in the tumor microenvironment. In the tumor microenvironment, LYVE-1 is more specific than VEGFR-3 for detecting lymphatic endothelial cells. No significant difference was observed in LVD between nontumorous areas and peripheral regions of the tumor. The authors concluded that there was no positive correlation between LVD and either tumor progression or the status of the lymph node metastasis. In their subsequent study, more intense VEGF-C expression was detected in cancer samples than BPH [74]. The lymph node-positive group exhibited significantly more VEGF-C expression than the lymph node-negative group. However, no statistically significant correlation was found between the increase of VEGF-C expression and the LVD in tumor tissues. The authors concluded that VEGF-C-induced abnormal functioning of lymphatics is important for prostate cancer lymph node metastasis rather than lymphangiogenesis. Similar results were reported by Roma *et al.* [75]. A monoclonal antibody (D2-40) was used to detect a fixation-resistant epitope of podoplanin [76–79]. LVDs in intratumoral, peritumoral or normal prostate zone were not significantly different between cases with lymph node metastases and those without. Interestingly, peritumoral lymphatic vessel invasion, which is defined as the presence of prostate cancer cells within the lymphatic vessel, was correlated with lymph node metastases, Gleason score, seminal vesicle invasion and positive surgical margins. They found that, although VEGF-C, -D and VEGFR-3 were elevated in prostate adenocarcinoma, these were not correlated with LVD, and the authors concluded that they might not induce lymphangiogenesis. According to Roma *et al.*, peritumoral lymphatic vessel invasion should be considered as a pathological parameter to look for lymph node metastasis in prostate cancer [75]. A study by Cheng *et al.* further supported these observations, where radical prostatectomy specimens containing invasive prostatic adenocarcinoma were analyzed

with D2-40 monoclonal antibody [80]. The LVD in intratumoral regions was found to be considerably lower than that of peritumoral or normal regions. LVDs in peritumoral and normal compartments were not significantly different. LVD did not correlate with lymph node metastasis, Gleason score, tumor volume, extraprostatic extension, seminal vesicle invasion or surgical margin positivity, suggesting that active lymphangiogenesis did not play a role in prostate cancer progression or lymph node metastasis. The authors hypothesized that prostate tumors secrete inhibitors of lymphangiogenesis, which might explain the lack of lymphangiogenesis in prostate cancer despite the presence of VEGF-C or -D. Although intriguing, there is no experimental verification to support this hypothesis.

One major difference between these conflicting studies is the technique used to stain lymphatic vessels. The studies that demonstrated an association between the lymphangiogenesis and lymph node metastasis used anti-VEGFR-3 antibodies to stain lymphatic vessels. However, VEGFR-3, although specifically expressed in normal adult lymphatic endothelium, is also expressed in tumor-associated angiogenic blood vessel endothelium cells [51]. Therefore, anti-VEGFR-3 antibodies may not be ideal to specifically stain the tumor-associated lymphatic endothelium. Thus, most investigators currently use antibodies against other lymphatic endothelial markers, such as LYVE-1 or D2-40. Interestingly, when D2-40 and VEGFR-3 antibodies were used to stain tumor lymphatic vessels, the staining pattern differed between VEGFR-3 and D2-40 [55]. The number of VEGFR-3-positive vessels was much lower than that of D2-40-positive vessels in corresponding sections. D2-40 staining recognized a significant number of lymphatic vessels, which were negative for VEGFR-3 staining. This suggests a diversity of lymphatic endothelial cell types in prostate tissue, where each type expresses a specific set of nonoverlapping lymphatic marker proteins. Therefore, it is important to understand the functions of different lymphatic endothelium cells in prostate cancer and their role in lymph node metastasis. The authors also reported peritumoral lymphatic invasion in a majority of prostate cancers with known lymph node metastases [55]. They found a significant increase in peritumoral LVD adjacent to tumors. Peritumoral LVD was significantly increased in prostate cancers with known lymph node metastases. However, they observed no correlation between intratumoral lymphatic invasion and lymph node status. One unexpected finding was that there was no evidence of active lymphatic proliferation in prostate cancer, as determined by Ki-67 staining, which was surprising, especially when there was an increase in peritumoral LVD.

