

Lymphatic vessel invasion detected by the endothelial lymphatic marker D2-40 (podoplanin) is predictive of regional lymph node status and an independent prognostic factor in patients with resected esophageal cancer

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Abstract: The discovery of markers to lymphatic endothelial cells and the development of novel antibodies to these markers have brought increasing attention to the lymphatics and progress in the understanding of lymphangiogenesis and cancer metastasis. In this study, we investigate the presence of lymphatic vessel invasion (LVI) detected by D2-40 immunohistochemical staining in resected esophageal cancer and correlated with clinicopathologic data and patient survival. Sixty nine patients, who had a primary resection of esophageal cancer, were analyzed by univariate and multivariate logistic regression, and univariate and multivariate survival analysis. The total rate of LVI was 72% (50/69). Positive LVI was significantly correlated with lymph node metastasis ($p < 0.001$), tumor size ($p < 0.001$), histological grading ($p = 0.017$), tumor depth ($p = 0.001$), and stage ($p < 0.001$). Multivariate logistic analysis identified LVI ($p = 0.036$) as a predictor of regional lymph node metastasis. On univariate survival analysis, patients with LVI had a significantly shorter disease-free survival, cancer-specific survival and overall survival. Multivariate analysis proved that LVI diagnosed by D2-40 is an independent prognostic factor of both disease-free survival ($p = 0.04$) and overall survival ($p = 0.032$) in resected esophageal cancer. These results show that LVI assessment identifies patients at high risk for regional lymph node metastasis and that LVI is an independent prognostic factor in patients with esophageal cancer. (*Folia Histochemica et Cytobiologica* 2011; Vol. 49, No. 1, pp. 90–97)

Key words: esophageal cancer, lymphatic vessel invasion, podoplanin, D2-40, lymph node metastasis, prognosis

Introduction

The presence of lymph node metastasis is known to be the most important prognostic factor in resectable esoph-

ageal cancer. More than 65% of patients with localized esophageal cancers and negative resection margin have a positive lymph node at the time of surgery [1]. However, despite complete tumor resection and extensive lymphadenectomy, systemic and local recurrence is common [2] and the five-year survival rate is 15–39% [3].

The discovery over the last decade of markers of lymphatic endothelial cells like podoplanin (D2-40) [4], LYVE-1 [5] and Prox 1 [6], and the development of novel antibodies to these markers, have generated

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increasing attention on lymphatics and progress in the understanding of lymphangiogenesis and cancer metastasis [7–9]. These markers enable the identification of lymphatic vessels immunohistochemically.

Lymphatic vessel invasion (LVI) is known to be an independent prognostic factor in patients with gastric and colorectal cancer [10, 11]. It is also a predictor of lymph node metastasis and indicates a poor prognosis, even in node-negative patients with cervical and breast cancer [12, 13]. Some studies have reported that LVI is one of the histological risk factors for nodal metastasis of esophageal cancer [14, 15]. The standard method of assessing LVI is light microscopic examination of hematoxylin and eosin (HE) stained sections. However, it is difficult to detect LVI with conventional HE staining. Controversies over the detection of LVI arise mainly from the difficulty in visualizing the lymphatic vessel wall. According to Alexander-Sefre et al. [16] accurate detection of LVI has some limitations. First, tumor cells that completely fill out the lymphatic channels make it difficult to differentiate between LVI and stromal invasion. Second, the artifactual spaces caused by tissue retraction due to fixation complicate the identification of true LVI.

The recently developed monoclonal antibody, D2-40, recognizes a mucin-like transmembrane glycoprotein, podoplanin, which is expressed in lymphatic endothelial cells and enables the identification of lymphatic vessels in paraffin section [17, 18]. It has been shown that D2-40 is the most sensitive and specific antibody for the detection of lymphatic endothelium in different malignancies like gastric [19], colorectal [20], vulvar [21], breast [22] and endometrial cancer [23].

In this study, we investigated the presence of LVI detected by D2-40 immunohistochemical staining in resected esophageal cancer and correlated with clinicopathologic data and patient survival.

