Expression of Membrane Type 1-Matrix Metalloproteinase and CD44 Variant 6 in Superficial Esophageal Squamous Cell Carcinoma

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Lymph node metastasis or vessel invasion may occur in superficial esophageal squamous cell carcinoma when it invades the muscularis mucosae. Membrane type 1-matrix metalloproteinase (Mtl-MMP) and CD44 variant 6 (CD44v6) are reported to play an important role in cancer invasion and metastasis. Therefore, we investigated whether Mtl-MMP and CD44v6 may be useful factors for prognosis and indication of endoscopic mucosal resection (EMR) in superficial esophageal carcinoma and whether the two parameters have any relationships. Seventy patients with superficial squamous cell carcinomas curatively resected without additional treatment between 1991 and 2000 at the Kanagawa Cancer Center were analyzed for Mtl-MMP and CD44v6 expression by immunohistochemical staining. The expression of Mtl-MMP was 28.6%, and it significantly correlated with depth of invasion and lymph node metastasis. The expression of CD44v6 was 44.3% and it was closely associated with depth of invasion and lymphatic permeation. There was no significant relationship between the expression of Mtl-MMP and CD44v6, but patients with expression of Mtl-MMP positive and CD44v6 negative had a high tendency of lymph node metastasis and poor prognosis. We conclude that the expression of Mtl-MMP and CD44v6 may be a significant indicator of EMR in superficial esophageal carcinoma.

Key Words : superficial esophageal cancer, membrane type 1-matrix metalloproteinase, CD44 variant 6, endoscopic mucosal resection

Introduction

When esophageal squamous cell carcinoma invades the submucosal layer, cases in which vessel invasion and lymph node metastasis also occur have been recognized, and in most of these cases, surgical therapy is required¹⁾. In the case of radical esophagectomy accompanying lymphadenectomy, a high incidence of postoperative complications is recognized and the patient must be added great stress²⁾. On the

other hand, with progress in recent years in endoscopic diagnosis using iodine stain, it has become possible to detect esophageal carcinoma at an early stage, and endoscopic mucosal resection (EMR), a low-invasive therapy, has spread rapidly^{3)~7)}. At present, in cases of superficial esophageal carcinoma in which the muscularis mucosae has not been reached, it is an exceptionally good indication for EMR because there is virtually no lymph node metastasis or vessel invasion⁸⁾⁹⁾. However, in cases

where invasion has gone deeper than the muscularis mucosae, it is difficult to predict whether there is lymph node metastasis or vessel invasion, and the merits of EMR in such cases are controversial¹⁰⁾¹¹⁾.

Various mechanisms are involved in the process of cancer invasion or metastasis. Early in the process, as cancer cells invade surrounding tissue and propagate, the decomposition of extracellular matrix (ECM) such as basement membrane or connective tissue plays a vital role¹²⁾¹³⁾. In 1994, membrane type 1-matrix metalloproteinase (Mt1-MMP) was identified as a membrane transfixing type MMP, and it became known for activation of MMP-2, which decomposes type IV collagen, an important compositional ingredient in basement membrane¹⁴⁾. Meanwhile, CD44, a type of glycoprotein, acts as a cell surface molecule to contribute to cell-to-cell or cell-to-ECM adhesion, to T cell activation, to cancer cell metastasis. The gene locus of CD44 is approximately 60 kD in size, is located in the region of human No. 11 chromosome p13, and is comprised of 20 exon at least. The existence of CD44 variant (CD44v), in which variants are inserted through alternative splicing in the extracellular domain, has also become evident¹⁵⁾¹⁶⁾. From recent research, it has been reported that in gastric cancer, if Mt-MMP expression proliferates, the prognosis becomes poor¹⁷⁾. A significant correlation has also been reported between Mt-MMP expression and tumor invasion in lung cancer and breast cancer¹⁸⁾. In head and neck cancer, a significant correlation has been identified between down regulation of Mt-MMP expression and tumor invasion or lymph node metastasis¹⁹⁾²⁰⁾. For this reason, Mt-MMP is considered effective in marking tumor and in assessing the effects of chemotherapy²¹⁾.

In the present study, a comparison was made between Mt1-MMP and CD44v6 expression and

clinicopathological factors in superficial esophageal carcinoma. An investigation was also made to determine if Mt1-MMP and CD44v6 can serve as prognostic factors in superficial esophageal carcinoma, or as forecast factors in lymph node metastasis or vessel invasion, important factors in deciding upon EMR therapy.

