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.

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.

Key Words: epithelial to mesenchymal transition; tumor budding; esophageal cancer; prognosis

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 cell lines 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% CO2.

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 resectable 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 RNaseasy 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‐GGTGGATGACACA-3' (reverse), which amplified a 78-bp product; vimentin: 5'-AAAACACCTTGAATCCCTTCA‐3' (forward) and 5'-GATTCCACTGTGCCAGAG-3' (reverse), which amplified a 78-bp product. RNA expression was determined using real-time quantitative PCR (qPCR). For standardization, expression of GAPDH according to the manufacturer 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 χ2 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
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 \(\geq 0.85\) 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 (\(\geq 3\) 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 I. Patient Demographics

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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 \(\geq 0.85\) (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 (\(\geq 3\) 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 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 \( (p\text{Stage}) \ (P = 0.021) \), 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 \( (\geq 65) \), gender (male), pathological N category \( (pN) \) \( (N2 \) and \( N3) \), lymphatic invasion, EMT

![Fig. 2](https://example.com/fig2.png)

**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.

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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 immunohistochemically 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 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).

**REFERENCES**


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