DOI: 10.5114/kitp.2013.36131

Intratumoral lymphatic vessel density, and intratumoral and peritumoral lymphatic vessel invasion as predictive factors of lymph node metastasis and prognostic factors in esophageal cancer

Mirosław Kozłowski[1], Wiesława Niklińska[2], Wojciech Naumnik[3], Wojciech Laudański[1], Robert Milewski[4], Jacek Nikliński[1,5]

[1]Department of Thoracic Surgery, Medical University of Białystok, Białystok, Poland
[2]Department of Histology and Embryology, Medical University of Białystok, Białystok, Poland
[3]Department of Pulmonology and Tuberculosis, Medical University of Białystok, Białystok, Poland
[4]Department of Statistics and Medical Informatics, Medical University of Białystok, Białystok, Poland
[5]Department of Clinical Molecular Biology, Medical University of Białystok, Białystok, Poland

Kardiochirurgia i Torakochirurgia Polska 2013; 10 (2): 120–129

Abstract

In esophageal cancer, metastases in regional lymph nodes occur early and are a poor prognostic factor. We studied intratumoral and peritumoral lymphatic vessel density, and intratumoral and peritumoral lymphatic vessel invasion using D2-40 immunohistochemical staining in 74 resected esophageal cancer specimens and correlated results with patients' clinicopathologic features and outcomes. Results were analyzed by univariate and multivariate logistic regression as well as univariate and multivariate survival analysis. The peritumoral lymphatic vessel density was significantly higher than the intratumoral lymphatic vessel density ($p < 0.001$). The peritumoral lymphatic vessel density and intratumoral lymphatic vessel density positively correlated with lymph node metastasis, tumor stage, depth of tumor invasion, and residual tumor. Positive intratumoral lymphatic vessel invasion and peritumoral lymphatic vessel invasion were significantly correlated with lymph node metastasis, tumor size, tumor depth, stage, and residual tumor. Multivariate logistic regression analysis identified intratumoral lymphatic vessel density ($p = 0.028$) and peritumoral lymphatic vessel invasion ($p = 0.036$) as risk factors of lymph node metastasis. Cox regression analysis showed that intratumoral lymphatic vessel invasion and peritumoral lymphatic vessel invasion are independent prognostic factors of disease-free survival ($p = 0.018$, $p = 0.021$), cancer-specific survival ($p = 0.016$, $p = 0.025$), and overall survival ($p = 0.017$, $p = 0.02$). Intratumoral lymphatics and peritumoral lymphatic vessel invasion are predictive factors of lymph node metastasis. Intratumoral and peritumoral lymphatic vessel invasion are independent prognostic factors in locally advanced esophageal cancer.

Streszczenie

Przerzuty do regionalnych węzłów chłonnych w raku przełyku występują wcześnie i są niekorzystnym czynnikiem prognostycznym. Przebadano gęstość naczyń limfatycznych wewnątrz i wokół guza oraz inwazję naczyń limfatycznych wewnątrz i wokół guza metodą immunohistochemiczną w 74 resekowanych guzach raka przełyku, wybarwiając naczynia przeciwciałem D2-40, i skorelowano uzyskane wyniki z cechami kliniczno-patologicznymi i przeżyciem pooperacyjnym chorych. W analizie statystycznej zastosowano testy jedno- i wieloczynnikowej regresji logistycznej, jedno- i wieloczynnikowej analizy przeżycia. Gęstość naczyń limfatycznych wokół guza była znamiennie statystyczne wyższa niż wewnątrz guza ($p < 0{,}001$). Gęstość naczyń limfatycznych wokół i wewnątrz guza pozytywnie korelowała z przerzutami do węzłów chłonnych, stopniem zaawansowania, głębokością nacieku nowotworowego i doszczętnością resekcji. Inwazja naczyń limfatycznych wewnątrz i wokół guza pozytywnie korelowały z przerzutami do węzłów chłonnych, wielkością guza, głębokością nacieku nowotworowego, stopniem zaawansowania i doszczętnością resekcji. W analizie wieloczynnikowej regresji logistycznej stwierdzono, że gęstość naczyń limfatycznych wewnątrz guza ($p = 0{,}028$) i inwazja komórek nowotworowych w naczyniach limfatycznych wokół guza ($p = 0{,}036$) są niezależnymi czynnikami predykcyjnymi ryzyka wystąpienia przerzutów w regionalnych węzłach chłonnych. W modelu wieloczynnikowej regresji Coxa stwierdzono, że inwazja naczyń limfatycznych wewnątrz guza i wokół guza stanowią niezależne czynniki prognostyczne czasu wolnego od nawrotu choroby ($p = 0{,}018$, $p = 0{,}021$), czasu przeżycia zależnego od choroby ($p = 0{,}016$, $p = 0{,}025$) i czasu całkowitego przeżycia ($p = 0{,}017$, $p = 0{,}02$) chorych. Naczynia limfatyczne wewnątrz guza i inwazja naczyń wokół guza są czynnikami predykcyjnymi przerzutów

Address for correspondence: Mirosław Kozłowski, MD, PhD, Department of Thoracic Surgery, Medical University of Białystok, 24A Sklodowskiej-Curie St., 15-276 Bialystok, Poland, tel. +48 85 746 82 73; fax +48 85 746 85 17, e-mail: miroslaw@cksr.ac.bialystok.pl

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

do węzłów chłonnych. Inwazja komórek nowotworowych w naczyniach limfatycznych wewnątrz i wokół guza jest niezależnym czynnikiem prognostycznym w miejscowo zaawansowanym raku przełyku.

Słowa kluczowe: rak przełyku, D2-40, gęstość naczyń limfatycznych i inwazja naczyń limfatycznych, przerzuty do węzłów chłonnych, prognoza.

Introduction

In esophageal cancer, the presence of neoplastic cells in lymph nodes is one of the best clinical criteria in the evaluation of tumor advancement, a significant prognostic indicator for outcome, and important in the selection of appropriate combined treatment [1]. However, even for patients in the same stage, the clinical course can be quite variable. Patients with lymph node involvement have an increased risk of disease recurrence and reduced overall survival even after extended radical esophagectomy with three-field lymphadenectomy [2].

Lymphatic vessel density (LVD) is one of the ways to evaluate lymphangiogenesis. Several types of cancer can induce the formation of lymphatic vessels [3], whereas other cancers do not actively induce lymphangiogenesis and simply invade existing lymphatic vessels [4]. Studies of intratumoral and peritumoral LVD and lymphatic vessel invasion (LVI) have been hampered by the lack of specific lymphatic markers. Recently, the assessment of lymphangiogenesis as well as intratumoral and peritumoral lymphatic vessel invasion of neoplasms has become possible with the development of lymphatic vessel-specific markers such as lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) [5], human transcription factor Prox-1 [6], and glomerular podocyte membrane mucoprotein podoplanin [7]. The D2-40 antibody detects a fixation-resistant epitope on podoplanin, which is a selective marker for lymphatic endothelium, allowing for the identification of lymphatic vessels in formalin-fixed, paraffin-embedded tissue sections and the study of LVD and LVI in solid tumors [8].

