residual tumor cells within the entire cancerous tissue was assessed as follows: grade 3, no viable residual tumor cells (pathological CR); grade 2, less than 1/3 residual tumor cells; grade 1b, 1/3 to 2/3 residual tumor cells; grade 1a, more than 2/3 residual tumor cells; grade 0, no significant response to preoperative therapy.[11,26,27] Patients with grade 2 and grade 3 were considered to have a major response, while those with grade 0, grade 1a, and grade 1b were considered to have a minor response.

The histopathological findings were classified according to the UICC TNM classification.[28]

PET-CT Protocol

All patients underwent ^{18}F-FDG-PET before chemotherapy and 2–3 weeks after the completion of chemotherapy in our hospital, using the protocol described previously.[29,30] PET/CT was performed with an integrated scanner (Gemini GXL; Philips). Blood glucose level was measured each PET study and confirmed to be less than 150 mg/dl. Whole-body images, generally from the top of the skull to midthigh, were acquired about 60 minutes after intravenous injection of ^{18}F-FDG at a dose of 3.7 MBq (0.10 mCu)/kg body weight. PET was performed using the following parameters: 3-dimensional emission scan, 2-min scan per bed position × 11 positions, ordered-subset expectation maximization reconstruction, and 4.0-mm slice thickness per interval. Acquisition for parameters for CT were as follows: breath-hold during normal expiration from the level of apex of lungs to the lower pole of kidneys; no intravenous or oral contrast medium; 120 kVp and 50 effective mAs; 16 slices; 1.5-mm detector collimation; and 5.0-mm slice thickness, with a 4.0-mm interval. Coronal and sagittal CT images were reconstructed using axial thin-section CT images with 1.5-mm slice thickness.

In semiquantitative analysis, regions of interests were placed over the primary tumors demonstrating maximum FDG uptake on baseline scan. SUV_{max} was calculated according to the following formula: PET count of the most intense point × calibration factor (MBq/kg)/injection dose (MBq)/body weight (kg). The FDG uptake of the tumor is visible when the SUV_{max} is above approximately 2.0. Thus, cases of SUV_{max} for the primary tumor or lymph nodes of $\geq$2.0 were judged as PET positive.

Statistical Analysis

The paired-*t* test was used to analyze the difference between pre-SUV_{max} and post-SUV_{max}. Analysis of variance (ANOVA) was used to compare SUV_{max}-DR and post-SUV_{max} value according to pathological response. Receiver operating characteristic (ROC) analysis was performed to identify a threshold value of SUV_{max}-DR and post-SUV_{max} value for predicting pathological major response. Sensitivity, specificity, positive and negative predictive values and the area under the ROC curve (AUC) was calculated. Overall survival was calculated from the date of neoadjuvant treatment to the occurrence of the event or to the last known date of follow-up. Actual survival was calculated by the Kaplan–Meier method and statistically evaluated by the log-rank test. The Cox proportional hazards regression model was used to analyze the simultaneous influence of prognostic factors. A p value of less than 0.05 was considered to indicate statistical significance. These analyses were carried out using the JMP Ver. 9.0 software (SAS institute, Cary, NC, USA).

RESULTS

Pathological Response to Preoperative Treatment and Survival

Table 1 summarizes the characteristics of 211 patients enrolled in this study. Of the 211 patients, 180 received preoperative ACF chemotherapy and the remaining 31 received preoperative DCF chemotherapy. With regard to the pathological response, pathological CR (grade 3) was experienced in 15 patients (7.1 %), grade 2 in 32 patients (15.2 %), while 138 patients (65.4 %) were classified as grade 1 (grade 1a; 98 patients, grade 1b; 29 patients) and 26 patients (12.3 %) were classified as grade 0. Thus, 47 patients (22.3 %) were considered to have a major response, while 164 patients (77.7 %) were considered to have a minor response. Patients with major response had significantly longer survival, compared to those with minor response (5-year overall survival 59.2 vs. 45.5 %, $p = 0.0447$, Fig. 1).

