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Epithelial to Mesenchymal Transition Correlates With Tumor Budding and Predicts Prognosis in Esophageal Squamous Cell Carcinoma

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Background and Objectives: Epithelial to mesenchymal transition (EMT) is considered to play an important role in cancer invasion. Tumor budding is a prognostic factor in esophageal squamous cell carcinoma (ESCC). The aim of this study was to explore the correlation between EMT and tumor budding.

Methods: Surgical specimens from 78 cases of ESCC resected without preoperative treatment between 2001 and 2013 were enrolled in the study. The mRNA expressions of E-cadherin and vimentin were measured in cancerous tissues using real-time PCR, and each tumor was classified into either epithelial or mesenchymal group. Tumor budding was evaluated in H&E-stained slides and divided into two groups; low-grade budding (<3) and high-grade budding (≥ 3).

Results: The 5-year survival rate in the epithelial group was significantly higher than that in the mesenchymal group (62.0% vs. 31.5%, $P = 0.021$). Survival rate of patients in the low-grade budding group was significantly higher than that of patients in the high-grade budding group (75.1% vs. 25.9%, $P < 0.001$). High-grade tumor budding was significantly associated with the mesenchymal group ($P = 0.009$).

Conclusion: EMT was found to occur in ESCC and was significantly associated with tumor budding. Tumor budding was identified as a significant independent prognostic factor among the current population of ESCC.

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KEY WORDS: epithelial to mesenchymal transition; tumor budding; esophageal cancer; prognosis

INTRODUCTION

Esophageal cancer is well known to be one of the cancers with a high malignant potential [1,2]. Most esophageal cancers in the Far East are histologically squamous cell carcinomas (ESCCs) and the most advocated therapy for this disease continues to be complete surgical resection. Despite treatment of patients with esophagectomy and lymph node dissection survival outcomes have not been encouraging [3].

Epithelial to mesenchymal transition (EMT) is literally characterized by a gain of mesenchymal cell markers (e.g., vimentin) and a loss of epithelial markers (e.g., E-cadherin) [4,5]. In this process, cells lose their epithelial characteristics, including their polarity and specialized cell–cell contacts, and acquire a migratory behavior that allows them to move away from their epithelial cell community and integrate into the surrounding tissue, even at remote locations. EMT and its reversal, mesenchymal to epithelial transition (MET), are fundamental processes involved in tumor cell invasion and metastasis [6,7]. This phenomenon is thought to be reactivated during the progression of cancers of cutaneous, prostatic, mammary, hepatic, gastric, pancreatic, and colorectal origin [8–13]. More recently, ESCC was found eligible to join this long list [14–16], and a better understanding of the role of EMT in invasion and metastasis of ESCC is expected to provide new insight to combat this fatal disease.

On the other hand, tumor budding has been reported to be a valuable prognostic indicator reflecting a tumor's malignant potential in colorectal cancer [17–19]. This pathologic entity refers to isolated single cancer cells or microscopic clusters of undifferentiated cancer cells, composed of fewer than five cancer cells found outside the invasive margin of a tumor [18]. More recently, we reported on relevance of this pathologic entity in ESCC as an independent prognostic factor that correlated also with lymph node metastasis, venous invasion and tumor depth, reflecting the biological activity of the tumor [20,21].

In colorectal cancer, EMT-derived tumor cells were found to be represented histopathologically by the presence of tumor buds and were reported to occur in 20–40% of tumors [18,22]. However, there has been no study establishing the correlation between tumor budding and EMT in ESCC. In the current study, the correlation between EMT status [23] of the surgical specimen, clinicopathological factors, and prognosis was examined in patients with ESCC. Furthermore, the association between tumor budding and EMT status was also explored.

Abbreviations: EMT, epithelial to mesenchymal transition; ESCC, esophageal squamous cell carcinoma; PCR, polymerase chain reaction; MET, mesenchymal to epithelial transition; H&E, hematoxylin and eosin; HR, hazard ratio; CI, confidence interval; UICC, Union for International Cancer Control; Ut, upper thoracic esophagus; Mt, mid-thoracic esophagus.

