Use of Immunostaining for the Diagnosis of Lymphovascular Invasion in Superficial Barrett’s Esophageal Adenocarcinoma

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Abstract

Background: The prevalence of Barrett’s esophageal adenocarcinoma (BEA) is increasing in Japan. Accurate assessment of lymphovascular invasion (LVI) after endoscopic resection or surgery is essential in evaluating treatment response. This study aimed to assess the usefulness of immunostaining in determining the extent of LVI in superficial BEA.

Methods: We included 41 patients who underwent endoscopic resection or surgery between January 2007 and July 2018. In all cases, 3-µm serial sections from paraffin-embedded resected specimens were used for hematoxylin and eosin (H-E) staining and immunostaining for D2-40 and CD31. A specialized gastrointestinal pathologist (T.Y.), blinded to clinical information, evaluated the extent of LVI from these specimens. The LVI-positivity rate was evaluated with respect to the depth of invasion, changes in the positivity rate on immunostaining, pathological characteristics of patients with LVI, lymph node metastasis or relapse, and course after treatment.

Results: H-E staining alone identified LVI in 7 patients (positivity rate: 17.1%). Depths of invasion were categorized based on extension to the submucosa (SM) or deeper. On immunostaining for D2-40 and CD31, additional positivity was detected in 2 and 1 patients with SM1 and 1 SM3, respectively; LVI was detected in 10 patients (positivity rate: 24.4%). LVI-positivity rates with invasion of the superficial muscularis mucosa (SMM)/lamina propria (LPM)/deep muscularis mucosa (DMM), and SM 1, 2, and 3 were 0%, 75%, 28.6%, and 55.6%, respectively.

Conclusions: Combined H-E staining and immunostaining is useful in diagnosing LVI in superficial BEA, particularly in endoscopically resected specimens.

Introduction

In Barrett’s esophagus (BE), columnar epithelium replaces normal squamous epithelium in
the distal esophagus owing to repeated esophageal inflammation, injury, and repair caused by regurgitation of gastric acid or bile [1, 2]. The longitudinal extension of Barrett’s mucosa covering the entire circumference of the esophagus for at least 3 cm and less than 3 cm is termed long-segment Barrett’s esophagus (LSBE) and short-segment Barrett’s esophagus (SSBE), respectively [3]. Adenocarcinoma originating from BE is termed Barrett’s esophageal adenocarcinoma (BEA). In Europe and the US, BEA accounts for approximately 60% of all esophageal cancer cases [4], and recent reports suggest a rapid rise in incidence, exceeding that of esophageal squamous cell carcinoma [5]. Meanwhile, BEA is less frequent in Japan, comprising only 4.7% of all esophageal cancer cases [6]. However, the incidence of gastroesophageal reflux disease (GERD) has recently increased in Japan owing to the introduction of a Western-style diet and a decrease in the incidence of Helicobacter pylori infection [7]. This change may increase the incidence of BE, and consequently, BEA. Indeed, several studies have reported a slight increase in the incidence of BEA in Japan [8, 9]. The 5-year survival rate for advanced BEA without distant metastases is only < 20% [10]; thus, early diagnosis and treatment are essential.

Superficial BEA is primarily treated with surgery and endoscopic treatment as it has low risk for lymph node metastases. In Europe and the US, the primary treatment modality for BEA is endoscopic mucosal resection (EMR) combined with radiofrequency ablation (RFA) [11], while it is total resection using endoscopic submucosal dissection (ESD) in Japan. Additional treatment may be considered in cases extending to the deep muscularis mucosa (DMM) or deeper or with lymphovascular invasion (LVI). ESD is more beneficial than EMR as it facilitates total resection, allowing fractional excision and RFA. ESD has been gradually introduced in Europe and the US [12].

However, given the rarity of BEA in Japan, no guidelines have been established for endoscopic resection of superficial BEA. Currently, endoscopic treatment is performed
according to the guidelines for esophageal squamous cell carcinoma. With the increase in the number of indications for ESD, a multicenter cooperative study reported that in the absence of LVI and components of poorly differentiated carcinoma, lymph node metastases were not noted in patients with lesions measuring ≤ 30 mm in the maximum diameter and ≤ 500-µm infiltration to the SM. However, D2-40 or CD31/CD34 immunostaining was not performed to examine the presence of LVI. Furthermore, no central pathological diagnosis was obtained [13]. To date, no study has investigated the extent of LVI using immunostaining in superficial BEA treated by endoscopic resection or surgery. Therefore, we aimed to evaluate the use of immunostaining in identifying LVI in patients with superficial BEA.

