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Detailed Analysis of Lymphatic Invasion Using D2-40 Immunostaining in Early Gastric Adenocarcinoma: Proposal of the Classification of Lymphatic Invasion by D2-40 Immunostaining

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Abstract

Aims: Lymphatic invasion by D2-40 immunostaining has been evaluated based on the presence of carcinoma cells in the lymphatic lumen, but the entering of carcinoma cells with an invading style into the lymphatic lumen of a lymphatic vessel was reported in early gastric adenocarcinoma (EGAC), in which carcinoma cells localize within mucosa or submucosa. Here, a detailed examination of lymphatic invasion by D2-40 immunostaining is made in EGAC.

Methods: A total of 204 EGAC patients who underwent endoscopic submucosal dissection was examined. Lymphatic invasion was classified as intra-lumen type and invading type by D2-40 immunostaining. In the intra-lumen type, carcinoma cells are present in the lymphatic lumen. In the invading type, carcinoma cells invade the lymphatic vessel with destruction of the lymphatic wall and enter the lymphatic lumen.

Results: Lymphatic invasion was noted in 15 cases. The sites of the invasion were mucosa (1 case), mucosa and submucosa (7 cases), and submucosa (7 cases). Intra-lumen-type invasion was present in all 15 cases, and invading-type invasion was noted in 2 cases (mucosa in 1 case and mucosa and submucosa in 1 case). These data indicate that carcinoma cells entered from both mucosal and submucosal lymphatic vessels and carcinoma cells in the mucosal lymphatic vessels remained in the mucosal lymphatic vessels or moved to submucosal lymphatic vessels. The carcinoma cells in the lymphatic vessels of the submucosa consisted of carcinoma cells that moved from the mucosal lymphatic vessels and carcinoma cells entered from submucosal lymphatic vessels, or they consisted of carcinoma cells that entered from submucosal lymphatic vessels only.

Conclusion: The classification of lymphatic invasion consisting of intra-lumen type and invading type offers an increase in diagnostic accuracy of lymphatic invasion, and it clarifies the entry and movement system of carcinoma cells in lymphatic vessels.

Keywords: Lymphatic invasion • D2-40 immunostaining • Intra-lumen type • Invading type

Introduction

Immunostaining of lymphatic vessels, in which D2-40 immunostaining is the most reliable marker, is used for the evaluation of lymphatic invasion in the daily diagnosis of biopsy and surgically resected organs with various carcinomas. In our previous published studies using D2-40 immunostaining for examination of the relationship between lymphatic invasion and lymph node (LN) metastasis, lymphatic invasion confirmed with D2-40 immunostaining was related to LN metastasis in various carcinomas, including uterine cervical carcinoma, uterine corpus carcinoma, esophageal carcinoma, and extra-mammary Paget's disease [1-5]. In our studies, lymphatic invasion using D2-40 immunostaining was diagnosed by the presence of carcinoma cells in the lymphatic lumen. Yonemura Y, et al. published an excellent study on lymphatic invasion using D2-40 immunostaining in gastric carcinoma, and they evaluated lymphatic invasion by carcinoma cells presenting in the lymphatic lumen [6]. Sako A, et al. examined lymphatic invasion using D2-40 immunostaining in 131 patients with early gastric adenocarcinoma (EGAC), in which carcinoma cells localize within mucosa or submucosa, who underwent gastrectomy with LN dissection, and they reported that adenocarcinoma cells invaded the wall and entered the lymphatic lumen in a lymphatic vessel of one case [7]. In the present study, we performed a detailed examination of lymphatic invasion using D2-40 immunostaining with the classification of lymphatic invasion by D2-40 immunostaining, consisting of intra-lumen type and invading type, in cases of EGAC with treatment by endoscopic submucosal dissection (ESD).