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MATERIALS AND METHODS

Cell Lines and Culture Conditions

Human ESCC cell lines, NUEC1, NUEC2, and NUEC3, were established and maintained at the Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine. Additional human ESCC cell lines, T.T and T.Tn, were obtained from the Japanese Collection of Research Bioresources, Japan, and WSSC was obtained from the American Type Culture Collection (Manassas, VA). TE1, TE2, and TE3 were donated by Tohoku University. All cells were grown in DMEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY), and incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2.

Patients and Sample Collection

From December 2001 to October 2013, a total of 78 specimens were collected from ESCC patients, who had been operated on in the Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine. The criterion for eligibility in this study was histologically proven ESCC in patients who underwent radical esophagectomy. Patients who received any chemotherapy or radiotherapy before surgery and those who had locally advanced unresectable cancer or synchronous malignancy derived from another organ were excluded. The median follow-up period was 21.2 months (range: 1–138 months). The tumors were staged according to the seventh edition of the UICC (Union for International Cancer Control) TNM staging system [24], and the tumor grade was classified according to the WHO classification of histological differentiation [25]. Collected samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was isolated from each of the frozen samples using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Written informed consent was obtained from all patients.

Real-Time Quantitative PCR Analysis

Total RNA, isolated from human ESCC cell lines, was used to generate complementary DNA and then amplified using polymerase chain reaction (PCR) primers as follows: E-cadherin: 5-GAAGGTGACAGAGCCTCTGGAT-3 (forward) and 5-CATTCCC-GTTGGATGACACA-3 (reverse), which amplified a 79-bp product; vimentin: 5-AAAACACCCTGCAATCTTTCAGA-3 (forward) and 5-GATTCCACTTTGCGTTCAAGGT-3 (reverse), which amplified a 78-bp product. RNA expression was determined using real-time quantitative PCR (qPCR). For standardization, expression of GAPDH in each sample was quantified using the primer set 5-AACGGCT-CCGGCATGTGCAA-3 (forward) and 5-GGCTCCTGTGCAGAGA-AAGC-3 (reverse). All PCR reactions were performed under the following conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, then 40 cycles at 95°C for 15 sec and 60°C for 1 min. Real-time detection of the emission intensity of SYBR Green was performed on an ABI prism 7000 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA). Each qPCR was performed in triplicate, including a no-template negative control.

Western Blotting

Cell lysates were prepared and electrotransferred from the gel to the PVDF membrane (Millipore, Darmstadt, Germany). After blocking membranes in Tris-buffered saline (TBS)–Tween containing 5% non-fat milk for 1 hr at room temperature under agitation, the membranes were incubated overnight at 4°C with the primary antibodies in a 5% solution of non-fat powdered milk in TBS–Tween. The following primary antibodies were used: rabbit anti-E-cadherin and rabbit anti-vimentin (1:1,000, Cell Signaling Technology, Beverly, MA).

Immunohistochemical Analysis

Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 μm and stained with hematoxylin and eosin (H&E). Slides were immunostained with the anti-E-cadherin antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-vimentin antibody (Nichirei Bioscience, Tokyo, Japan). 3,3′-Diaminobenzidine (DAB, Sigma–Aldrich, St. Louis, MO) was used for visualization of E-cadherin and vimentin staining. Membranous E-cadherin and cytoplasmic vimentin expression were then analyzed.

Two authors (Y.N. and M. K.) independently evaluated tumor budding at the invasive front in all specimens. In case of a disagreement on the grading of pathologic findings, we reviewed the slide together and reached a consensus diagnosis. Isolated single cancer cells and clusters composed of fewer than five cancer cells were defined as budding foci. These scattered foci were observed at the stroma in the active invasive front. To semi-quantify this finding, a microscopic field in which the budding intensity was considered maximal was selected on the slide containing the deepest portion of tumor penetration, and the number of budding foci was counted using a 20× objective lens. Patients were classified into the following two groups based on the number of tumor budding foci; a high-grade budding group in which the budding intensity was ≥ 3, and a low-grade budding group in which the budding intensity was <3.