Materials And Methods

Patients

This retrospective study evaluated 41 patients with superficial BEA who underwent endoscopic resection or surgery between January 2007 and July 2018 at the Nagoya University Hospital. Those treated at other hospitals and who received preoperative chemotherapy were excluded. Data on clinical information, endoscopic findings, treatments, histopathological findings, and course after treatment were collected from the electronic charts.

Diagnoses

Pathological diagnoses were made according to the Japanese Classification of Esophageal Cancer 11th edition, published by the Japan Esophageal Society [3]. The depth of tumor invasion was subclassified into 6 groups as follows: superficial muscularis mucosa (SMM); lamina propria (LPM); DMM; SM1; SM2; and SM3 involving ≤ 1/3 of the superficial, middle, and deep layers of the resected specimen, respectively. Among the endoscopically resected specimens, those with an SM infiltration of ≤ and > 200 µm were regarded as
SM1 and 2, respectively. In patients who underwent endoscopic resection or surgery, the results were compared between groups with an SM infiltration of < and ≥ 500 µm, respectively.

Immunostaining and LVI assessment

To evaluate the presence of LVI, 3-µm serial sections were prepared from paraffin-embedded blocks of resected specimens, stained with H-E, and subjected to immunostaining for Podoplanin (Clone D2-40, Dako) [14, 15] and CD31 (JC70, Roche Tissue Diagnostics) [16]. LVI was microscopically assessed using the H-E- and immunostained specimens by a specialized gastrointestinal pathologist (T.Y.) blinded to clinical information. LVI was defined as endothelial cells recognizable on D2-40- and CD31-positive cells and the presence of tumor cells in a space surrounded by these cells (Fig. 1).

The LVI-positivity rate was evaluated for the depth of invasion, changes in positivity rate on immunostaining, pathological characteristics of patients with LVI, lymph node metastasis or relapse, and treatment outcomes (overall, disease-specific, and relapse-free survival rates).

Statistical analyses

Continuous and categorical variables were presented as median (region) and number (percentage), respectively. Clinical parameters were compared using the Mann-Whitney U test and Fisher’s exact test for continuous and categorical variables, respectively. The log-rank test was used to investigate the survival rate. A p-value of 0.05 was regarded as significant. All statistical analyses were performed using the IBM SPSS Statistics software version 25 (IBM SPSS, Chicago, IL, U.S.A.) package.

Results

Patient characteristics
The median age of the 41 patients was 67 years; the patient characteristics are detailed in Table 1. Macroscopically, protruding tumors were detected in 31 patients, and the median maximum tumor diameter was 20 mm. ESD and surgery were performed as initial treatments in 13 and 28 patients, respectively. The histological types in 21, 17, and 3 patients were well differentiated (tub1), moderately differentiated (tub2), and poorly differentiated (por), respectively. Clinicopathological characteristics did not differ significantly between patients with SSBE and with LSBE.

Histopathological findings

Table 2 shows the histological type and number of patients with LVI on H-E staining and immunostaining for D2-40/CD31 according to the depth of invasion. Overall, 21 and 20 patients had pT1a and pT1b lesions, respectively, and 12 of the 21 patients with pT1a had DMM lesions. The depth of SM infiltration in endoscopic resection exceeded 200 μm in 3 patients, and the depths were 400, 800, and 1,300 μm, respectively. These patients were diagnosed as SM2. Among them, 2 patients underwent additional surgery, which revealed no residual cancer or lymph node metastases. The remaining patient preferred follow-up; no relapse has been observed during the 3-year follow-up after ESD. The incidence of tub2 and por histological subtypes increased with the depth of invasion.

In 7 patients, LVI positivity was noted using H-E-stained specimens alone (positivity rate: 17.1%), and the depth of invasion was evaluated to be SM1 or deeper. LVI was found in 10 patients (positivity rate: 24.4%) who were additionally diagnosed with LVI positivity on immunostaining for D2-40 and CD31. The LVI-positivity rates in SMM, LPM, DMM, SM1, SM2, and SM3 lesions were 0, 0, 0, 75, 28.6, and 55.6%, respectively. Overall, between H-E staining alone and immunostaining, LVI was consistently absent in 85.4% (35/41) cases. LVI was additionally detected on immunostaining in cases with SM1 (Fig. 2), in which the lymphatic endothelial cells were very thin near the tumor margin (site where LVI diagnosis
is relatively easy), making recognition difficult, and in cases with SM3 (Fig. 3), in which the tumor volume was large, making the identification of LVI at the site of tumor infiltration impossible.

