

Lymphatic Vessel Density, Nodal Metastases, and Prognosis in Patients With Head and Neck Cancer

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Objective: To examine the relationship between intratumoral lymphatic vessel density and clinical and pathological variables in patients with head and neck squamous cell carcinoma.

Design: Archived paraffin-embedded biopsy specimens were sectioned and stained with hematoxylin-eosin and anti-LYVE-1 antibody, a highly specific marker for lymphatic endothelium. Tumor grade, infiltrating margin, inflammatory infiltrate, and percentage of tumor necrosis were noted and lymphatic vessel density measured using Chalkley point counting.

Setting: Tertiary care center at a university hospital.

Patients: A total of 168 previously untreated patients with advanced squamous cell carcinoma (73, larynx; 62, oropharynx; and 33, hypopharynx) treated with primary radiation (with or without planned neck dissection) and salvage surgery from 1992 to 1999.

Interventions: Measurement of intratumoral lymphatic vessel density in pretreatment tissue biopsy specimen.

Main Outcome Measures: Disease-free and disease-specific survival, tumor occurrence, and nodal status.

Results: In patients with laryngeal carcinoma there was a significant relationship between the presence of intratumoral lymphatics and nodal metastases at presentation ($P = .02$) and poorly differentiated tumor grade ($P = .02$). Patients with high lymphatic vessel density also had a significantly worse disease-specific survival ($P = .03$). However, this difference was not significant with multivariate analysis. No significant relationship existed between the presence of intratumoral lymphatics and any of the clinical or pathological variables in oropharyngeal and hypopharyngeal carcinoma.

Conclusions: In this patient sample, the development of intratumoral lymphatics in laryngeal carcinoma, but not in oropharyngeal or hypopharyngeal carcinoma, is associated with a spread of the tumor to regional lymph nodes. Detecting tumor lymphatic vessel proliferation is another step in the understanding of tumor biology, and the targeting of lymphatic growth may be of potential therapeutic benefit in selected patients with head and neck squamous cell carcinoma.

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THREE HUNDRED OF THE 800 lymph nodes of the human body are located in the head and neck region, and cancer mortality is most frequently associated with regional metastasis.^{1,2} Lymph node metastasis is associated with a 50% reduction in a favorable prognosis. Tumor spread from the primary site to the regional nodes can occur by direct extension, hematogenous spread, or the lymphatic system. The third route is the most common for tumor dissemination. Nonetheless, a controversy continues with regard to the exact mechanism. Do the neoplastic cells travel through already existing lymph channels, or is there creation and invasion of new lymphatics at the primary tumor site?¹⁻⁴ In the past, it was thought that high interstitial intratumoral pressure would destroy new lymphatic vessel growth. How-

ever, more recently, confirmation of intratumoral lymphatic network suggests that lymphangiogenesis is an integral part of tumorigenesis.⁵⁻⁸

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In 1996, vascular endothelial growth factor receptor 3 (VEGFR-3) was one of the first lymphatic markers to be identified in healthy tissues. However, VEGFR-3 was subsequently found to stain blood vessels in some tumors, thus compromising its use as a lymphatic marker in cancer studies.⁹⁻¹¹ More recently, markers limited to the lymphatic system have been identified. These include Prox1, a homeobox gene product that regulates budding and sprouting of lymphatic endothe-

lial cells^{7,12}; podoplanin, an approximately 38-kDa glomerular podocyte membrane mucoprotein used principally to study Kaposi sarcoma¹³ and angiosarcoma¹⁴; and LYVE-1, a lymphatic vessel endothelial hyaluronan receptor 1. More than 80% of tissue hyaluronan is degraded in lymph nodes,^{11,15} with the remainder being recirculated and endocytosed by liver endothelial sinusoidal cells.^{16,17} Because it is degraded in the liver sinusoids, LYVE-1 is also found in these cells but not in the hepatic arterial or venous endothelium.¹⁶ Previous studies^{8,11} have established that LYVE-1 remains specific for lymphatic endothelium in tumors, including head and neck squamous cell carcinoma, where no evidence of tumor blood vessel staining has been observed.