In a recent study, Kuroda *et al.* also used the D2-40 antibody to stain lymphatic vessels in biopsy samples of radical prostatectomy specimens [81]. They evaluated the predictive value of peritumoral LVD and intratumoral lymphatic (ITLs) for lymphatic spread. Tissue specimens from patients who underwent radical prostatectomy for clinically localized prostate cancer were analyzed. ITLs were significantly less when compared with peritumoral lymphatics. Higher levels of average peritumoral LVD was significantly associated with higher positive core rates and lymphatic invasion. Higher levels of maximal peritumoral LVD were significantly associated with higher positive core rates, advanced pathological T stage, lymphatic invasion and pelvic lymph node metastasis. Lymphatic parameters in prostate biopsy cores were significantly associated with lymphatic invasion of prostatectomy specimens. Gleason score and average peritumoral LVD were significant predictors of lymphatic invasion, as determined by multivariate logistic regression analysis. The authors concluded that peritumoral LVD, but not the presence of ITLs, is an independent predictor of lymphatic invasion, confirming previous findings that peritumoral lymphatics, rather than ITLs, is more important for the lymphatic spread of prostate cancer.

Experiments with tissue culture & animal models of prostate cancer

Experiments with tissue culture and animal models of prostate cancer have revealed the mechanisms of the synthesis of lymphangiogenic growth factors in prostate cancer and their important contribution to lymphangiogenesis in prostate cancer lymph node metastasis.

Although results are contradictory with respect to the involvement of lymphangiogenesis in prostate cancer metastasis, most of the studies, either with prostate cancer tissue specimens or with animal models of prostate cancer, recognized the contribution of VEGF-C in prostate cancer lymph node metastasis. Thus, defining the molecular mechanisms by which VEGF-C synthesis is regulated in prostate cancer cells is important. Our laboratories observed a negative regulation of VEGF-C synthesis in prostate cancer cells by androgen. While withdrawal of androgen inhibited VEGF-A synthesis in prostate cancer cells, it favored increased synthesis of both VEGF-C protein and mRNA (Figure 2) [82,83]. The glucocorticoid receptor, another steroid hormone receptor, has also been shown to inhibit VEGF-C synthesis in glucocorticoid receptor-expressing DU145 cells upon binding with ligand [84]. The prostate-specific homeobox protein, NKX3.1, was found to negatively regulate VEGF-C synthesis by binding to a specific promoter site on the *VEGF-C* gene, recruiting histone deacetylase 1 and thereby inhibiting its transcription [85]. Loss of NKX3.1 protein has been observed in tissues from advanced-stage prostate cancer patients [86–88]. NKX3.1 was also found to be negatively regulated by androgen [89]. Thus, withdrawal of androgen reduces the NKX3.1 levels in prostate cancer, thereby facilitating VEGF-C synthesis. Androgen withdrawal also activates Ras-like oncogene, RalA, and FOXO-1 in prostate cancer cells, which induce VEGF-C synthesis [82,83]. Therefore, events such as the loss of NKX3.1 along with the activation of RalA and FOXO-1 act in concert to promote high levels of VEGF-C synthesis in prostate cancer (Figure 2) [85]. It will be important to determine whether tissue levels of VEGF-C are increased in prostate cancer patients treated with androgen-deprivation therapy, especially in the tumor tissues of those patients with castration-resistant disease. Other mechanisms have been reported for VEGF-C synthesis in prostate cancer. In a recent microarray-based study, it has been demonstrated that *VEGF-C* is one of the top six upregulated genes when NF- κ B2/p52 is overexpressed in LNCaP cells. Interestingly, ectopic expression of NF- κ B2/p52 made LNCaP cells castration resistant [90], suggesting its link with VEGF-C expression. COX-2 has also been implicated for the regulation of VEGF-C synthesis in prostate cancer [91]. Decreased synthesis of the cell adhesion molecule, CEACAM1, in prostate gland also upregulates the synthesis of VEGF-C and VEGF-D [92].