Material and methods

Patients and tissues

Tumor specimens were obtained from 69 patients with primary esophageal cancer who underwent an esophagectomy at the Department of Thoracic Surgery, Medical University of Białystok. The primary goal of the surgical approach was a complete resection of the primary tumor and its lymphatic drainage. None of the patients had received preoperational chemotherapy or radiotherapy treatment. The study population consisted of 56 men (82%) and 13 women (18%). The average age at the time of diagnosis was 64.2 years (range 44 to 77 years). The clinical features are

summarized in Table 1. None of the 69 patients died within 30 days of the operation or during their hospital stay. Thirty patients received chemotherapy, radiotherapy, or both, post-operatively. All patients have been followed up regularly at our institution with routine physical and laboratory examination after discharge. Follow-up examinations, such as computed tomography, ultrasonography of the abdomen and neck, and upper gastrointestinal (GI) series were carried out every three or six months. Endoscopic examination and PET-CT were performed when appropriate. The median follow-up period was 24 months (range 3–101 months). Recurrence occurred in 35 cases. Nine patients had pulmonary metastasis, six patients liver metastasis, five patients has pulmonary and liver metastasis, 12 had mediastinal and neck lymph node metastasis, and two cases relapsed in the esophagus after operation. Thirty-one patients died of esophageal cancer. Normal esophageal tissues were collected as control specimens.

Analysis of protein expression by immunohistochemistry (IHC)

The surgical specimens were fixed in 10% buffered formalin solution for 24 h, embedded in paraffin and handled in the Department of Pathology at the Medical University of Białystok for further processing. The tissue samples were obtained from the peripheral invasion front of the tumor. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex technique (ABC-technique). 4 μm -thick paraffin-embedded slides were cut from each study block. For antigen retrieval, the slides were heated in a microwave oven containing 0.01 mmol/L sodium citrate (pH 6.0). The sections were treated with 0.3% H_2O_2 for 10 min at room temperature. The slides were incubated for one hour at room temperature in a humidity tray with primary antibodies — D2-40 (mouse monoclonal antibody, 1:10, ABD-Serotec). The slides were rinsed twice in 0.1 mmol/L PBS (pH ~7.4) for 5 min, and incubated for 30 min at room temperature with anti-goat biotinylated secondary antibody (Vectastain ABC Kit, Vector), and anti-mouse biotinylated secondary antibody (Peroxidase Detection System, Novocastra) to identify the target. The sections were stained by 3'-diaminobenzidine (DAB) to visualize antigen-antibody complex. The nuclei were then stained with Mayer hematoxylin. Positive controls were made using tissue samples, as proposed by the antibody manufacturer, which showed a high expression of the proteins. Negative controls were made with the same tissue without antibody.

Table 1. Characteristics of esophageal cancer patients

Parameters		Number of patients
Esophageal cancer patients		69
Age	< 64 years	22
	≥ 64 years	47
Sex	Female	13
	Male	56
Location	Upper	4
	Midthoracic	25
	Lower	40
Tumor size	< 4 cm	33
	≥ 4 cm	36
Histological type	Squamous cell carcinoma	32
	Adenocarcinoma	37
Histological grade	Well-G1	7
	Moderate -G2	29
	Poor-G3	33
Tumor depth	T1	6
	T2	13
	T3	45
	T4	5
Stage	I	6
	IIA	19
	IIB	5
	III	39
Lymph node metastasis	N0	22
	N1	47
Residual tumor	R0	57
	R1	10
	R2	2

G1 — well differentiated; G2 — moderately differentiated; G3 — poorly differentiated; T1 — tumor invades lamina propria or submucosa; T2 — tumor invades muscularis propria; T3 — tumor invades adventitia; T4 — tumor invades adventitia; N0 — no regional lymph node metastases; N1 — regional lymph node metastases; R0 — no residual tumor; R1 — microscopic residual tumor; R2 — macroscopic residual tumor

Definition of lymphatic vessel invasion (LVI)

Lymphatic vessel invasion (LVI) was evaluated by microscopic examination of the slides. The presence of at least one tumor cell cluster in a podoplanin (D2-40)-positive vascular channel indicates LVI [19].