The goal of the present study was to determine whether a relationship exists between lymphatic vessel density (LVD) in oropharyngeal, laryngeal, and hypopharyngeal carcinomas and several clinicopathological variables, including survival. A previous smaller study⁸ revealed an association between LVD and nodal metastases in oropharyngeal carcinoma, but the relationship with survival was not reported.

METHODS

One hundred sixty-eight previously untreated patients with head and neck squamous cell carcinoma at stages III or IV were identified from the Princess Margaret Hospital, Toronto, Ontario, clinical and pathological databases. Seventy-three patients had laryngeal carcinoma; 62, oropharyngeal carcinoma; and 33, hypopharyngeal carcinoma. All patients with laryngeal carcinomas were at stages T3 or T4, and therefore the tumor involved either the lymphatic-rich areas of the supraglottis or subglottis, or both. All patients were treated from 1992 to 1999 with primary external beam radiation (with or without planned neck dissection) and salvage surgery. Each patient had corresponding archived paraffin-embedded tissue specimens available from the pretreatment tissue biopsy. All patients were followed up with clinical and radiological examinations to detect recurrence. Clinical data collection included the patient's age, sex, nodal status, recurrence, and survival time between diagnosis and the last date of follow-up.

Sections (4 μ m) of formalin-fixed paraffin-embedded tissue were cut onto silanized glass slides, cleared of paraffin in CitrocLEAR, a nonhazardous xylene substitute (HD Supplies, Aylesbury, England), and rehydrated through graded alcohol baths. Hematoxylin-eosin-stained sections of all the specimens were examined at low (original magnification \times 40) and medium (original magnification \times 100) power by 2 observers (N.J.B. and C.M.). Tumors were graded as either well, moderately, or poorly differentiated. Tumor invasion into surrounding normal tissue was identified as either pushing or infiltrating. A pushing margin was one with a defined border between tumor and stroma. An infiltrating margin was one without clearly defined tumor borders in which invasion occurred in irregular nests or strands. The entire tumor was examined and the percentage of tumor necrosis identified, and the inflammatory infiltrate was assessed as either scant or patchy.

A second set of sections was incubated in 0.03% hydrogen peroxide for 15 minutes to inactivate endogenous peroxidases before pressure-cooking for 3 minutes in 0.1M citrate buffer, pH 6.0, and then blocking in 10% normal human serum for 15 minutes. Subsequently, slides were then incubated (30 minutes) with 1:100 rabbit polyclonal anti-LYVE-1 antibody¹⁸ in total serum bilirubin and 5% normal human serum, rinsed twice

in total serum bilirubin, and then developed using the HRP Envision System (Dako Cytomation, Mississauga, Ontario). This system is based on a unique enzyme-conjugated polymer backbone, which also carries secondary antibody molecules. Slides were counterstained with hematoxylin (Sigma Diagnostics, St Louis, Mo) and mounted with Aquamount mountant (BDH Chemicals, Poole, England). Intratumoral LVD was determined in tumor vessel "hot spots" using a Chalkley point counting grid at high power (original magnification \times 250) by 2 observers (N.A. and N.J.B.), as described.¹⁹ The mean of the vessel counts in 3 hot spots per section was recorded.

For statistical analysis, the patients were divided by age (<60 years and \geq 60 years), nodal stage (N0 or N+), and the percentage of tumor necrosis (absent, if \leq 5%; or present, if \geq 10%). Intratumoral lymphatic vessels were described as either absent or present. The association between the presence and absence of intratumoral lymphatic vessels and each patient's sex, age, nodal stage, grade, margin of invasion, inflammatory infiltrate, and tumor necrosis was examined using the χ^2 test. Disease-free and disease-specific survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test by dividing the 2 groups by their LVD at the median value to give 2 similar-sized groups. A Cox proportional hazards model, using a forward variable selection technique (entry, $P < .1$; removal, $P > .20$) was used to identify the variables that were of independent statistical significance in predicting recurrence and survival. Statistical analysis was done using SPSS statistical software (version 10.0; SPSS Inc, Chicago, Ill).

