Acceleration of the Proliferative Activity of Esophageal Carcinoma with Invasion beyond the Muscularis Mucosae; Immunohistochemical Analysis Using MIB-1 for the Ki-67 Antigen

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Squamous cell carcinoma of the esophagus with cancer invasion beyond the muscularis mucosae is known to have lymph node metastasis and lymphatic or blood vessel invasion compared with intramucosal carcinoma. In submucosal and T2-3 carcinoma, lymph node and lymphatic/vascular involvement are shown more frequently, leading to a poor prognosis. Therefore, we examined proliferative activity of esophageal squamous cell carcinoma including early carcinoma in relation to clinicopathological findings. 77 cases of esophageal squamous cell carcinoma, including 23 cases of mucosal carcinoma (Tis+T1a), 35 cases of submucosal carcinoma (T1b) and 19 cases of advanced invasive carcinoma (T2+T3) undergoing surgical resection without preoperative treatment were studied using monoclonal antibody MIB-1 for Ki-67 antigen immunohistochemically, and the labeling index (LI) was calculated. The LI of MIB-1 positive nuclei correlated with the depth of cancer invasion was significantly increased in the cancer invading beyond the musculais mucosae. The LI at the invasive tip was significantly higher than that at the core of differentiated carcinoma. The LI values at both invasive tip and core of poorly differentiated carcinoma were higher than those of differentiated carcinoma with significant difference. The LI at the invasive tip of the carcinoma with lymph node metastasis or lymphatic invasion was significantly higher than that without them. Proliferative activities of esophageal cancer cell, immunostaining with MIB-1, had correlations to depth of tumor invasion, differentiation, lymph node metastasis and lymphatic invasion with significant difference. But if invading deeper than m3, the proliferative activity did not increase anymore.

Key words: esophageal squamous cell carcinoma, tumor proliferation, monoclonal antibody MIºB-1, immunohistochemistry

INTRODUCTION

Squamous cell carcinoma is the most common malignant tumor occurring in the esophagus. In the vast majority of cases, it is diagnosed at an advanced stage and is consequently characterized by a very poor prognosis because of rapid continuous tumor extension beyond the esophageal wall [1,2]. Many clinicopathological studies have been performed on advanced esophageal cancers. Similarly to other epithelial malignancies, however, esophageal carcinogenesis involves a multistage progression from normal mucosa to dysplasia, to carcinoma *in situ*, and subsequently to early and advanced invasive cancer [3,4]. We have clinicopathologically examined a number of surgically resected advanced and superficial esophageal cancers including early carcinoma, and analyzed their tumor growth, progression and prognosis [5-9].

Detection of superficial esophageal carcinoma has been increased recently along with the progress in diagnostic techniques [10, 11]. In Japan, it has been proposed that mucosal and submucosal esophageal carcinoma should be classified by the depth of invasion into the following 6 types: m1: carcinoma confined to the epithelium or extending slightly beyond the basement membrane (Tis); m2: carcinoma invading the lamina propria mucosae but not the muscularis mucosae (T1a); m3: carcinoma in contact with the muscularis mucosae (T1a); sm1: carcinoma minimally invading the upper submucosa (T1b); sm2: carcinoma with definite invasion of the submucosa (T1b); sm3: carcinoma invading the deep submucosa (T1b) (Fig. 1) [11]. It has been reported that lymph node metastasis and lymphatic or blood vessel invasion are uncommon in m1 or m2 carcinoma and occur after progression to m3 or sm1 disease [6, 11]. In sm2 and sm3 carcinoma, lymph node, lymphatic/vascular involvement occur more frequently, leading to a poor prognosis [6].

Various monoclonal antibodies have been developed and used to monitor the cell cycle and the proliferative activity of cancer cells [12,13]. Monoclonal antibody MIB-1 recognizes the Ki-67 antigen, which is expressed exclusively in the nuclei of proliferating cells (i.e., cells in the G1, S, G2, and M phases) [14]. MIB-1 can be