The patients in the SM group were sub-divided into two groups based on the depth of infiltration as follows: <500 μm and ≥500 μm. The former subgroup had 5 patients with SM1 lesions (4 and 1 underwent surgery and endoscopic resection, respectively), with a depth of infiltration of 400 μm. The latter subgroup included 15 patients. LVI was present in 3 (60%) and 7 (46.7%) patients. No specific pattern of distribution of LVI sites was observed.

Lymph node metastasis and relapse

Table 3 shows the pathological findings in 30 surgically treated patients with superficial cancer, including 2 who underwent additional treatment after ESD. In total, 3/41 (7.3%) patients showed lymph node metastases. These patients had protruding SSBE-derived lesions, invading at least up to SM2 (depth of infiltration: >1,000 μm). The tumor diameters were ≥25 mm, and they contained poorly-differentiated components. LVI was identified in 2 of 3 patients.

Overall survival rate and relapse-free survival

There were no significant differences in overall, disease-specific, and relapse-free survival between patients with T1a and T1b disease (Fig. 4). However, the recurrence rate was slightly higher among patients with T1b lesions. Relapse occurred in 3 patients with T1b disease, with a median follow-up of 46 months. In all 3 patients, LVI was present, the depth of invasion was evaluated to be at least SM2, poorly differentiated components were observed, and the tumor diameter was ≥20 mm. Among them, 1 patient died of primary disease. The 3-year disease-specific survival rate in those with T1a and T1b disease was 100% and 95.0%, respectively.
Discussion/conclusion

The results of this study show that combined H-E staining and immunostaining is useful in diagnosing LVI in superficial BEA, particularly in endoscopically resected specimens. LVI is directly related to lymph node/remote metastases in cancer patients [17–21]. Therefore, LVI may be useful in predicting the metastasis risk. In Japan, few studies have reported on the incidence of LVI positivity in BEA patients. Osumi et al. identified LVI in 18/55 lesions (32.7%) with DMM [22]. Furthermore, Nishi et al. observed in lymphatic invasion in 10.3% of cases with DMM invasion. Further, also reported that the LVI-positivity rate increased with the depth of invasion [8].

In this study, LVI was present in patients with depths of invasion of at least SM1. These differences was probably due to the use of immunostaining to identify LVI in all patients and the evaluation of all specimens by a single pathologist. Additional immunostaining increased the LVI-positivity rate by 7% than H-E staining alone. In patients with SM1 lesions, this rate increased from 25–75%. Although the number of SM1 patients was small (n = 4), the high positivity rate is noteworthy. LVI is usually assessed using H-E-stained specimens. In patients in whom assessment is exceptionally difficult, the results may depend on the pathologist’s subjective assessment [23, 24]. Particularly, it is difficult to evaluate fine lymphatic/venous invasion; difficult-to-identify lymphovascular endothelial cells; desmoplastic reaction of interstitial cells [25, 26]; and artifacts related to tissue specimen preparation [24, 27, 28]. Here, LVI diagnosis was also difficult in some patients. Particularly, the difficulty in recognizing lymphatic/blood vessels may increase with a reduction in the grade of tumor differentiation. These factors limit LVI assessment using H-E-stained specimens alone. Additional immunostaining may have increased the LVI-positivity rate among SM infiltrating lesions in this cohort.

Japanese guidelines recommend endoscopic treatment for esophageal cancer and early
gastric cancer. Conversely, no treatment guidelines for BEA have been developed owing to lack of data. In this study, LVI was absent in patients with infiltration up to the DMM. In SM1 lesions, no lymph node metastases were observed when the criteria proposed by Ishihara et al. were fulfilled [12]. This suggests that ESD may be increasingly employed in these cases. Notably, LVI, invasion to SM2 or deeper, presence of poorly differentiated components, and a maximum tumor diameter of ≥ 20 mm were common among patients with relapse. The patients with SM or superficial lesions had relatively favorable prognosis, and only few patients had relapse. Therefore, the risks of lymph node metastasis and relapse may be low in SM (infiltration: ≤500 µm) lesions with a maximum diameter of ≤ 20 mm, absence of LVI, and absence of poorly differentiated carcinoma components. This suggests that after ESD, follow-up is a feasible option in patients ineligible for surgery. However, LVI is detected on immunostaining in some patients with SM1 invasion. Therefore, pathological findings should be carefully evaluated with additional immunostaining.

