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Prognostic Significance of Peritumoral Lymphatic Vessel Density and Vascular Endothelial Growth Factor Receptor 3 in Invasive Squamous Cell Cervical Cancer

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Abstract

Cervical cancer is known to metastasize primarily by the lymphatic system. Dissemination through lymphatic vessels represents an early step in regional tumor progression, and the presence of lymphatic metastasis is associated with a poor prognosis. In patients who have undergone a radical hysterectomy, lymphovascular space invasion (LVSI), assessed on hematoxylin and eosin–stained slides, is a major factor for adjuvant therapy in patients with cervical cancer. With the advent of a lymphatic endothelial cell–specific marker, such as D2-40, it is now possible to distinguish between blood and lymphatic space invasion (LSI). In this study, the utility of D2-40 was assessed for the detection of lymphatic vessel density (LVD) and identification of LSI. The expressions of vascular endothelial growth factor receptor-3 (VEGFR-3), VEGF-C, tyrosine receptor kinase-2, and angiopoietin-1 were assessed by immunohistochemical methods on 50 patients with squamous cell carcinoma of the cervix. Clinicopathologic characteristics, including pelvic lymph node metastasis, were correlated with the above histochemical findings. We found that lymphangiogenesis, measured by an increase in peritumoral LVD, was significantly associated with positive lymph node status ($P < .005$). VEGFR-3 expression was significantly associated with LVD ($P < .05$). D2-40 staining verified LSI ($P = .03$) and surpassed that of hematoxylin and eosin–identified LVSI ($P = .54$). In conclusion, lymphangiogenic markers, specifically LVD quantified by D2-40 and VEGFR-3, are independently associated with LSI and lymph node metastasis in patients with early squamous cell carcinoma of the cervix treated with radical hysterectomy and pelvic lymphadenectomy.

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Introduction

Lymph node metastasis is a major cause of death from cervical carcinoma. Lymphatic vessels provide natural drainage, and they are the preferential routes for metastatic spread of many cancers, including most carcinomas that arise from vulva, vagina, and cervix [1]. Dissemination through lymphatics represents an early step in regional tumor progression, and the presence of lymphatic metastasis is associated with more aggressive tumors, with a poor prognosis. The clinical and pathological nodal status guides important treatment decisions about adjuvant treatment options when surgery is performed.

It was previously thought that lymphatic metastases involved the constant migration of malignant cells along preexisting lymphatic channels originating near the tumor, that is, the Halstead hypothesis [2]. Dissemination of cancer cells is now considered to be more episodic, occurring through emboli, rather than the steady march of cells, and may be through newly formed lymphatic vessels [3].

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Lymphatic vessel density (LVD) is an emerging prognostic factor associated with regional lymph node metastases, distant metastasis, and poor overall survival [4,5]. Although peritumoral lymphatic vessels contribute to tumor metastasis, there is the question as to whether intratumoral lymphatics have a role in tumor metastasis [6]. Thus, the location of tumor lymphatics may be an important factor in the metastatic spread of cancer cells.

The precise mechanisms whereby the newly formed lymphatics promote cancer progression are not well understood. However, several mechanisms have been postulated. A change of lymphatic function and an increase in lymphatic density alter the tumor microenvironment and its propensity for metastasis. Furthermore, cytokines direct from the lymphatic endothelial cells may facilitate tumor spread by promoting tumor cell migration toward, and invasion into, the vessel [7].

Vascular endothelial growth factor C (VEGF-C), a novel VEGF member, has been found to induce lymphangiogenesis through activation of the tyrosine kinase VEGF receptor 3 (VEGFR-3). VEGFR-3 is expressed predominantly on the surface of adult lymphatic endothelial cells and is therefore considered a major regulator of lymphangiogenesis [2,8]. The monoclonal antibody D2-40 [9] was recently reported to be selective for lymphatic endothelium, and it has identified lymphatic invasion in many malignant tumors [10]. There have been few studies to date on lymphangiogenesis due to the lack of these specific lymphatic endothelial markers and lymphatic-specific growth factors.