Statistical Analysis

Differences in the numerical data between the two groups were evaluated using Fisher's exact test or χ^2 test. Overall survival rates were calculated using the Kaplan–Meier method, and the difference in survival curves was analyzed using the log-rank test. Independent prognostic factors were analyzed using the Cox proportional hazards regression model in a stepwise manner. Data are expressed as the mean ± standard deviation. A P-value of less than 0.05 was considered a statistically significant difference. Data were analyzed using JMP version 10 software (SAS Institute, Cary, NC).

RESULTS

Characterization of EMT in Human ESCC Cell Lines

Expression of E-cadherin and vimentin was assessed for each of the human ESCC cell lines to determine the extent of EMT. Five of the cell lines (T.T, TE3, TE2, NUEC3, and NUEC1) were classified as epithelial based on their high-level mRNA expression of E-cadherin and low-level mRNA expression of vimentin using qPCR. Conversely, four of the cell lines (NUEC2, TE1, WSCC, and T.Tn) were considered to be mesenchymal, because these cell lines expressed mRNA for vimentin and E-cadherin at high and low levels, respectively (Fig. 1A). Protein expression using Western blot analysis was consistent with mRNA expression results (Fig. 1B). That is, the expression level of E-cadherin protein was relatively high in the same five cell lines (T.T, TE3, TE2, NUEC3, and NUEC1), whereas that of vimentin was high in the other four cell lines (NUEC2, TE1, WSCC, and T.Tn).

Clinical Implication of EMT in ESCC Patients

Patient backgrounds are summarized in Table I. EMT status was determined using a V/E ratio (vimentin mRNA expression divided by E-cadherin mRNA expression in cancerous tissues) in clinical ESCC specimens [23,26]. The median value of V/E ratio at 0.85 was tentatively