The risk of lymph node metastasis must be adequately evaluated. Many studies reported that LVI, detected on immunostaining for D2-40, was an independent prognostic factor for lymph node metastasis [19, 20, 29, 30]. LVI diagnosis may predict subsequent lymph node metastasis. In this study, only few patients had lymph node metastasis or relapse, making detailed statistical analysis unreliable.

In this study, as a result of immunostaining, LVI was newly diagnosed in some patients and ruled out in some cases despite positivity on H-E staining. Although we were unable to conclude whether additional immunostaining significantly increased the LVI-positivity rate in comparison with H-E staining alone, immunostaining may be useful in individual patients. The LVI-positivity rate was high among those with SM1 invasion; this should be considered while selecting patients for ESD. Furthermore, in patients with SM 2 or 3
invasion, the presence of LVI predicted future relapse; thus, it should also be considered during treatment. Positive findings on additional immunostaining in endoscopically resected specimens may facilitate decision-making for further treatment and prevent unnecessary surgery. However, immunostaining is cost and effort intensive and should be considered carefully in limited-resource settings.

The limitations of this study are its single-center retrospective design and small sample size. However, immunostaining for D2-40 and CD31 was performed in all patients with superficial BEA who underwent ESD or surgery, and the presence of LVI was examined. Furthermore, a single pathologist evaluated all cases, and the proportion of surgically treated patients was relatively high; the number of evaluable cases with lymph node metastases was also large.

In conclusion, immunostaining for D2-40 and CD31 is useful for identifying the presence of LVI in patients with superficial BEA. This is essential for evaluating the need for additional treatment, particularly in endoscopically resected specimens. Multicenter cooperative studies on ESD and surgery as treatment options for superficial cancer, are needed.

Declarations

Acknowledgments

None

Statement of Ethics

The study protocol was approved by the ethics review board of the Nagoya University (Approval Number: 2017-0392), and the work performed in this study was in accordance with the principles of the Declaration of Helsinki.

Conflicts of Interest

Isao Hosono, Ryoji Miyahara, Kazuhiro Furukawa, Kohei Funasaka, Tsunaki Sawada, Keiko Maeda, Takeshi Yamamura, Takuya Ishikawa, Eizaburo Ohno, Masanao Nakamura, Hiroki
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**Author Contributions**

Study concept and design: Isao Hosono and Ryoji Miyahara; Study supervision: Mitsuhiro Fujishiro; Data acquisition: Kazuhiro Furukawa, Kohei Funasaka, Tsunaki Sawada, Keiko Maeda, Takeshi Yamamura, Takuya Ishikawa, Eizaburo Ohno, Masanao Nakamura, and Hiroki Kawashima; Data analysis and interpretation: Isao Hosono and Ryoji Miyahara; Drafting of the manuscript: Isao Hosono; Critical revision of the manuscript for important intellectual content: Takio Yokoi and Yoshiki Hirooka; Technical, or material support: Takio Yokoi. All authors approved the final version of the manuscript.

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Tables

Table 1. Characteristics of 41 Patients with Superficial Barrett’s Esophageal Adenocarcinoma Treated with ESD or Surgery
| Characteristics                        | All patients (n=41) | SSBE (n=30) | LSBE (n=11) | SSBE VS. LSBE P-value |
|----------------------------------------|---------------------|-------------|-------------|----------------------|
| Age, median (range)                    | 67 (39-81)          | 66 (39-88)  | 68 (44-79)  | 0.757                |
| Sex (%)                                |                     |             |             |                      |
| Male                                   | 32 (78.0)           | 23 (76.7)   | 9 (81.8)    | 1.000                |
| Female                                 | 9 (22.0)            | 7 (23.3)    | 2 (18.2)    |                      |
| Body mass index (kg/m²), median (range)| 23.0 (16.7-32.6)    | 23.1 (16.7-32.6) | 23.0 (16.7-32.6) | 0.596                |
| Tumor size (mm), median (range)        | 20 (6-60)           | 17.5 (6-35) | 20 (10-60)  | 0.223                |
| Macroscopic type (%)                   |                     |             |             | 0.164                |
| Protruding type                        | 31 (75.6)           | 25 (83.4)   | 6 (54.5)    |                      |
| Flat type                              | 2 (4.9)             | 1 (3.3)     | 1 (9.1)     |                      |
| Depressed type                         | 8 (19.5)            | 4 (13.3)    | 4 (36.4)    |                      |
| Initial treatment (%)                  |                     |             |             | 0.127                |
| ESD                                    | 13 (31.7)           | 12 (40.0)   | 1 (9.1)     |                      |
| Operation                              | 28 (68.3)           | 18 (60.0)   | 10 (90.9)   |                      |
| Histological type (%)                  |                     |             |             | 0.181                |
| well differentiated (tub1)              | 21 (51.2)           | 18 (60.0)   | 4 (36.4)    |                      |
| moderately differentiated (tub2)       | 17 (41.5)           | 11 (36.7)   | 5 (45.4)    |                      |
| poorly differentiated (por)            | 3 (7.3)             | 1 (3.3)     | 2 (18.2)    |                      |