Procedures of ESD for EGAC have progressed, so ESD for the treatment of EGAC is selected in many cases at present. Compared with advanced gastric adenocarcinoma, the lymphatic structure is well-preserved in EGAC because advanced gastric carcinoma, in which carcinoma cells invade to the muscularis propria and subserosa, frequently leads to marked fibrosis, which obscures the lymphatic structure [8]. Moreover, the wall structure of the mucosa (lamina propria mucosae and muscularis mucosae) and submucosa is well-recognized in EGAC, compared with advanced gastric adenocarcinoma. Therefore, examination of EGAC lymphatic invasion using D2-40 immunostaining in ESD cases offers precise information on the entry and movement system of carcinoma cells in the lymphatic vessels of mucosa and submucosa, which has not been clearly defined. The recognition of this system offers useful information to advance understanding of the process of LN metastasis.

Materials and Methods

A total of 204 patients with EGAC who underwent ESD of the stomach between 2007 and 2014 in Juntendo University Nerima Hospital were examined. The patients consisted of 140 men and 64 women, and their ages ranged from 51 to 92 years (mean, 73 years). Among them, 23 patients received gastrectomy and LN dissection after ESD. Among the 15 patients with lymphatic invasion based on the pathological examination of ESD tissue, 11 patients were followed up for a few years or more than 5 years after ESD or following the operation performed after ESD.

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The ESD tissues were fixed with 10% formalin. After fixation of the tissues, serial 2-3-mm-thick sections from the entire ESD tissues were cut, and the cut sections were fixed with 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) using the routine method. Sections from selected paraffin blocks were stained with D2-40 immunostaining, using monoclonal mouse anti-human D2-40 (DAKO, Tokyo, Japan), with the LSAB method using XT Benchmark (Ventana, Yokohama, Japan). The antibody was used at a dilution of 1:100. Sections for D2-40 immunostaining were treated by incubating in EDTA buffer at 100o C for 60 min. After washing in 0.01 mol/L PBS, endogenous peroxidase activity was blocked by treating for 4 min with 3% aqueous hydrogen peroxidase. Visualization was performed with DAB (Dako Japan, Kyoto, Japan).

Classifications of the depth of invasion and type of adenocarcinoma were based on Japanese Classification of Gastric Carcinoma (15th edition) 2017 [9]. The depth of invasion consisted of pT1a (M) (mucosa, including muscularis mucosae), pT1b1 (SM1) (submucosa, <0.5 mm from muscularis mucosae), and pT1b2 (SM2) (submucosa, ≥ 0.5 mm from muscularis mucosae). The types of adenocarcinomas consisted of papillary adenocarcinoma (pap), tubular adenocarcinoma, well-differentiated (tub1), tubular adenocarcinoma, moderately differentiated (tub2), poorly differentiated adenocarcinoma, solid type (por1), poorly differentiated adenocarcinoma, non-solid type (por2), signet-ring cell carcinoma (sig), and mucinous carcinoma (muc). The well-differentiated adenocarcinoma group consisted of pap, tub1, and tub2 and the poorly differentiated adenocarcinoma group consisted of por1, por2, sig, and muc according to Gastric Cancer Treatment Guidelines 2021 [10]. Cases of adenocarcinoma with enteroblastic differentiation, hepatoid adenocarcinoma, and adenocarcinoma with the fundic gland type were excluded from the examination. The examined cases consisted of 167 pT1a (M) cases (tub1, 107 cases; pap+tub1, 31 cases; tub1+tub2, 14 cases; others, 15 cases), 16 pT1b1 (SM1) cases (tub1, 5 cases; tub1+tub2, 4 cases; pap+tub1, 3 cases; others, 4 cases), and 21 pT1b2 (SM2) cases (tub1+tub2, 7 cases; pap+tub1, 4 cases; tub2+por2, 4 cases; others, 6 cases). The 23 cases with gastrectomy and lymph node dissection after ESD consisted of pT1a (M) (2 cases), pT1b1 (SM1) (7 cases), and pT1b2 (SM2) (14 cases).

