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Esophagus

Peritumoral CD16b positive-neutrophil accumulation strongly correlates with regional lymph node metastasis in thoracic esophageal squamous cell cancer

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ABSTRACT

Background: The mechanism underlying cancer cell metastasis from the tumor to regional lymph nodes is not yet fully understood. We hypothesized that peritumoral neutrophil accumulation promotes regional lymph node metastasis in thoracic esophageal squamous cell cancer.
Methods: Between 2010 and 2019, 126 thoracic esophageal squamous cell cancer patients received curative (R0) esophagectomy without preoperative treatment in our hospital. Using paraffin-embedded resected tumors, we performed immunohistochemical analysis of CD16b-positive neutrophil accumulation in the peritumoral area, which was defined as a 1-mm region centered on the border separating the malignant cell nests from the host tissue. The relationship between the density of peritumoral CD16b staining and pathological lymph node metastasis or 5-year overall survival was evaluated.
Results: Although the clinicopathological characteristics of CD16b-high and CD16b-low patients did not differ, greater pathological lymph node metastasis ($P < .001$) and lymphatic invasion by the tumor ($P = .024$) and a poorer 5-year survival ($P = .010$) were seen in CD16b-high patients. Moreover, CD16b-positive neutrophil density was generally higher in the peritumoral area than within the tumor itself. Univariate and multivariate analyses showed that CD16b-positive neutrophil accumulation was an independent factor for lymph node metastasis with an odds ratio >25 ($P < .001$). On the other hand, blood neutrophil counts did not correlate with lymph node metastasis.
Conclusion: Peritumoral accumulation of CD16b-positive neutrophils is an independent factor strongly correlated with lymph node metastasis in thoracic esophageal squamous cell cancer.

Introduction

Squamous cell carcinoma is the dominant histological subtype of esophageal cancer worldwide (~90%).[1] Thoracic esophageal squamous cell cancer (TESCC) with regional lymph node metastasis is an advanced cancer with a poor prognosis. However, it differs significantly from cancers with distant organ metastasis because there is still a high probability for cure through esophagectomy with extensive lymph node dissection and neoadjuvant/adjuvant chemotherapy or chemoradiotherapy. For that reason, lymph node metastasis has been a major concern and primary focus of research for surgeons. Generally, lymphatic vessels within the tumor or the peritumoral area are the primary route by which tumor cells migrate to regional lymph nodes. Even today, however, the mechanism underlying tumor cell invasion of peritumoral lymphatic vessels and then regional lymph nodes has not been fully elucidated.

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Many studies, including ours,[2,3] have focused on the relationship between the tumor microenvironment and lymph node metastasis. Within the tumor microenvironment, various types of immune cells as well as fibroblasts are involved in lymphangiogenesis and lymph node metastasis.[4] This raises a key question: What are the roles tumoral and peritumoral immune cells in lymph node metastasis? To address that question, one focus has been intratumoral neutrophils.[5] In the present study, we hypothesized that neutrophil accumulation is essential for peritumoral inflammation and immune responses, which promote lymph node metastasis. To test that hypothesis, we investigated the relationship between peritumoral neutrophil density and lymph node metastasis in TESCC patients.

Methods

Patients

This study was approved by the Ethics Committee of Akita University Graduate School of Medicine #2355 and #2617. All participants provided informed consent and signed human subject institutional review board consent forms.

Between January 2010 and September 2019, 423 patients with esophageal cancer received esophagectomy at Akita University Hospital. Of those, we excluded 11 patients with cervical esophageal cancer and 38 with cancers other than squamous cell carcinoma (ie, adenocarcinoma, neuroendocrine carcinoma). The remaining 374 patients were diagnosed with TESCC. Our standard treatment strategy for cStage IA TESCC is upfront esophagectomy or definitive chemoradiotherapy, and for cStage IIA–IIIC TESCC it is neoadjuvant chemotherapy or chemoradiotherapy followed by esophagectomy or definitive chemoradiotherapy. Likewise, patients with metastasis in a supraclavicular lymph node but without distant organ metastasis (M1-lymph node) are treated using the above-described strategy. Upfront esophagectomy is also used to treat patients for whom preoperative treatment is inappropriate due to physical or other reasons. Among this group, endoscopic submucosal dissection was performed as a preoperative treatment in 29 patients, and 197 received neoadjuvant chemotherapy or chemoradiotherapy. Ultimately, 148 TESCC patients received curative (R0) upfront esophagectomy without preoperative treatment. Because in this study we evaluated the association between peritumoral neutrophil accumulation and regional lymph node metastasis, we considered it appropriate to include cases without preoperative treatment. We therefore enrolled 126 of the 148 TESCC patients without preoperative treatment in this study. The other 22 patients were not enrolled in the analysis due to the poor condition of the paraffin blocks or for other reasons. Clinical tumor stages and the treatment strategies were decided by a board composed of radiologists, oncologists, gastroenterologists, and surgeons. Pathological findings from resected tumors were reviewed by pathologists, and stages were determined according to the UICC International Union Against Cancer tumor-node-metastasis (TNM) Classification of Malignant Tumors (eighth edition).

