

Neoangiogenesis of gastric submucosa-invasive adenocarcinoma

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Abstract. Early gastric cancer may be defined as mucosal or submucosal invasive carcinoma, and exhibits a good prognosis: 90% of patients survive >10 years. Early gastric cancer infrequently exhibits lymph node metastasis, although submucosal invasion, the presence of vascular invasion and/or lymphatic permeation are independent risk factors for lymph node metastasis in early gastric cancer. The analysis of tumor lymphangiogenesis and angiogenesis are important to determine the extent of invasive progression and metastasis in patients. Previously, the presence of vessels expressing the D2-40 antibody and the factor-VIII protein has been identified immunohistochemically. The vessels that are immunoreactive for D2-40 and factor-VIII are morphologically similar to lymphatic vessels or small-size veins, also termed venules. In the present study, the association between tumor invasion and neoangiogenesis in early gastric cancer was examined. The D2-40/factor-VIII double-stained vessel (DSV) density was analyzed, in addition to lymphatic and blood vessel (vein and artery) density, using 46 submucosa-invasive and 50 mucosal carcinomas, and 20 non-neoplastic gastric tissues. The lymphatic density and DSV density of submucosa beneath the carcinoma and submucosa of the surrounding region in submucosa-invasive carcinoma were significantly increased ($P<0.001$) in comparison with those in mucosal carcinoma or non-neoplastic gastric tissue. No significant difference was observed in blood vessel density between non-neoplastic gastric, mucosal carcinoma and submucosa-invasive carcinoma tissues other than that of mucosa. The present study suggests the potential for the presence of D2-40/factor-VIII

DSV and the importance of this vessel for neoangiogenesis in early gastric cancer.

Introduction

Early gastric cancer may be defined as mucosal carcinoma and/or submucosa-invasive carcinoma, regardless of the nodal status. If left untreated, the majority of cases of early gastric cancer will progress over a number of months to several years (1). Mucosal carcinoma and submucosa-invasive carcinoma exhibit the potential for lymph node metastasis (2-4). The presence of vascular invasion and lymphatic permeation are independent risk factors for lymph nodal metastasis in early gastric cancer, in addition to a tumor diameter >3.0-3.5 cm, depressed or ulcerated lesions and undifferentiated histology (5). Lymphangiogenesis is hypothesized to be one of the early stages of invasion, and the characterization of this process is important for understanding the pathways of progression in human cancer. However, several reports have discussed the associations between lymphangiogenesis and cancer progression in early gastric cancer (6-9). Previously, the presence of morphologically intermediate vessels, which are similar to lymphatic vessels or small size veins, or venules, have been identified with hematoxylin & eosin (H&E) staining. It has been hypothesized that these intermediate vessels exhibit characteristics of lymphatic vessels and venules, and may serve important roles in tumor lymphangiogenesis/angiogenesis. However, there have been no studies to confirm this. Lymphangiogenesis in early gastric cancer remains incompletely characterized.

In the present study, immunohistochemical staining was performed with D2-40 and factor-VIII for the histological evaluation of lymphangiogenesis/angiogenesis. The intermediate vessels stained with D2-40 and factor-VIII were also analyzed, and termed double-stained vessels (DSVs). The densities of the lymphatic vessels stained with D2-40 only, the blood vessels stained with factor-VIII only and the DSV stained with D2-40 and factor-VIII were examined in 50 mucosal carcinoma lesions (44 patients), 46 submucosa-invasive carcinoma lesions (45 patients) and 20 non-neoplastic gastric tissues by double immunohistochemical staining with D2-40 and factor-VIII.

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Abbreviation: DSV, double-stained vessel