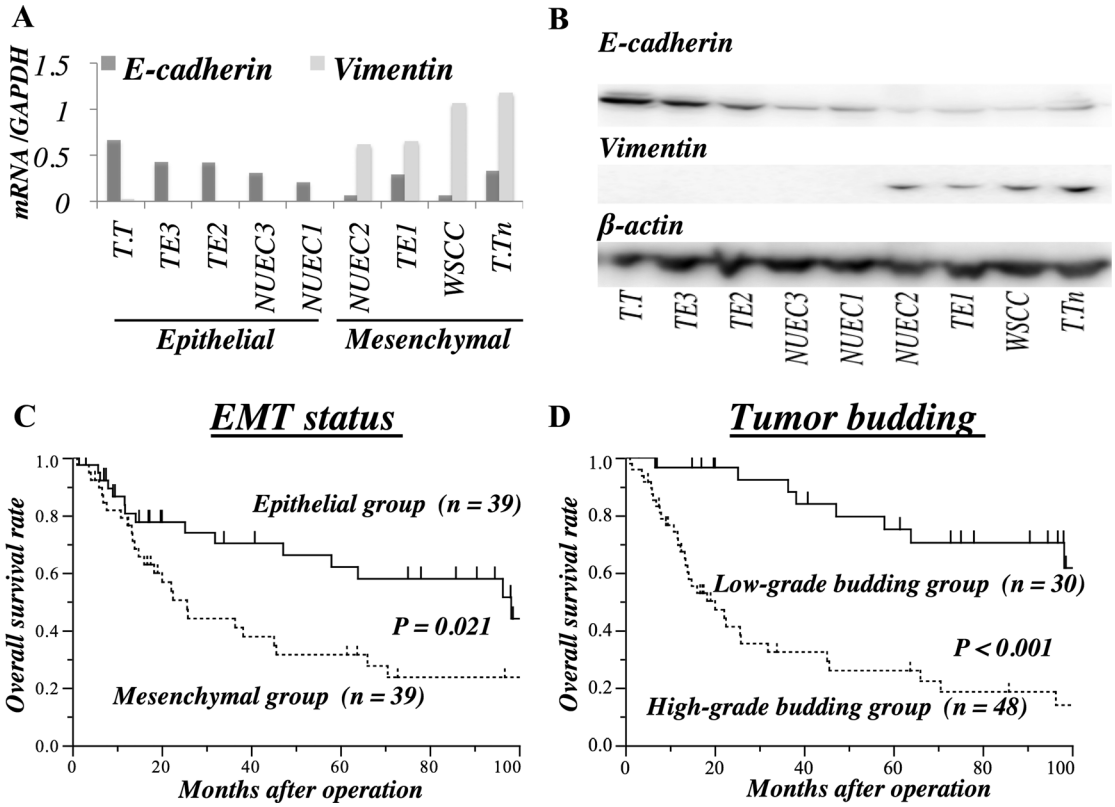

Fig. 1. **A**: Profiling of E-cadherin and vimentin expression in a panel of ESCC cell lines. The mRNA expression of E-cadherin and vimentin in nine ESCC cell lines was examined using real-time quantitative RT-PCR with *GAPDH* serving as a loading control. **B**: Protein expression was confirmed using Western blot analysis with β-actin as a loading control. **C**: Overall survival was evaluated based on EMT status. EMT status of each patient was determined as follows: V/E ratio (vimentin mRNA expression divided by E-cadherin mRNA expression in cancerous tissues) <0.85 (median) was epithelial; V/E ratio ≥0.85 (median) was mesenchymal. The difference in survival between groups was significant (31.5% vs. 62.0%, HR = 2.01; 95% CI, 1.11–4.04; $P = 0.021$). **D**: Overall survival was evaluated based on high-grade (≥3 tumor buds) and low-grade (<3 tumor buds) budding. The difference in survival between groups was significant (25.9% vs. 75.1%, HR = 5.33; 95% CI, 2.55–12.5; $P < 0.001$). HR, hazard ratio; CI, confidence interval.

TABLE I. Patient Demographics

Age (years, mean ± standard deviation)	64.8 ± 8.0
Gender	
Male	63
Female	15
Tumor location	
Upper thoracic esophagus	6
Middle thoracic esophagus	32
Lower thoracic esophagus	40
Operative method	
Subtotal esophagectomy	73
Distal esophagectomy	2
Transhiatal esophagectomy	3
Pathological stage	
IA	9
IB	5
IIA	14
IIB	6
IIIA	15
IIIB	13
IIIC	12
IV	4

determined as a cutoff value, as in the previous report [27]. Patients with a V/E ratio <0.85 were assigned to the epithelial group (n = 39), whereas those with a V/E ratio ≥0.85 were assigned to the mesenchymal group (n = 39). Table II showed the correlation between clinicopathological variables and EMT status, which revealed that the mesenchymal group was significantly associated with age. When survival was analyzed based on EMT status, the 5-year survival rate of patients in the mesenchymal group was significantly lower than that of patients in the epithelial group (31.5% vs. 62.0%, hazard ratio [HR] = 2.01; 95% confidence interval [CI], 1.11–4.04; $P = 0.021$) (Fig. 1C).

Intensity of Tumor Budding and Correlation With Clinicopathological Variables

H&E staining was performed on 78 ESCC cases. As illustrated in Figure 2A–C, tumor buds were identified based on standard H&E staining. Tumor budding counts ranged from 0 to 25 buds (mean: 5.8, median: 4). Among 78 ESCC cases examined, 48 cases (61.5%) were in the high-grade budding group (≥3 tumor buds), whereas 30 cases (38.5%) were in the low-grade budding group (<3 tumor buds). Of the 30 cases with low-grade budding, no tumor bud was observed in 5 cases.

