Evaluation of an Indicator for Lymph Node Metastasis of Esophageal Squamous Cell Carcinoma Invading the Submucosal Layer

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Lymph node metastasis is a major prognostic factor for esophageal squamous cell carcinoma (ESCC). In recent years, endoscopic mucosal resection (EMR) has been developed with excellent results for the treatment of the superficial ESCC. To make the EMR treatment successful, it is important to establish a good indicator to identify ESCC patients at a high risk of lymph node metastasis. In this study, we examined clinicopathological and immunohistochemical factors to investigate the factors involved in lymph node metastasis of ESCC invading to the submucosal layer (sm-ESCC). Surgical specimens from 84 sm-ESCC patients were examined. Among 84 sm-ESCC patients, 33 (39.3%) had lymph node metastases. Clinicopathologically, tumor depth, lymphatic invasion and blood vessel invasion showed significant correlations with lymph node metastasis by univariate analysis. Tumor depth and lymphatic invasion showed significant correlations by multivariate analysis of these factors. Immunohistochemically, P53 accumulation was observed in 45 cases (53.6%), cyclin D1 overexpression in 25 (29.8%), and pRB in 65 (77.4%). P53 accumulation, cyclin D1 overexpression and MIB-1 Labeling Index were significantly associated with lymph node metastasis by univariate analysis, and P53 accumulation showed a significant correlation with lymph node metastasis by multivariate analysis. Among tumor depth, lymphatic invasion and P53 accumulation, tumor depth and lymphatic invasion were significantly correlated with lymph node metastasis ($P=0.0023$ and $P=0.0092$, respectively) by multivariate analysis. These data suggest that tumor depth and lymphatic invasion can be considered as good indicators for lymph node metastasis among patients with sm-ESCC. In addition, P53 accumulation could be helpful to identify the patients who need additional treatment after EMR.

Key words: Esophageal squamous cell carcinoma — Submucosal layer — Lymph node metastasis — P53

The prognosis for esophageal squamous cell carcinoma (ESCC) remains unsatisfactory, despite the recent advances in diagnosis and treatment. This is partly because ESCC with lymph node metastasis frequently develops recurrence even after curative resection. Currently, chemotherapy and/or radiation therapy are performed for the recurrence of ESCC, but the outcome is not satisfactory. Therefore, the status of lymph node metastasis at the operation is a major prognostic factor after surgical treatment for ESCC.[1]

It has been reported that only 1–5% of ESCC patients in whom ESCC remains within the mucosal layer exhibit lymph node metastasis. However, 30–50% of ESCC patients exhibit lymph node metastasis when the carcinoma cells have invaded the submucosal layer.[2–6] In the course of progression, carcinoma cells change their genetic characteristics. It is possible that genetic changes occur during invasion from the mucosal layer to the adventitia in ESCC. From this point of view, investigation of factors involved in lymph node metastasis of ESCC invading to the submucosal layer (sm-ESCC) could lead to the identification of potent factors regulating lymph node metastasis of ESCC.

In recent years, endoscopic mucosal resection (EMR) for the treatment of mucosal ESCC has been developed.[7–9] This technique is beneficial because it is safe, easy, and above all, esophagus-preserving. As an indication for EMR, the detection of lymph node metastasis is critical. Even though the use of computed tomography and endoscopic ultrasonography is helpful to diagnose lymph node metastasis, it is not possible to predict the future risk for lymph node metastasis. Therefore, it is important to establish a good indicator to predict ESCC patients with a high risk of lymph node metastasis, using sections from the primary lesion.

Therefore, in this study, we examined clinicopathological factors and the status of P53, cyclin D1, pRB, and MIB-1 immunohistochemically to investigate the factors involved in lymph node metastasis of sm-ESCC.

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PATIENTS AND METHODS

Patients The subjects of this study were 84 sm-ESCC patients who underwent esophagectomy with lymph node dissection at the Tokyo Medical and Dental University Hospital between 1985 and 1995. Their informed consent for the study was obtained. The ages of the 75 male and 9 female patients ranged from 34 to 78 years (mean±SD, 62.5±9.0 years), and none of them had received chemotherapy and/or radiation therapy prior to surgery. Twenty-eight tumors were located in the upper third of the esophagus, 42 in the middle third, and 14 in the lower third.