Key words: early gastric cancer, progression, D2-40, and factor-VIII

Materials and methods

Patient samples. The pathological features of each lesion were evaluated using paraffin-embedded tissue specimens from 45 patients (46 lesions) with submucosa-invasive carcinoma and 44 patients (50 lesions) with mucosal carcinoma who underwent endoscopic submucosa dissection in the period between January 2007 and June 2013. Informed consent was obtained from each patient regarding the use of their clinical records and pathology specimens at the Hirosaki University Hospital (Hirosaki, Japan). A total of 20 non-neoplastic gastric tissues were also examined, which were taken from patients with surgically resected gastric cancer. The area of deepest invasion was selected as a representative histological specimen of each lesion. The selected sections were stained with H&E and double immunohistochemical staining with D2-40 and factor-VIII. For histopathological examination, the early gastric cancer specimens were fixed using formalin, embedded in paraffin, thinly sectioned and stained with H&E. Carcinoma lesions were histologically graded according to the Guidelines for Clinical and Pathologic Studies on Carcinoma of the Stomach (10). Categorical variables such as gender, age, tumor size, histological classification, depth of invasion, lymphatic invasion, venous invasion and endoscopic growth patterns are presented by frequency (Table I). Quantitative variables are presented by mean, range and standard deviation. The lesions were classified according to endoscopic growth patterns (10,11). Early gastric cancer is divided into three groups: Protruded, type 0-I; superficial, type 0-II and excavated, type 0-III (10). The superficial type is further subdivided into elevated type, 0-IIa; flat type, 0-IIb and depressed type, 0-IIc (12).

Double immunohistochemical staining with D2-40 and Factor-VIII. Serial sections of H&E staining were selected for double immunohistochemical staining. The distinguishing abilities of double immunohistochemical staining using D2-40 combined with factor-VIII were studied. The BenchMark[®] automated slide-processing system (Roche Diagnostics, Tokyo, Japan) was used according to the manufacturer's protocol. The deparaffinization step was performed for 16 min at 75°C using EZ Prep (Roche Diagnostics). The heat treatment step was performed for 60 min at 100°C using Cell Conditioning 1 solution (Roche Diagnostics). The slides were incubated with D2-40 antibody (cat. no. M3619, clone D2-40, monoclonal mouse; dilution, 1:6; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 32 min at 42°C. The D2-40 protein was visualized using the iVIEWTM 3,3'-Diaminobenzidine Detection Kit (Roche Diagnostics). The same slides were incubated with factor-VIII antibody (cat. no. 518101206, Factor-VIII-related antigen[®], polyclonal rabbit antibody; dilution, 1:4; Roche Diagnostics) for 32 min at 42°C subsequent to 100°C heat treatment using Cell Conditioning 1 solution (Roche Diagnostics) for 8 min. Factor-VIII protein was visualized using the ultraVIEWTM Universal Alkaline Phosphatase Red Detection Kit (Roche Diagnostics) followed by counterstaining with Hematoxylin II (Roche Diagnostics) and Bluing Reagent (Roche Diagnostics).

Three-dimensional (3D) analysis of DSVs. The slides of submucosa-invasive carcinoma that contained DSVs were

selected. The slides were sectioned to a thickness of 15 μ m and stained by double immunohistochemistry with D2-40 and Factor-VIII. Serial images of 0.25- μ m thick DSV were captured using TOCO[®] whole slide imaging system with multi-layer/fusion application (Claro, Inc., Aomori, Japan). The serial images were edited using Adobe Photoshop (Adobe[®]Photoshop[®]CS2 Windows[®], USA) and were converted into 3D images using plugins (3D viewer, StackReg) of ImageJ software (ImageJ v1.48; <https://imagej.nih.gov/ij/>) (13,14).

Calculation of lymphatic density, blood vessel density, and DSV density in early gastric cancer and non-neoplastic gastric tissue. DSVs were defined as the vessels stained by D2-40 and factor-VIII. The ratio of D2-40 to factor-VIII was not considered whilst counting the number of DSVs. The submucosa beneath the carcinoma (SMB) was defined as the submucosal layer just beneath the area affected by carcinoma tissue, and submucosa of the surrounding region (SMs) as the tissue at a certain distance from the area affected by carcinoma tissue in the representative sections. The mucosa (M) was defined as the proper M of carcinoma and non-neoplastic gastric tissue. SMB and SMs data were same in the non-neoplastic gastric tissue. A total of three regions each in M, SMB and SMs were chosen for the counting x20 (Fig. 3). The immunostained sections were scanned by light microscopy at a low magnification, x100, at the adequate areas for vessel density counting. The lymphatic vessels, blood vessels and DSVs were counted at three points each in M, SMB and SMs at a high magnification, x400. The mean number of lymphatic vessels, blood vessels and DSVs was determined to be the lymphatic density, blood vessel density and DSV density, respectively.

Statistics. The lymphatic density, blood vessel density and DSV density in M, SMB and SMs were analyzed by Turkey's test (T). IBM[®] SPSS[®] Statistics v. 22 (IBM SPSS, Armonk, NY, USA) was used for statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Three-dimensional (3D) analysis of DSVs. Images of a 0.25- μ m DSV captured in continuity (Fig. 1A). The lymphatic vessel was stained brown by D2-40, and the blood vessels were stained red by factor-VIII. DSVs were stained red and brown by Factor-VIII and D2-40 (Fig. 1A). The DSV of 3D image was showed in Fig. 1B.