Statistical analysis

Distribution was analyzed by the Shapiro–Wilk test. Categorical data was compared by the χ^2 or Fisher's exact probability test. Logistic regression analysis was used to identify univariable predictors of lymph node metastasis. Variables that were significant in the univariable analysis at $p < 0.05$ (and that made clinical sense to include in a model to predict lymph node involvement) were considered in a stepwise logistic regression model. The results for the final multivariable model are summarized as the p value, odds ratio, and 95% confidence interval for the odds ratio. Survival analysis was performed, including overall survival, disease-free survival and cancer-specific survival.

Overall survival, disease-free survival and cancer-specific survival were calculated from the date of surgery to last contact for living patients, to the date of the last follow-up for disease-free patients, and to the date of esophageal-cancer-induced death, respectively. The Kaplan–Meier method was used to estimate the probability of survival as a function of time. The differences in the survival of the subgroups of patients were compared using the log-rank test. The prognostic value of lymphatic vessel invasion was examined in univariate and multivariate analysis with Cox's proportional hazard model. All p values were based on two-tailed statistical analysis, and a p value of less than 0.05 was considered significant. Statistical analyses were carried out using the Statistica 8.0 PL program (StatSoft Inc., Tulsa, OK, USA) and the Graph-Pad Prism 5.01 program (GraphPad Software, San Diego, CA, USA).

In accordance with the Declaration of Helsinki, the study protocol was approved by the local Ethics Committee (No R-1-002/188/2008) and written informed consent was obtained from all participants before analysis.

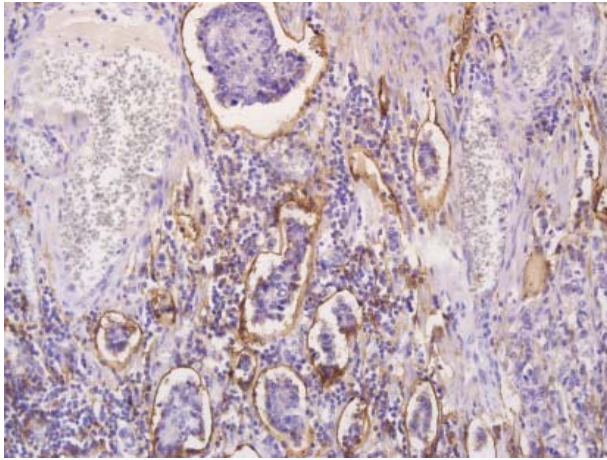


Figure 1. Lymphatic vessels with massive invasion (LVI) detected by podoplanin (D2-40). The endothelial cells of blood vessels did not stain with D2-40 (magnification $\times 400$)

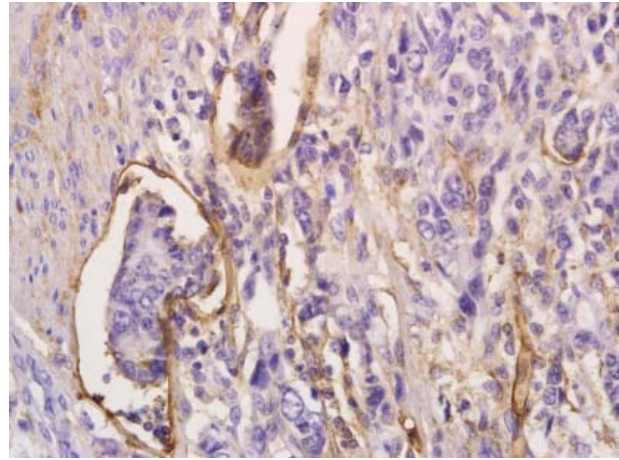


Figure 2. Cancer cell embolus that obliterated the lumen of lymphatic vessels is clearly outlined by D2-40 positive lymphatic vessels (magnification $\times 600$)

Table 2. Clinical and histopathologic factors associated with lymphatic vessel invasion (LVI) detected by podoplanin (D2-40)

