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Original article

Impact of immunohistochemically identified lymphatic invasion on nodal metastasis in early gastric cancer

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Abstract

Background. **Among various clinical and pathological findings, lymphatic invasion (Ly) is the strongest risk factor for nodal metastasis in gastric cancer. However, the diagnosis of Ly is subjective and often inaccurate because of the difficulty of detecting lymphatic vessels with conventional hematoxylin and eosin (HE) staining.**

Methods. **The distribution of lymphatics in the normal gastric wall was immunohistochemically characterized using a new selective marker of lymphatic endothelium, D2-40, in surgical specimens resected for early gastric cancer (EGC). Then, Ly in the primary lesion was reevaluated, and the positive (PPV) and negative (NPV) predictive values for nodal metastasis were comparatively examined for Ly detected by HE staining (Ly-HE) and by immunohistochemical staining (Ly-IM) in 131 cases of EGC.**

Results. **D2-40-positive lymphatic vessels were observed in the deep proper mucosal layer, and the lymphatic vessel density (LVD) was extremely high in the muscularis mucosa (MM) layer. The number of Ly-IM-positive cases (15/131) was higher than the Ly-HE-positive cases (10/131). In 48 cases of intestinal-type cancer, Ly-IM had a PPV of 33.3% (2/6) and anNPV of 100% (42/42), which was more accurate than the corresponding figures for Ly-HE (25% and 98%, respectively). In contrast, the accuracy of Ly-IM was similar to that of Ly-HE in 83 cases of diffuse-type cancer.**

Conclusion. **Lymphatic vessels are most densely distributed in the MM layer in the gastric wall. Immunohistochemical identification of lymphatics is useful to increase the accuracy of diagnosing Ly in resected gastric EGCs. Ly-IM is superior to Ly-HE as a predictor of nodal metastasis, at least for intestinal-type EGC.**

Key words Gastric cancer · Lymphatic invasion · D2-40 · Lymph node metastasis · Histological type

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Introduction

The prognosis of patients with early gastric cancer (EGC), defined as being confined to the mucosa or submucosa irrespective of the presence of regional lymph node (LN) metastasis, has improved with radical gastrectomy with complete removal of regional LN [1,2]. However, recent technical improvements in endoscopic mucosal resection (EMR), especially the insulated-tipped (IT) needle knife, have enabled endoscopic en bloc resection of large submucosal lesions [3,4]. Although nodal metastasis is one of the most important prognostic factors, the incidence has been reported to be approximately 3% in intramucosal EGC and 20% in submucosal EGC [5,6]. Therefore, theoretically, many EGCs can be treated with EMR (thereby avoiding radical gastrectomy) and conventional lymphadenectomy.

Numerous studies have reported the risk factors for lymph node metastasis in EGC, including tumor size, depth, histological type, ulceration, and lymphatic invasion (Ly), from the clinicopathological data from cases of surgically resected cancers [5,6]. Assessment of these risk factors, except for Ly, can mostly be achieved by endoscopic investigation. In contrast, evaluation of Ly can be performed only after EMR; and positive Ly commonly requires additional extended surgery based on various criteria for local treatment [5,7,8]. Indeed, retrospective data have indicated that the rate of lymphatic metastasis is markedly higher in Ly-positive than in Ly-negative EGC [6,9,10]. However, the diagnosis of Ly is usually performed on the basis of conventional hematoxylin-eosin (HE) staining, and sometimes lacks objectivity possibly because of the inability to distinguish lymphatics from blood vessels accurately.

Recently, several selective markers of lymphatic endothelium have been identified: podoplanin [11], prox-1 [12], LYVE-1 [13], desmoplakin [14], vascular endothelial growth factor receptor-3 (VEGFR-3) [15,16], and

D2-40 [17,18]. In this study, we used a new monoclonal antibody (mAb) to D2-40 and immunohistochemically characterized lymphatic vessels in each layer of the normal gastric wall in paraffin sections. Thereafter, we re-evaluated Ly of the primary lesion and examined the relation between immunohistochemically identified Ly and lymph node metastasis.