DSV. A representative DSV is demonstrated in Fig. 2. DSV was stained brown by D2-40 and red by factor-VIII. Lymphatic vessels were stained brown (D2-40) and the blood vessels were stained red (factor-VIII).

Calculation of lymphatic density, vessel density and DSV density in early gastric cancer and non-neoplastic gastric tissue. The analysis areas in M, SMB and SMs in early gastric cancer cases are demonstrated in Fig. 3. Capillary blood vessels were primarily observed in M. Lymphatic vessels were infrequently seen in M. Blood vessels and lymphatic vessels were mainly observed in SMB and SMs. DSVs were observed or not observed by case in SMB and SMs. DSVs were rarely seen in the M.

Table I. Clinicopathological characteristics of mucosal carcinoma and submucosa-invasive carcinoma.

Characteristics	Mucosal carcinoma (n=50, 100%)	Submucosa-invasive carcinoma (n=46, 100%)
Gender (%)		
Men	32 (73)	33 (73)
Female	12 (27)	12 (27)
Age (range)	73±8.4 (46-85)	72±8.0 (50-85)
Growth pattern (%)		
0-I	1 (2)	3 (7)
0-IIa	32 (64)	19 (41)
0-IIb	6 (12)	2 (4)
0-IIc	8 (16)	17 (37)
0-IIa+0-IIc	1 (2)	5 (11)
0-IIb+0-IIc	2 (4)	0 (0)
Tumor size (mm)	17±10 (4.0-52)	25±13 (5.0-57)
Histological classification (%)		
Well	38 (76)	22 (48)
Mod	12 (24)	23 (50)
Poor	0 (0)	1 (2)
Depth of invasion (μm)		757±670 (20-3,000) ^a
Lymphatic invasion (%)		
Positive	0 (0)	10 (22)
Negative	50 (100)	36 (78)
Venous invasion (%)		
Positive	0 (0)	19 (41)
Negative	50 (100)	27 (59)

Well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma.
^aDepth of submucosa-invasive carcinoma from muscularis mucosa.



Figure 1. (A) Images of a 0.25- μm DSV captured in continuity. DSV was stained with D2-40 (3,3'-Diaminobenzidine, brown) and factor-VIII (FAST RED, red). (B) 3D merges of images were captured in continuity. The color of the vessel walls changed from red to brown. DSV, double-stained vessel.

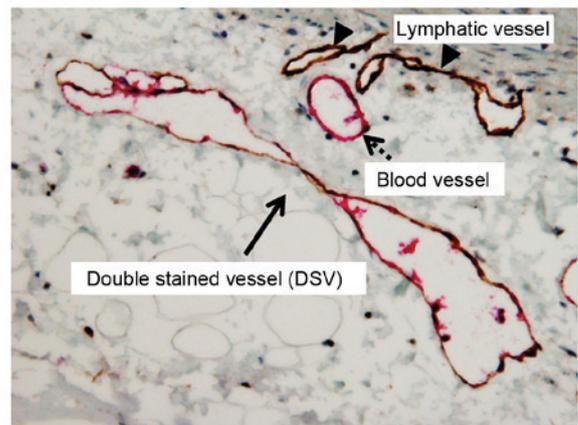


Figure 2. Lymphatic vessels were stained with DAB (arrow head), blood vessel was stained with FAST RED (dashed arrow), and DSV were stained with DAB and FAST RED (arrow) (magnification, x400). DSV were morphologically similar to lymphatic vessels. DAB, 3,3'-Diaminobenzidine; FAST RED, factor VIII; double-stained vessels, DSV.

Double staining with D2-40 and factor-VIII. Representative sections of double immunohistochemical staining for submucosa-invasive carcinoma and mucosal carcinoma are demonstrated in Fig. 4. Veins and arteries were stained red

by factor-VIII. Lymphatic vessels were stained brown by D2-40. DSVs were stained red and brown by Factor-VIII and D2-40. Red and brown were dyed stripe in DSVs (Fig. 4).