Lymphovascular space invasion (LVSI), defined as the identification of "any tumor inside endothelial-lined spaces in the primary tumor site by microscopy" [11], has been found to significantly increase the risk of lymph node involvement [12,13]. However, this is a combined parameter unable to distinguish invasion of lymphatic and blood capillaries at the primary tumor site. Despite its strong prognostic relevance, there has been no standardization in the measurement of true lymphatic space invasion (LSI) or LVD.

Methodical problems arise first in the identification and visualization of the lymphatic vessel wall after hematoxylin and eosin (H&E) staining using conventional light microscopy [14,15]. Difficulty also arises with the ability to discern "viable" tumor emboli within lymphatic lumens [16] and to identify retraction artifact, due to fixation, *versus* true lymphatic vessel space [17]. Previous studies have assessed peritumoral LVD by measuring the area with highest LVD, the so-called hot spot, and have not evaluated the entire stroma's vessel density; this generates selection bias and does not account for the entire tumor microenvironment [7,18]. The accurate assessment of LSI and LVD requires specific immunohistochemical markers for lymphatic vessels based on a standard protocol for microvessel count.

The mechanism by which newly formed lymphatics promote cancer metastasis is not well understood. Therefore, the aims of the present study were to apply the novel antibody D2-40 to squamous cell cervical cancers and evaluate its sensitivity for LVSI, to develop a mathematical assessment to quantify LVD, to compare LSI as detected by D2-40 immunostaining with that of conventional LVSI as detected by H&E staining, to investigate whether LVD intratumorally or peritumorally is a reliable risk factor for lymph node metastasis and cancer recurrence, and, finally, to investigate whether VEGF-C, VEGFR-3, angiopoietin-1 (ANG-1), and tyrosine receptor kinase-2 (TIE-2) are independent prognostic markers for cervical cancer metastasis.

Materials and Methods

Cervical Tissue Specimens and Patient Characteristics

We extracted 40 cases of invasive squamous cell carcinoma of the cervix from the Department of Surgical Pathology database at the University of Texas Medical Branch, Galveston, Texas, between the years 2007 and 2008. All patients had undergone radical hysterectomy with lymph node dissection. H&E–stained slides were reviewed, and the diagnoses were verified. Tissue samples and patient information were collected with full institutional review board approval.

Cases were evaluated for patient's age, clinical stage, clinical tumor size, and treatment. Staging was defined according to the clinical staging system of Federation Internationale des Gynaecologistes et Obstetristes [19].

Histopathologic Examination

Tissues from the specimens were initially fixed in 10% buffered formalin, paraffin-embedded, processed, and stained with H&E, by the Surgical Pathology Department as a part of the routine intake procedure. H&E slides of all cases were reviewed to confirm the diagnosis and the histopathologic characteristics including histologic type, pathological tumor size, depth of invasion, the presence of lymphatic and LVSI, grade, pelvic, and para-aortic lymph node metastasis. Four tumor-containing paraffin-embedded tissue blocks, identified on the histologic H&E review, were then selected from each patient for the purpose of this article's immunohistochemical studies. The clinicopathological features of the tumors are summarized in Table 1.

Immunohistochemical Analysis

Serial 5-μm sections were cut from formalin-fixed, paraffin-embedded tissue and were mounted on precoated slides for immunohistochemical detection of D2-40, VEGF-C, VEGFR-3, ANG-1, and TIE-2. Four goat polyclonal antibodies and one mouse monoclonal antibody were used for this study. The goat polyclonal antibodies, anti–VEGF-C (AF752), anti–VEGF receptor-3 (AF349), anti–ANG-1 (AF923), and anti–TIE-2 (AF313) were purchased from R&D Systems (Minneapolis, MN) and used at the concentrations of 7.5, 2.0, 5.0, and 7.0 μg/ml, respectively. Mouse monoclonal antibody D2-40, which reacts with an *O*-linked sialoglycoprotein found on lymphatic endothelium, was purchased from Signet Laboratories (Dedham, MA) and was used at a dilution of 1:200.