In the present study we evaluated LVD and LVI detected by D2-40 staining in intratumoral and peritumoral areas in order to clarify their significance in nodal metastasis and prognosis in patients with esophageal cancer.

Material and methods

Patients and tissues

Tumor specimens were obtained from 74 patients with primary esophageal squamous cell cancer and adenocarcinoma who underwent esophagectomy at the Department of Thoracic Surgery, Medical University of Bialystok. Only distal esophageal adenocarcinomas (adenocarcinoma of the esophagogastric junction type I according to the Siewert classification) were included, whereas adenocarcinoma types II and III were strictly excluded. All patients underwent peri-esophageal and perigastric lymph node dissections. None had received preoperative chemotherapy or radiotherapy. The study population consisted of 60 men (81%) and 14 women (19%). The median age at the time of diagnosis was 66 years (range: 42 to 78 years). Pathological staging was based on the AJCC TNM classification (7^{th} edition) [9]. No deaths occurred in the study population within 30 days of the operation or during the hospital stay. Follow-up information was available for all patients. Evaluations were performed every 3-6 months by means of a clinical history, physical examination, laboratory analysis, fiberoptic esophagoscopy, ultrasound examination of the neck and abdomen, barium esophagram, computed tomography, endoscopic ultrasound (EUS), PET-CT, and endobronchial ultrasound (EBUS) if necessary. The median follow-up period was 28.5 months with a maximum of 107 (range: 3-107). Analysis was performed on overall survival, disease-free survival, and cancer-specific survival. Overall survival, disease-free survival, and cancer-specific survival were all calculated from the date of the surgery to the last contact made with all living patients, to the date of the last follow-up for disease-free patients, and to the date of esophageal cancer-induced death, respectively. Patients who died from causes unrelated to the esophageal carcinoma, with no evidence of the disease, were death-censored. The sites of recurrent tumors were documented in 48 patients as they were determined by clinical and/or radiographic procedures. None were identified at autopsy or by reoperation of asymptomatic patients. Recurrence categories were subsequently expressed as the first site of recurrence rather than the cumulative (total) incidence of recurrence. Local recurrence was defined as the reappearance of cancer in the tumor bed and/or at the site of anastomosis. Forty-one patients received chemotherapy, radiotherapy, or both postoperatively and during follow-up. In total, thirty-seven patients died of cancer. Normal esophageal tissues three cm away from the tumor were collected as control specimens.

Analysis of protein expression by immunohistochemistry (IHC)

Surgical specimens were fixed in a 10% buffered formalin solution for 24 h, and then 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 tumor and adjacent benign peritumoral tissue. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex technique (ABC-technique). Paraffin-embedded slides were prepared from each study block by cutting slices at a thickness of four

micrometers. The slides were heated in a microwave oven containing a 0.01 mmol/l sodium citrate (pH 6.0) solution for antigen retrieval. Sections were then treated with 0.3 % H_2O_2 for 10 min at room temperature. 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). 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 complexes. The sections were stained with 3'3-diaminobenzidine (DAB) to visualize antigen-antibody complex. Nuclei were then stained with Mayer's hematoxylin. Positive controls were made using tissue samples provided by the antibody manufacturer which showed high expressions of proteins. Negative controls were made with the same tissue but without any antibodies.

Definition of lymphatic vessel density and lymphatic vessel invasion

Lymphatic vessel density (LVD) was evaluated according to methods described by Weidner *et al.*[10] with slight modifications. Any brown stained endothelial cell or cell cluster clearly separated from the adjacent cells, tissue elements and microvessels was defined as a lymph channel. All slides were screened using a low-magnification lens to identify areas that contained the highest number of D2-40 positively stained lymphatic vessels within the desmoplastic stroma (hot spot). The total number of vessels was then counted in five fields of hot spots at 400× magnification and converted to the number of vessels per 0.15 mm^2. LVD was determined as the mean value of vessel count for each case. Peritumoral lymphatic vessels were defined as D2-40-positive vessels in adjacent benign peritumoral tissue. Intratumoral lymphatic vessels were defined as D2-40-positive vessels located within the tumor mass and not confined by invagination of normal tissue. Intratumoral lymphatic vessel density (itLVD) and peritumoral lymphatic vessel density (ptLVD) were assessed separately. Scoring and counting were performed independently by an investigator who had no patient clinical information. The median ptLVD and itLVD were calculated.

Lymphatic vessel invasion (LVI) was evaluated by microscopic examination of the slides. The presence of at least one tumor cell cluster in a D2-40-positive vascular channel indicated LVI. Assessment of LVI was performed both intratumorally (itLVI) and peritumorally (ptLVI).

Statistical analysis

Distribution was analyzed by the Shapiro-Wilk test. Categorical data were compared by the χ^2 or Fisher's exact probability test. The correlations of itLVD and ptLVD with clinicopathologic parameters were analyzed by the Mann-Whitney U test or the Kruskal-Wallis one-way ANOVA. Related factors pertaining to lymph node metastasis were calculated using univariate and multivariate logistic regression analyses. 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 a log-rank test. The prognostic value of lymphatic vessel density and 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 analyses, and a p value less than 0.05 was considered significant. Statistical analyses were carried out using Statistica 8.0 PL software (StatSoft Inc., Tulsa, OK, USA) and SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL, 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 informed written consent was obtained from all patients before participation.

Results

In the majority of the esophageal tumors, lymphatic vessels identified with D2-40 staining were different in size and shape, with thin walls and no perivascular cells, and were found in both tumor and peritumoral areas. Intratumoral lymphatic vessels were usually small, irregular, had a tortuous or collapsed lumen, and were located in the close vicinity of tumor cells. Peritumoral lymphatic vessels were often enlarged and dilated with widely opened lumina. Peritumoral lymphatic vessels were more numerous than the intratumoral channels. Their size and shape varied from case to case and even in the same microscopic field of a single sample. Examples of intratumoral lymphangiogenesis, intratumoral lymphatic vessel invasion, and peritumoral lymphatic vessel invasion are shown in Figs. 1A and 1B.

Lymphatic vessel density in peritumoral areas (median 38, range 20-51) was significantly higher than that in intratumoral areas (median 28, range 12-42, $p < 0.001$). Lymphatic vessel density was divided into a high LVD group and a low LVD group according to the median. High ptLVD was associated with lymph node metastasis, stage, depth of invasion, tumor size, residual tumor, and sex. Higher itLVD significantly correlated with lymph node metastasis, stage, depth of invasion, tumor size, residual tumor, and histological type (Table I).