Association between Pathological Response and SUV_{max}-DR or Post-SUV_{max}

The mean SUV_{max} of the primary tumors was 11.4 before treatment, but decreased significantly after preoperative chemotherapy to 5.8 ($p < 0.0001$). Consequently, the mean SUV_{max}-DR during chemotherapy was 49.4 %. A mean SUV_{max}-DR was 30.8 % in grade 0, 45.4 % in grade 1, 73.5 % in grade 2 and 75.8 % in grade 3 ($p < 0.0001$, Fig. 2a). The ROC analysis identified a SUV_{max}-DR of 56 % as the cutoff value that optimized sensitivity and specificity for predicting pathological major response. The AUC for the ROC analysis was 0.803 for SUV_{max}-DR. With a SUV_{max}-DR cutoff value of 56 %, the sensitivity, specificity, positive and negative predictive value for predicting pathological major response was 92.9, 60.4, 36.8 and 97.1 %, respectively. Although there was a significant difference in SUV_{max}-DR among pathological response,

TABLE 1 Patient characteristics

Characteristic	Value[a]
Age (years)	64.6 ± 8.0
Gender	
Male	173 (82)
Female	38 (18)
Tumor location	
Upper third	29 (14)
Middle third	99 (47)
Lower third	83 (39)
Histology of SCC	
Well differentiated	40 (19)
Moderately differentiated	110 (52)
Poorly differentiated	61 (29)
Clinical T	
cT1	7 (3)
cT2	41 (20)
cT3	124 (59)
cT4	39 (18)
Clinical N	
cN0	25 (12)
cN1–3	186 (88)
Clinical stage	
0–I	5 (3)
II	46 (21)
III	118 (56)
IV	42 (20)
Pathological T	
pT0–1	55 (26)
pT2	39 (18)
pT3	107 (51)
pT4	10 (5)
Pathological N	
pN0	75 (36)
pN1	66 (31)
pN2	41 (19)
pN3	29 (14)
Pathological stage	
0–I	32 (15)
II	71 (34)
III	66 (31)
IV	42 (20)

SCC squamous cell carcinoma

[a] Data are presented as mean ± SD or *n* (%) of subjects

SUV_{max}-DR could not distinguish pathological complete response (grade 3) from good responder (grade 2).

A mean post-SUV_{max} was 9.5 in grade 0, 6.0 in grade 1, 2.6 in grade 2 and 2.3 in grade 3 ($p < 0.0001$, Fig. 2b). The ROC analysis identified a post-SUV_{max} of 3.8 as the cutoff value that optimized sensitivity and specificity for predicting pathological major response. The AUC for the ROC analysis was 0.795 for post-SUV_{max}. With a post-SUV_{max} cutoff value of 3.8, the sensitivity, specificity, positive and negative predictive value for predicting pathological major response was 94.7, 57.1, 33.3 and 98.0 %, respectively. Although there was a significant difference in post-SUV_{max} among pathological response, post-SUV_{max} could not distinguish pathological complete response (grade 3) from good responder (grade 2).

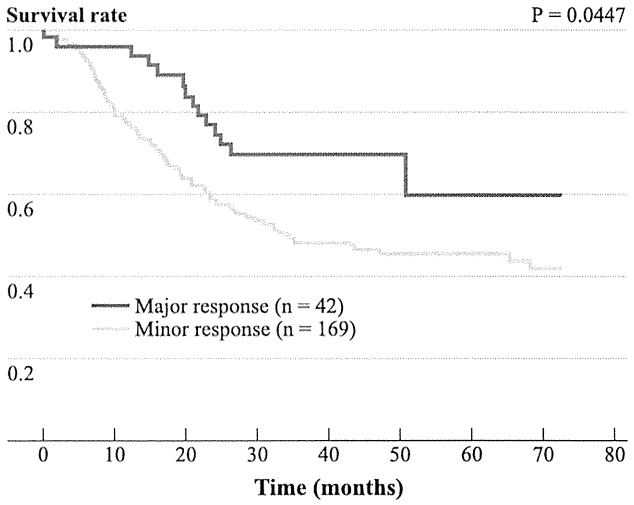

FIG. 1 Overall survival rate in 211 patients with esophageal cancers who underwent preoperative chemotherapy followed by surgery according to pathological response