Retraction artifacts due to tissue shrinkage during fixation produce lymphatic-like space formation around the carcinoma cell nests, so the confirmation of lymphatic endothelium with positive D2-40 immunostaining around the spaces is important for the diagnosis of lymphatic invasion. Lymphatic invasion using D2-40 immunostaining is classified as intra-lumen type and invading type. In the intra-lumen type, carcinoma cells are present in the lymphatic lumen without destruction of lymphatic endothelium surrounding the lumen (Figure 1). In the invading type, carcinoma cells invade the lymphatic vessel with destruction of the lymphatic endothelium comprising the lymphatic wall structure, and enter the lymphatic lumen (Figure 2). The destruction hole becomes wider with an increase in the number of entering carcinoma cells (Figure 2C and 2D).

The degree of lymphatic invasion is usually evaluated by a grading system: ly0 (no lymphatic invasion), ly1 (minimal lymphatic invasion), ly2 (moderate lymphatic invasion), and ly3 (marked lymphatic invasion), according to the Japanese Classification of Gastric Carcinoma (15th edition) 2017 [9]. Evaluation of the grading system according to the number of lymphatic vessels with invasion was not performed in previous studies of EGAC. We reported on the usefulness of the grading system of lymphatic invasion according to the number of lymphatic vessels with invasion for the prediction of nodal metastasis in carcinoma of the uterine cervix [1]. Therefore, in the current study, based on our previous study of uterine cervical carcinoma, we devised a grading system of lymphatic invasion according to the number of lymphatic vessels with invasion: ly0, none; ly1, 1-3 lymphatic vessels; ly2, 4-9 lymphatic vessels, ly3, ≥ 10 lymphatic vessels. The number of lymphatic vessels with invasion was counted on entire glass slides stained with D2-40 immunostaining.

Lymphangiogenesis is not described in the Japanese Classification of Gastric Carcinoma (15th edition) 2017 [9], so the grading of lymphangiogenesis was made as follows: lyg0, status of lymphatic vessels being the same compared with non-neoplastic areas; lyg1, definite increase of lymphatic vessels compared with non-neoplastic areas and the absence of a dense aggregation of lymphatic vessels; lyg2, definite increase of lymphatic vessels compared with non-neoplastic areas and the presence of a dense aggregation of lymphatic vessels.

Statistical analysis of the relationship between lymphatic invasion and several factors, including depth of invasion, differentiation of adenocarcinoma, and lymphangiogenesis, was assessed using the chi-square test. Factors related to LN metastasis were examined using a logistic regression model in 23 patients receiving gastrectomy and LN dissection after ESD. Significant differences were accepted at P<0.05. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 28.0.