Positive LVI was observed intratumorally in 58% (43/74) of cases and peritumorally in 62% (46/74) of cases. Tissue samples with both positive intratumoral LVI and peritumoral LVI were observed in 47% (35/74) of cases. Positive itLVI was significantly correlated with tumor size, histological grade, tumor depth, stage, lymph node metastasis, and residual tumor. Positive ptLVI was associated with lymph node metastasis, stage, depth of invasion, tumor size, residual tumor, and sex (Table I). High itLVD and ptLVD showed a significant correlation with the presence of itLVI and ptLVI ($p = 0.001$ and $p < 0.001$ respectively).

A logistic regression analysis was performed to determine which of the parameters studied best predicted

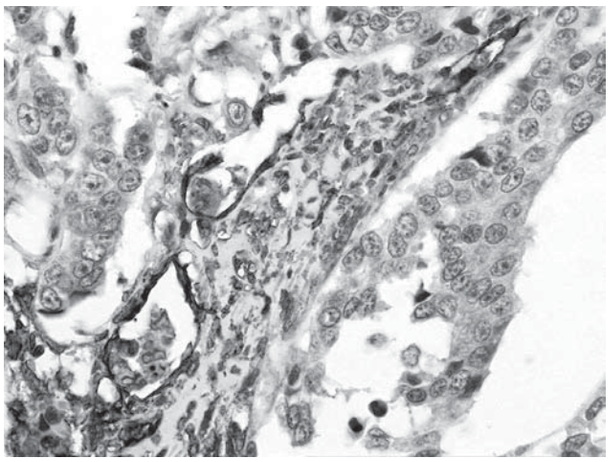


Fig. 1A. Intratumoral lymphangiogenesis and intratumoral lymphatic vessel invasion. The endothelial cells of small lymphatic vessels stained with D2-40. Cancer cells are clearly outlined by D2-40 positive lymphatic vessels (squamous cell carcinoma; magnification 600×)

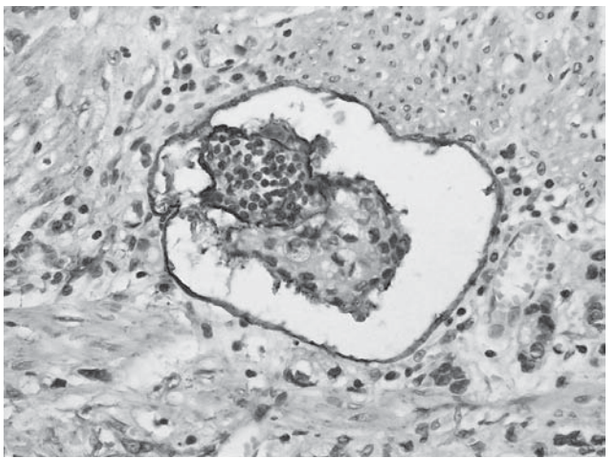


Fig. 1B. Peritumoral lymphatic vessel invasion. Cancer cell embolus of the lymphatic vessel is clearly outlined by D2-40 positive lymphatic endothelial cells. Many dispersed lymphatic endothelial cells can be seen in the surrounding extracellular matrix (squamous cell carcinoma; magnification 400×)

Tab. I. Association between intratumoral LVD, peritumoral LVD, intratumoral LVI, peritumoral LVI, and clinicopathological criteria

Clinicopathological criteria	Frequency *n* (%)	it LVD Median (range)	*p* value	pt LVD Median (range)	*p* value	itLVI negative (%)	itLVI positive (%)	*p* value	ptLVI negative (%)	ptLVI positive (%)	*p* value
Age (years)											
< 66	37 (50)	28 (18-41)	0.961[a]	38 (23-49)	0.879[a]	14 (37)	23 (63)	0.319[c]	14 (38)	23 (62)	0.595[c]
≥ 66	37 (50)	29 (12-42)		39 (20-51)		17 (46)	20 (54)		14 (38)	23 (62)	
Sex											
F	14 (19)	26 (12-33)	**0.046[a]**	30 (20-46)	**0.005[a]**	9 (64)	5 (36)	0.057[c]	10 (72)	4 (28)	**0.005[c]**
M	60 (81)	29 (15-42)		39 (23-51)		22 (37)	38 (63)		18 (30)	42 (70)	
Histological type											
Sqcc	32 (43.3)	27 (15-41)	**0.026[a]**	38 (24-49)	0.328[a]	15 (47)	17 (53)	0.301[c]	13 (41)	19 (59)	0.424[c]
Adc	42 (56.7)	29 (12-42)		39 (20-51)		16 (38)	26 (62)		15 (36)	27 (64)	
Location											
upper	4 (5.4)	31 (22-33)	0.311[b]	41 (23-44)	0.745[b]	2 (50)	2 (50)	0.938[d]	1 (25)	3 (75)	0.505[d]
midthoracic	26 (35.1)	28 (17-33)		38 (25-51)		11 (42)	15 (58)		8 (31)	18 (69)	
lower	44 (59.5)	29 (12-42)		37 (20-47)		18 (41)	26 (59)		19 (43)	25 (57)	
Tumor size											
< 4 cm	36 (48.6)	24 (12-38)	**0.002[a]**	31 (20-45)	**< 0.001[a]**	22 (61)	14 (39)	**0.001[c]**	20 (55)	16 (45)	**0.002[c]**
≥ 4 cm	38 (51.4)	29 (22-42)		40 (27-51)		9 (24)	29 (76)		8 (21)	30 (79)	
Histological grade											
G1	7 (9.4)	24 (12-41)	0.753[b]	35 (20-43)	0.624[b]	7 (100)	0 (0)	**0.001[d]**	5 (71)	2 (29)	0.093[d]
G2	32 (43.2)	28 (15-42)		38 (23-51)		15 (47)	17 (53)		13 (41)	19 (59)	
G3	35 (47.4)	29 (15-38)		39 (22-47)		9 (26)	26 (74)		10 (28)	25 (72)	
Stage											
I + II	32 (43.2)	22 (12-41)	**< 0.001[a]**	29 (20-42)	**< 0.001[a]**	22 (69)	10 (31)	**< 0.001[c]**	21 (66)	11 (34)	**< 0.001[c]**
III	42 (56.8)	29 (24-42)		40 (27-51)		9 (22)	33 (78)		7 (17)	35 (83)	
T1 + T2	21 (28.4)	23 (12-41)	**0.001[a]**	28 (20-42)	**< 0.001[a]**	16 (76)	5 (24)	**< 0.001[c]**	14 (67)	7 (33)	**0.002[c]**
T3 + T4	53 (71.6)	29 (17-42)		39 (22-51)		15 (28)	38 (72)		14 (26)	39 (74)	
N0	25 (33.7)	21 (12-29)	**< 0.001[a]**	28 (20-40)	**< 0.001[a]**	17 (68)	8 (32)	**0.001[c]**	19 (76)	6 (24)	**< 0.001[c]**
N1	49 (66.3)	29 (24-42)		40 (27-51)		14 (28)	35 (72)		9 (18)	40 (82)	
R0	62 (83.7)	27 (12-42)	**0.027[a]**	37 (20-51)	**0.049[a]**	31 (50)	31 (50)	**0.001[c]**	27 (43)	35 (57)	**0.019[c]**
R1 + R2	12 (16.3)	30 (18-41)		40 (26-47)		0 (0)	12 (100)		1 (8)	11 (92)	