Histopathological examinations Histopathological examination was performed on the basis of the Guide Lines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus.[10] The depth of invasion of sm-ESCC was subclassified into 3 groups according to the following criteria: sm1, carcinoma invasion beyond the muscularis mucosae with invasion to the shallow strata (upper one-third) of the submucosal layer; sm2, carcinoma invasion into the middle strata (middle third) of the submucosal layer; sm3, massive carcinoma invasion into the lower strata (lower third) of the submucosal layer (Fig. 1).[2, 11–13]

Immunohistochemical staining Immunohistochemical staining for P53, MIB-1, pRB, and cyclin D1 was performed on serial sections containing the most invasive lesion in the tumors of all 84 patients. Anti-P53 monoclonal antibody (DO-7, DAKO, Glostrup, Denmark) was used at a dilution with 1:100, cyclin D1 (DCS-6, DAKO) 1:1000, pRB (G3-245, Pharmingen, San Diego, CA) 1:500, and anti-MIB-1 (Immunotech, Marseille, France) 1:50. Immunohistochemical staining was performed by the avidin-biotin-peroxidase complex method, using a Histofine SAB-PO (M) detector kit (Nichirei Co., Tokyo). In brief, paraffin-embedded specimens were sliced 3 μm thick, deparaffinized in xylene, then rehydrated through graded alcohol on glass slides. For P53 staining, the sections were heated in a microwave oven in 0.01 M citrate buffer (pH 6.0) at 100°C for 15 min. For cyclin D1, pRB and MIB-1 staining, sections were treated by heating at 121°C for 10 min in an autoclave. Following 30 min incubation in 0.3% H_2O_2 in 100% methanol to inhibit endogenous peroxidase activity, the sections were incubated in normal goat serum to reduce nonspecific binding. The sections were incubated overnight with the primary antibody at 4°C, and then incubated in biotinylated goat anti-mouse IgG at room temperature for 10 min. Following 3 washes with phosphate-buffered saline (PBS), streptavidin-biotin-peroxidase complex was applied and the sections were visualized using diaminobenzidine and counterstained with hematoxylin. Normal mouse serum was used as a negative control for the primary antibody.
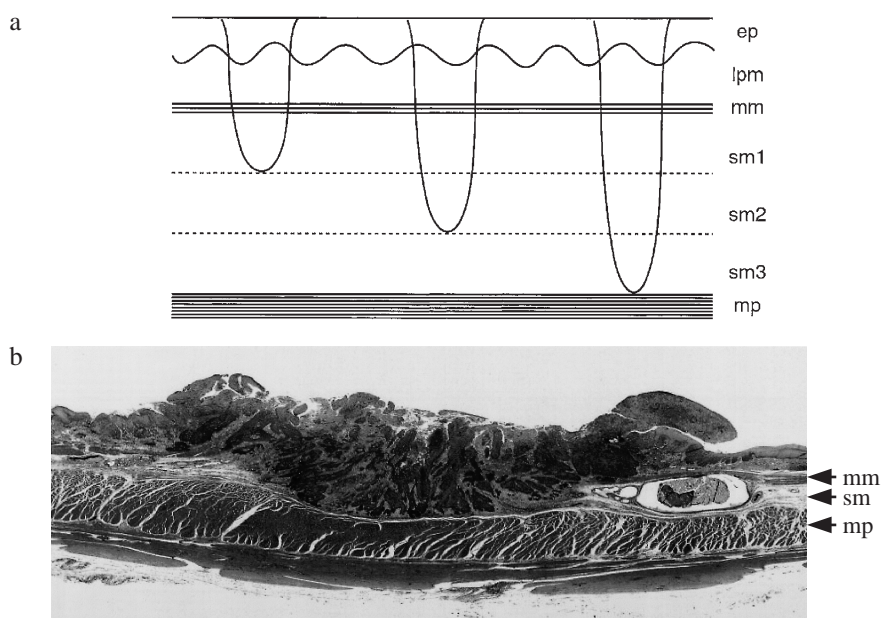