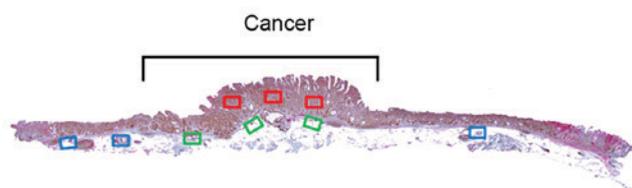


Figure 3. The countable areas of vessels in an early gastric cancer tissue. The red squares indicated mucosal layer (M). Green squares indicated submucosa beneath the carcinoma (SMb). Blue squares indicated submucosa of the surrounding region (SMs). Three points were selected in each area for counting vessels (x20).

Lymphatic density. The lymphatic density of the SMb in the submucosa-invasive carcinoma tissues was higher compared with that in mucosal carcinoma tissues ($P < 0.001$) and the non-neoplastic gastric tissues ($P < 0.001$). The lymphatic density of the SMs in the submucosa-invasive carcinoma tissues was also higher compared with that in the mucosal carcinoma tissues ($P < 0.001$) and the non-neoplastic gastric tissue ($P < 0.001$). There was no significant difference observed between the SMb and SMs in the mucosal carcinoma and the non-neoplastic gastric tissues ($P = 0.211$ and $P = 0.211$, respectively). In addition, there was no significant difference observed in the M (non-neoplastic vs. mucosal carcinoma tissues, $P = 0.211$; non-neoplastic vs. submucosa-invasive carcinoma tissues, $P = 0.213$ and mucosal carcinoma vs. submucosa-invasive carcinoma tissues, $P = 0.168$) (Fig. 5A).

Blood vessel density. The blood vessel density of M in the submucosa-invasive carcinoma tissues was higher compared with that in the mucosal carcinoma tissues ($P < 0.001$). There was no significant difference in the M between the non-neoplastic gastric and the submucosa-invasive carcinoma tissues ($P = 1.00$). No significant difference was observed in the M between the non-neoplastic gastric and mucosal carcinoma tissues ($P = 1.00$). In addition, no significant difference was observed in the SMb between the non-neoplastic gastric, mucosal carcinoma and submucosa-invasive carcinoma tissues (non-neoplastic vs. mucosal carcinoma tissues, $P = 0.841$; non-neoplastic vs. submucosa-invasive carcinoma tissues, $P = 1.000$; mucosal carcinoma vs. submucosa-invasive carcinoma tissues, $P = 0.717$, respectively). In addition, there was no significant difference observed in the SMs blood vessel density between the non-neoplastic gastric, mucosal carcinoma and submucosa-invasive carcinoma tissues (non-neoplastic vs. mucosal carcinoma tissues, $P = 0.999$; non-neoplastic vs. submucosa-invasive carcinoma tissues, $P = 0.801$; mucosal carcinoma vs. submucosa-invasive carcinoma tissues, $P = 0.101$) (Fig. 5B).

DSV density. The DSV density of the SMb and SMs in the submucosa-invasive carcinoma tissues was higher compared with that in the non-neoplastic gastric and the mucosal carcinoma tissues (SMb in submucosa-invasive carcinoma vs. SMb in non-neoplastic tissues, $P < 0.001$; SMb in submucosa-invasive carcinoma vs. SMb in mucosal carcinoma tissues, $P < 0.001$; SMs in submucosa-invasive carcinoma vs. SMs in the non-neoplastic tissues, $P < 0.001$; SMs in submucosa-invasive carcinoma vs. SMs in mucosal carcinoma tissues, $P < 0.001$).