Association Between Survival Rate and SUV_{max}-DR or Post-SUV_{max}

Univariate analysis of the prognostic factors demonstrated that pathological response, SUV_{max}-DR, post-SUV_{max} value, pathological response, pathological stage and number of metastatic lymph nodes were associated with patient survival (Table 2). SUV_{max}-DR of 50 %, post-SUV_{max} value of 3.5, and number of metastatic lymph nodes of three were utilized as the cutoff value because those cutoff values yield the largest difference in survival between the two groups with the highest hazard ratio and the lowest p value. Patients who experienced SUV_{max}-DR of >50 % demonstrated significantly longer survival compared with those with SUV_{max}-DR of <50 % (5-year overall survival 56.5 vs. 39.6 %, $p = 0.0137$, Fig. 3a). The 5-year overall survival rate of patients with post-SUV_{max} of <3.5 was significantly better than that of patients with post-SUV_{max} of >3.5 (62.2 vs. 35.1 %, $p < 0.0001$, Fig. 3b). Multivariate analysis that included the above parameters identified post-SUV_{max} value as independent and significant prognostic factor, together with the number of metastatic lymph nodes and pathological stage, whereas

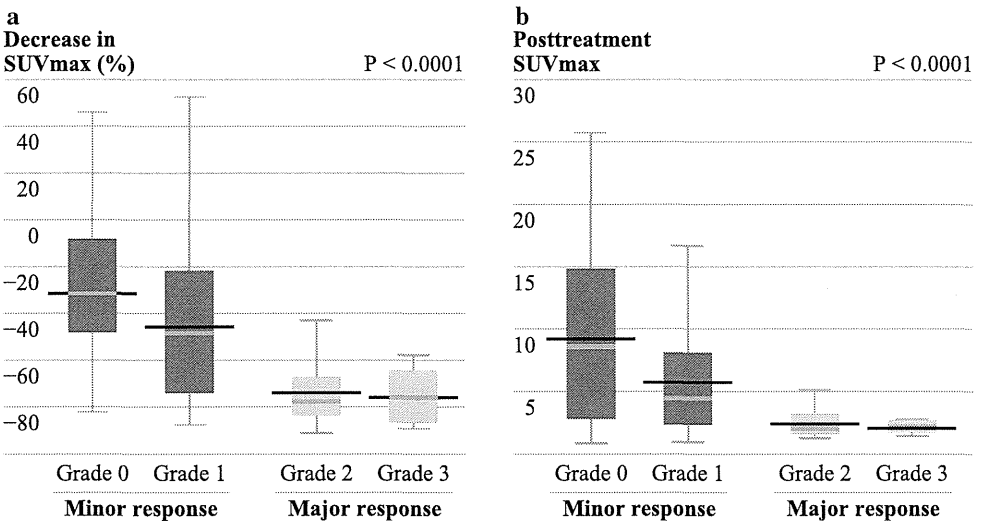

FIG. 2 Association between PET evaluation of response to therapy and pathological response. **a** SUV_{max}-DR during chemotherapy. **b** Absolute value of posttreatment SUV_{max}. *Symbols* are *box* plots representing medians, first and third quarter, and minimum and maximum. Each *horizontal line* indicates the mean value

TABLE 2 Results of univariate and multivariate analyses of the prognostic factors in patients with esophageal cancer