Characteristics	Number	LVI		p
		Negative	Positive	
Overall	69	19	50	
Age				
< 64	22	8	14	0.385 ^a
≥ 64	47	11	36	
Sex				
Female	13	6	7	0.164 ^a
Male	56	13	43	
Location				
Upper	4	1	3	0.291 ^b
Midthoracic	25	6	19	
Lower	40	12	28	
Tumor size				
< 4 cm	33	16	17	< 0.001 ^a
≥ 4 cm	36	3	33	
Histological grade				
G1	7	5	2	0.017 ^b
G2	29	8	21	
G3	33	6	27	
Stage				
I + II	30	16	14	< 0.001 ^a
III	39	3	36	
T1 + T2	19	11	8	0.001 ^a
T3 + T4	50	8	42	
N0	22	15	7	< 0.001 ^a
N1	47	4	43	
R0	57	18	39	0.157 ^a
R1 + R2	12	1	11	

^aFisher's exact test; ^b χ^2 test; G1 — well differentiated; G2 — moderately differentiated; G3 — poorly differentiated; T1 — tumor invades lamina propria or submucosa; T2 — tumor invades muscularis propria; T3 — tumor invades adventitia; T4 — tumor invades adventitia; N0 — no regional lymph node metastases; N1 — regional lymph node metastases; R0 — no residual tumor; R1 — microscopic residual tumor; R2 — macroscopic residual tumor

Table 3. Logistic regression analysis in relation to lymph node metastasis

Factors	Univariate p	Multivariate		
		Odds ratio	95% CI	p
Tumor size	< 0.001	1.631	0.934–4.834	0.004
Histological grade	0.050	0.675	0.313–1.453	0.306
Depth of invasion	0.001	1.431	1.131–6.334	0.016
Residual tumor	0.048	0.911	0.623–3.264	0.104
LVI	< 0.001	2.139	1.224–8.867	0.036

CI — confidence interval; LVI — lymphatic vessel invasion

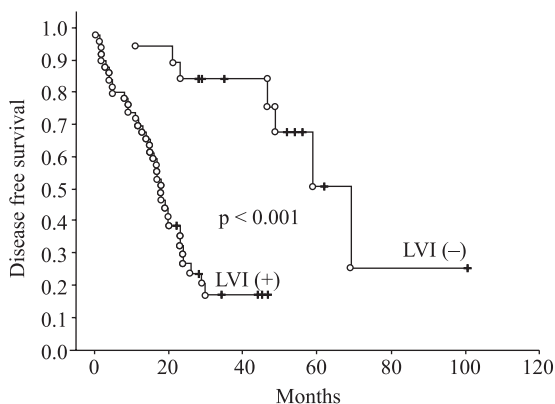


Figure 3. Kaplan–Meier analysis of disease-free survival according to lymphatic vessel invasion (LVI) detected by podoplanin (D2-40) in patients with esophageal cancer

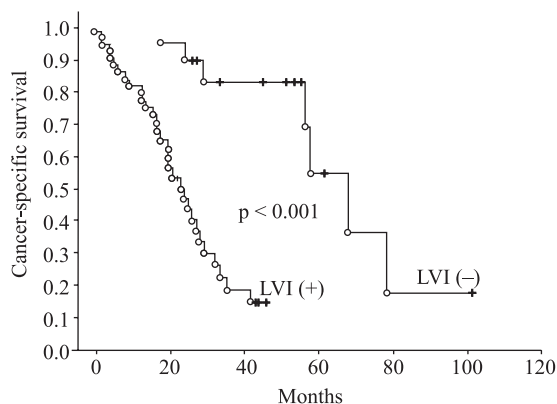


Figure 4. Kaplan–Meier analysis of cancer-specific survival according to lymphatic vessel invasion (LVI) detected by podoplanin (D2-40) in patients with esophageal cancer

Results

In esophageal tumors, the lymphatic vessels identified with podoplanin were different in size and shape, with a thin wall, without perivascular cells, and were found in both tumor and peritumoral areas. None of the podoplanin positive vessels were blood vessels. Most lymphatic vessels with LVI were greatly enlarged, sometimes with a tumor embolus (Figure 1). In Figure 2, a tumor embolus that obliterated the lumen of the lymphatic vessels is clearly outlined by D2-40 positive lymphatic vessels. The results of LVI invasion and clinicopathological features are summarized in Table 2. In this study, positive LVI with D2-40 immunostaining were detected in 72% (50/69) of the cases of esophageal cancer patients. In LVI-positive patients, lymph node metastasis was observed more frequently than in LVI-negative patients (43/50 vs. 4/19; $p < 0.001$). Of 47 patients with nodal involvement, four (8.5%) were not stained with D2-40. Positive LVI was significantly correlated with tumor size ($p < 0.001$), histological grade ($p = 0.017$), tumor depth ($p = 0.001$), and stage ($p < 0.001$). No significant difference was found between those with positive and negative LVI in terms of age, sex, tumor location, or residual tumor.