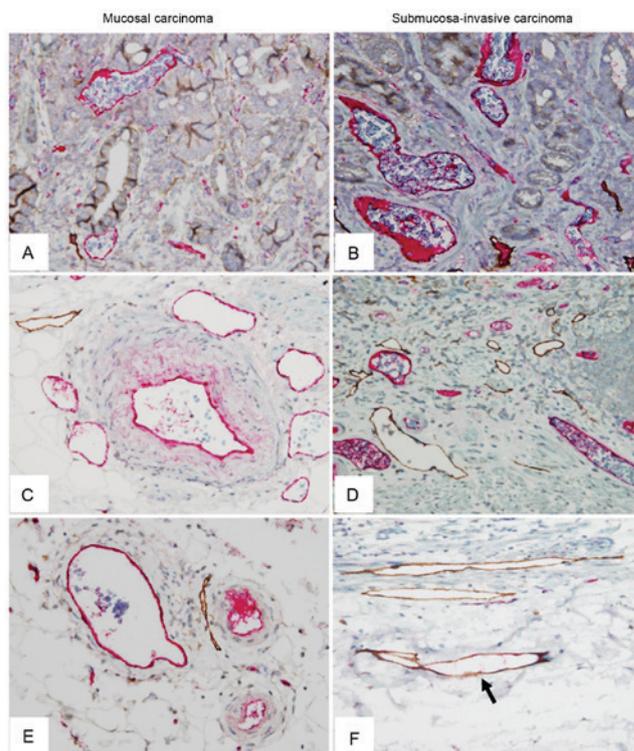


Figure 4. Representative images of double immunohistochemical staining by D2-40 and factor-VIII in mucosal carcinoma and submucosa-invasive carcinoma (x400). (A) and (B) were identified as mucosal layer. (C) and (D) were identified as submucosa beneath the carcinoma. (E) and (F) were identified as submucosa of the surrounding region. Blood vessels were stained with factor-VIII colored with red. Lymphatics were stained by D2-40 colored with brown. Double stained vessels were stained by both factor-VIII and D2-40, arrow.

There was no significant difference observed in the SMb between the non-neoplastic gastric and mucosal carcinoma tissues ($P = 1.000$). There was no significant difference observed in SMs between the non-neoplastic gastric and the mucosal carcinoma tissues ($P = 1.000$). In addition, the DSVs density of the M demonstrated no significant difference between the non-neoplastic gastric, the mucosal carcinoma and the submucosa-invasive carcinoma tissues (M in non-neoplastic gastric vs. M in mucosal carcinoma tissues, $P = 1.00$; M in non-neoplastic gastric vs. M in submucosa-invasive carcinoma tissues, $P = 0.925$; M in mucosal carcinoma vs. M in submucosa-invasive carcinoma tissues, $P = 0.97$) (Fig. 5C).

Discussion

Samples of mucosal carcinoma and submucosa-invasive carcinoma in which endoscopic submucosa dissection had been performed were analyzed. The present study demonstrated that the level of lymphatic density of the SMb and the SMs increased in submucosa-invasive carcinoma. Notably, no significant difference was observed in the SMb and the SMs lymphatic densities between the non-neoplastic gastric tissue and the mucosal carcinoma. This indicated that lymphatic density was associated with the depth of invasion and that lymphangiogenesis may begin with submucosal-invasion. Submucosa-invasive carcinoma may exhibit lymphatic invasion due to the lymphatic density of the SMb and the SMs.

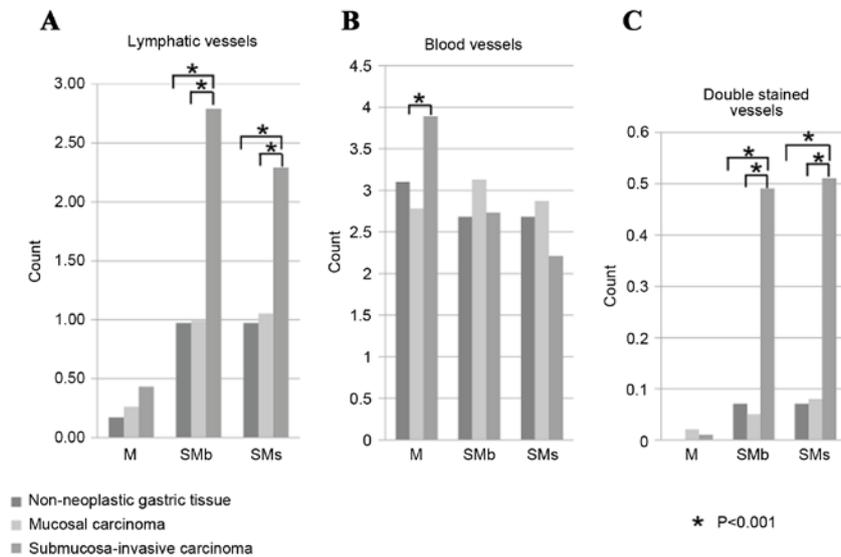


Figure 5. (A) Lymphatic density of the SMb in submucosa-invasive carcinoma was higher compared with that of mucosal carcinoma tissues ($P < 0.001$) and non-neoplastic gastric tissues ($P < 0.001$). Lymphatic density of SMs in submucosa-invasive carcinoma was also higher compared with that of mucosal carcinoma tissues ($P < 0.001$) and non-neoplastic gastric tissues ($P < 0.001$). (B) Vessel density of the M in submucosa-invasive carcinoma was higher compared with that of mucosal carcinoma tissues ($P < 0.001$). (C) Double stained vessel density of the SMb ($P < 0.001$) and the SMs ($P < 0.001$) in submucosa-invasive carcinoma were higher compared with those of the non-neoplastic gastric and the mucosal carcinoma tissues. * $P < 0.05$. M, mucosa; SMb, mucosa beneath the carcinoma; SMs, submucosa of the surrounding region.