Patients and methods

Patients

This study included 131 patients with EGC during 1993–2001 who underwent curative gastrectomy with standard lymph node dissection in the First Department of Surgery, Tokyo University Hospital. Another 37 advanced gastric cancers (AGC) were examined as well. In all cases with informed consent, serial step sections 3 μm thick were cut, fixed in 10% formalin solution, and embedded in paraffin. All of the resected primary tumors and regional lymph nodes were examined histologically using HE staining according to the Japanese Classification of Gastric Carcinoma [19], except for histological type. Tumors were histologically classified into two types: intestinal type (papillary and tubular adenocarcinoma with no component of diffuse type) and diffuse type (poorly differentiated adenocarcinoma and signet-ring cell carcinoma, including mixed type).

Immunohistochemistry

Serial 3-μm-thick sections were deparaffinized in xylene, hydrated through a graded series of ethanol, and then immersed in 3% hydrogen peroxide in 100% methanol for 30 min to inhibit endogeneous peroxidase activity. To activate the antigens, the sections were boiled in 10 mM citrate buffer pH 6.0 for 30 min. After rinsing in phosphate-buffered saline (PBS), the sections were incubated with normal rabbit serum for 10 min and then incubated overnight at 4 °C in humid chambers with a mouse mAb D2-40 (Signet, Dedham, MA, USA) with 1:100 dilution that reacts with human lymphatic endothelium [18,20] or CAM5.2 (Becton Dickinson, San Jose, CA, USA) with 1:8 dilution, which reacts with human cytokeratins 8 and 18 [17]. After washing three times with PBS, the sections were incubated with biotinylated anti-mouse immunoglobulin for 20 min. After washing again with PBS, the slides were treated with peroxidase-conjugated streptavidin tetrahydrochloride for 3 min. Light counterstaining with Mayer's hematoxylin was performed.

Quantification of lymphatic vessels

The number of D2-40-positive lymphatic vessels was assessed in normal gastric mucosa (>3 cm from the margin of the intramucosal cancer). The gastric wall levels were classified as mucosa (M), muscularis mucosa (MM), submucosa (SM), muscularis propria (MP), and subserosa (SS). The number of lymphatic vessels in a 1 mm-wide portion of each layer was counted, and the height of the layer was measured. Five serial spots were assessed in one sample, and six samples were examined. The mean lymphatic vessel density per square millimeter (LVD) was calculated from 30 data points in each layer.

Evaluation of lymphatic invasion

Lymphatics were detected by D2-40 staining, and tumor cells in the lymphatics were detected by cytokeratin staining using adjacent sections. Lymphatic invasion detected by HE staining and by immunostaining was expressed as Ly-HE and Ly-IM, respectively. The absence of lymphatic invasion was expressed as negative Ly and the presence of lymphatic invasion as positive Ly.

Statistical analysis

The association of LN metastasis with age and tumor size was assessed by Student's *t*-test. Other clinicopathological variables were assessed by the chi-squared test, and lymphatic vessel density was analyzed by analysis of variance (ANOVA), with $P < 0.01$ considered significant.

Results

Detection of lymphatic vessels in normal gastric wall

The specificity of D2-40 for lymphatic vessels was first assessed by immunostaining a normal area apart from the cancer tissue. This revealed positive staining of vessels with irregular morphology, an empty lumen, and thin monolayer endothelium. No vessels reactive with D2-40 contained erythrocytes (Fig. 1A). All these findings were compatible with the typical characteristics of lymphatic vessels.

