Significance of Lymphatic Invasion on Regional Lymph Node Metastasis in Early Gastric Cancer Using LYVE-1 Immunohistochemical Analysis

Ai Fujimoto, MD,[1,7] Yukio Ishikawa, MD,[1*] Yuri Akishima-Fukasawa, MD,[1] Kinji Ito, MD,[1] Yoshikiyo Akasaka, MD,[1] Seiichi Tamai, MD,[2] Tadaaki Maehara, MD,[3] Hideko Kiguchi, MD,[4] Kentaro Ogata, MD,[5] Chiaki Nishimura, PhD,[6] Kazumasa Miki, MD,[7] and Toshiharu Ishii, MD[1]*

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Abstract

It has been reported that lymphatic invasion is a predictor for lymph node metastasis in early gastric cancer (EGC); however, it has been impossible to differentiate between lymphatic invasion and blood vessel invasion using current staining techniques. We studied the significance of lymphatic invasion on regional lymph node metastasis in EGC by using human lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) antibody, specific to lymphatic vessels, and von Willebrand factor (vWF) antibody, specific to the blood vessels, to clearly distinguish these vascular tissues.

EGC tissues were obtained from 66 node-positive and 66 node-negative subjects and were matched by age and sex. These tissues were immunostained with antibodies against LYVE-1 and vWF. Multivariate logistic regression analysis demonstrated that lymphatic invasion was a significant independent predictor for regional lymph node metastasis (odds ratio, 4.667; P = *.0094), whereas blood vessel invasion was not. Thus, lymphatic invasion identified by LYVE-1 antibody could predict the existence of regional lymph node metastasis in EGC.*

The postsurgical 5-year survival rate for patients with early gastric cancer (EGC) is more than 90%[1-4]; however, in approximately 10.2% to 14.4% of EGC cases, cancer metastasizes to the regional lymph nodes, causing patient death due to subsequent systemic spread.[2,5-10] Lymph node metastasis has emerged as a significant independent indicator of poor long-term survival in EGC.[7,11]

Recent availability of endoscopic techniques and laparoscopic resection for patients with EGC has improved the quality of life by minimizing invasive procedures.[2,7,12-16] However, there is no method to precisely predict the existence of lymph node metastasis without using invasive procedures, thus limiting the use of minimally invasive techniques.

Among the routes by which EGC can metastasize to regional lymph nodes, metastasis through the lymphatics at the primary site is a major candidate. Although there are many small lymphatics and blood capillaries present at the primary site in gastric cancer, it is difficult to distinguish between these 2 vessels by using H&E staining.[17] However, we recently developed a polyclonal antibody against human lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1; to be released by DakoCytomation, Glostrup, Denmark, in 2007), an immunohistochemical marker that enables us to recognize lymphatics.[18] Thus, it has become possible to distinguish objectively between the 2 types of vascular tissues by immunohistochemical analysis for LYVE-1 and von Willebrand factor (vWF).

In this study, we attempted to examine the significance of lymphatic invasion as a predictor for regional lymph node metastasis by performing immunohistochemical techniques using the LYVE-1 antibody.

Materials and Methods

The materials were surgical samples of stomach and regional lymph nodes obtained from 66 patients who had undergone curative resection for gastric cancer at Toho University Hospital, Tokyo; the National Defense Medical College Hospital, Tokorozawa; Saiseikai Kanagawa-Ken Hospital, Yokohama; Hiratsuka Municipal Hospital, Hiratsuka; and Ida Municipal Hospital of Kawasaki City, Kawasaki, Japan, between January 1989 and December 2004. Histopathologic examination of all 66 node-positive specimens confirmed that although the cancer had invaded only the mucosa and submucosal layer of the stomach, it had metastasized to the regional lymph nodes. Among the cases with lymph node metastases, only 12 (18%) had intramucosal carcinomatous invasion and 54 (82%) had invasion extending to the submucosal layer.