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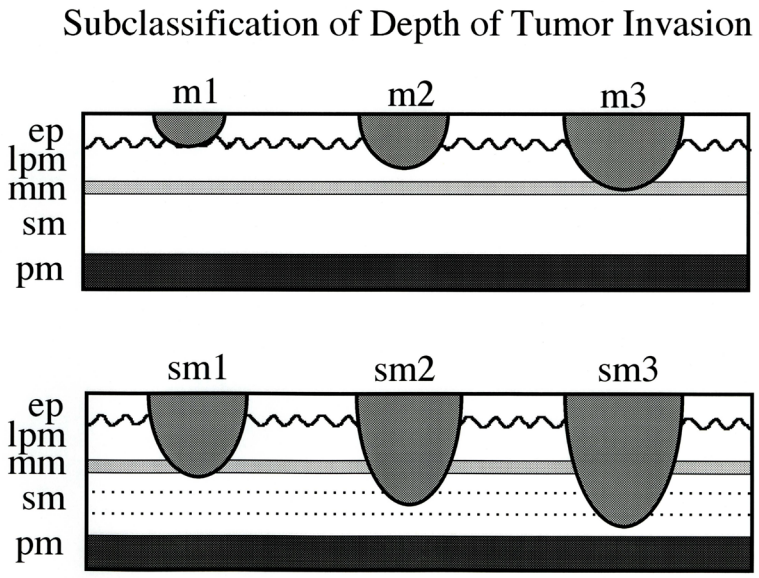

Fig. 1. Tis and T1 superficial carcinoma of the esophagus were divided into six subclassification as follows; m1 carcinoma limited to the epithelium; m2 carcinoma invading into the lamina propria mucosae; m3 carcinoma extending down to the muscularis mucosae; sm1 carcinoma minimally invading into the upper submucosa; sm2 carcinoma with definite invasion into to the middle submucosa; sm3 carcinoma massively invading into the deep submucosa

* : Core of the tumor
** : Invasive tip of the tumor

ep : epithelium
lpm : lamina propria mucosae
mm : muscularis mucosae
sm : submucosa
mp : muscularis propria

Table 1. Histopathological findings of esophageal squamous cell carcinoma

Depth of Invasion	No. of Cases	Differentiation			n		ly		v	
		Well	Mod.	Poorly	(-)	(+)	(-)	(+)	(-)	(+)
m1	6	–	–	–	6	0	6	0	6	0
m2	8	2	5	1	8	0	8	0	8	0
m3	9	3	5	1	8	1	3	6	6	3
sm1	12	1	9	2	7	5	3	9	12	0
sm2	11	2	6	3	4	7	3	8	7	4
sm3	12	5	6	1	6	6	4	8	7	5
T2 (mp)	8	4	3	1	3	5	2	6	5	3
T3 (ad)	11	6	3	2	2	9	2	9	0	11

Mod., moderately differentiated; **n, lymph node metastasis; ly, lymphatic** invasion; **v, blood vessel invasion; -, absent; +, present; m, mucosal carcinoma;** sm, submucosal carcinoma; **mp, muscularis propria; ad, adventitia.**

applied to paraffin-embedded sections after heating to invigorate the target antigen [15, 16].

In the present study, the proliferative activity of esophageal carcinoma including early carcinoma was assessed its clinicopathological significance using immunostaining with the monoclonal antibody MIB-1.

MATERIALS AND METHODS

Esophageal tissue specimens. All tissue specimens were obtained at surgical resection of esophageal squamous cell carcinoma at Tokai university Hospital. Seventy-seven patients with esophageal cancer were included in this study (74 men and 3 women); ranging in age from 45 to 73 (mean, 58.4) years. All esophageal specimens were rapidly fixed in 10% buffered formalin for histological and immunohistochemical analyses for 14-48 h, and routinely embedded in paraffin. The tumor invasion, venous invasion and lymphatic invasion were examined on 4μm thick sections stained with hematoxylin and eosin. Esophageal cancers we examined were classified according to the Japanese Guidelines for Clinical and Pathological Studies of the Esophagus (17). Also, we sub-classified Tis and T1 tumors (superficial carcinomas) into six groups as follows: (m1) carcinoma confined to the epithelium; (m2) carcinoma invading into the lamina propria mucosae; (m3) carcinoma in extending down to the muscularis mucosae; (sm1) carcinoma minimally invading into the upper submucosa; (sm2) carcinoma with definite invasion into the submucosa; (sm3) carcinoma massively invading the deep submucosa [6, 7, 11) (Fig. 1). The m1 cases were carcinoma in situ (Tis), m2 to m3 were intramucosal carcinomas (T1a), and sm1 to sm3 were submucosa invasive carcinomas (T1b). The depths of superficial carcinoma were as follows; m1 in 6 patients, m2 in 8 patients, m3 in 9 patients, sm1 in 12 patients, sm2 in 11 patients, sm3 in 12 patients, T2 in 8 patients and T3 in 11 patients. The histopathological characteristics (histological differentiation, lymph node metastasis, and lymphatic or blood vessel invasion) of the carcinomas are shown in Table 1.