Esophagectomy

Our standard operative procedure is right thoracoscopic or transthoracic (open) esophagectomy with 3-field lymph node dissection of the mediastinal (involving the paraesophageal region and areas around the trachea and bilateral main bronchus), abdominal (involving the perigastric region and areas around the celiac axis), and cervical (involving the bilateral paraesophageal region and supraclavicular region) lymph nodes. Some patients underwent 2-field (mediastinal and abdominal) lymph node dissection. Our standard reconstruction is with a gastric tube via the posterior mediastinal route. In some patients, however, reconstruction was with a free jejunal graft or pedicled colon.

Immunohistochemical staining

Paraffin-embedded tissue sections (4 μm) from the center of surgically resected tumors were examined immunohistochemically. Deparaffinized sections of specimens were initially incubated in citrate buffer (pH 6.0) for 10 minutes at 105°C in an autoclave for antigen retrieval. Endogenous peroxidase activity was inactivated by incubating the specimen for 30 minutes in 0.3% hydrogen peroxide in methanol at room temperature. Nonspecific reactions were blocked using 10% normal goat serum in tris for 30 minutes at room temperature. The sections were then first incubated overnight at 4°C with an anti-CD16b polyclonal antibody (1:100 dilution, LsBio), after which they were incubated for 30 minutes with Envision+ System-HRP labeled polymer anti-rabbit (K4003, Dako) and reacted with DAB peroxidase substrate. Lastly, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. Negative controls were treated using the same procedures except the primary antibody was omitted.

CD16b scoring in the peritumoral area

Immunohistochemical staining was imaged using a BZ-X800 microscope (Keyence) and was analyzed and scored using a BZ-X analyzer (Keyence). Immunoreactivity was analyzed quantitatively and automatically as follows. Regions where the anti-CD16b antibody bound were stained brown. The BZ analyzer used hybrid cell count software to judge color (immunoreactivity) based on hue, brightness, and saturation. Positive staining within a field was quantified using a set "hue threshold" that encompassed the entire brown signal within the image. This threshold was then applied to all specimens. Thereafter, we excluded areas that were clearly not CD16b-positive to the naked eye (eg, nonspecifically stained areas). Each section was chosen under a rule modified from the tumor-infiltrating lymphocyte scoring system.[6] Briefly, regions of interest (ROIs) were selected in the area of the tumor invasive margin, which was defined as a 1-mm wide region centered on the border separating the malignant cell nests from the host tissue. The ROI was defined as a 188 μm^2 area (500 × 500 pixels) involving both stromal and tumoral areas (Figure 1, *A–B*). Using the BZ-X analyzer, the tumor area within the square was masked, as were regions of the stroma area containing crush artifacts and regressive hyalinization. To then calculate the percent CD16b-positive cells, the CD16b-positive cells within the stromal area were measured automatically using the BZ-X analyzer (Figure 1, *C–D*) and divided by the entire stromal area inside the square within the ROI. We performed this calculation in 3 randomly selected peritumoral ROIs, after which the sum of the percentages was defined as the CD16b score (points) for the patient. Figure 2 shows representative calculations of CD16b scores in 2 patients.

CD16b scoring in the intratumoral area

To compare neutrophil accumulation between peritumoral and intratumoral areas, 5 patients with lymph node metastasis were randomly selected. Pathologists then identified areas of squamous cell carcinoma (within intratumoral areas) in