Materials and methods

Patients and materials

Among cases of esophageal squamous cell carcinoma excised at the Department of Surgery, Kanagawa Cancer Center, between 1991 and 2000, the subjects were 70 cases of superficial carcinoma in which the depth of tumor invasion was the mucosal or submucosal layer. In all these cases, esophagectomy together with lymphadenectomy was performed, and there was no chemotherapy, radiotherapy or other pretreatment before surgery. Excised specimens were fixed in formalin, embedded in paraffin, and cut into 4 μ m sections. The deepest portions of lesions were used for immunohistochemical examinations.

Histological classification

Of the 70 cases of superficial esophageal carcinoma, there were 19 cases of mucosal cancer and 51 cases of submucosal cancer. In the mucosal cancer cases, invasion depth was classified as follows: m_1 (carcinoma in situ), m_2 (invasion into the proper mucosal layer) and m_3 (invasion into the muscularis mucosae). The cases of submucosal cancer were classified into sm_1 (invasion into the upper third portion of the submucosal layer), sm_2 (invasion into the middle third portion of the submucosal layer) and sm_3 (invasion into the lower third portion of the submucosal layer) (Fig. 1). In addition, the invasion pattern of tumor presentation was classified into two types: the expansive type in

Fig. 1 Subclassification of depth of tumor invasion in superficial esophageal cancer.

which the cancer lesion is budding or comes with a drop nest but the basement membrane is not damaged and the infiltrative type in which invasion to the interstitial tissue is recognized accompanied by damage to the basement membrane.

Immunohistochemistry

Immunohistochemical staining was carried out by the method of Iezzoni et al²²⁾. Each paraffin-embedded specimen was cut into 4 μm sections, which were dewaxed in xylene and rehydrated through graded alcohols, and incubated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity for 30 min, and subjected to an antigen retrieval procedure (Dako, Glostrup, Denmark). Next, nonspecific binding of primary antibodies was blocked, sections were incubated with the primary antibody 113-5b7 (25 $\mu\text{m}/\text{ml}$, Fuji Chemical Industries Ltd, Takaoka, Japan) or DF1485 (12.5 $\mu\text{m}/\text{ml}$, Novocastra, UK) at 4°C overnight. After the primary antibody treatment, the sections were incubated with biotinylated anti-mouse IgG (Dako, Santa Barbara, USA). This was followed by incubation with horseradish peroxidase conjugated avidin-biotin complex (Dako, Glostrup, Denmark). Immunostaining was visualized with 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, USA). Finally, the sections were counterstained with Mayer's

haematoxylin.

Evaluation of immunohistochemical staining

In the evaluation of immunostaining, for Mt1-MMP, tumor cell staining at 10% or more was considered positive, and all staining below this rate was taken as negative. For CD44v6, tumor cell staining was taken as positive and no staining was taken as negative. Also investigated were the associations among Mt1-MMP and CD44v6 expression and invasion depth, vessel invasion and clinicopathological factors in prognosis.

Statistical analysis

All analyses were carried out using SPSS statistical software. For the analysis of the relationship between Mt1-MMP, CD44v6 expression and other clinicopathological factors, Fisher's exact probability test was used. For the analysis of cumulative survival rate, Kaplan-Meier's method was used together with the log-rank test. A p-value of less than 0.05 is considered to be a significantly statistical difference.

Results

Histological characteristics

Of the 70 cases of with superficial esophageal carcinoma, there were 19 cases of mucosal cancer (m_2 : 4 cases, m_3 : 15 cases) and 51 cases of submucosal cancer (sm_1 : 8 cases, sm_2 : 15 cases, sm_3 : 28 cases). In the m_2 cases, neither lymph node metastasis nor vessel invasion was recognized, but these did appear in cases of m_3 or deeper. In the sm_2 or sm_3 cases, lymph node metastasis, lymph vessel invasion and venous invasion increased significantly as compared with cases of m_3 or sm_1 ($p < 0.01$) (Table 1). Where there was infiltrating type invasion, lymph node metastasis tended to be extensive.

Table 1 Relationship between depth of invasion and clinicopathologic findings

Depth of invasion	LN metastasis	Lymphatic permeation	Venous invasion	Growth pattern (expansive/invasive)
m ₁ , m ₂ (n=4)	0	0	0	4 / 0
m ₃ , sm ₁ (n=23)	4 (17.8%)	3 (13.0%)	0	13 / 9
sm ₂ , sm ₃ (n=43)	22 (51.2%)	20 (46.5%)	11 (25.6%)	11 / 30

* p<0.01

Fig. 2 Mt1-MMP immunoreactivity was shown in cell membrane (a, Mt1-MMP staining×400). This reaction showed a tendency to be stronger in deeply invading nests (b, Mt1-MMP staining×100).