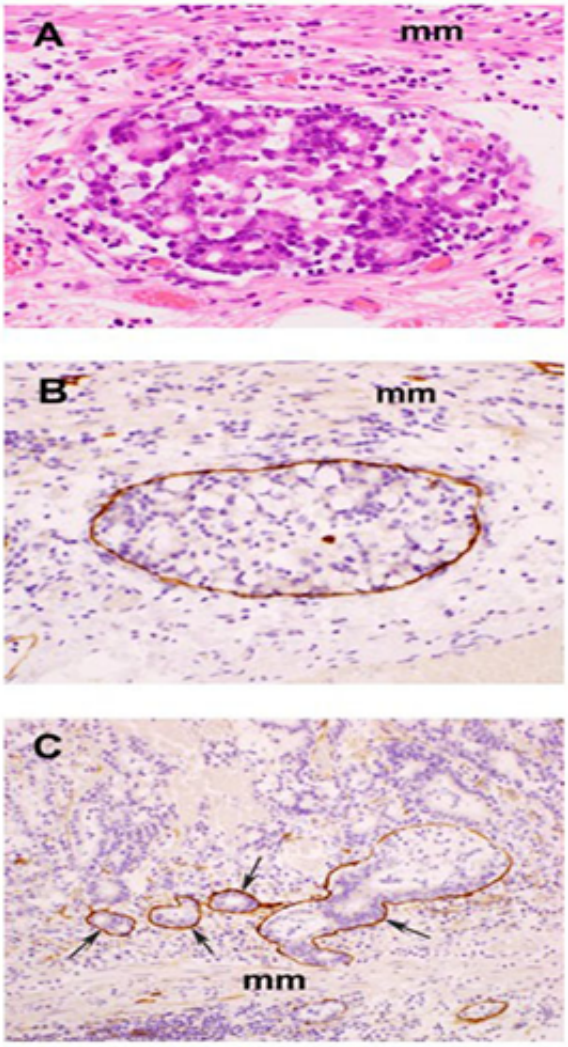

Figure 1. Lymphatic invasion of the intra-lumen type. **A,B)** Lymphatic invasion in a lymphatic vessel in the submucosa. The carcinoma cells are present in the lumen of a lymphatic vessel with a well-preserved wall structure. Note the positive staining in the endothelium of a lymphatic vessel by D2-40 immunostaining. A, H&E; B, D2-40 immunostaining. **C)** Lymphatic invasion in 4 lymphatic vessels (arrows) in the mucosa. The carcinoma cells are present in the lumen of the 4 lymphatic vessels. D2-40 immunostaining. mm: muscularis mucosae.

This was a retrospective study not interfering with diagnosis and patient management. This study was approved by the Research Ethics Committee of Juntendo University Nerima Hospital (no. 14-36).

Results

Lymphatic invasion was present in 15 cases (7.3%). Depth of invasion of carcinoma and status of lymphatic invasion including the layer of the invasion in the 15 cases with lymphatic invasion are shown in Table 1. Among the 15 cases, lymphatic invasion in the mucosa was present in 8 cases (53.3%): one case (case 1) had invasion in a mucosal lymphatic vessel (Figure 3) and 7 cases (cases 2-4, 6-9) had invasion in both mucosal and submucosal lymphatic vessels (Figure 4). In the 7 cases with lymphatic invasion of both mucosal and submucosal lymphatic vessels, the number of lymphatic vessels with invasion in the mucosa was greater than that in the submucosa in 2 cases (cases 2, 6), and the number of lymphatic vessels with invasion in the submucosa was greater than that in the mucosa in 5 cases (cases 3, 4, 7-9: Figure 4). In the 7 cases (cases 5, 10-15), lymphatic invasion was noted only in the submucosal lymphatic vessels (Table 1).