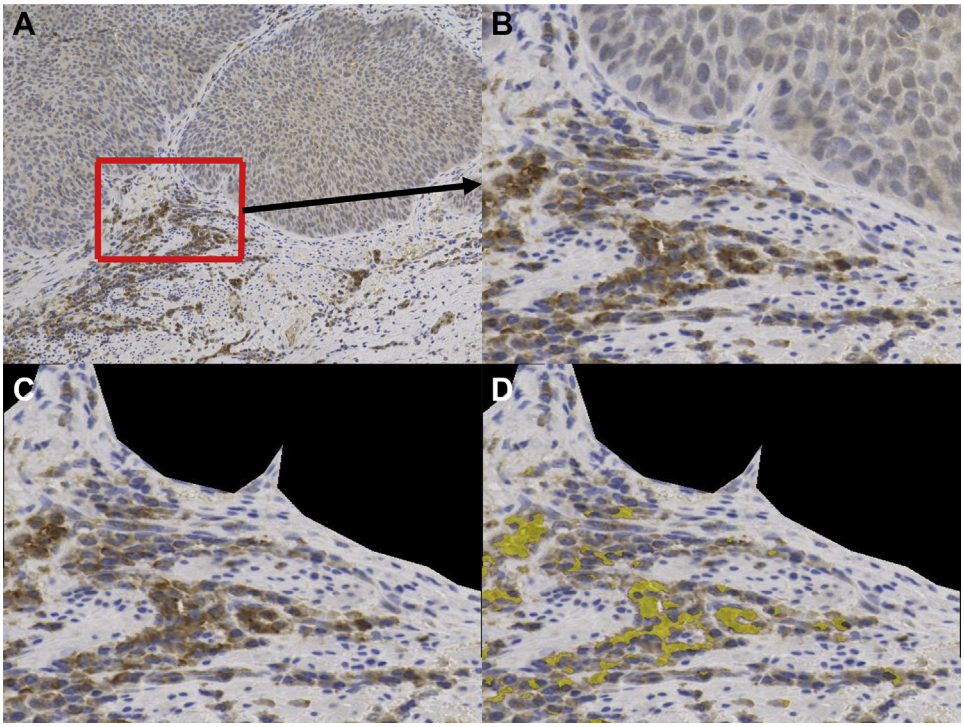

Figure 1. Method for determining the percent CD16b-positive area within a peritumoral region of interest. (A) A peritumoral area was randomly selected (red square) in which to determine the percent CD16b-positive area. (B) Higher magnification of the area with the red square in (A). The area within the red square was 188 μm^2 (500 × 500 pixels). (C) Tumoral areas were masked. (D) CD16b-positive cells (yellow area) were then counted using the analytical software.

hematoxylin/eosin-stained sections from each paraffin block from the 5 patients. To account for cancer heterogeneity, 3 randomly selected cores measuring 0.6 mm in diameter from within the tumor were collected from each paraffin block and placed on a slide glass. Immunohistochemical analysis was then performed as described above, and the CD16-positive neutrophil density (CD16b score) within the intratumoral areas were determined.

Statistical analysis

Continuous variables are presented as medians (minimum-maximum). To evaluate differences between the 2 groups, the Mann-Whitney-Wilcoxon test (for continuous variables) or Pearson's χ^2 test and Fisher exact probability test (for categorical variables) was used. Survival times were calculated from the date of esophagectomy to the patient's death or the date of the last clinical follow-up. The Kaplan-Meier method was used to construct survival curves. The log-rank test was used to assess differences between the curves. The relationship between CD16b density and confounders affecting the apparent likelihood of lymph node metastasis was tested using univariate and multivariate logistic regression analyses. All statistical analyses were performed using JMP15 (version 15.2.0, SAS Institute).

Results

Patient characteristics

The patients' median age was 70 (39–83) years. Of the 126 cancers treated, 17 (13%) were located in the upper thoracic esophagus, 69 (55%) in the middle thoracic segment, and 40 (32%) in the lower thoracic segment. There were 64 (51%) patients with pT1 tumors, 14 (11%) with pT2 tumors, and 48 (38%) with pT3 tumors. Sixty-eight (54%) patients had regional lymph node metastasis. Lymphatic and venous invasion were detected in 115 (91%) and 109 (87%) patients, respectively. Eighty-two (65%) patients received 3-field lymph node dissection, and 44 (35%) received 2-field dissection. The median follow-up period after surgery for censored cases was 53.4 (1.6–121.4) months.

CD16b immunostaining status

Figure 3 shows CD16b immunostaining in the intratumoral area around the invasive tumor margin (A) and near the center of the tumor (B). Within the intratumoral area around the invasive tumor margin, CD16b expression was sparsely distributed and generally not high (Figure 3, *A–B*). By contrast, CD16b expression was generally high in the peritumoral area. At the tumor margin, CD16b expression varied among patients; some patients were strongly positive for CD16b immunoreactivity (Figure 3, *C*), whereas others showed only weak signals (Figure 3, *D*).