Table 1. Association between Clinicopathological Variables and Lymphatic Metastases in Cervical Cancer (n = 35).

Parameters	No. Specimens	P
Total	35	
Stage		.704
1A/B	31	
2A	4	
Age, years		.2205
Mean	42	
Depth of invasion		.043
<2 mm free	12	
>2 mm free	23	
Tumor size		.244
>4 cm	14	
<4 cm	21	

Immunohistochemical assays were performed using the avidin-biotin-peroxidase complex (ABC) assay for all five primary antibodies. The 5-μm-thick sections were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity within the tissue was quenched by treating with 3% hydrogen peroxide (H_2O_2) in methanol for 20 minutes. Antigen retrieval was achieved by placing the sections in preheated (95-100°C) 0.01 M citrate buffer, pH 6.0, with 0.1% Tween 20 for 20 minutes. Nonspecific binding sites were blocked with rabbit normal serum (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) in phosphate-buffered saline for 20 minutes at room temperature. Primary antibodies for VEGF-C, VEGFR-3, and TIE-2 were used at the concentrations mentioned above, and the slides were incubated overnight at 4°C. Slides for ANG-1 were incubated 2 hours at room temperature. Subsequent incubation with the biotinylated secondary antibody, antigoat immunoglobulin G (Vectastain ABC kit; Vector Laboratories) was then performed for 30 minutes. The slides were then incubated with streptavidin-biotin-peroxidase complex (ABC) for 30 minutes. Finally, the peroxidase reactivity was visualized with 3,3′-diaminobenzidine (DAB; Vector Laboratories) according to the manufacturer's instructions. The sections were counterstained with Gill 2× hematoxylin (Fisher cat. no. 245-654) and mounted. Negative controls were performed by omitting the primary antibodies. The positive control was a single patient with squamous cell cervical cancer whose formalin-fixed paraffin-embedded sections were stained with for each batch of antibody.

For the detection of lymphatic channel endothelium, the same immunohistochemical procedures were followed except the use of different blocking serum, primary antibody, and secondary antibody. The tissue sections were blocked with normal horse serum (Vector Laboratories) for 20 minutes at room temperature and then incubated with primary D2-40 antibody (mouse monoclonal, 1:200 dilution; Signet Laboratories) for 1 hour at room temperature. Biotinylated secondary antibody, anti-mouse immunoglobulin G (Vectastain ABC kit; Vector Laboratories), was used.

Evaluation of Immunohistochemical Staining

The expressions of VEGF-C, VEGFR-3, ANG-1, and TIE-2 were scored by the staining intensity of the tumor cells. The intensity score was defined as follows: 1+, faint or equivocal immunoreaction; 2+, weak immunoreaction; 3+, moderate; and 4+, strong immunoreaction.

Demarcation of Peritumoral Region and Lymphatic Vessel Quantification

Whole-slide imaging of the entire D2-40–stained histopathologic section, as originally mounted on glass microscope slides, was obtained using the Aperio automated scanning robot (T2; Aperio Technologies, Vista, CA). This has a 20× microscope objective at a spatial sampling period of 0.47 μm per pixel. The images were subsequently viewed using the ImageScope software (Aperio Technologies).

For the purpose of this study, we strictly defined two peritumoral regions of interest. The first peritumoral region was defined as the area spanning 100 μm from the obvious tumor margin to 1 mm. The second peritumoral region was defined as the next 1 to 2 mm from the original tumor margin (Figure 1). LVD was defined as the number of D2-40–positive spaces per square millimeter for a specified region.