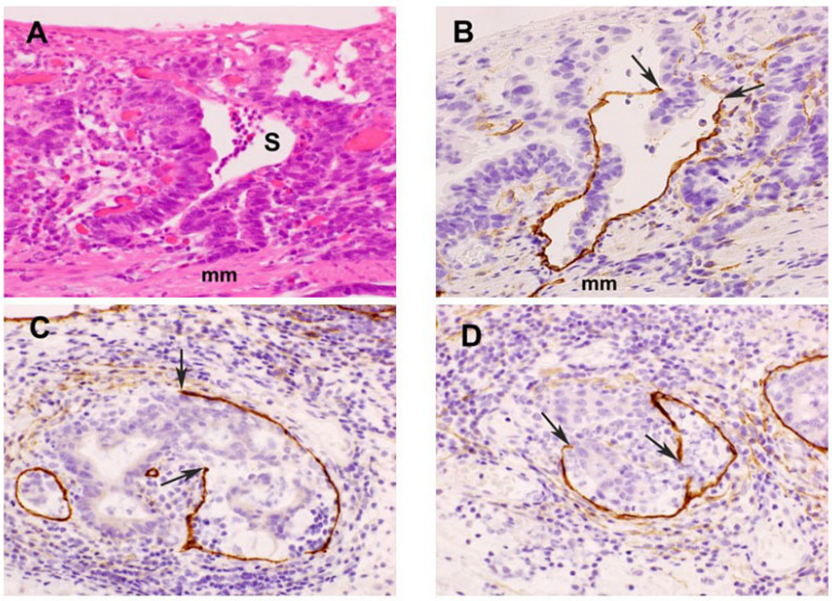

Figure 2. Lymphatic invasion of the invading type. **A,B)** Lymphatic invasion in a lymphatic vessel in the mucosa. A space (S) is present in the carcinomatous area, but the lymphatic vessel structure cannot be confirmed by H&E staining (A). B. The same site as A by D2-40 immunostaining. D2-40 immunostaining clarifies the lymphatic vessel structure, and the invading of carcinoma cells with destruction of the lymphatic wall into the lymphatic lumen is shown. Lymphatic endothelium around the destructive hole is indicated by arrows. mm: muscularis mucosae. **C,D)** Lymphatic invasion in a lymphatic vessel in the submucosa. The carcinoma cells invade through a destructive hole in the lymphatic wall into the lymphatic lumen. The destructive hole in C and D is wider than the hole in B. Lymphatic endothelium around the destructive hole is indicated by arrows. D2-40 immunostaining.

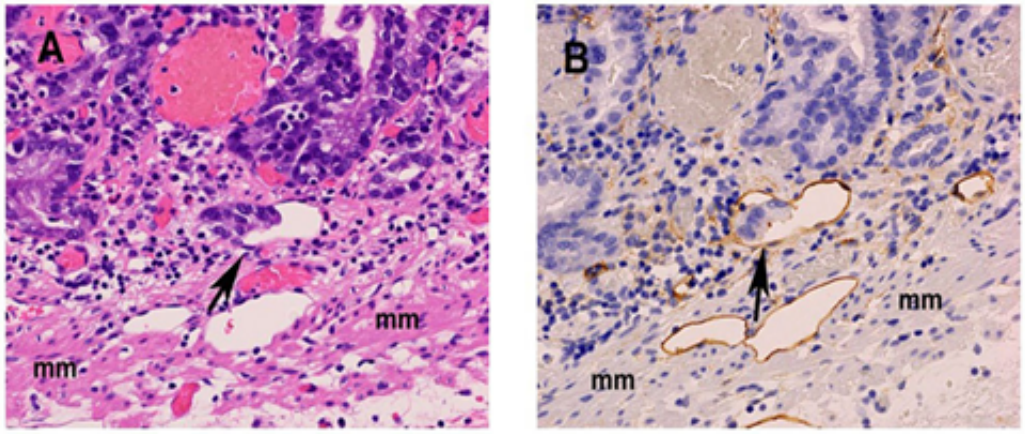

Figure 3. Lymphatic invasion of the intra-lumen type in the mucosa (case 1). A carcinoma cell nest is present in the lumen of a lymphatic vessel of the lamina propria mucosae (arrow). **A)** H&E and **B)** D2-40 immunostaining, mm: muscularis mucosae.

The intra-lumen type of lymphatic invasion was noted in all 15 cases. The invading type was present in 2 cases (a lymphatic vessel of the lamina propria mucosae in case 3 and lymphatic vessels of the lamina propria mucosae, muscularis mucosae, and submucosa in case 9). In case 9, the number of lymphatic vessels with the invading type in the submucosa was greater than that in the mucosa.

Lymphatic invasion was significantly correlated with the depth of invasion ($P<0.001$) and differentiation of adenocarcinoma ($P<0.001$). All 15 cases with lymphatic invasion showed lymphangiogenesis (lyg1 in 5 cases and lyg2 in 10 cases), and lymphatic invasion was significantly correlated with lymphangiogenesis ($P<0.001$).

Among the 15 cases with lymphatic invasion, 10 underwent gastrectomy and LN dissection. Carcinoma cells were not present in the resected stomach in any of the cases, and LN metastasis was noted in 3 cases (cases 3, 9, 10). Lymphatic invasion was significantly correlated with LN metastasis (Table 2, $P=0.007$), but the depth of invasion, differentiation of adenocarcinoma, and lymphangiogenesis were not significantly correlated with LN metastasis. A case (case 1) of mucosal adenocarcinoma, in which lymphatic invasion was present in a lymphatic vessel of the lamina propria mucosae, had been followed-up without an operation after ESD, and at more than 12 years after ESD, gastric carcinoma had not recurred.