High CD16b scores in the lymph node metastasis group

The median peritumoral CD16b score was 15 (0–40) points among patients with lymph node metastasis, which was significantly ($P < .001$) higher than the CD16b score of 5 (0–17) points among those without metastasis (Figure 4, *A*). Figure 4, *B* shows the receiver operating characteristic curve. The area under the receiver operating characteristic curve was 0.8477, and the optimum cutoff value was 11. We therefore defined CD16b expression as high when the score was ≥11 and low when it was <11.

The clinicopathological characteristics of the CD16b-high and -low groups are summarized in Table I. There were no

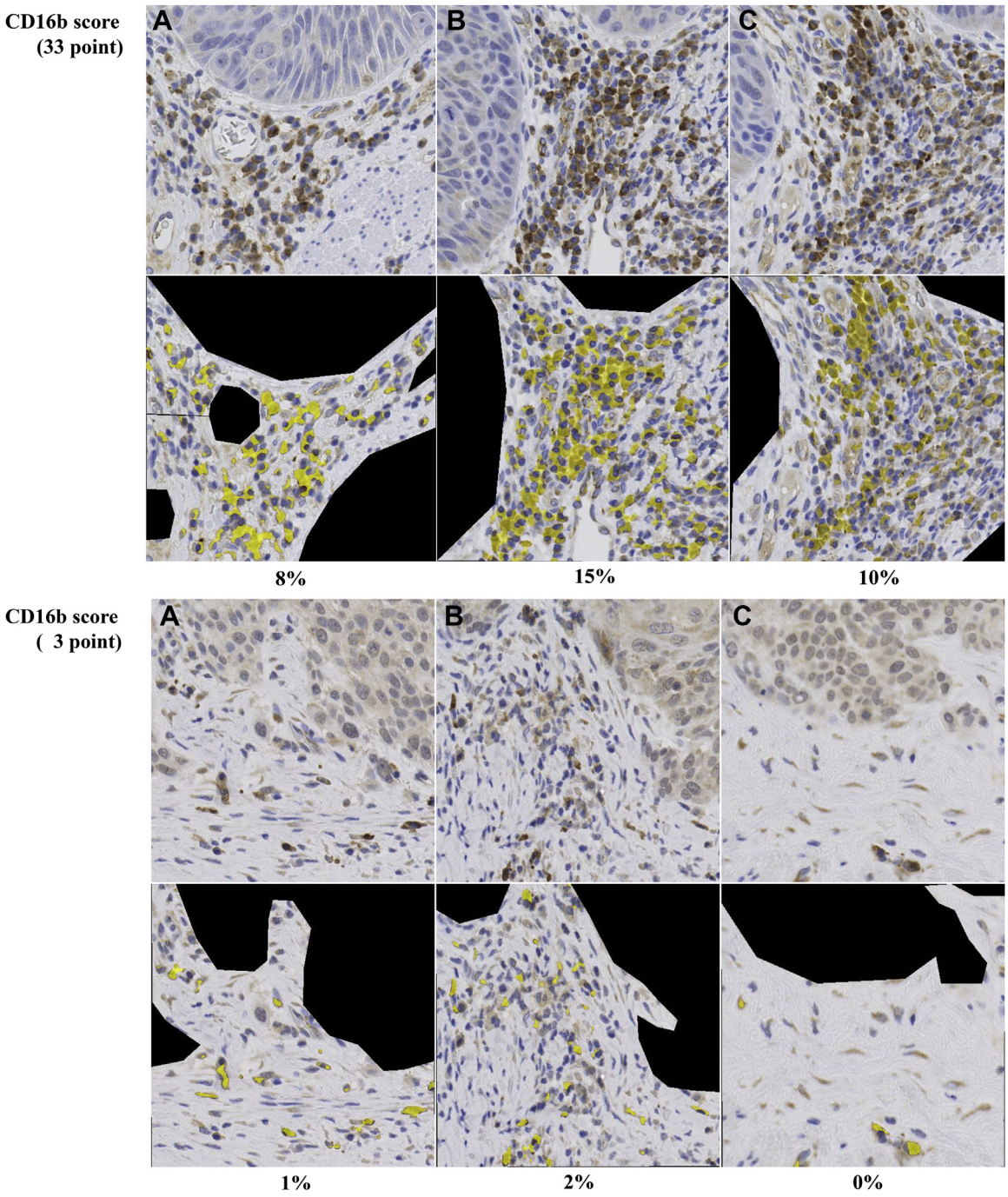

Figure 2. To calculate a CD16b score, 3 peritumoral areas were randomly selected (A–C), and the percent CD16b-positive area was determined in each. The 3 positivity rates were then summed to obtain the CD16b score (points). Upper panel: the CD16b score is 33 points; lower panel: the CD16b score is 3 points.

significant differences between the 2 groups with respect to sex, tumor location, pathological depth of tumor invasion (pT), tumor differentiation, field of lymph node dissection, or number of lymph nodes dissected. However, pathological lymph node metastasis status ($P < .001$) and lymphatic invasion ($P = .024$) significantly differed between the 2 groups, as did age ($P = .036$).