[a]Mann-Whitney U-test; [b]Kruskal-Wallis test; [c]Fisher's exact test; [d]χ^2 test; it LVD – intratumoral lymphatic vessel density; pt LVD – peritumoral lymphatic vessel density; itLVI – intratumoral lymphatic vessel invasion; ptLVI – peritumoral lymphatic vessel invasion; F – female; M – male; Sqcc – squamous cell carcinoma; Adc – adenocarcinoma; 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 adjacent structures; N0 – no regional lymph node metastases; N1 – regional lymph node metastases; R0 – no residual tumor; R1 – microscopic residual tumor; R2 – macroscopic residual tumor; bold values are statistically significant

the presence of lymph node metastasis in the current series of esophageal cancer cases. Univariate analysis showed that among the various clinicopathologic variables, tumor size, histological grade, tumor depth, residual tumor, ptLVD, itLVD, ptLVI, and itLVI were significant risk factors for lymph node metastasis. As indicated by multivariate analysis, itLVD (p = 0.028), ptLVI (p = 0.036), increasing depth of tumor invasion (p = 0.01), and tumor size (p = 0.042) were statistically significant independent risk factors in predicting lymph node metastases (Table II).

Kaplan-Meier curves showed an increased risk of poor disease-free survival, cancer-specific survival and overall survival associated with high itLVD, ptLVD, and D2-40 positive intratumoral and peritumoral LVI (Figs. 2A-C, 3A-C, 4A-C, 5A-C). The relative risk of poor disease-free survival, cancer-specific survival, and overall survival was significantly increased for positive ptLVI and positive itLVI in both univariate and multivariate analyses (Table III). Furthermore, itLVD and ptLVD were also shown to be significant unfavorable predictors for all three survivals by univariate analysis, but were not independent predictors for all three survivals by multivariate analysis. Moreover, lymph node metastasis, tumor stage, and residual tumor could serve as predictors by univariate analysis and as independent predictors by multivariate analysis for all three survivals. Tumor size, histological grade, and tumor depth were significant predictors for all three survivals only by univariate analysis (Table III).

Discussion

Lymphatic metastasis in esophageal cancer is characterized by early and widespread dissemination. Nodal involvement in the neck or abdominal cavity can be found irrespective of primary tumor location [11] and lymph node metastasis is closely related to long-term prognosis in patients who undergo esophagectomy [12].

Tab. II. Univariate and multivariate analysis of risk factors for lymph node metastases.

Factors	Univariate	Multivariate		
	p	Odds ratio	95% CI	p
age	0.051	13.021	1.30-130.13	0.071
sex	0.288	0.597	0.067-5.269	0.638
location	0.191	0.508	0.145-1.780	0.281
tumor size	**< 0.001**	1.294	0.713-3.394	**0.042**
histological grade	**0.050**	0.912	0.791-2.862	0.124
tumor depth	**< 0.001**	1.523	1.067-5.612	**0.010**
residual tumor	**0.042**	0.791	0.593-2.973	0.242
ptLVD	**0.001**	1.435	0.795-4.924	0.651
itLVD	**0.001**	1.312	0.02-3.568	**0.028**
ptLVI	**< 0.001**	1.623	0.516-4.911	**0.036**
itLVI	**0.002**	1.221	0.139-5.699	0.463

CI – confidence interval; pt LVD – peritumoral lymphatic vessel density; it LVD – intratumoral lymphatic vessel density; itLVI – intratumoral lymphatic vessel invasion; ptLVI – peritumoral lymphatic vessel invasion; bold values are statistically significant

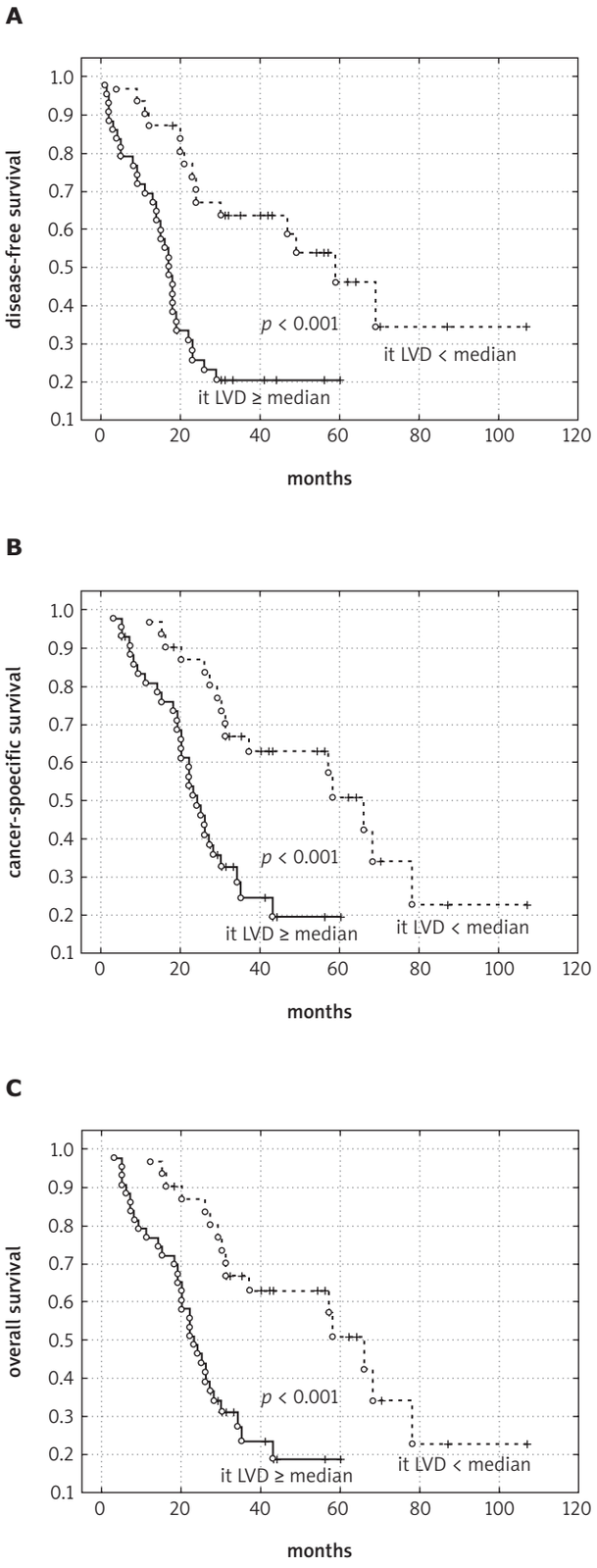
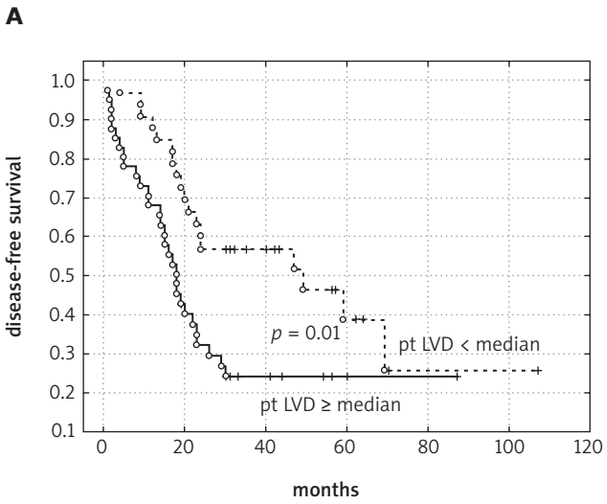
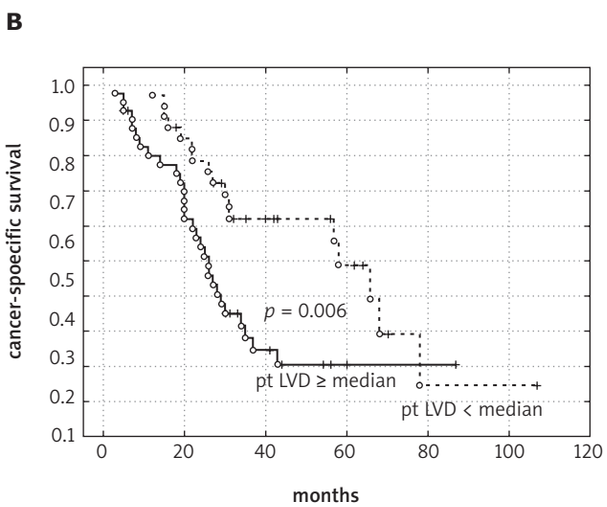