The lymphatics of the mucosal layer were found in the deep levels of the lamina propria layer (within 300 μm from the upper margin of the MM layer) (Fig. 1B). The average numbers of lymphatic vessels in each layer (M, MM, SM, MP, SS) per millimeter width were 1.80, 4.17, 1.40, 1.17, and 0.83, respectively; and the average heights (micrometers) of each layer were 863, 129, 771, 942, and 144, respectively. The lymphatics ves-

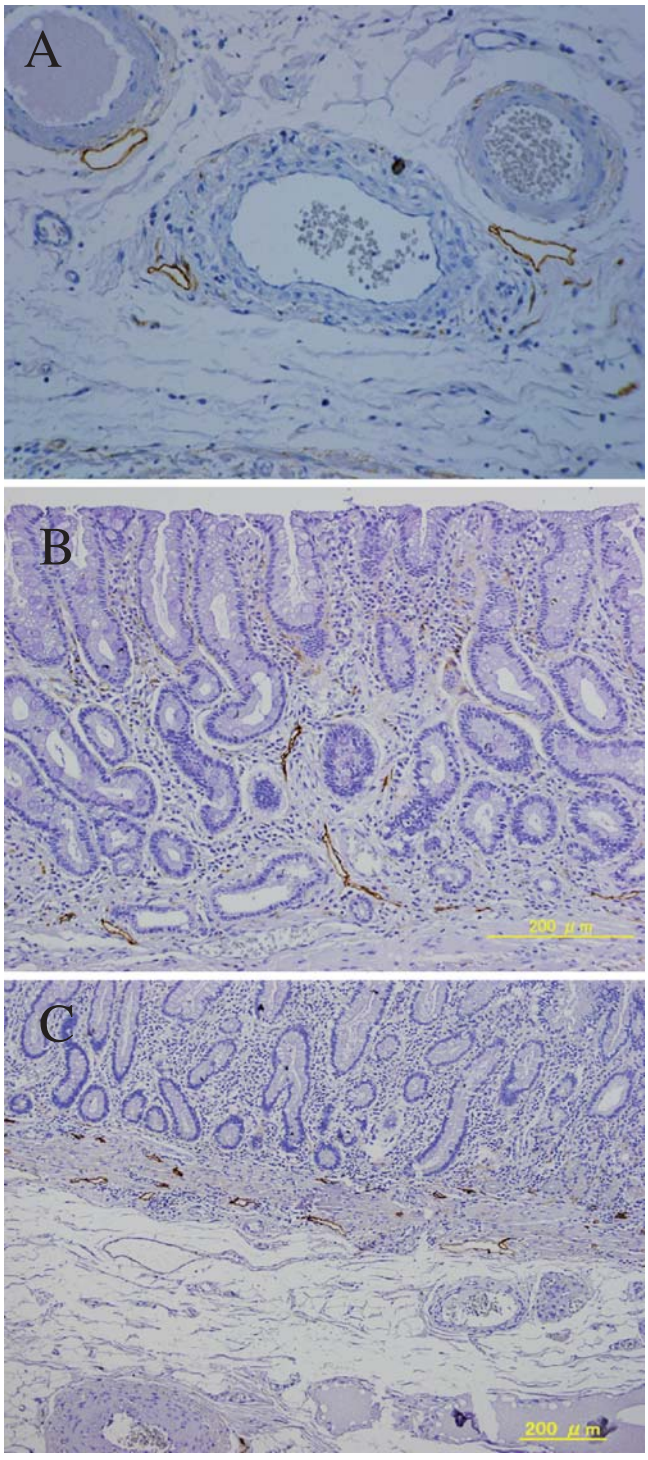

Fig. 1. Staining of normal gastric wall with D2-40. **A** Negative immunostaining of vascular vessels. (×400). **B** Positive immunostaining of lymphatics in mucosal layer. (×200). **C** Lymphatics in muscularis mucosa and submucosal layer. (×100)