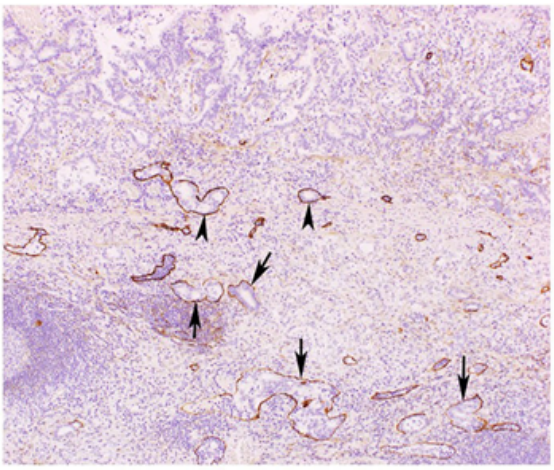

Figure 4. Lymphatic invasion of the intra-lumen type in the mucosa and submucosa (case 9). Lymphatic invasion in lymphatic vessels of the mucosa (arrowheads) and submucosa (arrows) is present. The number of lymphatic vessels with invasion in the submucosa is greater than that in the mucosa. D2-40 immunostaining.

Table 1. Depth of invasion of carcinoma and status of lymphatic invasion of 15 cases with lymphatic invasion.

Case	Depth of Invasion	Lymphatic Invasion (Layer of Invasion)
1	pT1a (M)	ly1 (m)
2	pT1b1 (SM1)	ly2 (m+sm, m>sm)
3	pT1b1 (SM1)	ly2 (m+sm, sm>m)
4	pT1b1 (SM1)	ly2 (m+sm, sm>m)
5	pT1b1 (SM1)	ly1 (sm)
6	pT1b2 (SM2)	ly2 (m+sm, m>sm)
7	pT1b2 (SM2)	ly3 (m+sm, sm>m)
8	pT1b2 (SM2)	ly2 (m+sm, sm>m)
9	pT1b2 (SM2)	ly3 (m+sm, sm>m)
10	pT1b2 (SM2)	ly1 (sm)
11	pT1b2 (SM2)	ly1 (sm)
12	pT1b2 (SM2)	ly2 (sm)
13	pT1b2 (SM2)	ly2 (sm)
14	pT1b2 (SM2)	ly1 (sm)
15	pT1b2 (SM2)	ly1 (sm)

Table 2. Relationship between lymphatic invasion and lymph node metastasis of 23 cases with gastrectomy and lymph node dissection after endoscopic submucosal dissection.

Lymph Node Metastasis	ly0	ly1	ly2	ly3
Absence	13 cases	4 cases	3 cases	0 case
Presence	0 case	1 case	1case	1 case

Discussion

The present study is the first trial of lymphatic invasion according to the classification consisting of intra-lumen type and invading type by D2-40 immunostaining in EGAC. To our knowledge, such studies have not been published in carcinomas of other organs. In the intra-lumen type, lymphatic invasion can be diagnosed by the detection of lymphatic endothelium with positivity by D2-40 immunostaining lining the lumen around the carcinoma cells. In the invading type, confirmation of the lymphatic structure using D2-40 immunostaining, which is shown in the figures (Figure 2B, 2C and 2D), is important. The recognition of various degrees of lymphatic wall destruction produced by carcinoma cells invading the lymphatic lumen, which is well-documented in the figures (Figure 2B, 2C and 2D), is needed for diagnosis of the invading type. The lymphatic invasion mainly consisted of the intra-lumen type, but diagnosis of the invading type increased the accuracy rate of lymphatic invasion. In daily pathological diagnosis, we evaluated lymphatic invasion, using D2-40 immunostaining, of endometrial adenocarcinoma in the uterine corpus according to our previous study, describing the relationship between lymphatic invasion and LN metastasis in endometrial adenocarcinoma in the uterine corpus [3]. Subsequently, we occasionally encountered cases of lymphatic invasion of the invading type. Thus, the diagnostic procedures for lymphatic invasion according to the classification consisting of intra-lumen type and invading type by D2-40 immunostaining can be applied to carcinomas of various organs.