Of the 66 patients, 51 underwent partial or subtotal gastrectomy and 15 underwent total gastrectomy. Curative surgery was defined as the removal of all gross cancers and the demonstration of tumor-negative surgical margins by microscopic examination of the total circumference. The patients were free of other types or degrees of invasion, distant visceral metastases, and complications due to other visceral cancers. The patients were not given chemotherapy preoperatively. Stomach specimens from 594 patients with EGC but without regional lymph node metastasis were extracted from the archives and used to identify age- and sex-matched control cases for each of the node-positive patients; 66 such control cases were finally sorted at random and used in this experiment. Of the 66 cases without lymph node metastases, 40 (61%) had invasion restricted to intramucosal carcinomatous invasion and 26 (39%) had invasion extending to the submucosal layer. Written informed consent to use the tissue samples was obtained from all patients.

The surgically resected stomachs were generally opened along the greater curvature, pinned on a cork board, and fixed in 10% formalin. After careful gross inspection and photography, each tumor was cut into 4-mm slices parallel to the major axis of the specimen and also cut parallel to the minor axis at the half-way mark of the major axis. If the tumors were smaller than 30 mm, all slices from the tumor were used; however, if the tumors were larger than 30 mm, only the slices obtained from the center of the tumor were used. The first cut was made in the middle of the tumor, followed by cuts above and below the middle mark to obtain the necessary slices. First, 1 horizontal row across all blocks and 2 rows each above and below the first row were sliced thin. The slices were embedded in paraffin, cut into 3-μm-thick sections, and treated by double staining with Victoria blue and H&E dyes to aid the identification of blood vessel structures, especially with regard to veins.

Immunohistochemical Analysis

Immunohistochemical staining with the LYVE-1 antibody, previously raised against a LYVE-1 polypeptide fragment,[19] was carried out after dewaxing and dehydration of the thin-sectioned specimens. The sections were pretreated with 10 mmol/L of citrate buffer solution (pH 6.0) for 15 minutes at 95°C and then with 40 μg/mL of Proteinase K (DAKO, Carpinteria, CA) for 3 minutes at room temperature. After washing in distilled water, the sections were incubated with LYVE-1 antibody (diluted 1:200) for 1.5 hours at room temperature, washed in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS) containing polysorbate 20, and treated with the Catalyzed Signal Amplification II kit (DAKO) according to the manufacturer's instructions. The immunostaining was visualized with diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin.

For vWF immunohistochemical staining, the sections were dewaxed, dehydrated, and pretreated with 10 mmol/L of citrate buffer solution (pH 6.0) for 15 minutes at 95°C. After washing in TBS, they were treated with 3% hydrogen peroxide for 10 minutes and then with 3% nonfat dried milk in TBS containing polysorbate 20 for 30 minutes. The sections were then incubated with antihuman vWF antibody (diluted 1:25; DAKO) for 2 hours at room temperature. A further wash in TBS was followed by treatment with a peroxidase-labeled polymer conjugated to goat and antirabbit or antimouse immunoglobulins (EnVision+ kit, DAKO) for 30 minutes at room temperature. The immunostaining was visualized with diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin.

Histopathologic Variables

We assessed the relationships between the following histopathologic variables: location, size, grade of differentiation, cancerous ulceration, and lymphatic and blood vessel invasion. For size of the cancer, the major axis of the primary EGC lesion was measured. For grade of differentiation, the histopathologic type at the primary site was categorized as papillary adenocarcinoma, well-differentiated adenocarcinoma, moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma, and signet-ring cell carcinoma according to the World Health Organization classification with Japanese modification.[19] For statistical treatments, we identified the first 3 types of differentiation as a low-grade malignancy group and the latter 2 types as a high-grade malignancy group according to the conventionally accepted relationship between the type of cancer and biologic behavior based on histopathologic classification.