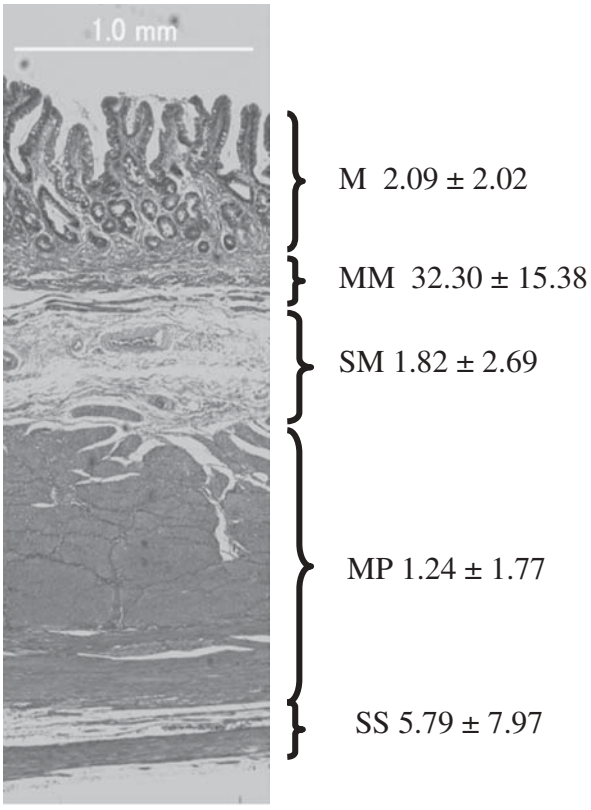

Fig. 2. Lymphatic vessel density (LVD) (per square millimeter) in each layer. Mean ± SD from 30 spots. LVD is significantly higher in the muscularis mucosa (*MM*). *M*, mucosa; *SM*, submucosa; *MP*, muscularis propria; *SS*, subserosa

sel densities (LVDs) were 2.09 ± 2.02/mm^2 (mean ± SD) and 6.00 ± 6.08 in the M layer and the deep levels of the M layer. In contrast, the MM layer contained a significantly higher number of lymphatics (32.30 ± 15.38/mm^2), with no alymphovascular area of more than 0.5 mm, as shown in Fig. 1C. The density of lymphatics found in the SM layer (1.82 ± 2.69/mm^2) was similar to that in the mucosal layer. However, the SM layer also contained alymphovascular areas and lacked lymphatics of more than 5 mm in length. LVDs in MP and SS were 1.24 ± 1.77/mm^2 and 5.79 ± 7.97/mm^2, respectively (Fig. 2).

Immunohistochemically identified lymphatic invasion

Lymphatic invasion was clearly demarcated by D2-40 staining, in contrast to HE staining (Fig 3A,B). Ly-IM was assessed by the combination of D2-40-expressing lumen and cytokelatin-expressing tumor cells (Fig. 3C,D). Ly was typically characterized by tumor cells that were completely surrounded by a lymphatic endothelium. In AGC, tumor cells were often observed to show a growing pattern in large lymphatic vessels (Fig.