Fig. 1.   (a) Schema of the subclassification of esophageal squamous cell carcinoma invading the submucosal layer. sm1, carcinoma invasion beyond the muscularis mucosae with invasion to the shallow strata (upper one-third) of the submucosal layer; sm2, carcinoma invasion into the middle strata (middle third) of the submucosal layer; sm3, massive carcinoma invasion into the lower strata (lower third) of the submucosal layer. (b) The microscopic finding of a tumor invading sm3. Hematoxylin and eosin stain (original magnification, ×2). ep, the mucosal epithelium; lpm, the lamina propria; mm, the muscularis mucosae; mp, the muscularis propria.
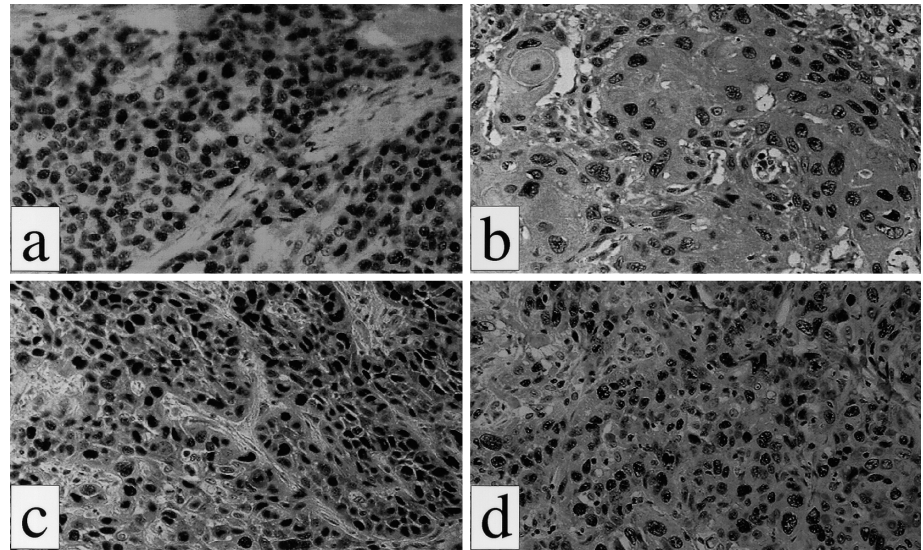
Fig. 2. Immunostainings with each antibody used in this study. (a) P53, (b) cyclin D1, (c) pRB, and (d) MIB-1 (original magnification, ×200).

Evaluation Protein expression was evaluated mostly at the invasive edge in the submucosal layer. For P53 and cyclin D1 staining, when more than 10% of the carcinoma cells were distinctly positive for nuclear staining, the specimen was scored as positive (Fig. 2, a and b).[14, 15] For pRB staining, when strong nuclear staining was recognized in fewer than 10% of the carcinoma cells, or when all carcinoma nests had 10–25% of pRB-positive staining cells showing a scattered staining pattern, the specimen was scored as negative (Fig. 2c).[16] MIB-1 Labeling Index was calculated as the number of distinct positive cells divided by the total number of examined cells (Fig. 2d). For the evaluation, more than 2000 carcinoma cells were counted by 2 experienced observers who were masked as to the clinical outcome. The results were compared, and any discrepancies were resolved by discussion.

Statistical evaluation Univariate analysis was performed by applying the χ^2 test and unpaired Student's t test. The values of Age and MIB-1 Labeling Index were expressed as the mean±SD. Multivariate analysis was performed by logistic regression analysis. All the statistical evaluations were performed by Stat View 5.0 for Macintosh (HULINKS Inc., Tokyo). $P<0.05$ was considered statistically significant.

RESULTS

Correlations between lymph node metastasis and clinicopathological findings Among our 84 sm-ESCC patients, 33 (39.3%) had lymph node metastasis. Table I shows the clinicopathological findings of the primary tumors. Sex, age, and tumor location showed no association with lymph node metastasis. Histopathologically,

univariate analysis showed that tumor depth, lymphatic invasion, and blood vessel invasion were significantly correlated with lymph node metastasis ($P<0.0001$, $P<0.0001$, and $P=0.0037$, respectively). Univariate analysis of clinicopathological factors showed that tumor depth was significantly correlated with lymphatic invasion and blood vessel invasion ($P=0.0002$ and $P<0.0001$, respectively, data not shown). Multivariate analysis of tumor depth, lymphatic invasion, and blood vessel invasion showed that tumor depth and lymphatic invasion were significantly correlated with lymph node metastasis ($P=0.0031$ and $P=0.0116$, respectively). These results suggested that the deeper the carcinoma cell invasion in submucosal layer, the higher the risk of lymphatic and blood vessel invasion and lymph node metastasis, and that tumor depth and lymphatic invasion could become good indicators to predict lymph node matastasis of sm-ESCC.

Correlation between lymph node metastasis and immunohistochemical findings Immunohistochemical examinations of P53, cyclin D1, pRB, and MIB-1 were performed for 84 sm-ESCC specimens. P53 accumulation was found in 45 cases (53.6%), and cyclin D1 overexpression was observed in 25 cases (29.8%). Positive immunostaining for pRB was observed in 65 cases (77.4%). The mean±SD value of MIB-1 Labeling Index was 34.37±12.65. Table II shows the correlations between lymph node metastasis and the immunohistochemical findings. Univariate analysis showed that P53 accumulation, cyclin D1 overexpression, and MIB-1 Labeling Index were significantly correlated with lymph node metastasis ($P=0.0046$, $P=0.0412$, and $P=0.0483$, respectively). Univariate analysis between clinicopathological factors and immunohistochemical findings showed that P53 accumu-