RESULTS

Numerous large, LYVE-1-positive, irregularly shaped, thin-walled lymphatic vessels were identified in the subdermal layer of normal squamous epithelium (**Figure 1A**). Two different types of intratumoral lymphatics were noted. The first type was cells similar in appearance to the subdermal lymphatics, located in the peritumoral stroma and within areas of connective tissue lying between clumps of tumor (**Figure 1B, D, and F**). The second, those cells intimately associated with the tumor cells, had a distinctive reticular architecture, with numerous tiny ill-defined lumina, as observed in an earlier study^{5,8} (**Figure 1C and E**). Lymphatic vessel density could not be accurately assessed in 8 of the 168 slides (1, oropharyngeal; 6, laryngeal; and 1, hypopharyngeal) because of poor staining, excess background, or a specimen that contained only a small fragment of tumor that was insufficient for evaluation.

We identified a significant relationship between the presence of intratumoral lymphatics and nodal metastases in patients with laryngeal carcinoma ($P = .001$) (**Table**). We did not find a similar relationship in patients with oropharyngeal or hypopharyngeal carcinoma (**Table**). A study power of 78% for the oropharyngeal and 60% for the hypopharyngeal group was computed. These calculations assume an α of .05 (2-tailed test) and a difference in proportion between the number of specimens with intratumoral lymphatics in the N0 group (10%) and the N+ group (50%) of 40%.

There was a significant relationship between the presence of intratumoral lymphatics and poorly differentiated tumors in the laryngeal carcinoma group ($P = .02$) (**Table**). This association was not present in the oropharyngeal or hypopharyngeal groups (**Table**). We did not find an association between the presence of intratumoral lymphatics