For this analysis, vascular invasion was defined as invasion and adherence of cancer cells to the inside cell walls of the lymphatic or blood vessels. Lymphatic and blood vessel invasion was considered to be present when we could observe

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at least 1 vessel invaded by cancer cells. We examined the location of lymphatic and blood vessel invasion by imaging an entire primary tumor. After immunostaining for endothelial cells of lymphatic and blood vessels, double staining with Victoria blue and H&E dyes was also used to aid the identification of elastic fibers in the vein.

For all histopathologic variables, each macroscopic record and microscopic slide was analyzed by pathologists (A.F., Y.I., and T.I.) to reach consensus.

Statistical Methods

Statistical analyses were performed using the χ^2 test, Fisher exact test, and the Mann-Whitney *U* test to assess the significance of the impact of each subset of histopathologic variables on lymph node condition. Univariate and multivariate logistic regression analyses were carried out to identify independent predictive factors for lymph node metastasis. Differences at a *P* value of less than .05 were considered statistically significant. The StatView program (SAS Institute, Raleigh, NC) was used for all analyses.

Table 1
Comparison of Histopathologic Parameters by Lymph Node Status in Early Gastric Cancer

Variables	Node Status		*P*
	Negative (n = 66)	Positive (n = 66)	
Location of primary cancer in gastric surface area			NS*
Upper third	10 (15)	13 (20)	
Middle third	41 (62)	29 (44)	
Lower third	15 (23)	24 (36)	
Mean size (mm)	26.9	37.1	.0005†
Cancerous ulceration	13 (20)	23 (35)	NS‡
Grade of cancer differentiation			.0235‡
Low-grade malignancy	43 (65)	33 (50)	
High-grade malignancy	23 (35)	33 (50)	
Lymphatic invasion	6 (9)	21 (32)	.0021‡
Blood vessel invasion	4 (6)	13 (20)	.0352‡

NS, not significant.
* χ^2 test.
† Mann-Whitney *U* test.
‡ Fisher exact test.

Results

Comparison of Variables Between Node-Negative and Node-Positive Groups

Differences in histopathologic variables between node-negative and node-positive groups are given in **Table 1**. For tumor size, the Mann-Whitney *U* test revealed a significant difference between the groups (*P* = .0005). There was a significant difference in cancer differentiation between the groups as assessed by the Fisher exact test (*P* = .0235). Furthermore, the node-negative group had differentiation predominantly in the low-grade malignancy range compared with the node-positive group.

The 2 varieties of vessels were easily distinguished by LYVE-1 **Image 1A** and vWF **Image 1B** immunostaining. The frequencies of lymphatic and blood vessel invasion were both significantly greater in the node-positive EGCs than in