Animal models of prostate cancer provided important clues for understanding the mechanism of prostate cancer lymph node metastasis (Table 2). In one report, Brakenhielm *et al.* provided evidence to suggest that prostate cancer metastasis correlates with the expression level of VEGF-C and tumor lymphangiogenesis [93]. The prostate cancer cell lines LAPC-4 (expresses high levels of VEGF-C) and LAPC-9 (expresses low levels of VEGF-C) were implanted subcutaneously in immune-deficient mice. After 100 days from the time of removal of primary tumors, most of the LAPC-4 tumors had meta-static lesions in locations distant from the primary site, including the regional lymph nodes and the lungs, while LAPC-9 tumors had very few metastatic lesions. LYVE-1- and VEGFR-3-positive lymphatic structures were observed in the LAPC-4 tumors, both peritumorally and intertumorally. Ectopic overexpression of VEGF-C in the LAPC-9 tumors significantly increased (~20-fold) the ILVs, with concomitant increase in lymph node metastasis. The authors suggested that VEGF-C-induced tumor lymphangiogenesis is sufficient to cause lymph node and lung metastasis in these animal models of prostate cancer. The detection of only intratumoral lymphatic density in this animal model is a concern, since, in many clinical studies discussed previously, intratumoral lymphangiogenesis was rarely detected in prostate cancer and did not correlate with lymph node metastasis. One explanation, as suggested by the authors, was that the subcutaneous

location of the tumor in these animal models might be responsible for the discrepancy with the clinical findings. In human prostate cancer, tumors in the prostate may have immediate access to pre-existing lymphatics. Therefore, tumor cells enter into the lymphatics by expressing lymphangiogenic growth factors that cause existing lymphatic vessels to enlarge and become hyper-permeable. On the other hand, tumor cells implanted subcutaneously in mice require the induction of lymphangiogenesis in order to gain access to the lymphatic network. In a subsequent study, the same investigators overexpressed a mutant form of VEGF-C in LAPC-9 cells [94]. VEGF-CC_{156S}, where cysteine at position 156 was mutated to serine, recognizes only VEGFR-3 and does not bind to VEGFR-2 [95]. Interestingly, VEGF-CC_{156S}-overexpressing tumors had reduced lymphangiogenesis (~ sixfold) compared with wild-type VEGF-C-expressing tumors (~20-fold), therefore suggesting the involvement of VEGFR-2 in prostate cancer lymphangiogenesis. Interestingly, lung metastases were only observed in animals with lymph node metastasis, which would suggest that lymph node metastases facilitated systemic metastasis. Significant inhibition of lymph node metastasis was also observed in orthotopic prostate tumors, which overexpress a soluble form of VEGFR-3. The soluble form of VEGFR-3 is a truncated form of VEGFR-3, which contains only the extracellular ligand-binding part of the receptor and lacks the intracellular and membrane-spanning domains. Soluble-VEGFR-3 binds to and blocks the functions of both VEGF-C and -D. A defect in the lymphatic vasculature in the periphery of tumors was found to be the cause for the reduced lymph node metastasis in soluble-VEGFR-3-expressing tumors. These results suggested that both VEGF-C and VEGFR-3 are major players in lymph node metastasis in prostate cancer. The authors also concluded that lymphangiogenesis, but not angiogenesis, is responsible for lymph node metastasis and systemic metastasis in prostate cancer.

A different approach was taken by Zeng *et al.* to address the involvement of VEGF-C, VEGFR-3 and lymphangiogenesis and ended up with different conclusions (Table 2) [96]. Lymphatic endothelial cells (LEC) were isolated from prostate tissues obtained from patients undergoing prostatectomy. Both VEGF-A and VEGF-C, but not VEGF-CC_{156S}, stimulated tube formation, migration and proliferation of prostate LEC. A neutralizing antibody against VEGFR-2 inhibited LEC migration and capillary-like network formation induced by PC-3 and LNCaP cells. These experiments suggest that a signaling event via VEGFR-2 rather than VEGFR-3 is involved in prostate LEC tube formation, migration and proliferation. Interestingly, an approximately 57-KDa VEGF-A protein band, distinct from the well-documented 46 KDa VEGF-A band, was observed and proposed to be important for VEGFR-2 activation in LEC. Further work is warranted to clarify the specific function of this higher molecular weight VEGF-A protein on prostate cancer lymph node metastasis.

Wong *et al.* also reported results that were contradictory to previously mentioned results [59]. They observed almost complete reduction of intratumoral lymphangiogenesis in both subcutaneous and orthotopic PC-3 xenografts following stable expression of VEGF-C siRNA and/or overexpression of soluble VEGFR-3. Metastasis to lymph nodes was not affected despite a decrease of ITLs. Pre-existing peripheral lymphatics at the tumor stroma border remained unchanged in the orthotopic tumors and might be sufficient for lymph node metastasis. One possible explanation of the conflicting results between Wong *et al.* and Burton *et al.* might be the choice of different prostate cancer cell lines in their studies, which differed in the expression of androgen receptor and ability of these cells to metastasize. In addition, the extent to which the lymphangiogenic growth factor axis is inhibited in these cell lines might be a crucial factor, since it has been reported by Lin *et al.* that inhibition of lymph node metastasis by soluble VEGFR3 is dose dependent [97]. A threshold level of soluble VEGFR3 in the serum was required for an efficient inhibitory effect. This is further supported by Tuomela *et al.*, who implanted PC-3 cells orthotopically into nude mice [98]. Mice were injected with a recombinant adenovirus, which expressed soluble VEGFR3 immunoglobulin (Ig). The mean size of PC-3 tumors was found to be significantly smaller in mice injected with VEGFR3-Ig