Previous studies of EGAC demonstrated that lymphatic invasion was related to LN metastasis [11-13]. Similar to previous studies, lymphatic invasion was related to LN metastasis in the current study. Gastric Cancer Treatment Guidelines 2021 recommend gastrectomy and dissection of LN for cases of EGAC showing lymphatic invasion in ESD tissue [10]. The current study indicates that the evaluation of lymphatic invasion by the classification consisting of intra-lumen type and invading type increases the accuracy rate of lymphatic invasion, compared with evaluation of intra-lumen type alone, which leads to a more appropriate treatment strategy after ESD in EGAC.

In the study of Sako A, et al. [7], which is described in Introduction, they report that carcinoma cells enter the lymphatic lumen by the invading style and reach the regional LN. However, in their study, the site (mucosa or submucosa) of the lymphatic vessels with carcinoma cells entering the lumen was not described [7]. The classification of lymphatic invasion consisting of intra-lumen type and invading type offers the detailed information regarding the entry and movement of carcinoma cells in the lymphatic vessels in EGAC. The carcinoma cells enter the lymphatic vessels of both mucosal and submucosal lymphatic vessels based on the presence of lymphatic invasion of the invading type in both the mucosa and submucosa. A case with lymphatic invasion of the intra-lumen type in a lymphatic vessel of mucosa did not show recurrence of gastric carcinoma at more than 12 years after ESD. Seven cases showed lymphatic invasion of the intra-lumen type in both the mucosa and submucosa. In 7 cases, lymphatic invasion of the intra-lumen type was noted in the submucosa only. These results indicate that carcinoma cells entered from the mucosal lymphatic vessels remained in the mucosal lymphatic vessels or moved to the submucosal lymphatic vessels. The carcinoma cells in the lymphatic vessels of the submucosa consisted of carcinoma cells that moved from the mucosal lymphatic vessels and carcinoma cells entered from submucosal lymphatic vessels, or they consisted of carcinoma cells that entered from submucosal lymphatic vessels only. In addition, in our hospital, among the 11 cases of EGAC treated with gastrectomy and LN dissection, a case of mucosal adenocarcinoma with lymphatic invasion of intra-lumen type in a lymphatic vessel of the mucosa without carcinoma cells in lymphatic vessels of the submucosa did not show LN metastasis and was without recurrence of gastric carcinoma for more than 5 years after the operation (un-published data). This data supports the opinion that carcinoma cells entered from the mucosal lymphatic vessels remained in the mucosal lymphatic vessels.

In the current study, 10 cases with lymphatic invasion in the submucosa, in whom gastrectomy and LN dissection were performed, divided into non-metastatic group (7 cases) and metastatic group (3 cases). In both groups, carcinoma cells were not present in the resected stomach. We speculated that carcinoma cells in the submucosal lymphatic vessels remain in the submucosal lymphatic vessels or move to lymphatic vessels of deeper layers, i.e., the muscularis propria and subserosa, of the stomach, and flow to lymphatic vessels connected to the extra-stomach lymph node. The confirmation of this speculation is needed to do a detailed examination of the advanced gastric adenocarcinomas using the proposed classification of lymphatic invasion by D2-40 immunostaining.

Conclusion

The classification of lymphatic invasion consisting of intra-lumen type and invading type by D2-40 immunostaining is proposed in the present study. This classification offers an increase in diagnostic accuracy of lymphatic invasion, and it clarifies the entry and movement system of carcinoma cells in lymphatic vessels.

Recommendation for Future Studies

Authors recommend future studies using the proposed classification of lymphatic invasion by D2-40 immunostaining on the carcinomas in various organs.

Author Contribution

The authors are responsible for the study design, data collection, data analysis, and preparation of the article.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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