Characteristic	Variable	Univariate			Multivariate (model A)			Multivariate (model B)		
		HR	95 % CI	*p* value	HR	95 % CI	*p* value	HR	95 % CI	*p* value
Age	1-year increase	1.04	0.99–1.04	0.386						
Gender	Male versus female	1.13	0.67–1.91	0.636						
Tumor location	Lower versus upper/middle	0.73	0.49–1.10	0.131						
Histology	Poorly differentiated versus well/ moderately differentiated	1.34	0.87–2.05	0.183						
SUV_{max}-DR	1 % decrease	1.00	1.00–1.01	0.039						
SUV_{max}-DR	<50 versus >50	1.66	1.11–2.37	0.015	1.34	0.88–2.04	0.170			
Post SUV_{max} value	1 unit increase	1.05	1.01–1.08	0.006						
Post SUV_{max} value	≥3.5 versus <3.5	2.24	1.51–3.32	<0.0001				1.75	1.13–2.86	0.015
Pathological response	Minor response versus major response	1.74	1.01–3.01	0.047	1.22	0.65–2.30	0.531	1.38	0.73–2.60	0.324
No. of metastatic LN	1 node increase	1.02	1.00–1.02	0.0009						
No. of metastatic LN	≥3 versus <3	2.86	1.95–4.22	<0.0001	1.67	1.06–2.64	0.028	1.79	1.13–2.86	0.014
pStage				<0.0001						
	0–I	0.10	0.04–0.29							
	II	0.33	0.20–0.55							
	III	0.81	0.51–1.28							
	IV (ref)									
	III/IV versus I/II	3.46	2.27–5.29		2.72	1.64–4.50	0.0001	2.35	1.39–3.98	0.002

HR hazard ratio, *CI* confidence interval, *SUV_{max}-DR* decreased ratio of maximal standardized uptake, *LN* lymph node

SUV_{max}-DR was not an independent prognostic factor (Table 2).

Of 211 patients, we divided 185 patients who had follow-up periods of more than 3 years into short- and long-term survivors on the basis of a cutoff of 3 years of overall survival. Of 83 long-time survivors, 50 (60.2 %) had post-SUV_{max} of <3.5, while 39 (38.2 %) of 102 short-time survivors had post-SUV_{max} of <3.5. This difference was statistically significant ($p = 0.0029$). On the other hand, 49 (59.0 %) of 83 long-time survivors had SUV_{max}-DR of >50 %, while 48 (47.1 %) of 102 short-time survivors had SUV_{max}-DR of >50 %, and this difference was not statistically significant ($p = 0.1047$)

DISCUSSION

Recent studies have suggested that ^{18}F-FDG-PET is useful for evaluating the response to preoperative chemotherapy for esophageal cancer. In many cases, a decrease in the FDG uptake or absolute value of posttreatment FDG