than control mice. Treatment with VEGFR3-Ig significantly decreased the density of both lymphatic and blood vessels in tumors as well as the incidence of lymph node metastasis.

Summary of the findings from patient samples & animal models of prostate cancer

A number of studies have begun to define the mechanism of lymph node metastasis in prostate cancer (Tables 1 & 2). Most of these studies demonstrated the involvement of lymphangiogenic growth factors, particularly VEGF-C, in this process. However, discrepancies between studies have been noted. The major differences in these studies can be attributed to how the prostate cancer cells enter the lymphatic vasculature. In some studies, an increase in LVD or lymphangiogenesis was reported as a reason for lymph node metastasis. Among these, some also observed an increase in intratumoral LVD. Since most of the clinical findings argued against an increase in ILV, the contribution of ILV in this process is likely to be a rare event. ILVs were primarily observed in animal models, when cells were implanted subcutaneously into immuno-compromised mice [94]. Therefore, it is possible that the subcutaneous tumor microenvironment influenced the development of ILVs. Increases in peritumoral LVD or invasion of tumor cells in existing peritumoral lymphatic vessels were primarily observed in prostate cancer patients with lymph node metastases [75,80,81]. Thus, an increase in peritumoral LVD may provide a greater opportunity for prostate cancer cells to enter into the lymphatic vessels. On the other hand, increased invasion of prostate cancer cells into the existing peritumoral lymphatics is probably a more direct process and requires specific communication between prostate cancer cells and lymphatic endothelial cells. Such communication has been reported in other cancer types [99,100] and needs to be verified in prostate cancer. The role of lymphangiogenic growth factors in establishing such communication needs to be elucidated. Interestingly, in most studies, lymph node metastasis was correlated with VEGFR-3 expression, whereas this was not always the case with LVD. Therefore, other cell types, which also express VEGFR-3 in tumor tissue, might be important for lymph node metastasis. It remains to be determined what these cell types might be in prostate cancer tissues and how they might facilitate lymph node metastasis.

Therapeutic implications

Regional lymph node metastases are found in up to 10% of men with clinically staged organ-confined prostate cancer. The clinical implication of these findings has remained elusive. As previously mentioned, studies have suggested that the 10-year cancer-specific survival is significantly worse in those prostate cancer patients with positive lymph nodes. It has also been reported that men with more than two positive nodes exhibit poor prognosis when compared with patients with one or two positive nodes. Therefore, detection of lymph node metastasis has prognostic significance for prostate cancer, since it might be an indicator of systemic disease. Interestingly, lymph node metastasis may also be viewed as a mediator of distant organ metastasis. The development of lymph node lymphangiogenesis in the sentinel lymph node before the arrival of cancer cells, in animal models, supports the concept that distant metastases arise from metastatic cancer cells in the sentinel lymph nodes. It has been hypothesized that lymphangiogenic growth factors not only facilitate the invasion of cancer cells into the lymph nodes but also modulate the lymph nodes in a way that supports growth, survival and further transformation of metastatic cancer cells. Therefore, dissection of lymph nodes should have therapeutic advantages. Blocking the lymphangiogenic growth factor pathways should also have therapeutic benefits. The issues related to the prognostic or therapeutic significance of lymph node dissection for prostate cancer patients can only be resolved by understanding the significance of lymph node metastasis in the overall metastatic process. This will be achieved through a comprehensive knowledge of the mechanism(s) of lymph node metastasis in prostate

cancer, the role of lymphangiogenic and other growth factors in this process and their relationship with the overall process of metastasis. Our current understanding in this regard is not complete and requires further studies with both human prostate cancer tissues and animal models of prostate cancer.