This strict assessment of LVD was performed using the Aperio slide-scanning software. All D2-40–immunoreactive spaces were counted within the two peritumoral regions of the full slide image and, subsequently, divided by the total area of the defined region, yielding LVD in spaces per square millimeter. Groups of immunoreactive endothelial cells, with or without patent lumens, were counted as a lymphatic space. LSI, detected by immunohistochemical staining, was considered positive if a tumor cell or cluster of tumor cells was clearly visible inside the D2-40–immunopositive space.

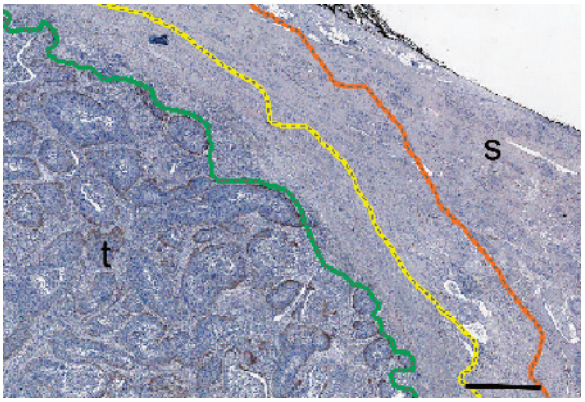

Figure 1. Designation of peritumoral regions. Aperio Scan Scope (Aperio Technologies) image of D2-40–stained formalin-fixed, paraffin-embedded squamous cell carcinoma of the cervix. Green line directly adjacent to tumor mass designates tumor boundary. Peritumoral region 1 was set as the region spanning 1 mm from the tumoral border, designated by the yellow line. Peritumoral region 2 spanned 1 mm from the boarder of region 1, designated by the orange line. *s* indicates stroma; *t*, tumor. Original magnification, ×2; scale bar, 1 mm.

Statistical Analysis

All statistical calculations were carried out using SAS Software, Version (9.2) of the SAS System for Windows (Cary, NC). The Cochran-Mantel-Haenszel test was used to compare and determine whether there was a significant difference in immunohistochemical expression within the VEGF-C, VEGFR-3, ANG-1, and TIE-2 groups and to determine whether there was a correlation between any of the above variables with clinicopathological parameters. Level of statistical significance was set at $P \leq 0.05$.

Results

Reclassification of Patients by D2-40 Immunohistochemistry

The D2-40 antibody, known to selectively stain lymphatic endothelial cells, identified lymphatic channels within tumor and in the peritumoral stroma. Endothelial cells lined spaces devoid of blood cells (indicative of lymphatic spaces) stained strongly, whereas obvious blood vessels showed no immunolabeling with D2-40 (Figure 2). Myoepithelial cells were also immunoreactive to D2-40, but differentiation of myoepithelium from lymphatic epithelium was uncomplicated based on size, location, and organization. One patient was removed from the study owing to diagnosis of lymphoepithelioma-like carcinoma. In addition, four patients were removed from the study owing to insufficient tissue to complete all immunohistochemical analysis.

D2-40–confirmed LSI was compared with the occurrence of LVSI identified on the H&E slide by pathology at the time of surgery and

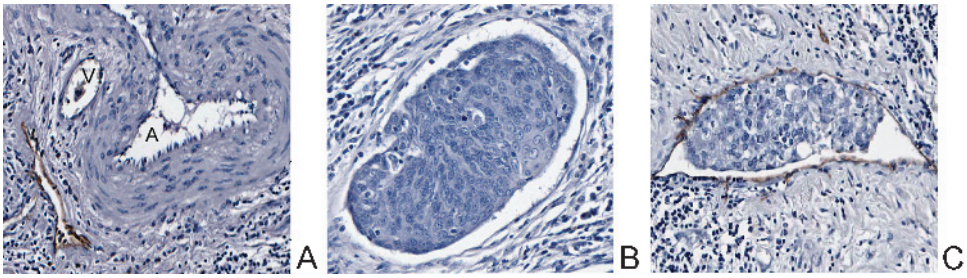

Figure 2. Utility of D2-40 immunohistochemical staining. (A) Typical results of immunostaining with the D2-40 antibody, brown staining selectively present in lymphatic endothelium. (B) Commonly seen retraction artifact mimicking LVSI. (C) D2-40–stained endothelial cells, stained brown, verify the presence of true LSI of the cancer cells. Without the use of the lymph epithelial–specific immunohistochemistry staining, differentiation between panels A and B would prove difficult and subjective. Original magnification, ×20.

verified independently by the study pathologist. LSI was found predominately in the stroma directly adjacent to the tumor, rarely extending past 1 mm from the tumor, regardless of tumor size.