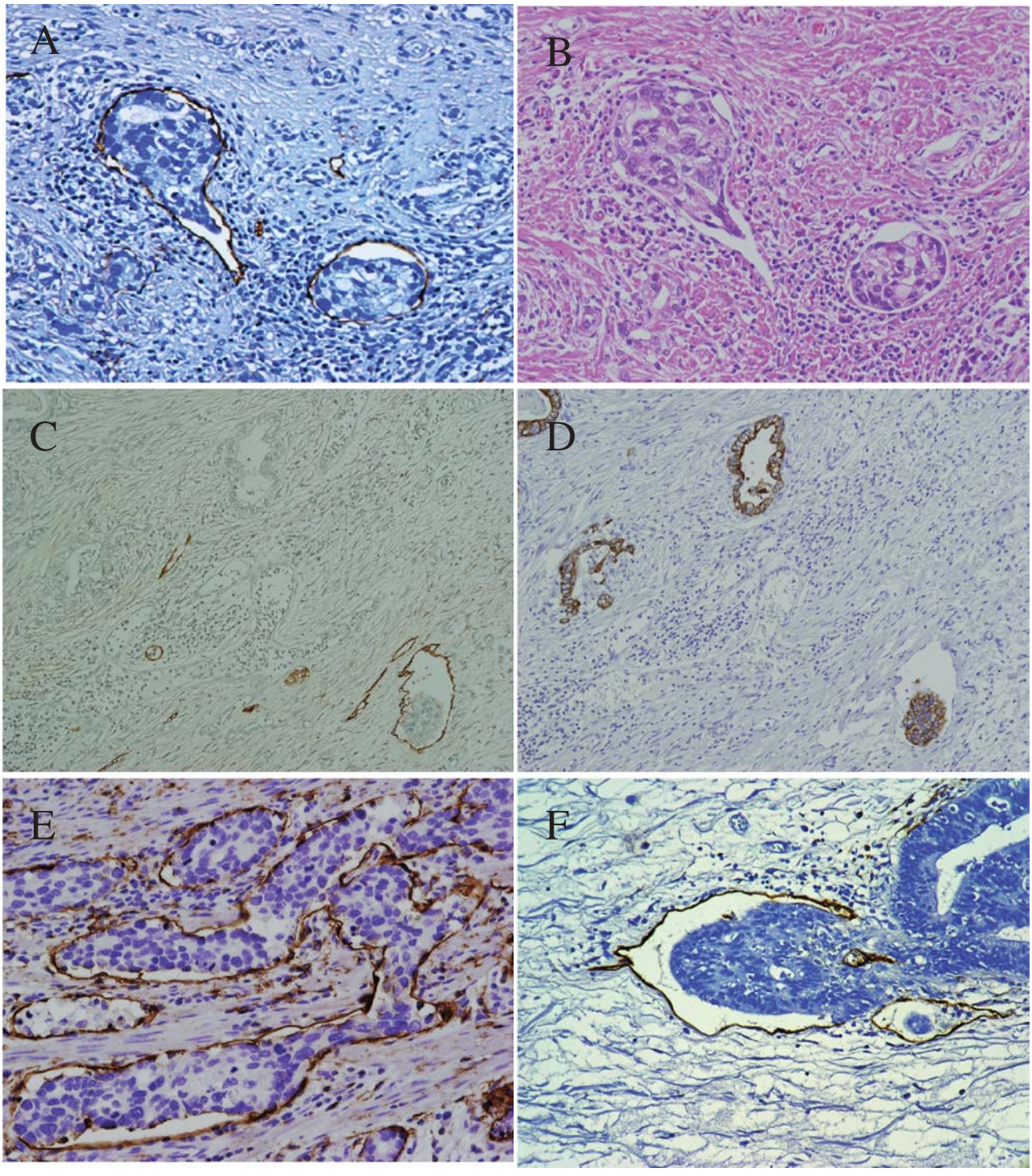

Fig. 3. Identification of lymphatic invasion after immunodetection of lymphatic vessels. **A** D2-40 staining (×200). **B** Staining of section adjacent to **A** (×200). **C** Positive immunostaining of lymphatics (×100). **D** Positive immunostaining of tumor cells with cytokeratin in the adjacent section (×100). **E** Tumor spreading in lymphatics (×400). **F** Tumor invading the lymphatic wall (×200)

Table 1. Clinicopathological factors and lymph node metastasis of early gastric cancer

Factor	Lymph node metastasis		
	Negative	Positive	*P*
Age	60.8 ± 10.5	53.6 ± 8.5	0.006
Sex			
Male	86 (87.8%)	12 (12.2%)	
Female	27 (81.8%)	6 (18.2%)	NS
Depth			
m	63 (99.98%)	1 (0.02%)	
sm	51 (75.0%)	17 (25.0%)	0.001
Macroscopic			
Elevated	16 (84.2%)	3 (15.8%)	
Ulcerated	97 (86.6%)	15 (13.4%)	NS
Size	3.8 ± 2.8	5.8 ± 3.0	0.005
Histology			
Intestinal	46 (95.8%)	2 (4.2%)	
Diffuse	67 (80.7%)	16 (19.3%)	0.015
Lymphatic invasion (HE)			
Negative	110 (91.0%)	11 (9.0%)	
Positive	3 (30.0%)	7 (70.0%)	0.001
Venous invasion			
Negative	106 (86.9%)	16 (13.1%)	
Positive	7 (77.8%)	2 (22.2%)	NS

Age and size: average ± standard deviation (SD)
Age and size were evaluated by Student's *t*-test, the others by the chi-squared test

Table 2. Relation between Ly-IM and Ly-HE in early gastric cancer

Ly-IM staining	Ly-HE negative	Ly-HE positive
Ly-IM negative	113	3
Ly-IM positive	8	7

Ly, lymphatic invasion; HE, hematoxylin and eosin staining; IM, immunohistochemical staining

3E). In one case, we could detect tumor cells that were clearly invading the lumen of a lymphatic vessel through its wall (Fig. 3F).