By contrast, the median intratumoral CD16b score in 5 randomly selected patients with lymph node metastasis was 0.2 (0.0–0.9) points, much lower than in the peritumoral area (15 [0–40], $n = 68$).

Positive relationship between CD16b-positive neutrophil accumulation in the peritumoral area and lymph node metastasis

Univariate analyses showed that CD16b expression (<11 vs ≥11) (odds ratio 25.440), pathological lymphatic invasion (positive versus negative) (odds ratio 6.061), and tumor differentiation (poorly versus not poorly) (odds ratio 3.036) were all significant factors affecting regional lymph node metastasis (Table II). Stepwise multivariate analysis, taking into consideration CD16b expression, lymphatic invasion, and tumor differentiation also showed CD16b expression and tumor differentiation status to be

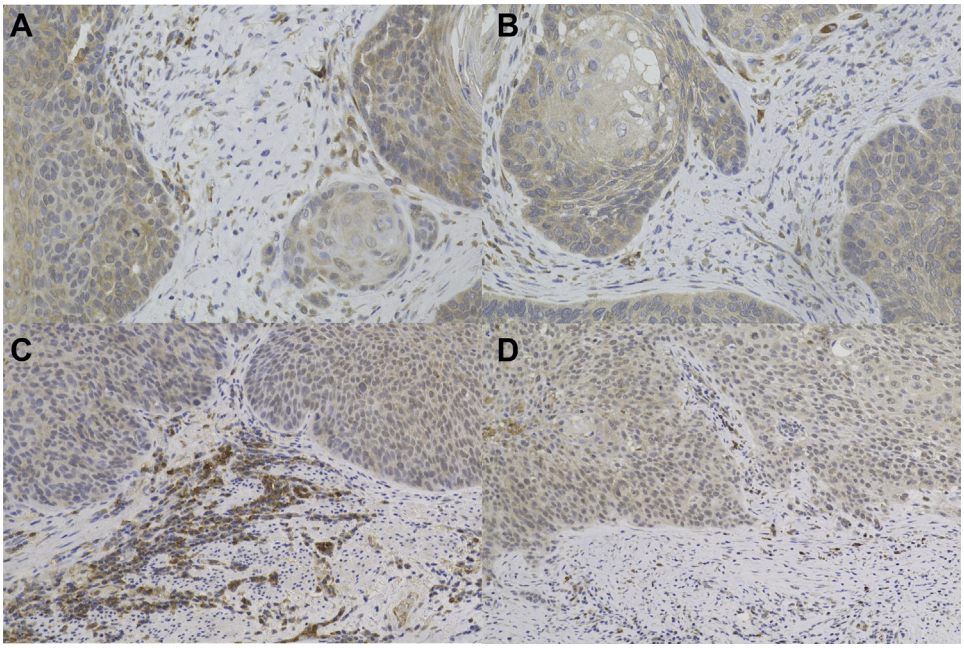

Figure 3. Intratumoral CD16b expression around the tumor invasive margin (A) and near the center of the tumor (B). In panel (C), strong CD16b-positive neutrophil accumulation was detected in the peritumoral area but is not seen in the panel (D).