Using the Cochran-Mantel-Haenszel test to compare the two groups based on the binary response, LSI or LVSI-positive or LVSI-negative, no association or no pattern of association was found between LSI and LVSI status. LSI was found to be independently statistically associated with nodal status, independent of LVSI status (P = .0009), whereas the converse was not true (Table 2).

Correlation of LVD with Clinicopathological Prognostic Factors in Cervical Cancer

D2-40 verified an increased presence of lymphatic vessels in stroma directly adjacent to tumor. Lymphatic vessels within tumor were rare and, if found, were collapsed. One tumor was found to have an extremely large number of patent intratumoral lymphatics: after further histologic review, the condition of this patient was diagnosed as lymphoepithelioma-like carcinoma. This patient's sample was then removed from the study set.

We found the LVD of peritumoral region 1, directly adjacent to the tumor, to be 1.25-fold (P < .05, analysis of variance [ANOVA]; Figure 3*A*) that of peritumoral region 2. Peritumoral LVD showed no correlation with patient age, tumor size, or depth of invasion. LVD in peritumoral region 1 (LVD1) showed a greater correlation with lymph node metastasis (P = .0005, ANOVA; Figure 3*C*) when compared with peritumoral region 2 (LVD2; P = .015, ANOVA; Figure 3*B*) and have a positive correlation with LSI (P = .015, ANOVA).

The median number of positive lymph nodes was 2 (range, 1-10). Lymph node metastasis was present in 14 cases (40%).

Cochran-Mantel-Haenszel test performed showed an increase in LSI or LVD1 to be statistically significant at predicting lymph node positivity at the time of surgery (P < .05; Table 2). In addition, LSI was found to have superior odds ratio estimate compared with LVD1 of 15.91 (95% confidence interval [CI], 1.29-195.1) and 1.78 (95% CI, 1.01-3.14), respectively.

Correlation of Immunohistochemical Markers with Lymphatic Space Density

Multiple regression analysis of the predictors of LVD, VEGFR-3, VEGF-C, ANG-1, and TIE-2 was performed. Significant positive correlation was found between VEGFR-3 (P = .006) and ANG-1 levels within the tumors with peritumoral LVD (P = .0298). Staining of both VEGFR-3 and ANG-1 was found to be homogenous within the tumor and present in advanced dysplastic epithelium (Figure 4). No statistical correlation was found between VEGF-C or TIE-2 and LVD.

Discussion

Combined parameters of lymphatic invasion, margin status, tumor size, depth of invasion, parametrial involvement, and stage all guide physicians in the recommendation of adjuvant treatment. Lymph node positivity has repeatedly been shown to have an ominous survival impact, with a 67% 5-year survival in stage IB cancer patients [20] to 91% in node-negative patients [21]. Adjuvant treatment of this cancer is, as a standard, a combination of chemotherapy and radiation therapy [22,23]. Identification of LSI has been shown to predict nodal metastasis, and the use of this novel antibody standardizes the histologic assessment of an important prognostic factor in squamous cell cervical cancer improving its predictive ability.

In early cervical cancer, lymphatic involvement has been one of the more difficult parameters to quantify owing to fixation artifact, tumor sampling discrepancy, and proper identification of lymphatic channels. We have used the antibody D2-40 to improve the identification of lymphatic vessels in radical hysterectomy specimens, aiding in the verification of LSI. We have found that the identification of D2-40–verified LSI is far superior than relying on H&E staining for the prediction of nodal metastasis.