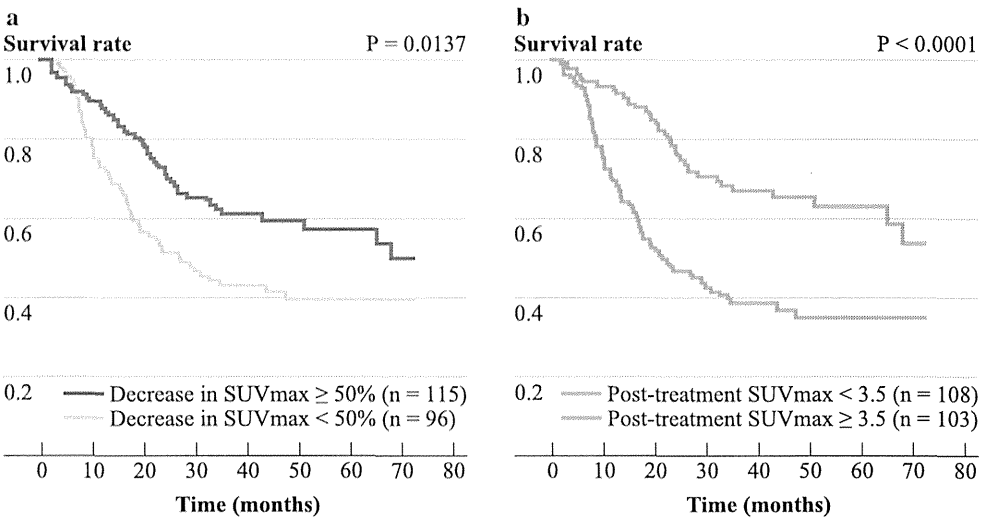

FIG. 3 Overall survival rate in 211 patients with esophageal cancers who underwent preoperative chemotherapy followed by surgery according to SUV_{max}-DR during chemotherapy (**a**) and according to the absolute value of post-SUV_{max} (**b**). *DR* decreased ratio

uptake after preoperative chemotherapy is used to reflect a positive response. However, there is no information on which of these two variables is more valuable in determining the response to neoadjuvant therapy and predicting prognosis of patients who receive neoadjuvant therapy followed by surgery. In the present study, which included a large number of patients with esophageal cancer, we found that the absolute value of post-SUV_{max} seems to be more valuable in predicting prognosis than SUV_{max}-DR, although SUV_{max}-DR and post-SUV_{max} equally correlate with pathological response.

Previous studies investigated the usefulness of decrease in FDG uptake during preoperative treatment in predicting the pathological response and prognosis of patients who receive multimodal therapy for esophageal cancer. Several studies demonstrated that early decrease in FDG uptake during preoperative treatment, such as 14 days after the start of chemotherapy or chemoradiotherapy, is predictive for both pathological response and prognosis.[17–19] In the above studies, the cutoff value for the decrease in FDG uptake applied to distinguish those whose disease responded to therapy and those whose disease did not respond to therapy was 30–35 %.[17–19,31,32] On the other hand, other studies also investigated the usefulness of late decrease in FDG uptake after completion of preoperative treatment in predicting pathological response and survival, but the results of these studies are controversial.[24,33,34] In the study of Port et al.[33] patients who demonstrated >50 % decrease in FDG uptake did not only experience a clinically good response and pathological down-staging, but also demonstrated better prognosis in preoperative chemotherapy followed by surgery for esophageal cancer. On the other hand, Vallböhmer et al.[34] found that the decrease in the FDG uptake at 2–3 weeks after completion of preoperative chemoradiation did not correlate with histopathological regression or prognosis. In our study, SUV_{max}-DR after completion of preoperative chemotherapy correlated significantly with patients survival in univariate analysis but it was not an independent predictor for survival in multivariate analysis.

Previous studies also investigated the utility of absolute value of FDG uptake after completion of preoperative treatment. For example, Swisher et al.[23] reported in their study of 83 patients who underwent preoperative chemoradiotherapy, a significantly correlation between posttreatment FDG uptake and pathological response. They also reported longer survival of patients with posttreatment FDG uptake of <4.0 compared to those with uptake of >4.0. In the study of Mamede et al.[24] FDG uptake of <4.35 after completion of preoperative treatment was the most reliable prognostic factor in patients who underwent preoperative chemoradiotherapy for esophageal cancer. In the study of Konski et al.[35] which included 81 patients who received definitive or preoperative chemoradiotherapy for esophageal cancer, posttreatment FDG uptake predicted disease-free survival in patients who had undergone definitive chemoradiotherapy, but not in patients who had undergone preoperative chemoradiotherapy. In our study, post-SUV_{max} correlated significantly with pathological response and multivariate analysis identified this parameters as an independent prognostic factor.