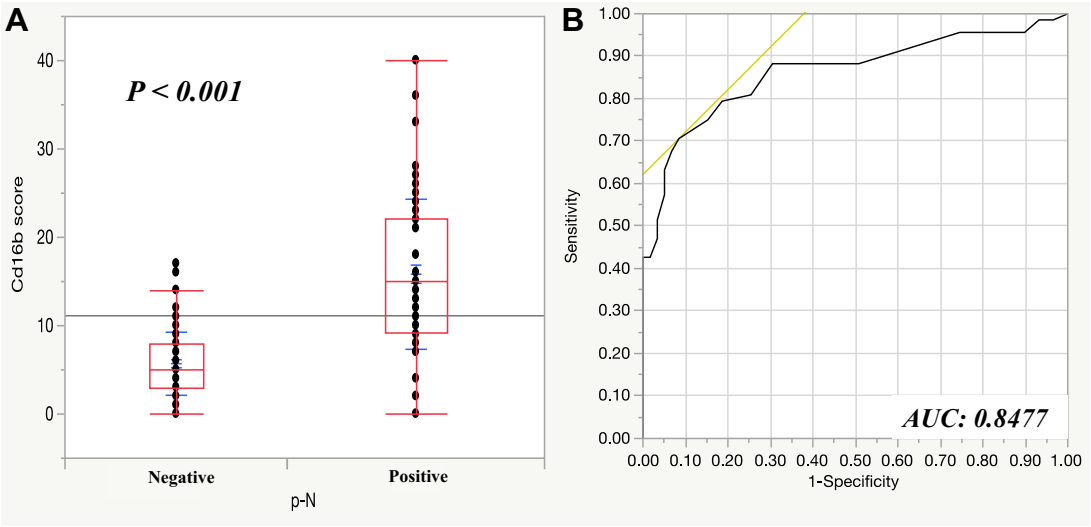

Figure 4. (A) CD16b scores in patients negative or positive for lymph node metastasis. Bars depict the median scores along with the interquartile ranges. (B) Receiver operating characteristic curve analysis of CD16b expression and lymph nodes metastasis. AUC, area under the curve.

independent factors (odds ratio 26.850 and 3.629) (Table II). Additional multivariate analysis with a crude model, age- and sex-adjusted model, and further adjusted models, the odds ratio for regional lymph node metastasis in patients with a CD16b score ≥11 was more than 25 (Table III).

Neutrophils and lymphocyte counts in the blood and the neutrophil-to-lymphocyte ratio (NLR) did not differ between CD16b-high and -low patients (Table I).

CD16b expression status affects 5-year overall survival

Kaplan-Meier curves illustrating the association between CD16b expression status and 5-year overall survival showed CD16b-high patients to have significantly poorer 5-year overall survival than CD16b-low patients (Figure 5).

Discussion

The present study yielded several noteworthy results. First, peritumoral accumulation of CD16b-positive neutrophils strongly corelated with regional lymph node metastasis in TESCC patients who did not receive preoperative treatment. Second, high neutrophil accumulation correlated with poor 5-year overall survival. Third, intratumoral neutrophil accumulation is much lower than in peritumoral regions. Fourth, we detected no relationship between peritumoral neutrophil accumulation

Table I
Clinical features of patients in the CD16b-high and CD16b-low groups

	CD16b-high ($n = 53$)	CD16b-low ($n = 73$)	P
Age at surgery	72 (49–83)	68 (39–80)	.036*
Sex			.790
Female	6 (11%)	10 (14%)	
Male	47 (89%)	63 (86%)	
Tumor location			.558
Upper	9 (17%)	8 (11%)	
Middle	29 (55%)	40 (55%)	
Lower	15 (28%)	25 (34%)	
Depth of tumor invasion (pT)			.385
T1	24 (45%)	40 (55%)	
T2	8 (15%)	6 (8%)	
T3	21 (40%)	27 (37%)	
Lymph node metastasis (pN)			<.001*
N0	5 (9%)	53 (73%)	
N1	33 (62%)	15 (21%)	
N2	11 (21%)	4 (5%)	
N3	4 (8%)	1 (1%)	
pN			<.001*
Negative	5 (9%)	53 (73%)	
Positive	48 (91%)	20 (27%)	
Pathological stage			<.001*
IA	2 (4%)	30 (41%)	
IB	3 (6%)	6 (8%)	
IIA	0 (0%)	17 (23%)	
IIB	22 (42%)	9 (12%)	
IIIA	17 (32%)	7 (10%)	
IIIB	4 (8%)	3 (4%)	
IVA	5 (9%)	1 (1%)	
Lymphatic invasion			.024*
Positive	52 (98%)	63 (86%)	
Negative	1 (2%)	10 (14%)	
Venous invasion			.606
Positive	47 (89%)	62 (85%)	
Negative	6 (11%)	11 (15%)	
Tumor differentiation			.482
Not poorly	34 (76%)	53 (82%)	
Poorly	11 (24%)	12 (19%)	
Extent of lymph node dissection			.705
2-field	17 (32%)	27 (37%)	
3-field	36 (68%)	46 (63%)	
Numbers of lymph nodes dissected	52 (16–116)	56 (13–108)	.519
Neutrophil counts in the blood	3.36 (0.98–9.94) $\times 10^3$	3.29 (1.09–9.39) $\times 10^3$	.848
Lymphocyte counts in the blood	1.40 (0.56–3.09) $\times 10^3$	1.60 (0.66–3.25) $\times 10^3$	.131
Neutrophil-to-lymphocyte ratio	2.30 (0.90–6.60)	2.20 (0.60–8.20)	.400
Postoperative adjuvant chemotherapy			.698
Done	2 (4%)	5 (7%)	
Not	51 (96%)	68 (93%)	

* Statistically significant.