Fig. 2. Kaplan-Meier analysis of disease-free survival (A), cancer-specific survival (B), and overall survival (C) according to intratumoral lymphatic vessel density (itLVD) detected by D2-40 in patients with esophageal cancer





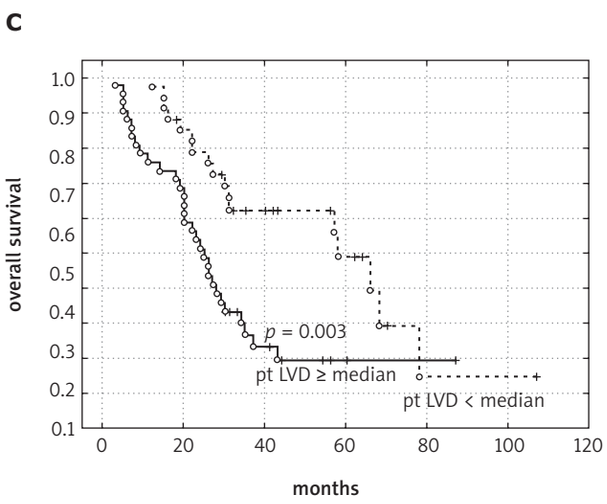
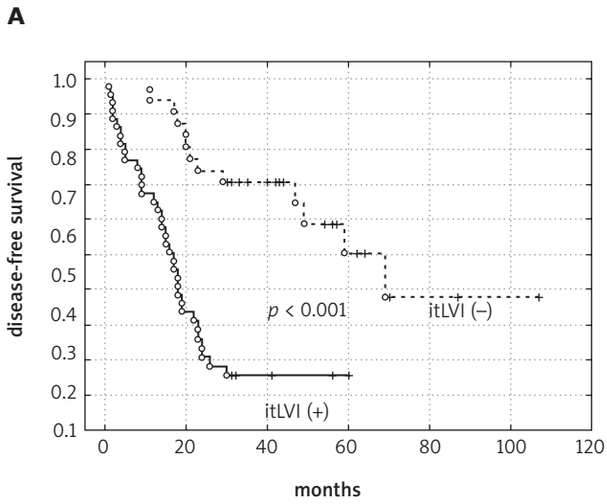
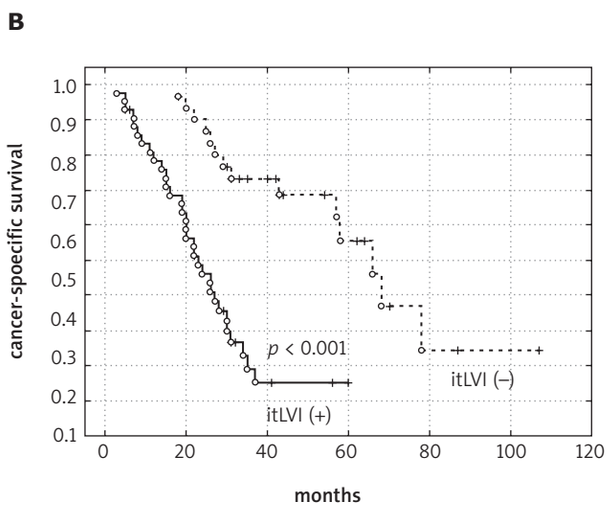


Fig. 3. Kaplan-Meier analysis of disease-free survival (A), cancer-specific survival (B), and overall survival (C) according to peritumoral lymphatic vessel density (ptLVD) detected by D2-40 in patients with esophageal cancer





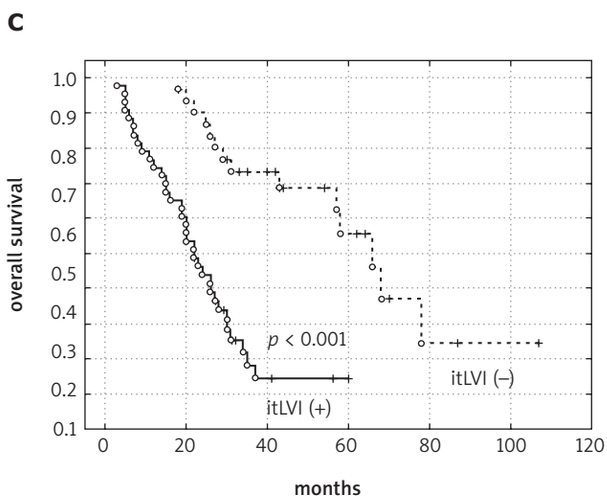


Fig. 4. Kaplan-Meier analysis of disease-free survival (A), cancer-specific survival (B), and overall survival (C) according to intratumoral lymphatic vessel invasion (itLVI) detected by D2-40 in patients with esophageal cancer