Immunohistochemical staining of Mt1-MMP and CD44v6

Mt1-MMP and CD44v6 were both expressed on the cell membrane surface. The rate of expression at the presentation of Mt1-MMP tumor is 28.6%. Expression tended to be strong

Fig. 3 CD44v6 was strongly stained in the basal layer of normal esophageal epithelium (a, CD44v6 staining×20), while it was weakly stained in squamous cell carcinoma of the esophagus (b, CD44v6 staining×20).

at locations near the tumor invasion front as compared with the tumor periphery (Fig. 2). On the other hand, CD44v6 was expressed positively in normal squamous cells, but in cancer cells its expression was weak as compared with normal squamous cells, and its positive rate was 44.3% (Fig. 3).

Relationship between Mt1-MMP, CD44v6 expression and clinicopathological factors

The associations between Mt1-MMP and CD44v6 expression and invasion depth are presented in Table 2. Between the m₃ or sm₁ cases and sm₂ or sm₃ cases, statistically significant differences were recognized in the expres-

Table 2 Relationship between Mt1-MMP, CD44v6 expression and depth of invasion

Depth of invasion	Mt1-MMP Positive	CD44v6 Negative
m ₁ , m ₂ (n=4)	1 (25%)	1 (25%)
m ₃ , sm ₁ (n=23)	3 (13.0%)	6 (26.1%)
sm ₂ , sm ₃ (n=43)	16 (37.2%)	24 (55.8%)

* p<0.01

Table 3 Relationship between Mt1-MMP, CD44v6 expression and clinicopathologic findings

	Mt1-MMP Positive (%)	CD44v6 negative (%)
Tumor size		
<5cm (n=49)	13 (26.5%)	22(44.9%)
>5cm (n=21)	7 (33.3%)	9 (42.9%)
Growth pattern		
Expansive type	6 (20%)	10 (33.3%)
Invasive type	14 (35%)	20 (50.0%)
LN metastasis		
Negative	9 (20.5%)	17 (38.6%)
Positive	11 (42.3%)	14 (53.8%)
Lymphatic permeation		
Negative	14 (29.8%)	17 (36.2%)
Positive	6 (26.1%)	14 (60.9%)
Venous invasion		
Negative	15 (25.4%)	26 (44.1%)
Positive	5 (45.5%)	5 (45.5%)
Recurrence		
Absent	16 (27.6%)	24 (41.4%)
Present	4 (33.3%)	7 (58.3%)

* : p<0.05.

sions of Mt1-MMP and CD44v6 (p<0.01). The associations between Mt1-MMP and CD44v6 expression and other clinicopathological factors are indicated in **Table 3**. Mt1-MMP expression increased significantly in cases of positive lymph node metastasis (p<0.05), and its expression tended to be frequent also in cases of infiltrative type invasion and cases of positive venous invasion. CD44v6 expression, on the other hand, was significantly down

Table 4 Relationship between Mt1-MMP expression and CD44v6 expression

	Mt1-MMP	
	Positive	Negative
CD44V6	Positive 14 / 42.9%	25 / 24%
	Negative 5 / 83.3%	25 / 36%

Number of patients / Percent of lymph node metastasis positive case (%)

regulated in cases of positive lymph vessel invasion (p<0.05), and it tended not to be recognized in cases of infiltrative type invasion, positive lymph node metastasis, or recurrence.

Relationship between Mt1-MMP and CD44v6 expression

No statistically significant differences were recognized in the expressions of Mt1-MMP and CD44v6. Of the cases of CD44v6 negative expression, 6 were Mt1-MMP positive expression, and lymph node metastasis was recognized in 5 of these cases (83.3%). Two of these cases resulted in recurrence and death (**Table 4**).

Relationship between Mt1 - MMP, CD44v6 expression and prognosis

The 5-year survival rate of cases of Mt1-MMP positive expression is 74.7%, while that of negative expression is 76.4%, so no statistically significant difference was recognized. The 5-year survival rate of cases of CD44v6 positive expression is 83.6%, whereas that of negative cases is 66.6%, so there was no statistically significant difference, but the prognosis tended to be poor in cases of CD44v6 negative expression.

Discussion

MMP decomposes basement membrane and connective tissue but its expression in suppressed by a variety of factors. With respect to the

activation of MMP-2, which forcefully decomposes type IV collagen, Sato et al. report that latent type MMP-2 is specifically activated on the cell membrane surface by Mt1-MMP¹⁴⁾. In addition, Will et al. report that among tissue inhibitors of metalloproteinase (TIMP), a MMP inhibitor, TIMP-2 and TIMP-3 exhibit inhibitive action against Mt1-MMP²³⁾, which suggests that the decomposition of ECM is regulated through a variety of pathways. The present study reveals that Mt1-MMP is expressed significantly in cases of sm₂ or sm₃ invasion depth, rather than in cases of m₃ or sm₁. Even in cases of superficial esophageal carcinoma, as invasion depth increases, Mt1-MMP expression is believed to be up-regulated, and it can serve as an index of malignancy.