D2-40 lymphatic vessels were almost exclusively found at the interface between the stroma and tumor and not within the tumor, a finding in good accordance with published studies [24–26]. One

Table 2. Correlation of LSI and Lymph Node Metastasis Independently Controlling for Either LVSI (A) or LSI (B).

	LSI−			LSI+	
	LVSI+	LVSI−		LVSI+	LVSI−
(A) LVSI by Lymph Node Status Controlling for LSI					
Lymph node metastasis					
pN+ primary tumors (n = 14)	0	1		8	5
pN0 primary tumors (n = 21)	5	9		4	3
P (Cochran-Mantel-Haenszel)			.9		
(B) LSI by Lymph Node Status Controlling for LVSI					
Lymph node metastasis					
pN+ primary tumors (n = 14)	5	1		8	0
pN0 primary tumors (n = 21)	3	9		4	5
P (Cochran-Mantel-Haenszel)			.0009		

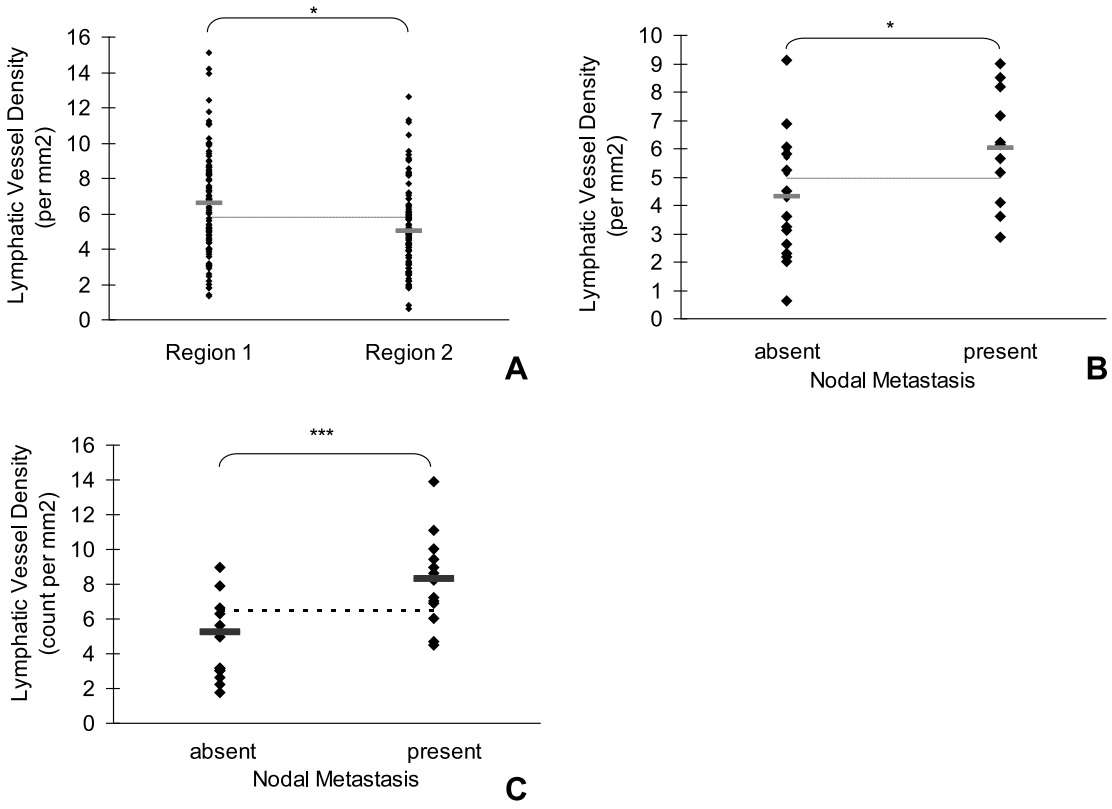

Figure 3. Peritumoral LVD in cervical squamous cell carcinoma. (A) LVD in peritumoral region 1, when compared with region 2, was found to be 1.25-fold higher. (B and C) Comparison of peritumoral LVD in region 2 (B) and region 1 (C) with the absence and presence of lymph node metastasis. Lines indicate median VEGFR-3 immunostaining score values. *$P < .05$; ***$P = .0005$, ANOVA.