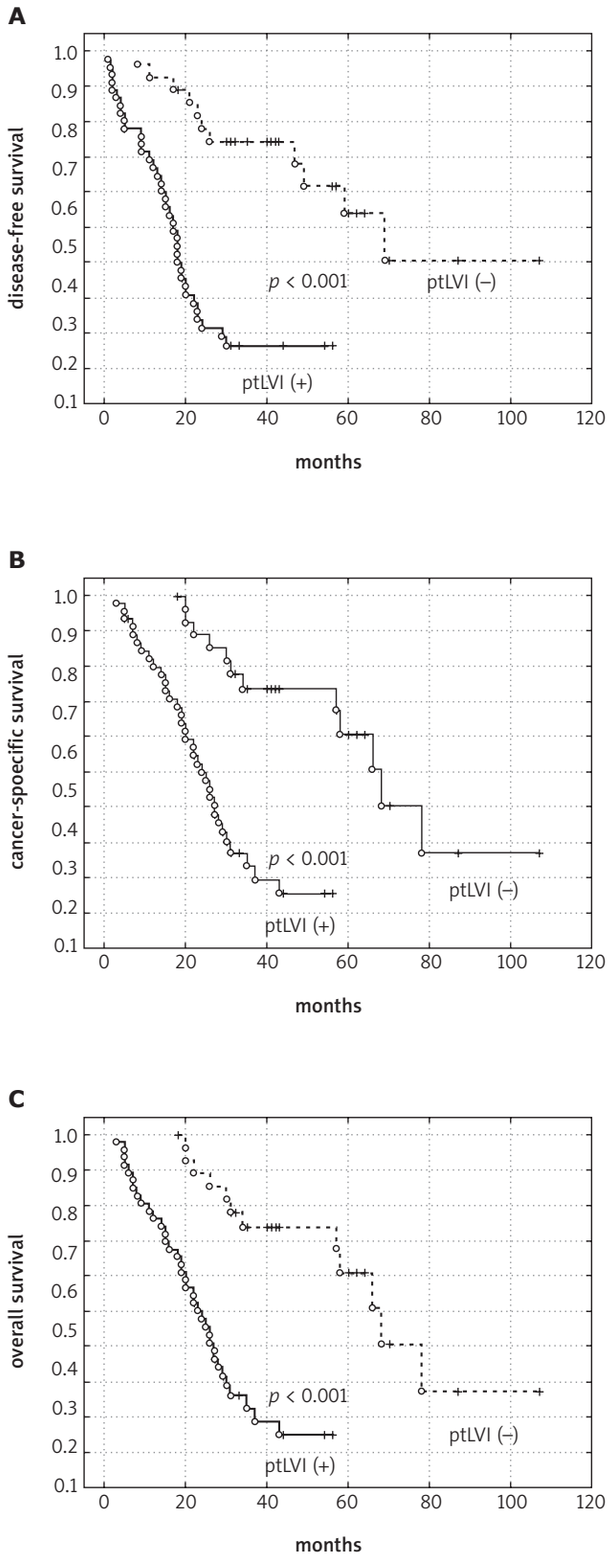


Fig. 5. Kaplan-Meier analysis of disease-free survival (A), cancer-specific survival (B), and overall survival (C) according to peritumoral lymphatic vessel invasion (ptLVI) detected by D2-40 in patients with esophageal cancer

In this study, we chose the lymphatic endothelium-specific marker D2-40 to evaluate LVD and LVI because it was found to be specifically expressed in lymphatics, which allows for objective identification [8]. Historically, the presence of intratumoral lymphatic vessels has been a subject of controversy in solid tumors in general, despite the consensus regarding the existence of peritumoral lymphatic channels. Previous studies reported that solid tumors do not have intratumoral lymphatic vessels [4] or the intratumoral lymphatics may be nonfunctional [13]. This study clearly demonstrated that D2-40-positive lymphatic vessels were detected in intratumoral and peritumoral areas in the majority of tumors. We observed that intratumoral lymphatics were disorganized, small and flattened in contrast with the widely open lymphatics in peritumoral regions. This was in accordance with previous studies which investigated tumor-associated lymphangiogenesis using the same antibody in head and neck, and esophageal cancer [14,15].

In this study, the median LVD in the peritumoral area was significantly higher than that in the intratumoral area. Both correlated significantly with sex, lymph node metastasis, tumor size, depth of invasion, stage, and residual tumor. In contrast, Mori *et al*. [15] did not find any correlation between LVD and pathological parameters. High itLVD was found to be significantly correlated with lymph node metastasis by Inoue *et al.* [16] and Imamura *et al.* [17] in esophageal cancer, and by Tomita *et al.* [18] only in subepithelial extension of esophageal cancer. A recent study suggested that intratumoral lymphatics play a greater role than peritumoral lymphatics in nodal metastasis of head and neck cancer [19]. Our results showed that intratumoral lymphatic vessels were found in large tumors and in tumors that had already spread to regional lymph nodes. Several reports have shown that increased ptLVD is positively associated with prognostic factors, including lymph node metastasis in patients with gastric, and esophageal cancer [20, 21]. This suggests that both high itLVD and high ptLVD have important roles in maintaining an appropriate environment for tumor cells and could enhance the entrance of tumor cells into lymphatic vessels by increasing the contact surface between lymphatic vessels and tumor cells.

During the course of dissemination through the lymphatics, lymphatic invasion by cancer cells is considered as a preliminary step in the development of lymph node metastasis. In our study lymph node metastasis was observed in 72% of itLVI-positive and in 82% of ptLVI-positive patients. Lymph node metastasis was significantly related to the tissue status of itLVI ($p = 0.001$) and ptLVI ($p < 0.001$) which were diagnosed on D2-40 staining. Lin *et al.* [22] have reported similar observations for colorectal cancer. Imamura *et al.* [17], Tomita *et al.* [18] and our earlier study [23] revealed that LVI in the entire tumor tissue of esophageal cancer was significantly linked with nodal metastasis. In contrast, Mori *et al.* [15] found that only ptLVI positively correlated with lymph node metastasis. Thus, it is likely that tumor cells metastasize to regional lymph nodes by invading intratumoral and peritumoral lymph vessels. We