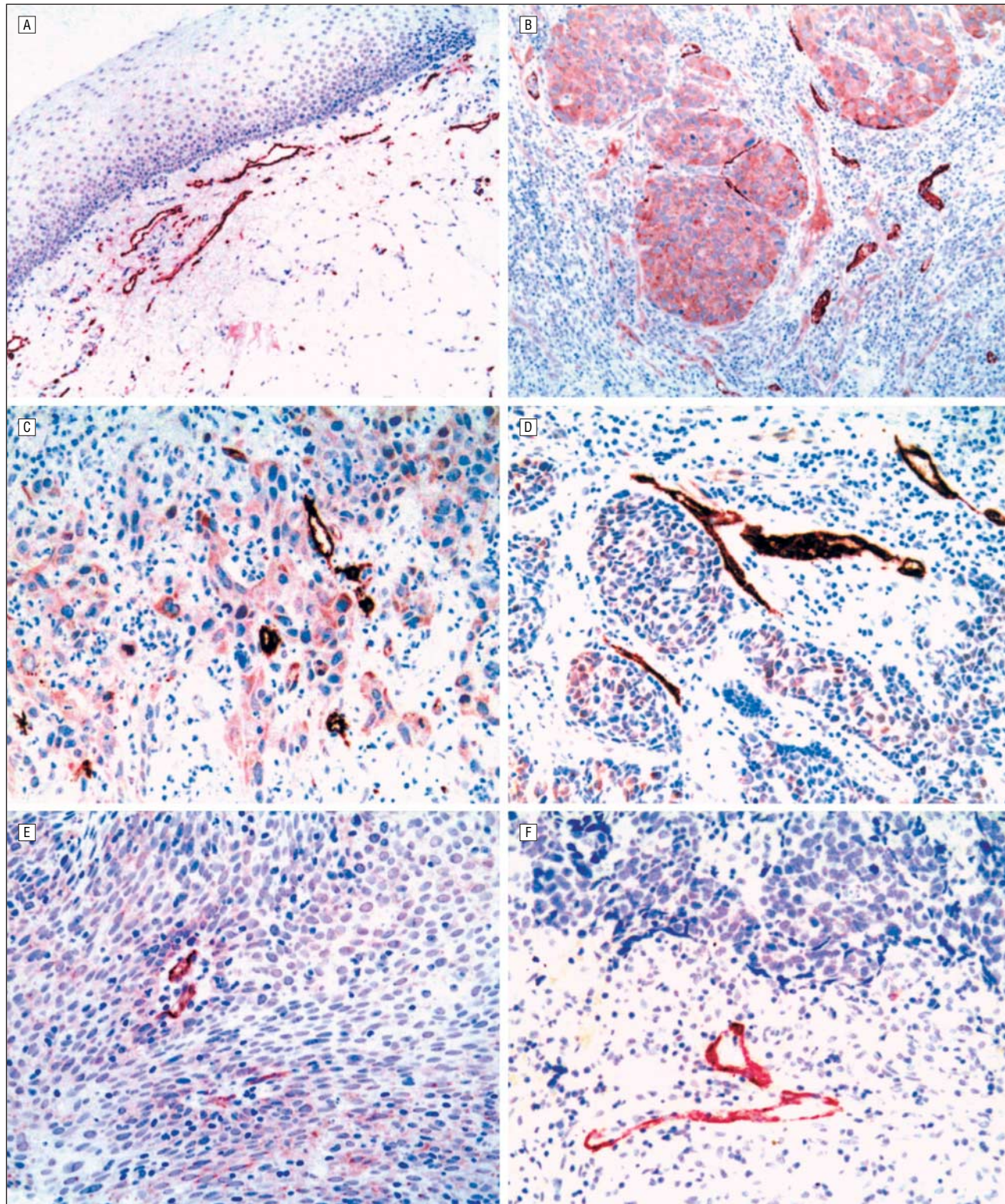


Figure 1. Lymphatics positive for LYVE-1 antibody in head and neck squamous cell carcinoma. A, Healthy subepithelial lymphatics stained brown with LYVE-1 antibody (original magnification $\times 40$); B, peritumoral and intratumoral lymphatics (original magnification $\times 40$); C, small reticular intratumoral lymphatics (original magnification $\times 400$); D, larger LYVE-1-positive lymphatic in the connective tissue between clumps of tumor cells (original magnification $\times 200$); E, tiny thin-walled intratumoral lymphatics (original magnification $\times 400$); and F, larger lymphatics positive for LYVE-1 antibody adjacent to a clump of tumor (original magnification $\times 400$).

and the patient's age, sex, tumor margin of invasion, inflammatory infiltrate, or percentage of tumor necrosis.

We found no significant relationship between disease-free survival and a high or low intratumoral LVD in

patients from any of the tumor site groups (oropharynx, $P = .32$; larynx, $P = .42$; hypopharynx, $P = .15$) (**Figure 2A, C, and E**). There was a statistically significant difference in disease-specific survival between patients

Table. Relationship Between the Presence of Intratumoral Lymphatics and Nodal Metastases

Variable	Larynx			Oropharynx			Hypopharynx		
	Absent	Present	χ^2 Value (P Value)	Absent	Present	χ^2 Value (P Value)	Absent	Present	χ^2 Value (P Value)
Sex									
Men	10	44	0.139 (.71)	10	40	2.631 (.11)	8	14	0.123 (.76)
Women	3	10		0	11		3	7	
Age, y									
<60	7	17	2.280 (.13)	3	18	0.104 (.75)	2	3	0.083 (.77)
>60	6	37		7	33		9	18	
N stage									
N0	13	23	12.285 (.001)	2	11	0.012 (.91)	3	8	0.375 (.54)
N+	0	27		8	40		8	13	
Tumor grade									
Well differentiated	4	4	7.558 (.02)	0	3	1.277 (.53)	1	0	2.222 (.33)
Moderately differentiated	8	41		9	38		8	15	
Poorly differentiated	0	9		1	10		2	6	
Tumor margin									
Infiltrating	3	25	1.961 (.16)	5	26	0.013 (.91)	6	10	0.139 (.71)
Pushing	9	28		5	24		5	11	
Inflammatory infiltrate									
Little or none	9	39	0.038 (.85)	6	32	0.057 (.81)	8	13	0.375 (.54)
Patchy or confluent	3	15		4	18		3	8	
Necrosis, %									
<5	8	32	0.226 (.64)	6	24	0.560 (.45)	6	5	3.023 (.08)
>10	4	22		4	27		5	16	

with a high or low intratumoral LVD in the laryngeal group ($P = .03$) (Figure 2B). This difference, however, was not significant when the presence of nodal metastases at presentation (N stage) was introduced into the Cox proportional hazards model. A computation of the disease-specific survival showed no statistical difference in the oropharyngeal ($P = .92$) and hypopharyngeal groups ($P = .79$) (Figure 2D and F). The median follow-up time of patients with laryngeal carcinoma was 41.5 months (range, 1-105 months); for oropharyngeal carcinoma, 30 months (range, 1-91 months); and for hypopharyngeal carcinoma, 23.5 months (range, 3-98 months).