There are basically two different ways to examine a second ^{18}F-FDG-PET taken during the course of preoperative treatment: early examination after initiation of preoperative therapy or late examination after completion of preoperative therapy. Regarding early examination of the second ^{18}F-FDG-PET, several studies demonstrated that ^{18}F-FDG-PET examination after 14 days of initiation of preoperative therapy can differentiate responding tumors from nonresponding tumors early in the course of therapy, allowing for early modification of the treatment protocol such as discontinuation of preoperative therapy for patients

who demonstrate no metabolic response early in the course of therapy.[17,18,31] Indeed, in the study of Lordick et al.[19] patients with metabolic response (>35 % decrease in FDG uptake) at the time of 2 weeks after initiation of chemotherapy continued to receive preoperative chemotherapy, whereas those who did not respond to metabolic therapy at the same time discontinued preoperative chemotherapy and proceeded to surgery; this suggested the feasibility of a PET response-guided treatment algorithm. Thus, early examination of the second ^{18}F-FDG-PET may be useful for selecting the best therapeutic strategy during the course of treatment. On the other hand, the absolute value of FDG uptake measured by late examination after completion of therapy can reflect the volume of the residual tumor after preoperative treatment, which is a well-known predictor of prognosis of patients who undergo preoperative therapy followed by surgery for esophageal cancer.[9,10,36,37] In fact, post-SUV_{max} correlated significantly with pathological response in our study. Moreover, the present results of the usefulness of the absolute value of post-SUV_{max} as a predictor survival allow us to propose the use of only a single posttreatment ^{18}F-FDG-PET examination to predict survival instead of two ^{18}F-FDG-PET examinations before and after therapy.

In the present study, both SUV_{max}-DR and post-SUV_{max} correlated significantly with pathological response, but those could not distinguish pathological complete response (grade 3) from pathological good response (grade 2). This finding is consistent with the results of previous studies.[23,33,34] Port et al.[33] demonstrated that complete absence of FDG uptake cannot be equated with complete pathological response although a decrease in FDG uptake after preoperative therapy is a useful marker for the prediction of the pathological response and survival in patients who underwent preoperative chemotherapy followed by surgery for esophageal cancer. Similar disappointing results were reported by Vallböhmer et al.[34] demonstrating that FDG-PET is not useful for distinguishing major response from minor response and pathological CR from major response. Thus, although FDG-PET is useful to predict pathological response, it is not helpful for detecting pathological complete response to avoid additional surgery, for patients who experience pathological complete response to preoperative therapy. Further studies are needed to develop a method that can distinguish pathological complete response from major response.

There are several limitations to this study. There was some selection bias. Patients who tolerated chemotherapy poorly and were not fit for surgery were not included in this study. Similarly, patients who did not undergo surgical resection because of distant metastasis during chemotherapy and those who received chemoradiotherapy after neoadjuvant chemotherapy because of tumor enlargement during chemotherapy were also excluded. Second, there was little difference in pretreatment SUV_{max} among the patients, because most of patients enrolled in this study had clinical T3 or T4 tumors. This may be the reason why a single posttreatment ^{18}F-FDG-PET examination (post-SUV_{max}) is useful to evaluate pathological response in this study.

In conclusion, the results of the present study demonstrated that the absolute value of post-SUV_{max} seems to be more valuable for predicting prognosis than late SUV_{max}-DR in patients with esophageal cancer who received preoperative chemotherapy followed by surgery, although SUV_{max}-DR and post-SUV_{max} equally correlate with pathological response. Further studies are needed to avoid subsequent surgery for patients who experience pathological complete response to preoperative therapy, because ^{18}F-FDG-PET cannot distinguish pathological complete response from microscopic residual tumors at this time.

Disclosure The authors declare no conflict of interest.

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Mesenchymal phenotype after chemotherapy is associated with chemoresistance and poor clinical outcome in esophageal cancer

JOHJI HARA, HIROSHI MIYATA, MAKOTO YAMASAKI, KEIJIRO SUGIMURA, TSUYOSHI TAKAHASHI, YUKINORI KUROKAWA, KIYOKAZU NAKAJIMA, SHUJI TAKIGUCHI, MASAKI MORI and YUICHIRO DOKI

Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan

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Abstract. The relationship between the epithelial-mesenchymal transition (EMT) and resistance to anticancer treatment has attracted attention in recent years. However, to date, there is no direct clinical evidence for a link between the mesenchymal phenotype and chemoresistance in human malignancies. The expression of EMT-related markers, including E-cadherin, Snail, vimentin, ZEB1, β-catenin and N-cadherin was examined immunohistochemically in 185 tissue samples from patients with esophageal cancer (including 93 patients who received preoperative chemotherapy followed by surgery and 92 patients who underwent surgery without preoperative therapy). The relationship between the expression of the above markers and clinical outcome including prognosis and response to chemotherapy was also examined. The expression of E-cadherin, a marker of epithelial cells, was significantly lower in residual tumors than chemo-naive tumors (P=0.003). The expression of Snail (P=0.028), ZEB1 (P<0.001) and N-cadherin (P=0.001), markers of mesenchymal cells, was higher in residual tumors than in chemonaive tumors. The expression of E-cadherin correlated inversely with that of Snail (P<0.001). Reduced expression of E-cadherin and increased expression of Snail in residual tumors from patients who received chemotherapy correlated significantly with poor response to chemotherapy and short survival time. Multivariate analysis identified Snail expression as an independent prognostic factor, along with tumor depth, in patients who received preoperative chemotherapy for esophageal cancer. The results suggest transition of residual esophageal cancer cells to mesenchymal phenotype after chemotherapy and this contributes to resistance to chemotherapy and poor prognosis in patients with esophageal cancer.

Correspondence to: Dr Hiroshi Miyata, Department of Gastroenterological Surgery, Osaka University, Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: hmiyata@gesurg.med.osaka-u.ac.jp

Key words: esophageal cancer, chemotherapy resistance, epithelial-mesenchymal transition, snail, E-cadherin

Introduction

Esophageal cancer is one of the most aggressive and lethal malignancies. Surgical treatment is considered the standard management approach for esophageal cancer. However, despite recent advances in surgical technique, the prognosis of patients who undergo surgery alone is poor (1-3). Thus, multimodal treatment such as surgery following neoadjuvant chemotherapy or chemoradiotherapy is advocated. In fact, several clinical trials have shown that such multimodal therapies prolonged survival of patients with esophageal cancer (4-7). However, the reported response rate to chemotherapy in esophageal cancer is only 19-40% (1,2,4,8-10) and chemoresistance has emerged as a serious problem. Thus, there is a need to understand the underlying mechanism of chemoresistance in esophageal cancer.

Epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal phenotype. The latter phenotype is characterized by enhanced migratory capacity, invasiveness, high resistance to apoptosis and enhanced production of components of the extracellular matrix (ECM) (11). EMT and the reverse process, termed mesenchymal-epithelial transition (MET), play a central role in embryogenesis (type 1 EMT). EMT is also associated with wound healing, tissue regeneration and organ fibrosis (type 2 EMT) (12-14). Moreover, EMT occurs in neoplastic cells that have previously undergone genetic and epigenetic changes, specifically in genes that favor clonal outgrowth and the development of localized tumors (type 3 EMT). Upon undergoing EMT, cancer cells acquire migratory and invasiveness properties that allow them to migrate through the ECM, resulting in increased metastatic potential (15,16).

Accumulating evidence suggests a direct link between EMT and acquisition of stem cell characteristics (17). Induction of EMT confers many of the properties of self-renewing stem cells (17,18). These findings suggest that EMT plays an impor-

tant role in resistance to chemotherapy, because cancer stem cells are considered responsible for resistance to anticancer treatment, such as chemotherapy and radiotherapy (19-21). A possible association between EMT and chemotherapy resistance is suggested by recent studies on various cancer cells. However, there is virtually no direct clinical evidence that links mesenchymal phenotype to chemoresistance in human malignancies. Moreover, the association between EMT and chemoresistance has not been elucidated in esophageal cancers.