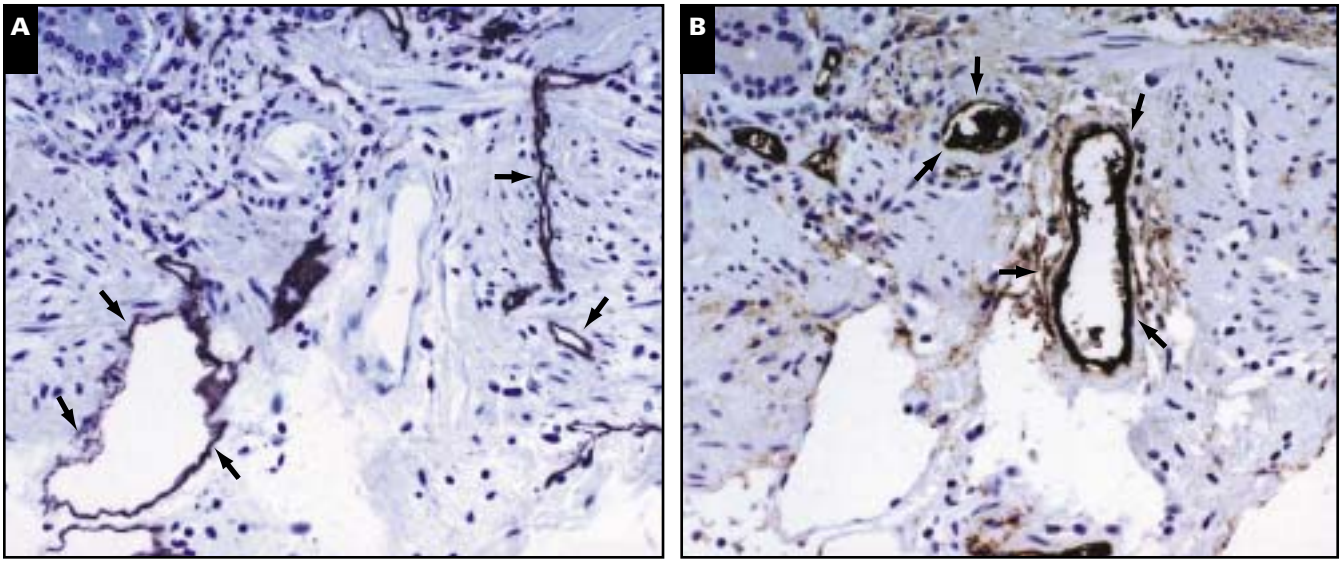

Image 1 Discrimination of lymphatic vessels from blood vessels at the same site of a normal mucosa. **A**, Lymphatic vessel is lined with lymphatic vessel endothelial hyaluronan receptor-1–positive endothelial cells just beneath the lamina muscularis mucosae (arrows) (×400). **B**, Blood vessel is lined with von Willebrand factor–positive endothelial cells at the same site as in **A**. There is a blood vessel containing blood components within its lumen (arrows) (×400).

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the node-negative EGCs ($P = .0021$ and $P = .0352$, respectively; Fisher exact test). On the other hand, there was no significant difference in the frequency of lymph node metastasis for location of cancer in the stomach and cancerous ulceration.

Univariate and Multivariate Logistic Regression Analyses of Histopathologic Variables as Predictors of Lymph Node Metastasis

The size of cancer, a low grade of cancer differentiation, lymphatic invasion, and blood vessel invasion were significant independent predictors for lymph node metastasis by univariate logistic regression analysis **Table 2**. When multivariate analysis was undertaken on these 4 factors, significant predictors were size of primary cancer and lymphatic invasion **Table 3**.

Observation of Lymphatic and Blood Vessel Invasion

Of the 66 cases with lymph node metastases, 21 had lymphatic invasion and 13 had blood vessel invasion. Of the 66 cases without lymph node metastases, 6 had lymphatic invasion and 4 had blood vessel invasion. In 9 cases, lymphatic and blood vessel invasion were present.

We studied the spatial distribution of lymphatic and blood vessel invasion in relation to structure of the gastric wall of the primary cancer for the 132 node-positive and node-negative EGCs. Lymphatic invasion was mainly recognized just beneath the lamina muscularis mucosae (15/27 [56%]) **Image 2A** and **Image 2B** and in the submucosa (only in submucosa, 8/27 [30%]; in lamina muscularis mucosae and submucosa, 3/27 [11%]) and rarely occurred only within the mucosa (1/27 [4%]). When lymphatic invasion was assessed in terms of the portion of the primary cancer, it was frequently seen at the cancer periphery (17/27 [63%]) and in the central and peripheral portions (6/27 [22%]) of the cancer. Lymphatic invasion alone was rare in the central part of the cancer (4/27 [15%]).

Blood vessel invasion was mostly recognized in large blood vessels that exhibited a venous structure, which exists within the submucosa (12/17 [71%]) **Image 3A**. When blood vessel invasion was assessed in relation to the portion of the primary cancer, invasion was seen in the central and peripheral portions (4/17 [24%]), in central portions (7/17 [41%]), and at the periphery (6/17 [35%]) of the primary cancer at almost equal frequency. vWF staining **Image 3B** was used to identify only 1 case of invasion by cancer cells in the blood vessel, whereas the other 16 cases were analyzed by double staining with Victoria blue and H&E dyes.