COMMENT

The present study demonstrates a relationship between the presence of intratumoral lymphatics and regional metastasis in laryngeal squamous cell carcinoma. This adds further support to previous studies^{8,20} reporting that intratumoral lymphatics are important in the spread of head and neck squamous cell carcinoma to regional lymph nodes. However, this support must be balanced with the negative findings in oropharyngeal and hypopharyngeal cancer in our study and in laryngeal cancer in the study by Beasley et al.⁸ This subgroup of their study consisted of a small number of patients ($n = 16$) presenting with early- and late-stage disease. In contrast, our laryngeal subgroup consisted of 73 patients with advanced disease (stages III and IV). The negative results in the oropharyngeal and hypopharyngeal groups underline the problem in predicting tumor behavior in the clinical setting. Factors that influence tumor metastatic potential include the ability of tumor cells to separate from the main

tumor mass, invade adjacent lymphatics, migrate to regional lymph nodes, and then divide and establish a separate group of viable tumor cells in a distant site that will proliferate.²¹ If any one of these steps is altered, the cell clone will not survive despite adequate lymphatics through which to migrate. In the laryngeal carcinoma group, the presence of intratumoral lymphatics seems to be a prerequisite for metastatic spread because no patients had neck node metastases at presentation in the absence of intratumoral lymphatics. In the other groups (those with oropharyngeal and hypopharyngeal cancer), some tumors had clearly metastasized without evidence of intratumoral lymphatics. This suggests that the lymphatics through which they had spread were too small to be identified, were missed on the tissue sections sampled for analysis, or were destroyed after the metastasis occurred. This study highlights some of the potential pitfalls in examining single sections of a tumor taken at a single point in time and the interaction of other biological systems on the process of tumor metastases.

The power of the study in the oropharyngeal group was 78%, which confirms that a sufficient number of patients were included in the analysis. However, the power of 60% in the hypopharyngeal group indicates that a significant result may have been missed and that a larger study sample may reveal significant findings.

We also identified an association between the presence of intratumoral lymphatics and a poorly differentiated laryngeal carcinoma. This may reflect a more aggressive nature of some tumors that develop and spread via the lymphatic system. Patients with laryngeal carcinoma and a high LVD showed a decrease in disease-specific survival. However, there was no statistical significance when nodal sta-