Future perspective

The expression of lymphangiogenic growth factors, especially VEGF-C, has been correlated with prostate cancer lymph node metastasis. Research with human prostate cancer tissues and animal models has presented conflicting interpretations with respect to the role of tumor lymphangiogenesis or the lymphatic invasion of tumor cells as the predominant mediator of lymph node metastasis. Interestingly, VEGF-C regulates both of these processes. Furthermore, a stress-resistant function of VEGF-C appears to be important for metastatic progression of prostate cancer cells. Thus, it is possible that all these functions of VEGF-C contribute simultaneously and, in varying degrees, to promote prostate cancer lymph node metastasis. The scenario is even more complicated when considering the heterogeneity in tumors for a single prostate cancer patient, which would result in inpatient variation of the involvement of the contributing factors for lymph node metastasis. Future research should address this complicated issue. This knowledge would be important for developing therapies that are more personalized in nature. The role of lymphangiogenesis in sentinel lymph nodes should be another area for future research. Such studies should address the importance of lymph nodes in systemic metastasis and the question of whether lymph nodes can serve as incubators for metastatic transformation of cancer cells. Such studies may help to resolve issues related to the prognostic or therapeutic significance of lymph node dissection in prostate cancer patients.

Executive summary

Tumor-associated lymphangiogenesis can facilitate lymph node metastasis

- The therapeutic importance of lymph node metastasis, especially its role in predicting disease progression and determining future post-operative therapies, underscores the necessity of understanding the process of lymph node metastasis.
- Many tumors secrete growth factors that function in a paracrine manner to stimulate the growth of new lymphatic vessels from a precursor, a process called lymphangiogenesis. Tumor-associated lymphangiogenesis is considered to be important for lymph node metastasis.
- VEGF-C, VEGF-D and their receptors in the lymphatic endothelial cells, such as VEGF receptor 3 and neuropilin-2, are the major mediators of tumor lymphangiogenesis.

Studies with human prostate cancer tissue samples that correlate lymphangiogenesis with lymph node metastasis

- Expression of VEGF-C and VEGFR-3 has been correlated with prostate cancer lymph node metastasis.
- Increased invasion of tumor cells into the peritumoral lymphatics has been suggested as the mechanism of lymph node metastasis.
- However, conflicting results from clinical studies, which examined the relationship between lymphangiogenesis and prostate cancer lymph node metastasis, have been reported.
 - The technique of detecting tumor-associated lymphatic vessels might lead to these conflicting results.

- Tumor-associated lymphatic endothelial cells might be heterogeneous in structural and functional aspect. Dissecting each type of endothelial cell may resolve such conflicting results.

Experiments with tissue culture & animal models of prostate cancer

- Tissue culture and animal models of prostate cancer yielded conflicting results relating to the involvement of lymphangiogenesis in prostate cancer lymph node metastasis.
 - Several gene products, such as RalA, FOXO-1, NKX3.1, NF- κ B2/p52, COX-2 and CEACAM1, serve either as positive or negative regulators for VEGF-C expression in prostate cancer cells.
 - Some animal studies suggested that lymph node metastasis is a precursor for distant systemic metastasis.
- The involvement of VEGF-A and VEGF receptor 2 in lymphangiogenesis and lymph node metastasis has also been reported.

Conclusion

- An increase in peritumoral lymphatic vessel density and/or invasion of tumor cells in existing peritumoral lymphatic vessels appears to be a major factor in lymph node metastasis of prostate cancer.
- VEGF-C and VEGFR-3 are important for prostate cancer lymph node metastasis.
- A comprehensive knowledge of the mechanism(s) of lymph node metastasis in prostate cancer, the role of lymphangiogenic and other growth factors in this process and its relationship with the overall process of metastasis, has clinical implication in resolving the issues related to the prognostic or therapeutic significance of lymph node dissection in prostate cancer patients.

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Figure 1. Tumor-associated lymphatic vessels serve as a route for lymph node metastasis
(A) Lymph node. (B) Metastases of tumor cells through lymphatic vessels to lymph nodes.

Figure 2. Signaling pathways for VEGF-C synthesis in prostate cancer

Table 1Studies of the correlation of lymphatic vessel density and lymph node metastasis[†].