Immunohistochemical analysis. Sections 4μm thick were deparaffinized, and endogenous peroxidase activity was quenched by incubation in 0.3% H_2O_2 in methanol for 30 min. Sections were microwaved (H2500, Energy Beam Sciences Inc., Agawan, MA) in 0.01M citrate buffer, pH 6.0, for 20 min for antigen retrieval (18). Nonspecific binding was blocked with normal sheep serum (Cosmo Bio Co.Ltd., Tokyo, Japan) in phosphate-buffered saline (PBS), then slides were incubated with a 100-fold dilution of the monoclonal

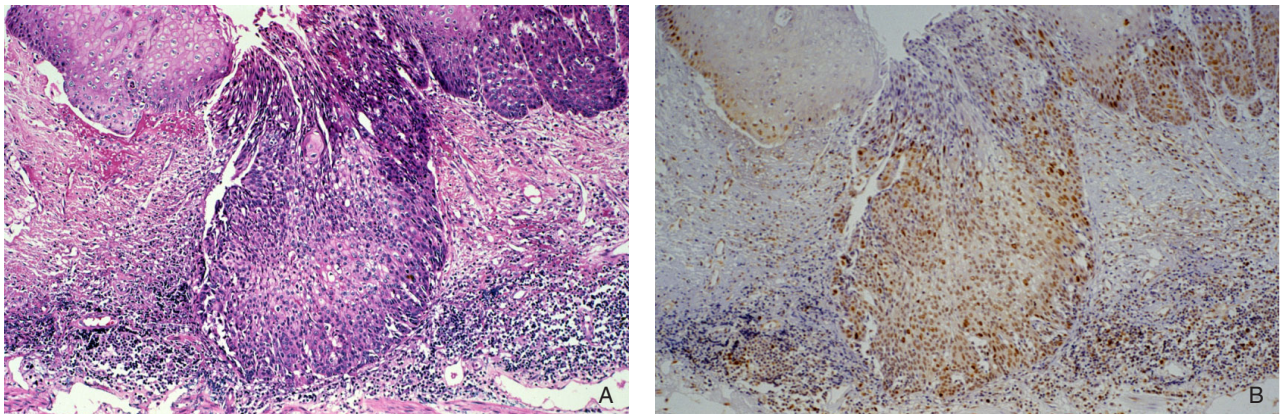

Fig. 2. Microscopic findings of MIB-1 positive esophageal squamous cell carcinoma. The esophageal carcinoma invaded the esophageal wall (A, hematoxylin and eosin, x25). Immunoreactivity of MIB-1 antibody was detected in the nuclei of carcinoma cells (B, LSAB method, x25).

Table 2. Correlation between Depth of Invasion and MIB-1 Labeling Index (n=77)

Depth of Invasion	No.of cases	Labeling Index (%) Invasive Tip		Core
m1	6	20.3± 6.3		—
m2	8	23.1± 3.4	*	—
m3	9	38.1± 9.5	*	—
sm1	12	36.5± 9.7	*	21.7±11.3
sm2	11	39.7±10.3	**	27.1±11.7
sm3	12	35.5±10.5	*	22.5±11.7
T2(mp)	8	36.3±6.8	*	20.9±6.9
T3(ad)	11	37.8±7.3	*	24.8±10.4

* p<0.01, ** p<0.05

antibody MIB-1 (IMMUNOTECH S.A., Marseille Cedex, France). Immunoreactivity was detected by the peroxidase-labeled streptavidin biotin (LSAB) method (DAKO A/S, Copenhagen, Denmark) [19]. Slides were subsequently incubated with a biotinylated anti-mouse Ig$(Fab)_2$ antibody (Amersham International Plc., Buckinghamshire, UK) at 1:100 for 60 min, followed by detection using streptavidin-conjugated horseradish peroxidase.

Assessment of Ki-67 expression. The tumor cells with nuclei containing brown immunoreactive products were counted as positive for Ki-67 expression. The site for assessment of proliferative activity was selected by examining hematoxylin-eosin stained sections by microscopic observation at a magnification of 40x (Fig. 2A). Using the adjacent serial section, tumor cells with nuclei containing brown reaction products were counted as positive (Fig. 2B). The MIB-1 labeling index (LI) was calculated as the number of positive nuclei per 1,000 nuclei counted. The growing edge of the carcinoma is defined as the invasive tip of the tumor. Proliferative activity was assessed at invasive tip for the mucosal carcinomas (Tis+T1a) and at both invasive tip and core of the nest for the invasive carcinomas(T1b, T2, T3).