Table I. Relationship between Lymph Node Metastasis and Clinicopathological Findings

	Lymph node metastasis positive (n=33, %)	Lymph node metastasis negative (n=51, %)	Univariate analysis	Multivariate analysis		
			P value	P value	Odds ratio	95% confidence intervals
Sex			0.7374	not analyzed		
Male	29 (87.9)	46 (90.2)				
Female	4 (12.1)	5 (9.8)				
Age	61.79±10.38	62.90±8.10	0.5835	not analyzed		
Tumor location			0.5227	not analyzed		
Upper	13 (39.4)	15 (29.4)				
Middle	14 (42.4)	28 (54.9)				
Lower	6 (18.2)	8 (15.7)				
Tumor depth			<0.0001	0.0031	6.380	1.870–21.769
sm1	0 (0)	9 (17.6)				
sm2	5 (15.2)	24 (47.1)				
sm3	28 (84.8)	18 (35.3)				
Lymphatic invasion			<0.0001	0.0116	15.675	1.849–132.908
Positive	32 (97.0)	28 (54.9)				
Negative	1 (3.0)	23 (45.1)				
Blood vessel invasion			0.0037	0.5707	1.397	0.440–4.438
Positive	23 (69.7)	19 (37.3)				
Negative	10 (30.3)	32 (62.7)				
Histopathological grading			0.942	not analyzed		
Well differentiated	4 (12.1)	7 (13.7)				
Moderately differentiated	20 (60.6)	29 (56.9)				
Poorly differentiated	9 (27.3)	15 (29.4)				

Table II. Relationship between Lymph Node Metastasis and Immunohistochemical Findings

	Lymph node metastasis positive (n=33, %)	Lymph node metastasis negative (n=51, %)	Univariate analysis	Multivariate analysis		
			P value	P value	Odds ratio	95% confidence intervals
P53 expression			0.0046	0.0043	4.383	1.588–12.098
Positive	24 (72.7)	21 (41.2)				
Negative	9 (27.3)	30 (58.8)				
Cyclin D1 expression			0.0412	0.0633	2.673	0.947–7.547
Positive	14 (42.4)	11 (21.6)				
Negative	19 (57.6)	40 (78.4)				
MIB-1 Labeling Index	37.85±12.71	32.28±2.24	0.0483	0.0533	0.962	0.924–1.001
pRB expression			0.7748	not analyzed		
Positive	25 (75.8)	40 (78.4)				
Negative	8 (24.2)	11 (21.6)				

Table III. Multivariate Analysis with Lymph Node Metastasis

Factor	Multivariate analysis A			Multivariate analysis B		
	P value	Odds ratio	95% confidence intervals	P value	Odds ratio	95% confidence intervals
Tumor depth	0.0023	6.610	1.958–22.320	not analyzed		
Lymphatic invasion	0.0092	17.691	2.036–153.743	0.0029	23.667	2.941–190.440
P53 expression	0.0603	2.979	0.954–9.306	0.0260	3.275	1.152–9.305

lation had no correlation with clinicopathological factors (data not shown). On the other hand, cyclin D1 overexpression showed a significant correlation with blood vessel invasion ($P=0.0317$, data not shown). In terms of MIB-1 Labeling Index, there was a significant relationship between sm2 and sm3 ($P=0.0233$, data not shown). Multivariate analysis of P53 expression, cyclin D1 expression, and MIB-1 Labeling Index showed that P53 accumulation was significantly correlated with lymph node metastasis ($P=0.0043$). These results suggested that P53 accumulation could be a good predictor for lymph node metastasis of sm-ESCC immunohistochemically.

Multivariate analysis of clinicopathological and immunohistochemical factors for lymph node metastasis of sm-ESCC In terms of clinicopathological findings, tumor depth and lymphatic invasion were significantly correlated with lymph node metastasis of sm-ESCC. On the other hand, P53 expression had a significant relationship to lymph node metastasis of sm-ESCC immunohistochemically. Therefore, these factors were examined by multivariate analysis to establish the best indicator for lymph node metastasis of sm-ESCC. Multivariate analysis showed that tumor depth and lymphatic invasion were significantly correlated with lymph node metastasis ($P=0.0023$ and 0.0092, respectively, Table III: analysis A). This suggested that tumor depth and lymphatic invasion could become better indicators to predict lymph node metastasis of sm-ESCC. On the other hand, in EMR samples, the evaluation of tumor depth is inadequate because the entire submucosal layer can not be resected. In such cases, lymphatic invasion could be the best indicator, and P53 expression could be helpful to predict lymph node metastasis of sm-ESCC ($P=0.0029$ and 0.0260, respectively, Table III: analysis B).