For CD44, which is expressed on the cell membrane surface, it was confirmed that in rat cancer tissue there is a significant correlation between abnormal expression and metastasis²⁴⁾. With respect to human cancer tissue, it has been reported that there is a relationship between CD44 and metastasis or prognosis in colorectal cancer²⁵⁾. In the expression of CD44v, there is organ specificity. Mulder et al., for example, report that in colorectal cancer, prognosis is significantly poor in cases of CD44v6 positive expression²⁶⁾. On the other hand, Harold-Mende et al. report that although CD44v is expressed constantly in normal squamous epithelium of the head and neck region, it is down-regulated significantly in squamous cell carcinoma²⁷⁾. In addition, Spafford et al. report that in laryngeal cancer, prognosis is significantly poor in cases of CD44v6 negative expression²⁸⁾. In the present study, it was found that CD44v6 expression is down-regulated in cases of superficial esophageal carcinoma, and although it correlates significantly with positive lymph vessel invasion, it could not serve as a useful prognostic

factor.

As for the relationship between Mt1-MMP expression and CD44 expression, Takahashi et al. report that in melanoma, CD44 regulates MMP-2²⁹⁾. By blocking CD44 expression, these investigators claim that they could observe MMP-2 secretion and sthenia of mRNA expression from MMP-2, and that tumor invasion and metastasis were promoted. Mt1-MMP is a type of protein that controls MMP-2, and it is suspected of having some type of relationship with CD44 expression, but in the present study, Mt1-MMP expression was low at 19.4% in cases of down-regulated CD44v6 expression, so it is hard to say that down-regulated CD44v6 expression promotes Mt1-MMP expression. Nevertheless, in 5 of the cases (83.3%), lymph node metastasis was recognized, and because recurrence and death were noted in 2 cases, it is believed that in superficial esophageal carcinoma, malignancy is high in cases of down-regulated CD44v6 expression and Mt1-MMP positive expression. In Mt1-MMP negative case, the percentage of lymph node metastasis is about 20%, and it may not be useful by itself. But in cases of down-regulated CD44v6 expression and Mt1-MMP positive expression, these are high tendency of lymph node metastasis, so that expressions may be useful marker for lymph node metastasis in EMR case.

From recent cases of superficial esophageal cancer it was discovered that a difference in prognosis can be recognized between mucosal cancer and submucosal cancer. The presence or absence of lymph node metastasis or vessel invasion becomes a factor in this difference³⁰⁾³¹⁾. Watanabe et al. report that aside from lymph node metastasis or vessel invasion, intramural metastasis is an independent factor in prognosis¹⁾. In addition, Tajima et al. report that invasion to the submucosal layer, heteromorphy of the nucleus, and invasion pattern are impor-

tant in deciding whether to pursue additional therapy after EMR³²⁾. In the application of EMR, at present it is difficult to judge whether there is lymph node metastasis or vessel invasion in m_3 or sm_1 cases of invasion depth, so it is considered a relative indication. In our investigation, lymph node metastasis or vessel invasion was recognized when m_3 invasion depth was reached, and in cases of sm_2 and sm_3 , lymph node metastasis and vessel invasion exceeded 40%. Lymph node metastasis was also significantly widespread in cases of Mt1-MMP positive expression, and in cases of CD44v6 negative expression, lymph vessel invasion was extensive. This suggests that lymph node metastasis and vessel invasion can be predicted. Of 23 cases of m_3 and sm_1 invasion depth, lymph node metastasis and vessel invasion were recognized in 5 cases, and of these 5 cases, Mt1-MMP positive expression or CD44v6 negative expression was recognized in 4 cases, which is a high rate (80%). In other words, in cases of m_3 and sm_1 invasion depth, considered to be a relative indication of EMR, if Mt1-MMP and CD44v6 expression in the deepest region of cancer invasion obtained by EMR is investigated, cases of Mt1-MMP positive expression or CD44v6 negative expression can be found. It is assumed that the potential for lymph node metastasis or positive vessel invasion is high in these cases, and that after EMR, additional treatment such as surgery and chemotherapy or radiation therapy, will be necessary.

In conclusion, these findings suggest that in superficial esophageal carcinoma Mt1-MMP and CD44v6 expressions vary significantly with invasion depth, lymph node metastasis and lymph vessel invasion, and that such expressions could serve as an index in deciding upon EMR application.

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