TABLE II. Correlation Between EMT Status and Clinicopathological Variables

Variables	Epithelial group	Mesenchymal group	*P*-value
No. of patients	39	39	
Age (≥65 vs. ≤64)	14/25	24/15	0.022
Gender (male vs. female)	32/7	31/8	0.774
Tumor location (Ut, Mt vs. Lt)	19/20	19/20	1.000
Histopathological grading (G1, G2 vs. G3, G4)	35/4	34/5	0.723
Pathological T category (T1, T2 vs. T3, T4)	12/27	14/25	0.631
Pathological N category (N0, N1 vs. N2, N3)	25/14	22/17	0.488
Venous invasion ((+) vs. (−))	19/20	14/25	0.292
Lymphatic invasion ((+) vs. (−))	30/9	29/10	0.792
Pathological stage (IA, IB, IIA, IIB vs. IIIA, IIIB, IIIC, IV)	19/20	15/24	0.361

Ut, upper thoracic esophagus; Mt, mid-thoracic esophagus; Lt, lower thoracic esophagus.

TABLE III. Correlation Between Tumor Budding and Clinicopathological Variables

Variables	Low-grade budding	High-grade budding	*P*-value
No. of patients	30	48	
Age (≥65 vs. ≤64)	11/19	27/21	0.108
Gender (male vs. female)	21/9	42/6	0.078
Tumor location (Ut, Mt vs. Lt)	16/14	26/22	0.642
Histopathological grading (G1, G2 vs. G3, G4)	28/2	41/7	0.470
Pathological T category (T1, T2 vs. T3, T4)	14/16	12/36	0.048
Pathological N category (N0, N1 vs. N2, N3)	22/8	25/23	0.062
Venous invasion ((+) vs. (−))	11/19	23/25	0.381
Lymphatic invasion ((+) vs. (−))	19/11	41/7	0.028
Pathological stage (IA, IB, IIA, IIB vs. IIIA, IIIB, IIIC, IV)	18/12	16/32	0.021
EMT status (mesenchymal vs. epithelial)	9/21	29/19	0.009

Ut, upper thoracic esophagus; Mt, mid-thoracic esophagus; Lt, lower thoracic esophagus.

Table III shows the correlation between the clinicopathological variables and tumor budding, which revealed that the high-grade budding group was significantly associated with EMT status ($P = 0.009$), pathological stage (pStage) ($P = 0.021$), lymphatic invasion ($P = 0.028$), and pathological T category (pT) ($P = 0.048$).

When survival was analyzed based on tumor budding, the 5-year survival rate of patients in the high-grade budding group was significantly lower than that of patients in the low-grade budding group (25.9% vs. 75.1%, HR = 5.33; 95% CI, 2.55–12.5; $P < 0.001$) (Fig. 1D).

Tumor Budding as a Prognostic Factor in ESCC Patients

Univariate analysis showed that age (≥65), gender (male), pathological N category (pN) (N2 and N3), lymphatic invasion, EMT