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

Factors	Disease-Free Survival				Cancer-Specific Survival				Overall Survival			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Tumor size	2.510 (1.381-4.563)	**0.003**	0.691 (0.338-1.535)	0.335	2.614 (1.392-4.908)	**0.003**	0.676 (0.312-1.463)	0.320	2.764 (1.484-5.149)	**0.001**	0.772 (0.361-1.652)	0.505
Histological grade	1.858 (1.162-2.971)	**0.010**	1.562 (0.873-2.794)	0.133	1.963 (1.212-3.182)	**0.006**	1.620 (0.895-2.931)	0.111	2.052 (1.270-3.317)	**0.003**	1.600 (0.892-2.870)	0.115
Tumor depth T1+2 vs T3+4	2.401 (1.174-4.910)	**0.016**	1.186 (0.356-3.948)	0.781	2.665 (0.601-6.445)	**0.011**	1.969 (0.601-6.445)	0.263	2.773 (1.304-5.896)	**0.008**	2.055 (0.658-6.420)	0.215
Tumor stage I+II vs III	2.641 (1.414-4.931)	**0.002**	1.226 (0.641-2.828)	**0.028**	2.539 (1.318-4.890)	**0.005**	1.113 (0.735-3.987)	**0.003**	2.401 (1.270-4.537)	**0.007**	1.301 (0.931-4.681)	**0.002**
Lymph node metastasis	4.671 (2.136-10.214)	**< 0.001**	13.544 (3.249-56.463)	**< 0.001**	4.942 (2.126-11.488)	**< 0.001**	17.752 (3.957-79.634)	**< 0.001**	4.301 (1.940-9.532)	**< 0.001**	12.150 (2.751-53.667)	**0.001**
Residual tumor	3.421 (1.701-6.879)	**0.001**	2.248 (1.001-5.051)	**0.050**	3.632 (1.756-7.512)	**0.001**	2.201 (0.963-5.030)	**0.048**	3.754 (1.865-7.556)	**0.001**	2.161 (0.973-4.801)	**0.049**
pt LVD	2.124 (1.176-3.838)	**0.013**	0.599 (0.262-1.367)	0.223	2.309 (1.250-4.263)	**0.008**	0.785 (0.331-1.859)	0.582	2.431 (1.326-4.456)	**0.004**	0.926 (0.394-2.172)	0.859
it LVD	3.323 (1.739-6.350)	**< 0.001**	0.916 (0.364-2.304)	0.853	3.241 (1.646-6.381)	**0.001**	0.714 (0.284-1.826)	0.482	3.388 (1.732-6.629)	**0.001**	0.769 (0.298-1.984)	0.587
ptLVI	5.058 (2.402-10.649)	**< 0.001**	2.814 (1.166-6.789)	**0.021**	5.763 (2.521-13.174)	**< 0.001**	3.006 (1.150-7.856)	**0.025**	5.969 (2.621-13.590)	**< 0.001**	3.081 (1.196-7.937)	**0.020**
itLVI	4.360 (2.223-8.548)	**< 0.001**	2.991 (1.221-7.325)	**0.018**	4.542 (2.220-9.293)	**< 0.001**	3.309 (1.252-8.746)	**0.016**	4.716 (2.317-9.597)	**< 0.001**	3.242 (1.237-8.492)	**0.017**

HR – hazard ratio; CI – confidence interval; pt LVD – peritumoral lymphatic vessel density; it LVD – intratumoral lymphatic vessel density; itLVI – intratumoral lymphatic vessel invasion; ptLVI – peritumoral lymphatic vessel invasion; T1 – tumor invades lamina propria or submucosa; T2 – tumor invades muscularis propria; T3 – tumor invades adventitia; T4 – tumor invades adjacent structures; bold values are statistically significant

also found that the presence of both itLVI and ptLVI significantly correlated with tumor size, tumor depth, TNM stage, and residual tumor. Similar to our present results, Saad *et al.* [21] and Imamura *et al.* [17] showed a significant correlation between lymphatic invasion in the entire tumor tissue detected on D2-40 staining and depth of tumor invasion, stage, and lymph node metastases. Our analysis suggests that both itLVI and ptLVI promote malignant progression of esophageal cancer.

With regard to lymphangiogenesis, some previous reports noted that high ptLVD could be a risk factor for lymph node metastasis in gastric cancer [20], and head and neck squamous cell carcinoma [14]. In esophageal adenocarcinoma, Saad *et al.* [21] reported that LVD correlated significantly with LVI and lymph node metastases in multivariate analysis. On a prediction model of lymph node metastasis, Gockel *et al.* [24] found that LVI only gained statistical significance in univariate analysis. In our univariate analysis of lymph node prediction, tumor size, histological grade, depth of invasion, residual tumor, ptLVD, itLVD, ptLVI, and itLVI were highly significant. However, only itLVD, ptLVI, tumor size, and depth of invasion were shown to be independent risk factors to predict lymph node metastasis in multivariate analysis.

A few studies have investigated the role of LVD by using D2-40 as a prognostic marker in esophageal cancer. Saad *et al.* [21] showed that LVD correlated with a short disease-free survival. Inoue *et al.* [16] and Imamura *et al.* [17] found that high itLVD was significantly associated with worse overall survival in univariate analysis. In the present study, the results of a univariate analysis indicated that increased both itLVD and ptLVD were associated with shorter disease-free survival, cancer-specific survival and overall survival. However, multivariate analysis did not prove them as independent prognostic predictors. A similar observation was also noted in a report by Imamura *et al.* [17]. In contrast, Inoue *et al.* [16] stated that itLVD could serve as an adverse independent prognostic factor for overall survival.

The relationship between LVI and prognosis has become evident in various cancers [22]. Previous studies have reported LVI detected by hematoxylin and eosin staining in the entire tumor tissue to be an independent predictor of poor overall survival in curative resected esophageal cancer [25]. Imamura *et al.* [17], using D2-40 staining, revealed that positive LVI correlated with worse prognosis in univariate analysis and was an adverse independent prognostic factor for overall survival in patients classified as node negative. Our earlier study found that LVI in the entire tumor is an independent prognostic factor of both disease-free survival and overall survival [23]. This study's results show that positive itLVI and ptLVI, like lymph node metastasis, tumor depth, stage, and positive resection margin, are significantly associated with worse disease-free survival, cancer-specific survival and overall survival in univariate and multivariate analyses.

These data provide indirect evidence for the presence of functional intratumoral lymphatic vessels and indicate that both intratumoral and peritumoral lymphatic vessels participate in the lymphatic spread of esophageal cancer. These results also suggest that itLVI and ptLVI are functionally similar in regard to their metastatic potential and therefore their evaluation is equally important. These data also suggest that itLVD, ptLVD, itLVI, and ptLVI detected by D2-40 staining could be useful in daily pathological or clinical practice. Evaluation of itLVD, ptLVD, itLVI, and ptLVI could be used as a complement to lymph node assessment. Esophageal cancer patients with itLVI or ptLVI have a worse prognosis than those without, which might be explained by more aggressive biological behavior and an increased probability of cancer recurrence in these tumors. The presence of itLVI or ptLVI indicates that tumor cells have already invaded the lymphatic system. Therefore, in each resectable tumor, radical esophagectomy with three-field lymphadenectomy should be performed. According to our survival analysis, we recommend that for patients with esophageal cancer with high itLVD and ptLVD, and positive itLVI and ptLVI, detailed follow-up should be carried out to detect recurrence. Earlier implementation and longer courses of adjuvant chemotherapy may need to be seriously considered.