Lymphatic invasion detected by hematoxylin-eosin or immunohistochemical staining

The clinical and pathological characteristics of 131 patients with EGC are summarized in Table 1. In addition to size, depth, and histology, Ly diagnosed both by HE staining (Ly-HE) and IM staining (Ly-IM) was significantly correlated with LN metastasis. Table 2 shows the positive correlation between Ly-HE and Ly-IM. However, among the 121 EGCs diagnosed as Ly-negative by HE staining, 8 showed positive lymphatic invasion after immunodetection of lymphatics. In some cases, HE diagnosis failed to identify surrounding lymphatics that were filled with tumor emboli (Fig. 4A), and in other cases HE diagnosis could not distinguish retraction artifacts that isolated tumor aggregates due to tissue shrinkage during fixation from true tumor emboli in lymphovascular spaces (Fig. 4B). In contrast, among the 10 Ly-positive tumors by HE diagnosis, 3 were diagnosed as Ly-negative by IM staining. In these cases, blood vessel invasion was misdiagnosed as lymphatic invasion.

The layers of lymphatic invasion observed in immunostained samples of 15 Ly-IM-positive cases (3 mucosal cancers, 12 submucosal cancers) are expressed in Table 3. In nine of these submucosal cancers, lymphatic invasion by tumor cells was found in the MM layer. Consistent with the distribution of lymphatics, lymphatic invasion was most frequently observed in the MM layer.

Predictive value of Ly-IM for nodal metastasis

The predictive value of nodal metastasis by Ly-IM in intestinal- and diffuse-type EGCs is shown in Table 4. Among 48 patients with intestinal-type cancer, 6 showed positive Ly-IM, 2 of whom were positive for lymph node metastasis; none of the 42 EGCs with negative Ly-IM showed lymphatic metastasis. Thus, the positive (PPV) and negative (NPV) predictive values were 33% (2/6) and 100% (42/42), respectively. These values were slightly higher than those of Ly-HE (25%, 98%) In contrast, Ly-IM showed a PPV of 67% (6/9) and an NPV of 87% (64/74) in diffuse-type cancers. This was not superior to the accuracy of Ly-HE in diffuse-type EGC.