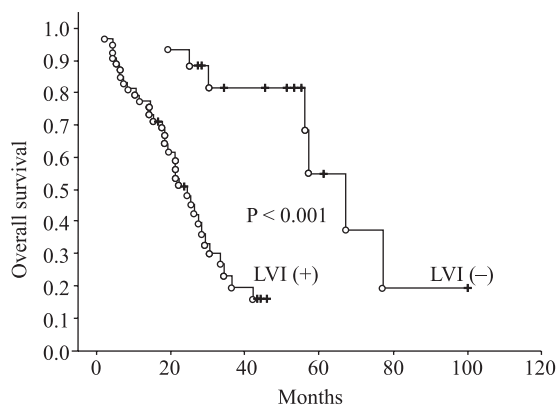


Figure 5. Kaplan–Meier analysis of overall survival according to lymphatic vessel invasion (LVI) detected by podoplanin (D2-40) in patients with esophageal cancer

To investigate the risk factor of lymph node metastasis, multivariate regression analyses were conducted to include tumor size, histological grade, depth of invasion, residual tumor and lymphatic vessel invasion. Logistic regression analysis identified LVI ($p = 0.036$), increasing depth of tumor invasion ($p = 0.016$) and increasing tumor size ($p = 0.004$) as multivariable predictors of regional lymph node metastasis. Other factors did not predict lymph node metastasis (Table 3).

Table 4. Cox regression analysis of independent factors affecting disease-free survival, cancer-specific survival and overall survival

Factors	Disease-free survival		Cancer-specific survival		Overall survival	
	HR (95% CI)	p	HR (95% CI)	P	HR (95% CI)	p
Tumor size	0.72 (0.311–1.691)	0.45	0.49 (0.193–1.268)	0.14	0.68 (0.271–1.740)	0.42
Histological grade	2.26 (1.200–4.285)	0.01	1.85 (0.940–3.641)	0.07	1.76 (0.903–3.433)	0.09
Tumor depth T1 + 2 vs. T3 + 4	1.08 (0.303–3.900)	0.89	1.51 (0.357–6.463)	0.57	1.90 (0.475–7.647)	0.36
Tumor stage I + II vs. III	0.34 (0.093–1.304)	0.11	0.20 (0.049–0.847)	0.02	0.18 (0.048–0.722)	0.015
Lymph node metastasis	16.78 (2.903–97.033)	0.002	21.37 (2.518–65.378)	0.001	12.83 (2.518–65.378)	0.002
Residual tumor	2.72 (1.064–6.990)	0.03	3.33 (1.228–9.028)	0.01	3.38 (1.289–8.875)	0.013
LVI	4.80 (1.044–22.069)	0.04	4.07 (0.648–25.684)	0.13	5.58 (1.031–9.517)	0.032

HR — hazard ratio; CI — confidence interval; LVI — lymphatic vessel invasion

On univariate survival analysis, LVI was associated with poor disease-free survival (Figure 3, $p < 0.001$), cancer-specific survival (Figure 4, $p < 0.001$) and overall survival (Figure 5, $p < 0.001$). Multivariate regression analysis indicated that LVI could be an independent prognostic factor for both disease-free survival ($p = 0.04$) and overall survival ($p = 0.032$), but not cancer-specific survival. Moreover, lymph node metastasis and residual tumor could serve as independent predictors for all three survivals (lymph node metastasis, $p = 0.002$, 0.002 , 0.001 ; residual tumor, $p = 0.03$, 0.013 , 0.01 , respectively). The TNM stage was the independent prognostic predictor for both overall survival ($p = 0.015$) and cancer-specific survival ($p = 0.02$). The histological grade could be an independent prognostic factor for disease-free survival ($p = 0.01$) (Table 4).