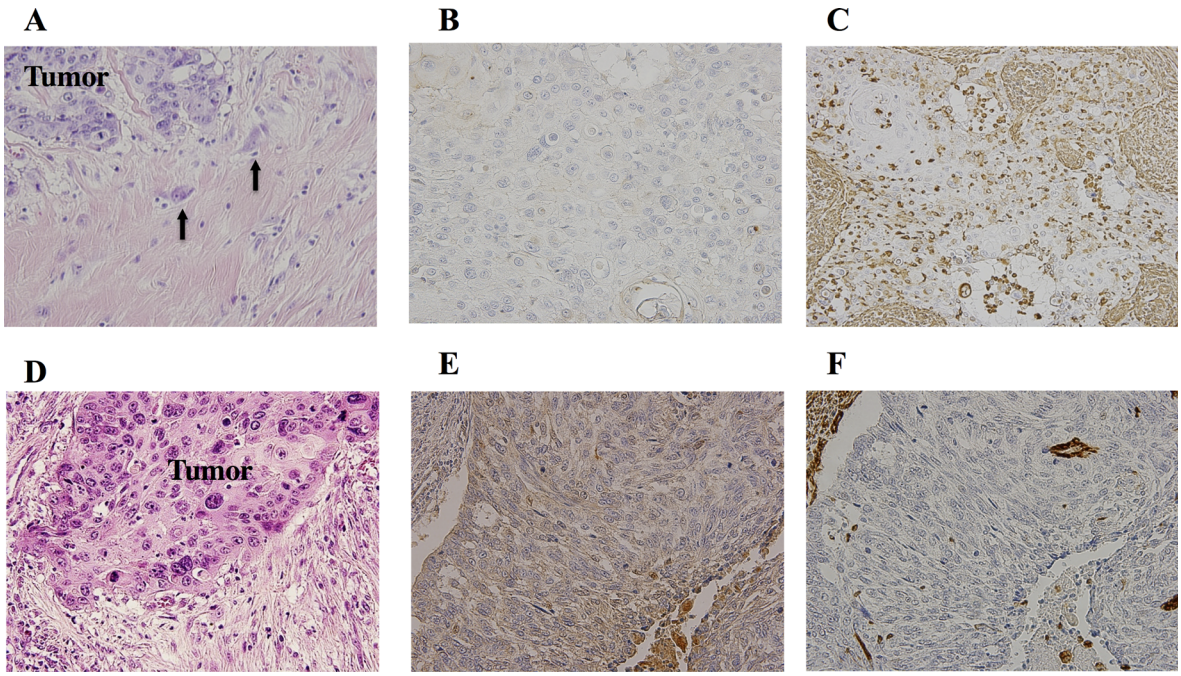

Fig. 2. Tumor budding in H&E-stained specimens and immunohistochemical analysis of E-cadherin and vimentin. Representative high-grade budding in ESCC is shown in (A)–(C). **A**: Tumor budding foci is shown by an arrow (magnification 400×). **B**: Immunohistochemical staining of E-cadherin. Tumor cells showed weak membranous E-cadherin expression. **C**: Strong cytoplasmic vimentin expression is shown. Representative low-grade budding in ESCC is shown in (D)–(F). **D**: Tumor front in a low-grade budding specimen is shown (magnification 400×). **E**: Immunohistochemical staining of E-cadherin. Tumor cells showed strong membranous E-cadherin expression. **F**: No cytoplasmic vimentin expression was observed.

TABLE IV. Multivariate Analysis of Predictive Factors of Survival

	Univariate			Multivariate		
Variables	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Age (≥65 years)	2.17	1.16–4.15	0.016	1.88	0.93–3.92	0.081
Gender (male)	2.83	1.13–9.47	0.024	2.00	0.74–7.00	0.183
Tumor location (Ut and Mt)	1.68	0.90–3.19	0.101			
Histopathological grading (G3 and G4)	1.66	0.63–3.67	0.228			
Pathological T category (T3 and T4)	1.51	0.78–3.17	0.279			
Pathological N category (N2 and N3)	2.94	1.58–5.58	<0.001	1.71	0.89–3.42	0.108
Venous invasion (+)	0.93	0.48–1.73	0.816			
Lymphatic invasion (+)	7.05	2.16–43.4	<0.001	2.74	0.86–12.1	0.091
EMT status (mesenchymal)	2.08	1.11–4.04	0.021	1.54	0.73–3.31	0.258
Tumor budding (high-grade)	5.33	2.55–12.5	<0.001	3.40	1.48–8.68	0.003

Ut, upper thoracic esophagus; Mt, mid-thoracic esophagus; HR, hazard ratio; CI, confidence interval.

status (mesenchymal), and tumor budding were significantly associated with poor survival. On multivariate analysis, high-grade tumor budding was an independent prognostic factor for ESCC patients (HR = 3.40; 95% CI, 1.48–8.68; $P = 0.003$) (Table IV).