Discussion

The present study is the first in which the significance of lymphatic invasion identification on regional lymph node metastasis in EGC was investigated by using immunohistochemical markers for lymphatic and blood vessels simultaneously. Immunostaining using LYVE-1 antibody enabled us to objectively distinguish the presence of lymphatic and blood vessels at the site that are apt to be overlooked as vacant interstitial spaces when using only H&E staining. In fact, by using this new antibody, we could observe considerably more lymphatics invaded by cancer cells compared with conventional staining methods.

The size of the primary tumor,[5,8,9,20,21] undifferentiated histopathologic features,[5,8,10,22] lymphatic or blood vessel invasion,[2,5,9,10,20,21,23,24] and cancerous ulceration[2,12,13] have been

Table 2
Univariate Logistic Regression Analysis of Node-Positive Early Gastric Cancers

Parameter	Odds Ratio	95% Confidence Interval	*P*
Location (middle and upper vs lower)*	1.990	0.926-4.277	NS
Size	1.028	1.008-1.047	.0046
Cancerous ulceration	2.181	0.989-4.806	NS
Grade of cancer differentiation (low vs high)	2.601	1.157-5.846	.0207
Lymphatic invasion	4.667	1.740-12.512	.0022
Blood vessel invasion	3.802	1.169-12.363	.0264

* Middle, upper, and lower thirds of the gastric surface area.

Table 3
Multivariate Logistic Regression Analysis of Node-Positive Early Gastric Cancers

Parameter	Odds Ratio	95% Confidence Interval	*P*
Size	1.030	1.010-1.049	.0023
Grade of differentiation (low vs high)	2.073	0.952-4.517	NS
Lymphatic invasion	3.987	1.404-11.325	.0094
Blood vessel invasion	3.646	0.996-13.353	NS

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considered as parameters of lymph node metastasis in EGC. In this study, the multivariate logistic regression analysis demonstrated that lymphatic invasion was a significant independent predictor of regional lymph node metastasis in EGC, whereas blood vessel invasion was not a significant predictor.

It is desirable that logistic analyses be conducted on a large sample and the results analyzed blindly, but it was not possible in the present study because EGC with lymph node metastases accounts for only 10% of reported cases in literature and although our data in the past 15 years on node-negative cases are vast, pathologic materials and information on patients' backgrounds were insufficient. Thus, subjects in 2 groups in the present study were matched by age and sex. Although we tried to avoid biases on comparative variables as much as possible, it is undeniable that unseen variables could have influenced our results. The results of the present study were also confirmed by results obtained in previous studies.

For lymphatic invasion, because the lymphatics are a route to lymph nodes, it would be reasonable to conclude that lymphatic invasion is one of the predictors of lymph node metastasis. However, previous studies have not been able to clarify the differences between lymphatic and blood vessel invasion.