Table II
Univariate and multivariate analyses of lymph node metastasis (logistic regression analysis)

	Univariate analysis				Multivariate analysis			
	95% CI		P	Odds ratio	95% CI		P	Odds ratio
Age (<71 vs ≥71)	−0.59	0.119	.195	-				
Sex (male vs female)	−0.442	0.624	.734	-				
CD16b score (<11 vs ≥11)	−2.201	-1.130	<.001*	25.440	−2.320	−1.09	<.001*	26.850
Tumor location (upper vs middle and lower)	−0.200	0.867	.248	-				
Depth of tumor invasion (pT1 vs pT2-3)	−0.651	0.060	.104	-				
Lymphatic invasion (positive vs negative)	0.196	1.856	.011*	6.061	−1.879	0.552	.458	
Venous invasion (positive vs negative)	−0.078	1.005	.096					
Neutrophil counts in the blood ($\leq 3.3\times10^3$ vs $>3.3 \times 10^3$)	−0.487	0.224	.470					
Tumor differentiation (poorly vs not poorly)	0.067	1.103	.025*	3.036	0.037	1.292	.037*	3.629

CI, confidence interval.
* Statistically significant.

and the preoperative NLR, a known marker of systemic inflammation.

The close link between inflammation and cancer is well documented, and many reports have shown a correlation between prognosis and systemic inflammation.[7] Among the blood parameters thought to reflect systemic inflammation (eg, C-reactive protein), evidence suggests the preoperative NLR associates with survival in several solid tumors.[8] Moreover, some reports suggest a

Table III
Multivariable logistic regression analyses of lymph node metastasis and the odds ratio in the various models

	P	Odds ratio	95% CI
Crude (CD16b score, ≥11%)	<.001*	25.440	1.130–2.201
Adjusted for age and sex	<.001*	25.360	1.121–2.208
Adjusted for age, sex, tumor location, and pT	<.001*	25.333	1.111–2.218
Adjusted for age, sex, tumor location, pT, lymphatic invasion, and venous invasion	<.001*	26.090	1.110–2.254
Adjusted for age, sex, tumor location, pT, lymphatic invasion, venous invasion, and tumor differentiation	<.001*	28.578	1.096–2.391

CI, confidence interval; *pT*, pathological depth of tumor invasion.
* Statistically significant.

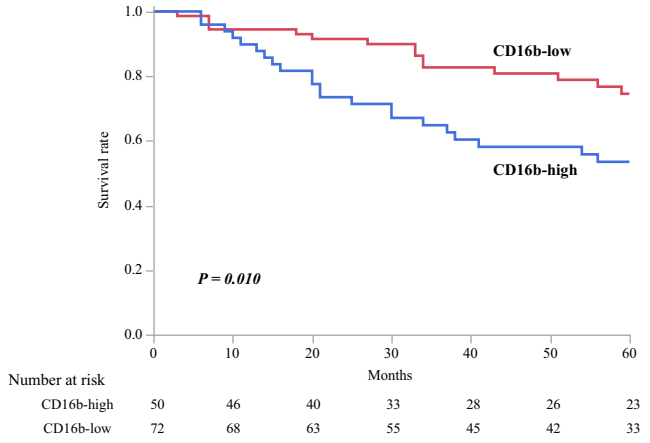

Figure 5. Kaplan-Meier analysis comparing 5-year overall survival between CD16b-high (CD16b score ≥11) and CD16b-low (CD16b score <11) patients. The CD16b score cutoff was determined from the receiver operating characteristic curve analysis.

relationship between the NLR and lymph node metastasis in various cancers, including advanced gastric cancer and esophageal squamous cell cancer.[7,9] Patients with lymph node metastasis and a high blood NLR had significantly poorer cancer-specific survival than those with a low NLR.[7] The present study suggests there is no significant association between the NLR and CD16b-positive neutrophil accumulation or lymph node metastasis. It is also noteworthy that peritumoral neutrophil numbers do not correlate with systemic inflammation.