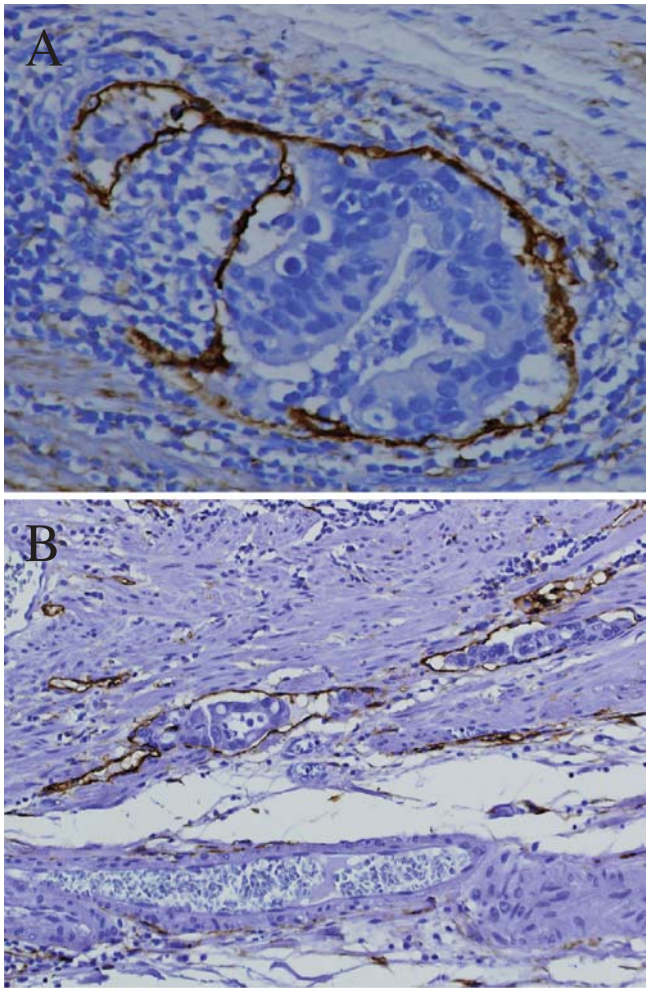

Fig. 4. Typical cases of lymphatic invasion missed with hematoxylin and eosin staining but detected after D2-40 staining. **A** Tumor emboli in lumen of lymphatics (×200). **B** Lymphatic invasion with retraction artifact (×100)

Table 3. Layer where lymphatic invasion was detected immunohistochemically

Case	Depth of invasion	Part of lymphatic		
		M	MM	SM
1	m	+	−	
2	m	+	−	
3	m	−	+	
4	sm	+	+	−
5	sm	+	+	−
6	sm	−	+	−
7	sm	−	+	−
8	sm	−	+	−
9	sm	−	+	−
10	sm	−	+	−
11	sm	−	+	−
12	sm	−	+	−
13	sm	+	−	+
14	sm	+	−	+
15	sm	−	−	+

+, lymphatic invasion positive; –, lymphatic invasion negative; m, M, mucosa; sm, SM, submucosa; MM, muscularis mucosa

Table 4. Ly-IM and lymph node metastasis from intestinal-type and diffuse-type early gastric cancer

Cancer type	Lymph node metastasis		
	Negative	Positive	*P**
Intestinal			
Ly-IM negative	42 (100%)	0	
Ly-IM positive	4 (66.7%)	2 (33.3%)	0.001
Diffuse			
Ly-IM negative	64 (86.5%)	10 (13.5%)	
Ly-IM positive	3 (33.3%)	6 (66.7%)	0.001

*By chi-squared test

Discussion

D2-40 is an mAb against a 40000-dalton O-linked sialoglycoprotein that was initially identified as the testicular oncofetal antigen [21]. Recent reports, however, have shown that the antibody reacts with a fixation-resistant epitope in lymphatic endothelium in various sites, including malignant tissues [18,22–24]. Our study of the stomach demonstrates that lymphatic microvessels can be clearly observed in the deeper part, but not in the upper area, of the M layer and were most densely distributed in the MM layer in normal gastric mucosa. In the SM layer, the density of lymphatic vessels was mostly the same as that in the M and MP layers. Previous transmission electron microscopy studies demonstrated that lymph capillaries were detected only in the deep lamina propria adjacent to and within the MM layer, although large lymph vessels were observed in the SM layer [25,26]. Our results are mostly consistent with those data, indicating that the D2-40 antigen is similarly expressed in both lymphatic capillaries and endothelium of larger lymphatics. These findings also suggest that mucosal lymphatic capillaries form the submucosal collecting lymphatics through a close network in the MM layer in the gastric wall.

Lymphatic spread of cancer is assumed to occur through cancer cells permeating into peritumorous lymphatics and reaching the regional lymph nodes. Thus, close contact between tumor cells and lymphatics is thought to be a step in lymphatic metastasis. Clinical studies on gastric cancer have shown that lymphatic metastasis was rare in mucosal cancer but was markedly increased in submucosal cancer [5,6]. This is reasonably explained by our results suggesting that cancer cells invading the MM layer have the most frequent chance to penetrate the lymphatic lumen. In fact, cancer cells invading lymphatic vessels were most frequently detected in the MM layer in our observation (Table 3).