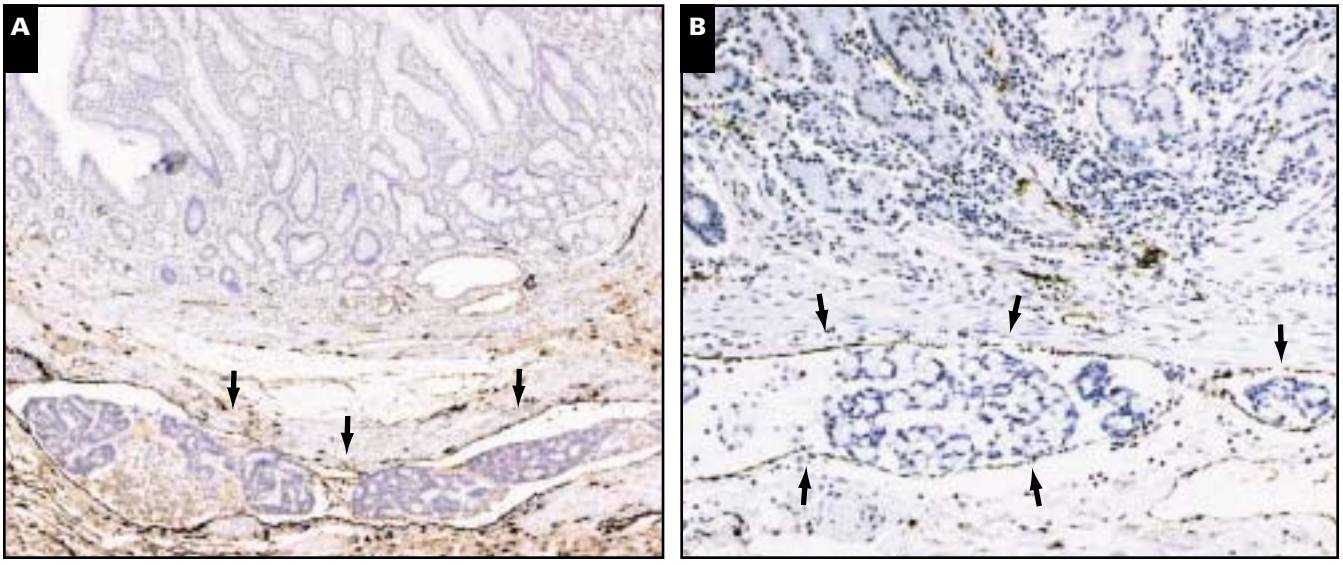

Image 2 The arrows in the image indicate the circumferences of the lymphatic vessels. **A** and **B**, Lymphatic invasion is evident just beneath the lamina muscularis mucosae (**A**, LYVE, ×100; **B**, LYVE, ×200). LYVE, lymphatic vessel endothelial hyaluronan receptor-1.