In summary, the present study suggests that the lymphatic system is an important pathway in the progression of esophageal cancer. Intratumoral lymphatic vessel density and peritumoral lymphatic vessel invasion are predictive factors of lymph node metastasis. Intratumoral and peritumoral lymphatic vessel invasion are independent prognostic factors in patients with esophageal cancer.

This paper was partly presented at the 20th European Conference on General Thoracic Surgery of the European Society of Thoracic Surgeons in Essen, Germany, June 10-13, 2012.

Acknowledgements

This work was supported by a grant from the Medical University of Białystok [No. 123-38772 L] and all authors declare that there is no conflict of interest in connection with this paper.

References

1. Ielpo B, Pernaute AS, Elia S, Buonomo OC, Valladares LD, Aguirre EP, Petrella G, Garcia AT. Impact of number and site of lymph node invasion on survival of adenocarcinoma of esophagogastric junction. Interact Cardiovasc Thorac Surg 2010; 10: 704-708.
2. Nakagawa S, Kanda T, Kosugi S, Ohashi M, Suzuki T, Hatakeyama K. Recurrence pattern of squamous cell carcinoma of the thoracic esophagus after extended radical esophagectomy with three-field lymphadenectomy. J Am Coll Surg 2004; 198: 205-211.
3. Straume O, Jackson DG, Akslen LA. Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. Clin Cancer Res 2003; 9: 250-256.
4. Williams CS, Leek RD, Robson AM, Banerji S, Prevo R, Harris AL, Jackson DG. Absence of lymphangiogenesis and intratumoural lymph vessels in human metastatic breast cancer. J Pathol 2003; 200: 195-206.
5. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol 1999; 144: 789-801.
6. Rodriguez-Niedenführ M, Papoutsi M, Christ B, Nicolaides KH, von Kaisenberg CS, Tomarev SI, Wilting J. Prox1 is a marker of ectodermal placodes, endodermal compartments, lymphatic endothelium and lymphangioblasts. Anat Embryol (Berl) 2001; 204: 399-406.
7. Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, Kerjaschki D. Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am J Pathol 1997; 151: 1141-1152.
8. Kahn HJ, Bailey D, Marks A. Monoclonal antibody D2-40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. Mod Pathol 2002; 15: 434-440.
9. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. AJCC cancer staging manual. Part III. Esophagus and esophagogastric junction. 7th ed. Springer, New York 2010; 103.
10. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N Engl J Med 1991; 324: 1-8.
11. Akiyama H, Tsurumaru M, Kawamura T, Ono Y. Principles of surgical treatment for carcinoma of the esophagus: analysis of lymph node involvement. Ann Surg 1981; 194: 438-446.
12. Clark GW, Peters JH, Ireland AP, Ehsan A, Hagen JA, Kiyabu MT, Bremner CG, DeMeester TR. Nodal metastasis and sites of recurrence after en bloc esophagectomy for adenocarcinoma. Ann Thorac Surg 1994; 58: 646-653.
13. Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, Munn LL, Jain RK. Lymphatic metastasis in the absence of functional intratumor lymphatics. Science 2002; 296: 1883-1886.
14. Franchi A, Gallo O, Massi D, Baroni G, Santucci M. Tumor lymphangiogenesis in head and neck squamous cell carcinoma: a morphometric study with clinical correlations. Cancer 2004; 101: 973-978.
15. Mori D, Yamasaki F, Shibaki M, Tokunaga O. Lateral peritumoral lymphatic vessel invasion can predict lymph node metastasis in esophageal squamous cell carcinoma. Mod Pathol 2007; 20: 694-700.
16. Inoue A, Moriya H, Katada N, Tanabe S, Kobayashi N, Watanabe M, Okayasu I, Ohbu M. Intratumoral lymphangiogenesis of esophageal squamous cell carcinoma and relationship with regulatory factors and prognosis. Pathol Int 2008; 58: 611-619.
17. Imamura Y, Watanabe M, Nagai Y, Baba Y, Hirashima K, Karashima R, Iwatsuki M, Yoshida N, Kinoshita K, Kurashige J, Iyama K, Baba H. Lymphatic vessel invasion detected by the D2-40 monoclonal antibody is an independent prognostic factor in node-negative esophageal squamous cell carcinoma. J Surg Oncol 2012; 105: 277-283.
18. Tomita N, Matsumoto T, Hayashi T, Arakawa A, Sonoue H, Kajiyama Y, Tsurumaru M. Lymphatic invasion according to D2-40 immunostaining is a strong predictor of nodal metastasis in superficial squamous cell carcinoma of the esophagus: algorithm for risk of nodal metastasis based on lymphatic invasion. Pathol Int 2008; 58: 282-287.
19. Frech S, Hörmann K, Riedel F, Götte K. Lymphatic vessel density in correlation to lymph node metastasis in head and neck squamous cell carcinoma. Anticancer Res 2009; 29: 1675-1679.
20. Wang XL, Fang JP, Tang RY, Chen XM. Different significance between intratumoral and peritumoral lymphatic vessel density in gastric cancer: a retrospective study of 123 cases. BMC Cancer 2010; 10: 299.
21. Saad RS, Lindner JL, Liu Y, Silverman JF. Lymphatic vessel density as prognostic marker in esophageal adenocarcinoma. Am J Clin Pathol 2009; 131: 92-98.

22. Lin M, Ma SP, Lin HZ, Ji P, Xie D, Yu JX. Intratumoral as well as peritumoral lymphatic vessel invasion correlates with lymph node metastasis and unfavourable outcome in colorectal cancer. Clin Exp Metastasis 2010; 27: 123-132.
23. Kozłowski M, Naumnik W, Nikliński J, Milewski R, Lapuć G, Laudański J. 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. Folia Histochem Cytobiol 2011; 49: 90-97.
24. Gockel I, Domeyer M, Sgourakis GG, Schimanski CC, Moehler M, Kirkpatrick CJ, Lang H, Junginger T, Hansen T. Prediction model of lymph node metastasis in superficial esophageal adenocarcinoma and squamous cell cancer including D2-40 immunostaining. J Surg Oncol 2009; 100: 191-198.
25. Brücher BL, Stein HJ, Werner M, Siewert JR. Lymphatic vessel invasion is an independent prognostic factor in patients with a primary resected tumor with esophageal squamous cell carcinoma. Cancer 2001; 92: 2228-2233.