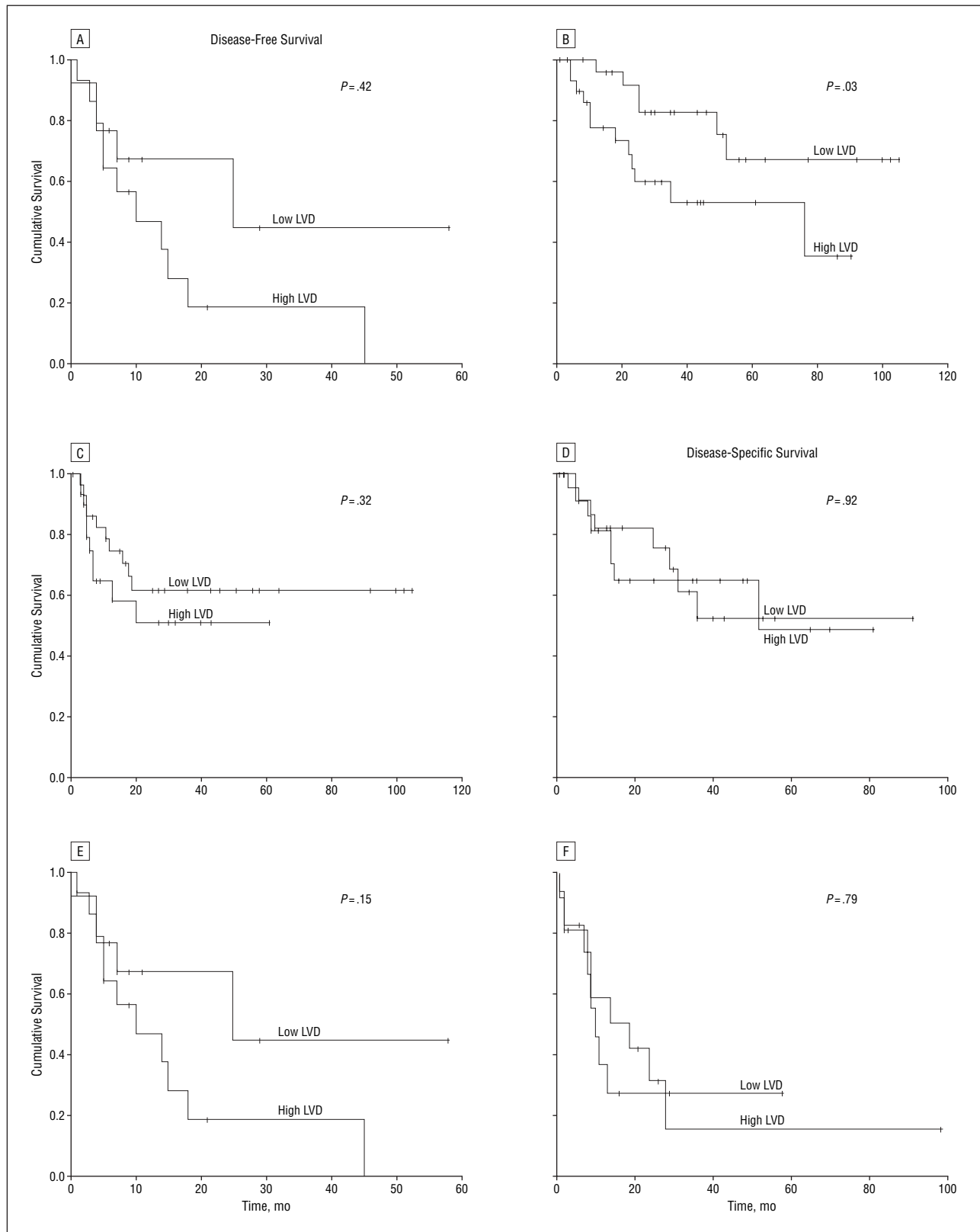


Figure 2. Disease-free and disease-specific survival in head and neck squamous cell carcinoma by high and low lymphatic vessel density (LVD). A, Disease-free survival in laryngeal carcinoma ($P = .42$); B, disease-specific survival in laryngeal carcinoma ($P = .03$); C, disease-free survival in oropharyngeal carcinoma ($P = .32$); D, disease-specific survival in oropharyngeal carcinoma ($P = .92$); E, disease-free survival in hypopharyngeal carcinoma ($P = .15$); and F, disease-specific survival in hypopharyngeal carcinoma ($P = .79$).

tus at presentation was included in the multivariate model of survival, probably because of the link seen between LVD and N stage. Nodal disease at presentation is also a powerful predictor of recurrence and survival.^{22,23}

Previous studies have demonstrated a relationship between VEGF-C/VEGF-D expression and nodal metastasis.^{7,20,21,24} The presence of morphologically abnormal lymphatics adjacent to tumor cells increases the potential for the primary tumor to migrate to local lymph nodes. Other hypotheses include the presence of paracrine factors secreted by tumor cells^{7,20} that facilitate lymphatic spread.

This study demonstrates that the presence of intratumoral lymphatics in laryngeal carcinoma may assist tumor spread to regional lymph nodes. Additional studies to evaluate the function of these intratumoral lymphatics and their 3-dimensional structure within the tumor may help to define this role more clearly. A highly lymphangiogenic neoplasm could, in theory, be identified using LYVE-1 antibody as a marker for lymphatic endothelium in biopsy specimens. Future studies may demonstrate whether this is likely to predict the presence of lymph node metastases in patients with stage N0 neck carcinoma and influence the decision to perform elective neck dissection. Lymphangiogenesis inhibitors targeted at VEGF-C, for example, could be used as an anticancer treatment and potentially decrease further regional metastases after primary therapy.

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