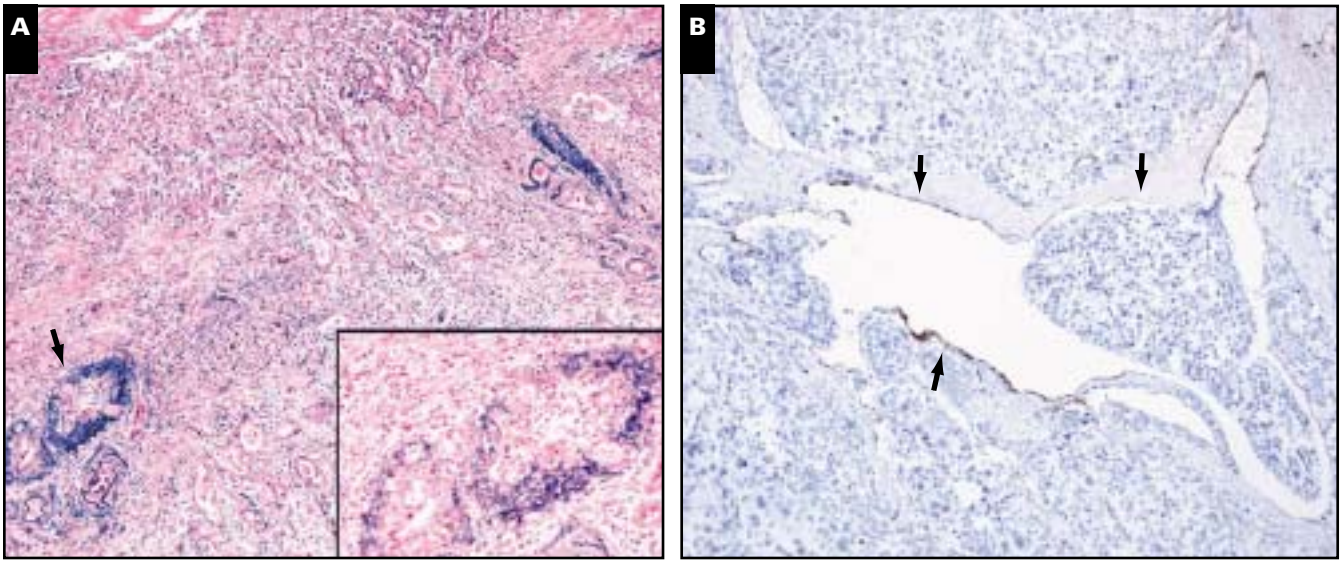

Image 3 **A**, Venous invasion as recognized by double staining with H&E and Victoria blue dyes (arrow) (×100). Inset, High-power view of the same vein (×400). **B**, Blood capillary invasion as recognized by von Willebrand factor immunostaining. The arrows indicate the circumference of the blood capillary (×150).

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In 1999, Banerji et al[25] identified the human LYVE-1 molecule as a major receptor of hyaluronan at the surface of the lymphatic endothelium. However, immunohistochemical detection using a previous LYVE-1 antibody was possible in only some pathologic tissue samples but difficult in most pathologic tissue samples.

We developed a new LYVE-1 antibody. Our LYVE-1 antibody has a higher specificity for lymphatic endothelial cells than previous LYVE-1,[25] podoplanin,[26] prox-1,[27] desmoplakin,[28] D6,[29] the mannose receptor,[30] and D2-40.[31] Furthermore, our LYVE-1 antibody could detect lymphatics under various conditions[18] that previously caused difficulty in detection.

By using these methods, we observed lymphatic invasion in EGC. Most lymphatics were found just beneath the lamina muscularis mucosae, and they were generally not seen within the mucosa. It has been accepted that the lymphatics arise only in the mucosa below the bases of gastric glands. Lymphatic vessels in the mucosal layer have no endothelial lining and so could be termed tissue channels rather than lymphatics.[32] Thus, because of these anatomic features, it is likely that lymphatics are not demonstrable in the mucosa by LYVE-1 even when a large number of such tissue channels are invaded by cancer cells in the gastric mucosa.

In addition, lymphatics were almost never found in the central portion of the primary cancer. However, lymphatics distributed at the periphery of the cancer, which remained intact, showed distinct LYVE-1 positivity. It is conceivable that the cancer cells make contact with lymphatics in the central portion of the primary tumor and destroy the lymphatic structure in that area. Microscopic examination of lymph vessel detected numerous lymph vessels, particularly those inside cancer masses with structural defects and fragmentation due to cancer cell invasion. The fragments from lymphangial epithelia were successfully stained with LYVE-1 antibody, suggesting the presence of lymphatic invasion. However, for the purpose of accuracy of the study, only cases with lymph vessels with complete structure were counted to confirm lymphatic invasion. Therefore, it is possible that because of the limitations of the antibody and statistical methods, only 30% are seen to have lymphatic invasion.

In recent years, minimal surgical procedures such as endoscopic techniques and laparoscopic resection have been developed to treat EGC; however, there remains a serious problem of ignoring the status of regional lymph nodes in such treatments. When we encounter lymphatic invasion at the primary site by microscopic examination of EGC, we need to consider the possibility of regional lymph node metastasis. Thus, the LYVE-1 antibody could be further explored by using it to identify lymphatic invasion in clinical situations by immunohistochemical analysis.

From the Departments of [1]Pathology and [6]Medical Informatics, School of Medicine, Toho University, Tokyo; [7]Gastroenterology and Hepatology, Ohmori Medical Center, Toho University, Tokyo; [2]Pathology and [3]Surgery, National Defense Medical College, Tokorozawa; [4]Pathology, Saiseikai Kanagawa-Ken Hospital, Yokohama; and [5]Pathology, Ida Municipal Hospital of Kawasaki City, Kawasaki, Japan.

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Address reprint requests to Dr Fujimoto: Dept of Pathology, School of Medicine, Toho University, 5-21-16 Ohmori-Nishi, Ohta-Ku, Tokyo 143-8540, Japan.

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* *Drs Fujimoto and Ishikawa contributed equally to complete the study.*

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