Recent studies with more detailed pathological analysis have indicated that the depth [27,28] and total volume [29] of submucosal invasion are closely correlated with the risk of lymphatic metastasis. However, our results suggest that the width or area of MM invasion may be more important for the evaluation of nodal status in submucosal gastric cancer.

In our series, we found some inconsistency between Ly-HE and Ly-IM. By immunodetection of lymphatic vessels with D2-40, Ly was additionally detected in 8 of 121 cases that were categorized as Ly-HE-negative, whereas 3 of 10 cases that were categorized as Ly-HE-positive turned out to be from blood vessel invasion. When Ly-IM was evaluated from the standpoint of the predictive value, Ly-IM clearly predicted nodal status more accurately in intestinal-type cancers, with 100% NPV. We evaluated 11 intestinal-type advanced gastric cancers (AGCs) among 37 AGCs and found that none of the negative Ly-IM AGCs was associated with lymphatic metastasis (data not shown). Therefore, negative Ly-IM is considered to be a reliable marker for no nodal metastasis in intestinal cancer.

In contrast, Ly-IM was not apparently superior to Ly-HE in diffuse-type cancers. With our method, most Ly was observed as tumor cells that were completely surrounded by lymphatics, whereas tumor cells invading a lymphatic vessel could rarely be found. Therefore, Ly as shown in Fig. 3A or Fig. 3E may be intralymphatic growth characteristic of intestinal-type cancer, as shown in the schema of Fig. 5, because intestinal-type cancers are apt to maintain *intercellular* contact more frequently than the diffuse type. On the other hand, diffuse-type cancers often lose glandular formation in the submucosal layer, and thus tumor cells may be more scattered in lymphatic vessels. Therefore, the morphological detection of intralymphatic tumor cells may be more difficult in diffuse-type than intestinal-type cancers, even though the lymphatic vessels are clearly detected on immunohistochemical staining.

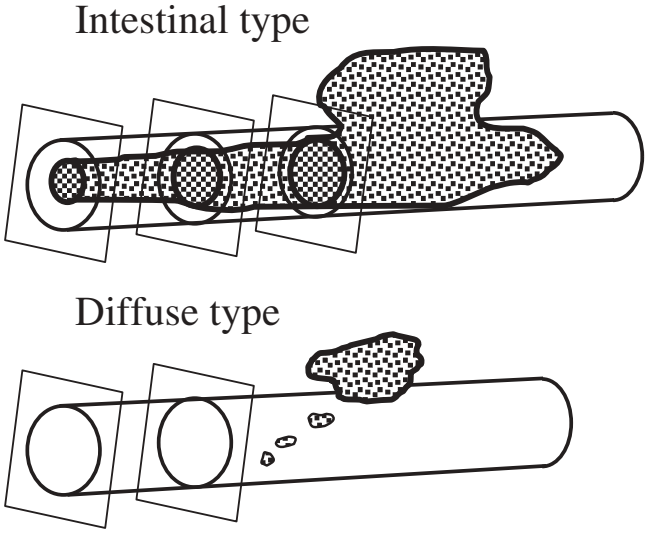

Fig. 5. Schema of lymphatic invasion. Lymphatic invasion of diffuse type has less chance to be detected morphologically

Conclusion

Lymphatic vessels in the gastric wall can be clearly and selectively identified by immunostaining for D2-40. Because lymphatics are most densely distributed in the MM layer, cancer cells appear to have the greatest chance to penetrate the lymphatics in the MM layer. Pathological evaluation of the area and length of MM invasion might be another important factor to predict nodal metastasis in EGCs. Selective identification of lymphatic vessels with this method can increase the accuracy of detecting lymphatic invasion by the primary tumor, and Ly detected by this method (Ly-IM) is an excellent predictor of nodal metastasis from intestinal-type EGCs. Our data suggest the clinical implication that intestinal-type EGCs, even those with submucosal invasion, might be safely treated with EMR alone when Ly-IM is not observed in the resected material.

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