Statistical analysis. The relationships between the LI of MIB-1 and various histopathological prognostic factors was assessed. Data are express as the mean ± standard deviation (SD). Student's t-test was used for comparisons between group frequencies. Differences were considered to be significant at $P<0.05$.

RESULTS

LI and histological invasion

The LI of MIB-1 for m3 carcinoma was significantly higher than that for m1 and m2 carcinoma, but was not significantly different from the values for T1b (sm1-3), T2 or T3 carcinomas (Table 2).

LI values at different tumor sites

The LI values obtained from the invasive tip and the core of the carcinoma beyond submucosal invasion were compared. The mean LI at the invasive tip was significantly higher than that in the core for 54 patients with T1b, T2 and T3 carcinomas (Table 2).

LI and tumor differentiation

The degree of differentiation was determined in 71 patients with T1a, T1b, T2 and T3 carcinoma and its relationship to the LI of MIB-1 was studied. For well and moderately differentiated carcinoma, the LI at the invasive tip was significantly higher than that in the core, while no apparent difference was observed for poorly differentiated carcinoma. The LI values at both the invasive tip and the core of poorly differentiated carcinoma were significantly higher than the respective values for well or moderately differentiated carcinoma (Table 3).

Table 3. Correlation between Histological Differentiation and MIB-1 Labeling Index (n=71)

Histological Differentiation	No.of cases	Labeling Index (%) Invasive Tip	Core
Well differentiated	23	35.6±7.1 —*—	20.2±5.7
Mod. differentiated	27	32.9±9.8 —*—	19.4±7.1
Poorly differentiated	11	45.8±8.8	42.3±7.3

* p<0.01

Table 4. Correlation between Lymph Node Metastasis, Lymphatic Invasion, Blood Vessel Invasion and MIB-1 Labeling Index of the Invasive Tip (n=71)

		No.of cases	Labeling Index of Invasive Tip (%)	
n	(-)	30	33.2± 9.5	*
	(+)	33	41.0± 7.4	
ly	(-)	17	31.3± 9.2	*
	(+)	46	39.5± 8.4	
v	(-)	37	35.9±10.0	
	(+)	26	38.7± 8.2	

* p<0.01

LI and lymph node metastasis

The LI values in the presence and absence of lymph node metastasis were studied in m3 and T1b, T2, T3 carcinomas which were considered to be potentially associated with nodal involvement. The LI at the invasive tip was significantly higher for the patients with lymph node metastasis than that without lymph node metastasis. (Table 4).

LI and lymphatic invasion

The LI values in the presence and absence of lymphatic invasion were also studied in m3 and T1b, T2, T3 carcinoma. The LI at the invasive tip of the carcinoma with lymphatic invasion was significantly higher than that without lymphatic invasion (Table 4).

LI and blood vessel invasion

Finally, the LI values in the presence and absence of blood vessel invasion were compared for m3 and T1b, T2, T3 carcinoma. The LI between the cases with blood vessel invasion and without blood vessel invasion showed no significant difference (Table 4).

DISCUSSION

We examined proliferative activity in 77 cases of human esophageal squamous cell carcinoma, including early-stage cancers, using monoclonal antibody MIB-1 for Ki-67 antigen immunohistochemically and analyzed its clinicopathological significance.

The Japanese Society for Esophageal Disease has proposed that Tis/T1 tumors (superficial esophageal carcinomas) should be classified into six sub-types based on the depth of invasion [6, 7, 11]. According to this classification, we previously analyzed the clinicopathological characteristics of esophageal carcinomas [6, 11]. Lymph node metastasis or lymphatic/venous invasion was uncommon in m1/m2 carcinomas and was infrequently found in m3/sm1 carcinomas. The sm2/sm3 and T2/T3 carcinomas more frequently showed lymph node metastasis or lymphatic/venous invasion, leading to poor prognosis. Many investigators have suggested that the depth of invasion is closely related to the incidence of lymph node and vascular involvement and is an important factor to be considered in determining the therapeutic approach [6,11]. Mucosal carcinoma classified as m1-m2 is usually treated by endoscopic mucosal resection [6,11], which is less invasive than open surgery. However, esophageal carcinoma with invasion beyond the mucosae requires radical surgical resection by thoracotomy and laparotomy.