Study	Marker	LVD correlation with LN metastasis	Major findings	Ref.
Tsurusaki <i>et al.</i> (1999)	VEGFR-3	+	VEGF-C mRNA expression was correlated with LN metastasis	[68]
Jennbacken <i>et al.</i> (2005)	VEGFR-3	+	VEGF-C protein expression was correlated with LN metastasis VEGFR-3 expression was correlated Gleason score	[69]
Zeng <i>et al.</i> (2004)	VEGFR-3/D2-40	+	VEGFR-3-positive vessels in prostate tumor were associated with Gleason grade, extracapsular extension and surgical margin VEGF-C was higher in cancer tissue than in BPH Strong VEGF-D staining was observed within the fibromuscular stroma of prostate cancer tissues	[70]
Li <i>et al.</i> (2004)	VEGFR-3	+	VEGFR-3 expression was correlated with Gleason score, pre-PSA levels, and LN metastasis	[72]
Trojan <i>et al.</i> (2004)	LYVE-1/CD34	–	LN positive group exhibited significantly more VEGF-C expression than the LN-negative group	[73]
Roma <i>et al.</i> (2006)	D2-40	– (lymphatic vessel invasion correlated with LN metastasis)	VEGF-C, -D and VEGFR-3 were elevated in prostate adenocarcinoma Intratumoral LVD is significantly lower than peritumoral LVD	[75]
Cheng <i>et al.</i> (2008)	D2-40	–	Intratumoral LVD is significantly lower than peritumoral LVD	[80]
Zeng <i>et al.</i> (2005)	D2-40 and VEGFR-3	+ peritumoral LVD	D2-40 staining recognized a significant number of lymphatic vessels, which were negative for VEGFR-3 staining Increased peritumoral lymphatic invasion was also suggested for increased LN metastasis	[55]
Kuroda <i>et al.</i> (2008)	D2-40	+ peritumoral LVD	Intratumoral LVD is lower than peritumoral LVD	[81]

[†]None of these studies reported a correlation of LVD with prostate-specific antigen relapse or cancer-specific survival.

+: Positive correlation; –: Negative correlation; BPH: Benign prostatic hyperplasia; LN: Lymph node; LVD: Lymphatic vessel density; LYVE: Lymphatic vessel endothelial hyaluronan receptor; Prostate-specific antigen; VEGFR: VEGF receptor.

Table 2

Studies of the correlation of lymphatic vessel density and lymph node metastasis in animal or tissue culture models of prostate cancer.

Study	Marker	Model used	LVD correlation with LN metastasis	Comment	Ref.
Brakenhielm <i>et al.</i> (2007), Burton <i>et al.</i> (2008)	LYVE-1, VEGFR-3	Subcutaneous implantation of LAPC-4 (high VEGF-C-expressing) and LAPC-9 (low VEGF-C-expressing) cells in nude mice	+ (intratumoral LVD was correlated with LN metastasis)	VEGF-C expression was correlated with LN metastasis LN metastases facilitated systemic metastasis VEGF-C and VEGFR-3 were suggested as the major players for LN and distant metastasis in prostate cancer	[93,94]
Zeng <i>et al.</i> (2006)		Studied the effect of VEGF-C, VEGF-A, VEGFR-2 and VEGFR-3 on the tube formation, migration and proliferation of primary prostate LECs		VEGFR-2 was detected to be important for prostate LEC tube formation, migration and proliferation An approximately 57-KDa VEGF-A protein band was proposed to be important for VEGFR-2 activation in LECs	[96]
Wong <i>et al.</i> (2005)	LYVE-1	Subcutaneous or orthotopic implantation of a subline of PC-3 cells VEGF-C/ VEGFR-3 axis was blocked by either VEGF-C siRNA or soluble VEGFR-3	-	Metastasis to LNs was not affected despite a decrease of intratumoral lymphatics	[59]
Lin <i>et al.</i> (2005)	LYVE-1	Subcutaneous implantation of PC-3 subline; VEGF-C axis was blocked by soluble VEGFR-3 expressed via a recombinant adeno-associated viral vector	+	A threshold level of soluble VEGFR-3 in the serum was required for an efficient inhibitory effect of lymphangiogenesis and LN metastasis	[97]
Tuomela <i>et al.</i> (2009)	LYVE-1	Orthotopic implantation of PC-3 cells; VEGF-C axis was blocked by soluble VEGFR-3 expressed via a recombinant adeno-associated viral vector	+	Blocking the VEGFR-3 axis inhibited primary tumor growth and tumor angiogenesis	[98]

+: Positive correlation; -: Negative correlation; LEC: Lymphatic endothelial cell; LN: Lymph node; LVD: Lymphatic vessel density; LYVE: Lymphatic vessel endothelial hyaluronan receptor; PSA: Prostate-specific antigen; VEGFR: VEGF receptor.