Discussion

Lymph node metastasis is a complex biological process initialized by tumor cells, also involving spread through the lymphatic vessels to the lymph node. Although lymphatic vessel invasion is included in the TNM system as a 'L' category, its mention in the histopathology report is optional. LVI has been little studied due to the difficulties associated with detecting and characterizing lymphatic markers. The development of selective immunohistochemical markers for staining lymphatic endothelial cells enable a more precise study of the lymphatic channels and the molecular mechanisms involved in lymphangiogenesis [24].

In this study, we evaluated LVI using podoplanin, a 38 kDa mucin-type transmembrane glycoprotein which is recognized by the monoclonal antibody D2-40 as a lymphatic marker. We showed that D2-40 was positive in the endothelial cells of lymph vessels and identified tumor cell clusters in lymph vessels,

whereas, D2-40 did not react with the endothelium of blood vessels. The distinction between lymph vessels and blood vessels in esophageal cancer has also been demonstrated using D2-40 [25].

The standard method for assessing LVI is light microscopic examination of hematoxylin and eosin (HE) stained section. We identified LVI using D2-40 in 72% (50/69) of the cases of resected esophageal cancer. In various studies, the total rate of positive LVI ranged from 39.1% to 49.9%. This is lower than in the current study, but von Rahden et al. [14] and Brücher et al. [15] assessed tumor cells in lymphatic channels on HE stained slides. Our results suggest that LVI in esophageal cancers occurs in more patients than is generally assumed.

In the current study, lymph node metastasis was observed in 86% of LVI-positive patients. Tomita et al. [26] obtained similar results. Lymph node metastasis was significantly ($p < 0.001$) related with the tissue status of lymphatic invasion, which were diagnosed by D2-40 immunohistochemistry. This suggests that LVI is thought to precede or occur coincidentally with lymph node metastasis. However, four (8.5%) patients who were negative for LVI on D2-40 had lymph node metastasis. Brücher et al. [15] showed that 75.8% of patients with LVI assessed on HE stained slides had lymph node involvement. The increase in sensitivity of detection of LVI is attributed to the demarcation of lymphatic endothelium that stains positively for D2-40 around the tumor cells. Nevertheless, D2-40 could be useful in distinguishing true lymphatic invasion from retraction artifact.

We also found that positive LVI significantly correlated with tumor size, histological grade, tumor depth, and stage. Our analysis clearly shows that LVI is associated with more advanced disease and histological grade. This reflects the aggressiveness of

esophageal cancer and suggests that many patients with locally advanced tumors may already have systemic, not just local, tumor disease.

In this study, we have shown that LVI, detected using immunostaining with monoclonal antibody D2-40, is a histological risk factor predictive of lymph node metastasis in patients with resected esophageal cancer. This is in accordance with studies investigating a risk factor predictive of nodal involvement in colorectal, vulvar and esophageal cancer [11, 21, 26]. Our observations indicate that the main source of lymph node metastasis may be delivered from the lymphatic invasion in esophageal cancer.

We also found that increasing depth of tumor invasion and tumor size were also predictors of lymph node metastases. Deeper invasion into the esophageal wall increases exposure to the lymphatics and the potential for longitudinal lymphatic extension and lateral metastasis to regional lymph nodes. Similar esophageal cancer data were reported by Rice et al. [27]. Age, sex, location, histological grade, or residual tumor were not predictive of regional lymph node metastasis.

The prognostic value of LVI has been shown in patients with solid tumors like breast cancer [28], colorectal carcinoma [29] and gastric carcinoma [30, 31]. In the current study, on univariate survival analysis, patients with LVI had a significantly shorter disease-free survival, cancer-specific survival and overall survival.

Although our analysis clearly shows that LVI is associated with more advanced disease, LVI is also present in many patients with early disease. Multivariate analysis proved that LVI diagnosed by D2-40 immunohistochemistry is an independent prognostic factor of both disease-free survival and overall survival in resected esophageal cancer.

In summary, LVI assessment identifies those patients at particularly high risk for regional lymph node metastasis. Univariate and multivariate analyses revealed the presence of LVI as an independent prognostic factor and LVI should be included with other established factors used in staging and treatment decision-making for patients with esophageal cancer.

Based on our results, we recommend the use of D2-40 immunoreactions for the routine evaluation of lymphatic invasion in esophageal cancer.

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