ESD, endoscopic submucosal dissection; SSBE, short segment Barrett’s esophagus; LSBE, long segment Barrett’s esophagus

Table 2. Histological Type/Presence of Lymphovascular Invasion (LVI) with Respect to the Depth of Invasion, and Comparison of LVI-positivity Rates between H-E- and D2-40/-CD31-stained Specimens
| Depth of invasion and number | Histological type | H-E staining | Immunostaining | P-value |
|-----------------------------|------------------|--------------|-----------------|---------|
|                             | tub1  | tub2  | por | Ly+ | V+ | LVI+ (%) | D2-40 Ly+ | CD31 V+ | LVI+ (%) |
| T1a SMM                     | 7     | 6     | 1    | 0   | 0   | 0 (0)    | 0         | 0       | 0 (0)    |
| LPM                         | 2     | 1     | 1    | 0   | 0   | 0 (0)    | 0         | 0       | 0 (0)    |
| DM                          | 12    | 10    | 2    | 0   | 0   | 0 (0)    | 0         | 0       | 0 (0)    |
| T1b SM1                     | 4     | 1     | 3    | 0   | 1   | 1 (25)   | 3         | 0       | 3 (75)   |
| SM2                         | 7     | 1     | 5    | 1   | 2   | 2 (28.6)| 2         | 2       | 2 (28.6) |
| SM3                         | 9     | 2     | 5    | 2   | 4   | 4 (44.4)| 4         | 2       | 5 (55.6) |
| Total                       | 41    |       |      |     | 7   | 10 (17.1)| 3         | 10      | 0.58     |

H-E, Hematoxylin and eosin; SMM, superficial muscularis mucosa; LPM, lamina propria; DMM, deep muscularis mucosa; SM, submucosa

Table 3. Pathological Findings in 30 Patients with Superficial Cancer who Underwent Surgery

| Depth of invasion and number | Histological type | Lymphovascular invasion | Lymph node metastasis | Recurrence |
|------------------------------|------------------|-------------------------|-----------------------|------------|
|                              | tub1  | tub2  | por | LVI+ | +   | +   |
| SMM                          | 3     | 3     | 0   | 0    | 0   | 0   |
| LPM                          | 2     | 1     | 1   | 0    | 0   | 0   |
| DMM                          | 6     | 5     | 1   | 0    | 0   | 0   |
| SM1                          | 4     | 1     | 3   | 0    | 3   | 0   |
| SM2                          | 6*    | 1     | 4   | 1    | 2   | 2   |
| SM3                          | 9     | 3     | 4   | 2    | 5   | 1   |
| Total                        | 30    | 10    | 3   | 3    |     |     |

*Including 2 who underwent additional surgery after ESD

SMM, superficial muscularis mucosa; LPM, lamina propria; DMM, deep muscularis mucosa; SM, submucosa
Figures

Figure 1

(a) Microphotograph of lymphovascular invasion (LVI) as assessed using hematoxylin and eosin (H-E) staining. (b) Microphotograph of lymphatic vessel invasion as assessed using D2-40 staining (positive). (c) Microphotograph of blood vessel invasion as assessed using CD31 staining (negative)

Figure 2

A case where immunostaining was useful for evaluating the presence of lymphovascular invasion (patient with SM1, in whom the lymphatic endothelial cells were very thin, making recognition difficult. Lymphovascular invasion was detected on immunostaining for D2-40).
A case where immunostaining was useful for evaluating the presence of lymphovascular invasion (patient with SM3, in whom the tumor volume was large, making the assessment of lymphovascular invasion at the site of tumor infiltration impossible. Lymphovascular invasion was detected on immunostaining with D2-40).

Survival curves in patients with T1a/T1b tumors. (a) Overall survival. (b) Disease-specific survival. (c) Relapse-free survival.