hypothesis for the lack of functional intratumoral lymphatic vessels is owing to the collapse by the physical stress exerted by the growing tumor [26]. Our studies revealed that cervical cancers with high peritumoral lymph angiogenesis, measured with peritumoral LVD, are significantly more often associated with nodal metastasis. The significant association between LVD and LSI could simply be explained through classic probability, where lymphangiogenesis induces increase of opportunities for the tumor cells to enter lymphatic vessels. We found a significant correlation in both LSI and LVD with regional nodal metastasis.

In our studies, we found that the LVD1 directly adjacent to the tumor (within 1 mm) showed a greater correlation with lymph node metastasis when compared with the second peritumoral region (LVD2), suggestive of a paracrine lymphangiogenic mechanism involving the malignant cells. Four known lymphangiogenic factors were studied in the cervical epithelium for their correlation to LVD and nodal metastasis. VEGFR-3 is expressed differentially by cervical epithelial cells. Whereas VEGFR-3 was found in both malignant and nonmalignant cells, expression of VEGFR-3 was negative or faint in normal nondysplastic cervical epithelium and unregulated in the highly dysplastic epithelium as well as carcinoma. Although the role of epithelial expressed VEGFR-3 in lymph angiogenesis remains to be established, VEGFR-3 expression in neoplasia may play an important role in facilitating lymphatic dissemination of cervical cancer. Our study revealed a statistically significant correlation between VEGFR-3 expression from neoplastic cells and regional lymph node metastasis. This finding is consistent

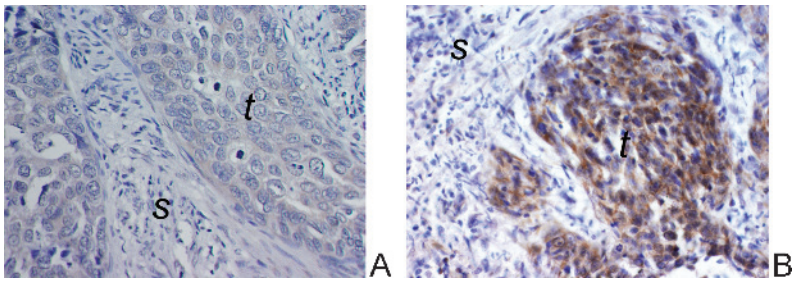

Figure 4. Immunohistochemical expression of VEGFR-3 in invasive cervical squamous cell carcinoma. VEGFR-3 immunopositivity was found in the cytoplasm of the tumor cells. Expression is heterogeneous within the tumor yet variable between patients. VEGFR-3 expression was graded from 1+ (faint staining; A) to 4+ (intense staining; B). Intensity of staining was found to have a statistically significant positive correlation with lymph node metastasis ($P < .05$, ANOVA). *s* indicates stroma; *t*, tumor. Original magnification, ×40.

with findings in prostate cancer [27,28], both of which found VEGF-3 expression associated with more aggressive poorly differentiated tumors.

In conclusion, immunohistochemically confirmed peritumoral LSI predicts regional lymph node metastasis with an odds ratio of 15.91, significantly improving the utility of LSI to guide therapeutic decisions. Correlation of peritumoral LVD with nodal metastasis suggests a role for lymph angiogenesis in the metastatic phenotype and a tumor-modulated paracrine lymphangiogenic mechanism. Finally, up*-regulation of VEGFR-3 early in the carcinogenic process may indicate a more aggressive phenotype with a higher propensity for early lymphatic dissemination.

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Conflict of Interest Statement

There is no conflict of interest with any author or product used in this translational research study.

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