DISCUSSION

In gastrointestinal carcinomas, the risk of lymph node metastasis increases when carcinoma cells invade deeper layers. As for ESCC, it has been reported that only 1–5% of ESCC patients in whom ESCC is restricted to the mucosal layer exhibit lymph node metastasis. However, 30–50% of ESCC patients exhibit lymph node metastasis when the carcinoma cells invade the submucosal layer.[2–6]

The 5-year survival rate for patients with lymph node metastasis was significantly poorer than that for patients without lymph node metastasis, and so lymph node metastasis is a major prognostic factor for sm-ESCC.[1] Therefore, we immunohistochemically investigated factors involved in lymph node metastasis of sm-ESCC invading to the submucosa. P53, cyclin D1, pRB, and MIB-1 were selected for immunohistochemical examinations, and univariate analysis of these factors showed that P53 accumulation could become a major risk factor for lymph node metastasis.

p53 is a tumor suppressor gene product that undergoes frequent alterations in various human carcinomas. Loss of function of P53 protein occurs as a consequence of missense point mutation, allelic loss on chromosome 17,[17–19] or inactivation by viral proteins such as human papilloma virus (HPV) E6,[20] SV40 large T antigen,[21, 22] and adenovirus E1B 55-kD.[23] As a result, the downstream targets of P53 are not appropriately regulated and such normal functions as growth arrest,[24] apoptosis,[25, 26] and control of genomic stability,[27] are lost. This functional loss of P53 protein leads to the inhibition of activation of mdm2 transactivation and hence mdm2 protein synthesis.[28] Degradation of P53 is regulated by the ubiquitin-proteasome pathway,[29] and mdm2 protein has been reported as an E3 ubiquitin ligase for P53 ubiquitination.[30, 31] In normal cells, this interaction between P53 and mdm2 keeps the P53 protein level at a low, often undetectable by immunohistochemistry, level.[18, 19] On the other hand, mutations of P53 prevent this interaction, lead to the accumulation of non-functional P53 protein, and as the result, allow detection by immunohistochemistry.[32, 33] However, immunohistochemically detected P53 protein does not always reflect the functional loss of P53.[34] With cellular stresses such as DNA damage, heat shock, and hypoxia, the phosphorylation status of P53 could be altered.[35, 36] This alteration results in modulation of P53 and mdm2 interaction, leading to accumulation of functional P53 protein, which causes cell death and/or cell cycle arrest. Furthermore, it has been reported that overexpression of ARF, which can bind to and negatively regulate mdm2, leads to abrogation of mdm2-targeted destabilization of P53, and accumulation of functional P53 protein.[37] In these cases, P53 can be detected immunohistochemically, and whether detected

P53 is functional or not is undistinguishable. These may be the reasons why P53 accumulation has been reported to show no correlation with the prognosis of ESCC patients,[38–45] and why in our study, multivariate analysis of clinicopathological and immunohistochemical factors for lymph node metastasis did not show a significant relationship between P53 accumulation and lymph node metastasis, although P53 accumulation showed significant relationship with lymph node metastasis by univariate and multivariate analysis focusing on immunohistochemical factors.

Our study demonstrated that tumor depth, lymphatic invasion, and P53 accumulation could be good indicators for lymph node metastasis of sm-ESCC. Recently, EMR has become important for patients with superficial ESCC because it is safe, easy, and above all, esophagus-preserving.[7–9] To make treatment with EMR successful, it is necessary to predict the future risk for lymph node metastasis and possible need for additional surgical resection. However, in terms of the pathological examination of tumor samples resected by EMR, the evaluation of tumor depth is inadequate because the entire submucosal layer can not be resected. Tajima *et al.*[46] classified the tumor depth of sm-ESCC as the distance from the lower edge of the lamina muscularis to the deepest portion of the invad-ing tumor, and examined the relationship with lymph node metastasis. They reported that lymphatic invasion correlated with lymph node metastasis, and tumor depth showed no significant relationship with lymph node metastasis. Therefore, it is necessary to establish a new classification of tumor depth to make treatment with EMR successful. At the same time, our study showed that P53 accumulation by immunohistochemistry could be helpful when tumor depth could not be examined.

In conclusion, tumor depth and lymphatic invasion are good clinicopathological indicators to predict lymph node metastasis of sm-ESCC. In addition, P53 accumulation by immunohistochemistry is considered helpful to identify the patients who need additional surgical resection after EMR.

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