It has been indicated that increased intratumoral lymphatic density correlates with lymph node metastasis in early gastric cancer (6). Gao *et al* (7) separately calculated the intratumoral and peritumoral lymphatic densities for early gastric cancer and advanced gastric cancer. The authors also stated that intratumoral and peritumoral lymphatic densities were positively correlated with lymph node metastasis in early gastric cancer. Only peritumoral lymphatic density was positively correlated with lymph node metastasis and poor patient outcome in advanced gastric carcinoma (7). These studies included analysis of surgical specimens of early gastric cancer. The present study concluded that there was no significant difference in mucosal lymphatic density between the non-neoplastic gastric, mucosal carcinoma and submucosa-invasive carcinoma tissues in the present study. Listorom *et al* (15) stated that lymphatics are not identified in the upper and middle regions of the lamina propria in normal, hyperplastic or neoplastic tissues. They also revealed that lymphatics formed a network immediately superficial to, within and below the muscularis mucosa in non-neoplastic gastric tissue. In the present study, a number of lymphatics were observed just below the muscularis mucosa in the early gastric cancer samples.

The blood vessel density of the mucosa in the submucosa-invasive carcinoma tissue was higher compared with that

in mucosal carcinoma tissues in the present study. Previous studies have suggested that high intratumoral vessel density is correlated with lymph node metastasis and distant metastasis in gastric cancer (16-18). Tomoda *et al* (9) stated that microvessel density was significantly higher in tumors with venous invasion, lymphatic vessel invasion and node metastases compared with tumors without venous invasion, lymphatic vessel invasion or lymph node metastasis. Conversely, Lee *et al* (6) analyzed not only lymphatic vessel density but also microvessel density in 141 patients with early gastric cancer and demonstrated that intratumoral micro-vessel density and peritumoral micro-vessel density did not correlate with lymph node metastasis. The effects of intratumoral micro-vessel density in early gastric cancer remains debatable. In the present study, there was no difference observed in the mucosa blood vessel density between submucosa-invasive carcinoma and non-neoplastic gastric tissues. The microvessels and small-to medium-sized vessels were counted. Counting systems differed in individual studies. The definition of blood vessels and definition of the peritumoral area also slightly differed in each study. A large study group and a repetitive test and validation method may be required for reliable and reproducible criteria for studies investigating blood vessel density.

D2-40 has been demonstrated to detect the lymphatic endothelium in formalin-fixed paraffin-embedded tissues and it does not react with blood vessel endothelium (19). Factor-VIII is a subunit of factor-VIII, and factor-VIII has been termed the von Willebrand factor. The von Willebrand factor is synthesized by endothelial cells (20) and megakaryocytes (21). It is present in plasma, stored in platelet α -granules (22) and located in endothelial cells (23). In the present study, lymphatic vessels were stained with D2-40 and blood vessels were stained with factor-VIII, and this revealed DSVs stained with both factor-VIII and D2-40 in early gastric cancer specimens using an automated slide processing system.

There have been few studies on double staining using an automated slide processing system. Automated slide systems allow every slide to be easily and equally stained. There have been no previous reports on DSVs. Therefore, it was difficult to decide the definition of DSVs. Morphologically, DSVs are similar to the lymphatic system. DSV density was significantly higher in the SMb and the SMs in submucosa-invasive carcinoma compared with the SMb and the SMs in non-neoplastic gastric and mucosal carcinoma tissues. It was hypothesized that DSV density was correlated with tumor progression in early gastric cancer. It may be that immature vasculatures are synthesized in carcinoma invasion. The immature vasculatures may have characteristics of lymphatic and blood vessel. DSVs were usually dilated and thin thickness in the present study. DSVs were observed to be weak vessels, therefore it may be easy to invade by cancer cells. The function and characteristics of DSVs require additional investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

SM, TT analyzed and interpreted the present study. HSa made substantial contribution to acquisition of data and analysis. Hse provided substantial contributions to analysis and interpretation of data for the present study. TY, HH, TH, YW and HK provided substantial contributions to analysis and interpretation of data for the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of Hirosaki University Graduate School of Medicine (organization number; 2017-1149).

Consent for publication

The patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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