The interplay between immune component cells, including neutrophils, and the tumor microenvironment is an area of intense research. Macrophages and lymphocytes have been a major focus in tumor immunology research. Recently, however, there has been a focus on the role of neutrophils, another myeloid-derived white blood cell. Mediators present within tumors and the tumor microenvironment recruit neutrophils to the region, where they promote tumor progression or growth control.[10] Infiltrating neutrophils are found within many solid tumors. For example, dense populations of CD66b-positive, tumor-associated neutrophils have been identified in head and neck squamous cell carcinomas, gastric and pancreatic adenocarcinomas, and transitional bladder cancer.[11] Interestingly, Wu et al assessed the localization of neutrophils in cervical cancer patients and found that the highest neutrophil density was in the peritumoral area, which is consistent with our result.[12] Furthermore, Carus et al reported that in cervical cancer patients peritumoral areas have higher median densities of CD66b-positive neutrophils than tumoral or stroma areas.[13] On the other hand, neutrophil accumulation has also been detected within metastatic lymph nodes as well as the primary tumor itself.[14] In gastric adenocarcinoma, the numbers of CD15-positive intratumoral neutrophils at the primary site and regional lymph nodes are positively correlated with the depth of primary tumor invasion and lymph node metastasis.[15] CD16b, also known as FcγRIIIb, is a low-affinity Fcγ receptor exclusively expressed on neutrophils, which plays a key role in cancer immunotherapy.[16,17] We found that the number of peritumoral CD16b-positive neutrophils is not associated with the pathological depth of tumor invasion (pT). Nonetheless, peritumoral accumulation of CD16b-positive neutrophils was accompanied by the appearance of lymph node metastasis. Seth et al reported that in breast cancer patients, neutrophil depletion decreased multiorgan metastasis in the early phase, but not the late phase.[18] This suggests peritumoral accumulation of CD16b-positive neutrophils may be associated with the early stages of lymph node metastasis.

Neutrophil accumulation within tumors was classically considered to be a defensive immune response.[19] However, several studies have shown that infiltrated tumoral neutrophils promote cancer cell migration and invasion.[20] Neutrophils contribute to tumor invasion and angiogenesis through production of matrix metalloproteinase-9, vascular endothelial growth factor, myeloperoxidase, and other cytokines and chemokines.[21] As we reported previously,[22] tumor-associated macrophages can be divided into an antitumorigenic M1 phenotype and protumorigenic M2 phenotype. Similarly, intratumoral neutrophils are capable of polarization into an antitumorigenic N1 phenotype and a protumorigenic N2 phenotype.[23] N2 neutrophils are thought to support tumor expansion by expressing arginase, matrix metalloproteinase-9, vascular endothelial growth factor, and various chemokines. Moreover, transforming growth factor (TGF)-β signaling reportedly functions to drive neutrophils toward the N2 phenotypes.[24] However, blocking TGF-β failed as a therapeutic strategy as a result of significant side effects reflecting the numerous physiological pathways that involve TGF-β.[25]

In a mouse spontaneous mammary carcinoma model, tumor cells were observed to secrete interleukin (IL)-1 to induce IL-17 expression in γδT cells, which led to the granulocyte colony-stimulating factor (G-CSF)-dependent expansion and polarization of neutrophils. This tumor-induced neutrophil expansion and differentiation led to the inhibition of cytotoxic T-cell function and facilitation of lymph node and distant organ metastasis.[18] Moreover, the absence of γδT cells or neutrophils profoundly reduced lymph node and pulmonary metastases without influencing primary tumor progression. In addition, Stefanie et al reported that neutrophils aid tumor cell colonization in distant organs and lymph nodes by releasing leukotrienes, which can be blocked by pharmacological inhibition of arachidonate 5-lipoxygenase, a leukotriene-generating enzyme.[26] Although it might seem that targeting neutrophils and associated chemokines could be a potential new antitumor therapy, this would likely result in self-defeating immunosuppression, as neutrophils are essential for host defense against infection. Additional studies are therefore needed to gain a more detailed understanding of relationship between tumors and neutrophils.

The present study has several limitations. First, the cellular interactions between tumor cells and CD16b-positive neutrophils were not directly observed. Second, we did not determine how

CD16b-positive neutrophils cause lymph node metastasis. Third, we did not compare CD16b-positive neutrophils with other neutrophil types, such as CD15-positive or CD66-positive neutrophils.

In conclusion, our findings suggest that peritumoral accumulation of CD16b-positive neutrophils is an independent factor strongly correlated with lymphatic invasion and regional lymph node metastasis in TESCC. Further studies are needed to confirm this finding.

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Conflict of interest/Disclosure

The authors declare no related conflicts of interest.

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