Correlation Between E-Cadherin and Vimentin Expression and Tumor Budding

In normal tissues, immunohistochemical expression of E-cadherin is detected in epithelial cells, whereas vimentin expression is observed in stromal cells, but not in the epithelium. E-cadherin and vimentin expression were also evaluated immunohistologically in representative eight cases, four cases with high-grade budding and another four cases with low-grade budding. A representative finding at the invasive front of a high-grade budding specimen was shown in Figure 2A, tumor cells showed weak membranous E-cadherin expression (Fig. 2B) and strong cytoplasmic vimentin expression (Fig. 2C). Immunohistochemical staining of the invasive front of a low-grade budding specimen was shown in Figure 2D, tumor cells showed strong membranous E-cadherin expression (Fig. 2E) with no cytoplasmic vimentin expression (Fig. 2F).

DISCUSSION

The term "tumor budding" denotes the presence of individual cells and small clusters of tumor cells at the invasive front, and this morphological feature has been increasingly recognized as a strong and robust adverse prognostic factor in various cancers, including ESCC [17–21,26,28]. On the other hand, EMT is a process whereby tumor cells gain migratory and invasive properties as mesenchymal cells during the cancer pathological process. Therefore, tumor budding could morphologically reflect the process of EMT [22]. In fact, the association between tumor budding and EMT has been reported in colorectal cancer, tongue squamous cell carcinoma, pancreatic cancer, endometrial cancer, and breast cancer [29–32]. However, there has been no report that has studied the correlation between tumor budding and EMT in ESCC.

In the current study, we attempted to classify ESCC into either the epithelial or mesenchymal type, based on their extent of mRNA expression of E-cadherin, the epithelial marker, and vimentin, the mesenchymal marker [23,26]. The classification was possible not only with cell lines but also with surgically resected specimens. EMT status was found also to be a prognostic factor, and this was compatible with previous reports which found through real time PCR [27] and immunohistochemical staining [33] using molecular markers such as vimentin and fibronectin that the mesenchymal phenotype is predictive of poor prognosis in ESCC.

On the other hand, ESCC patients could also be classified into high-grade or low-grade budding groups, and tumor budding was significantly associated pN, pT, and overall survival, compatible with past studies [34,35]. As in previous studies that looked at other types of cancer [22,25], we evaluated EMT status using RNA extracted from surgical specimens incised from bulky primary lesions; not necessarily limited to the tumor-invasive front. Nevertheless, EMT status of the primary lesion was significantly associated with the grade of tumor budding; high-grade tumor budding was significantly associated with the mesenchymal phenotype.

The detailed mechanism of the EMT process has not been fully clarified despite numerous investigations of signal transduction pathways. Our results potentially implied that ESCC of epithelial phenotype can acquire a mesenchymal phenotype through EMT, and that tumor budding at the invasive front could be characteristic of that phenotype. However, functional analyses with relevant genes are mandatory to clarify the mechanism behind EMT and its relation with budding. One further weakness of the current observation is the small sample size. We looked only at chemonaive patients since chemotherapy could influence the phenotype and morphology of the primary lesion. This approach led to a shortage of surgical specimens because neoadjuvant chemotherapy is the current standard of care in Japan for Stage II/III ESCC. However, since preoperative chemotherapy or chemoradiotherapy has been given not only in Japan but in several countries and regions, the current approach may provide a precious opportunity to gain insight regarding biology of the disease.

CONCLUSIONS

In conclusion, we determined the EMT status by calculating the vimentin to E-cadherin expression ratio, and demonstrated that EMT could occur in clinical specimens from ESCC patients. Furthermore, tumor budding in ESCC patients was an independent prognostic factor among chemonaive patients and was also associated with EMT status. The validation of our results in a large series is warranted, as is elucidating the underlying mechanisms through further investigation.

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