Proliferating cells are in G1, S, G2, or M phase of the cell cycle, while non-proliferating cells are in G0 phase. In 1983, Gerdes et al. developed a monoclonal antibody that reacted with Ki-67 antigen [20, 21]. The antibody can identify proliferating cells (i.e., cells in G1, S, G2, and M phases) by recognizing this antigen, which is exclusively expressed in the nuclei of such cells. Ki-67 immunostaining has already been widely applied to other organ cancers, and the prognostic significance of this marker has been well documented. However, the application of this technique is restricted to fresh frozen sections. In contrast, the MIB-1 antibody that we used in the present study can be applied to paraffin-embedded sections after heating to enhance the antigenicity of Ki-67. Immunostaining with MIB-1 has a high reproducibility as the staining pattern is independent of the method of fixation and shows little variability [16]. Therefore, MIB-1 appears to be suitable for the quantitative assessment of cell proliferation in esophageal carcinoma.

In the normal esophagus, immunostaining with MIB-1 was predominantly seen exclusively in the basal layer of the epithelium, while the superficial layer is not stained. Cells in the superficial layer are better differentiated, parakeratinized, and have lost their nuclei, while the basal layer consists of proliferating cells [22]. The basal layer of the epithelium is proliferative zone of the esophagus. Therefore, Ki-67 expression may be closely related to the proliferative activity of esophageal cells. This manuscript shows the analysis of the superficial cancer with adding T2 and T3 advanced

cancer. Previous analysis on superficial cancer showed the significant increase of MIB-1 LI in the cancer invading deeper than m3, compared to m1, m2 [7, 23]. But the present study beyond m3 did not show the significant increase of LI according to the depth level. The proliferation of m3 to T3 was shown to be similar. The significantly higher proliferative activity of m3 and more advanced carcinoma compared to m1 or m2 carcinoma may contribute to the increase of lymph node, lymphatic, or blood vessel invasion due to accelerated tumor growth. These results suggest that m3 carcinoma may be borderline between treatments by endoscopic mucosa resection or radical open surgery, and should remain for further studies in more details in the future.

The LI at the invasive tip of the carcinoma was higher than that of the core. Esophageal carcinoma usually undergoes differentiation and keratinization towards the center of the tumor focus. This finding is supported by our result that the invasive tip showed significantly higher proliferative activity than the core of the tumors.

Proliferative activity was also analyzed in relation to tumor differentiation. The LI at the invasive tip of well to moderately differentiated carcinoma was significantly higher than that in the core. In contrast, proliferative activity did not differ significantly between the invasive tip and core of poorly differentiated carcinoma. Thus, tumor cells in poorly differentiated carcinoma had a higher proliferative activity compared to the both regions of differentiated carcinoma. Some authors have found no difference of LI in relation to the degree of differentiation [24], while others have reported a higher positive rate in poorly differentiated carcinoma compared to differentiated carcinoma [7, 25].

Regarding the relationship between proliferative activity and lymph node metastasis, some studies on esophageal carcinoma have also shown that the LI of MIB-1 differs significantly between tumors with and without lymph node metastasis [7, 24]. In the present study, the LI with lymph node metastasis was significantly higher than that of tumors without nodal involvement. It has been reported that significant prognostic factors for esophageal carcinoma include the presence of lymph node involvement and the number of the metastatic lymph nodes. Therefore, the LI for MIB-1 may be a useful prognostic factor when determining the therapeutic approach for esophageal carcinoma, since a high LI value may be predictive of lymph node metastasis.

The LI data for the carcinomas with and without lymphatic or blood vessel invasion were analyzed. Previous study showed that positive rate for MIB-1 had significant correlation with the degree of lymphatic invasion [7, 23]. In the present study, the LI of tumors with lymphatic invasion was significantly higher than that of tumors without it, although no significant difference between tumors with and without blood vessel invasion. This suggests that a high proliferative activity is predictive of lymphatic involvement, and the LI of MIB-1 may be a useful prognostic factor.

Ki-67 expression as identified by immunostaining with monoclonal antibody MIB-1 may indicate the grade of malignancy of esophageal squamous cell carcinoma. Immunostaining with MIB-1 may allow histological grading of esophageal carcinoma and provide a useful guide to the selection of cancer therapy.

CONCLUSIONS

The correlation between histopathologic characteristics and proliferative activity of esophageal carcinoma was investigated. Proliferative activities of cancer cells in esophageal squamous cell carcinoma, immunostaining with the MIB-1, were related to the depth of invasion, differentiation, lymph node metastasis and lymphatic invasion with statistical significance. Cancer cell proliferative activity was accelerated in esophageal carcinoma with cancer invading beyond the muscularis mucosae. The significantly higher proliferative activity of m3 carcinoma compared to m1 or m2 carcinoma may contribute to the increase of lymph node metastasis and lymphatic invasion due to accelerated tumor proliferation. But if invading deeper than m3, the proliferative activity did not increase anymore.

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