The present study was designed to determine the expression of EMT-related markers, including E-cadherin, snail, ZEB1 and vimentin, in residual tumors after chemotherapy using samples obtained from patients who underwent preoperative chemotherapy for esophageal cancers. The study also investigated the relationship between the expressions of such EMT markers with prognosis of patients who underwent chemotherapy.

Materials and methods

Patients and tissue samples. The 185 tissue samples were obtained from patients who underwent radical esophagectomy with lymph node dissection for thoracic esophageal cancer between 1999 and 2007 at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University (Osaka, Japan). Informed consent was obtained from each patient prior to participation in the study. Of these patients, 93 received preoperative chemotherapy followed by surgery while the remaining 92 patients underwent surgery without preoperative therapy. In 65 of the 93 patients who underwent preoperative chemotherapy followed by surgery, endoscopic biopsy samples were obtained before treatment and used for immunohistochemical analysis. Two courses of 4-week preoperative chemotherapy with cisplatin at 70 mg/m^2, adriamycin at 35 mg/m^2 by rapid intravenous infusion on Day 1 and 5-FU at 700 mg/m^2 by continuous intravenous infusion on Days 1-7 followed by 3-weeks off were scheduled before surgical treatment (6,22). The median duration of the follow-up period was 46 months (range, 18-78 months). Furthermore, 107 patients (57.8%) died during the follow-up.

Immunohistochemistry and evaluation. Resected tumor specimens were fixed with 10% formalin in phosphate-buffered saline (PBS). The paraffin-embedded tissue blocks were sectioned at 4-μm slices. The sections were deparaffinized in xylene and dehydrated in graded ethanol. For antigen retrieval, they were incubated in 10 mM citrate buffer at 95°C water bath for 40 min. The endogenous peroxidase activity in the tissue specimens was blocked by incubating the slides in 3% hydrogen peroxide (H_2O_2) solution in methanol at room temperature for 20 min. After treatment of the sections with 1% bovine serum albumin for 30 min at room temperature to block nonspecific reactions, all sections were incubated with a primary antibody at working dilution in a humidified chamber at 4°C overnight. The antibodies used in the study were anti-E-cadherin monoclonal antibody (mAb, dilution 1:100, buffer pH 9.0; Dako, Corp., Carpinteria, CA), anti-Snail polyclonal antibody (pAb, dilution 1:100, buffer pH 9.0; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-vimentin mAb (dilution 1:100, buffer pH 9.0; Santa Cruz Biotechnology, Inc.), anti-ZEB1 mAb (dilution 1:500, buffer pH 6.0; Dako, Corp.), anti-β-catenin mAb (dilution 1:100, buffer pH 9.0; Dako, Corp.), anti-N-cadherin pAb (dilution 1:200, buffer pH 9.0; Millipore, Bedford, MA). After incubation with secondary antibodies for 20 min, the reactions were visualized using Vectastain ABC immunoperoxidase kit (Vector Laboratories, Burlington, VT) with 3,3'-diaminobenzidine, which stained the antigen brown, and hematoxylin counterstaining.

Two investigators (J.H. and H.M.) independently evaluated the immunohistochemical sections. The deepest invaded area, called the invasive front, was recorded. The degree of E-cadherin and β-catenin immunostaining was graded as reduced, negative or cytoplasmic immunoreactivity; preserved, strong linear immunoreactivity on the cell membrane (23). The expression levels of nuclear-Snail and cytoplasmic-vimentin, cytoplasmic-ZEB1, membrane- or cytoplasmic-N-cadherin were scored as negative, ≤10% positive tumor cells; positive, >10% positive tumor cells (Fig. 1).

Clinical and histopathological evaluation of response to chemotherapy. Two weeks after 2 cycles of neoadjuvant chemotherapy, all patients were re-assessed to evaluate the clinical response to chemotherapy by endoscopy, computed tomography (CT) and positron emission tomography (PET). The World Health Organization response criteria for measureable disease and the criteria of the Japanese Society for Esophageal Diseases were used to assess clinical response (24,25). A complete response (CR) was defined as disappearance of all lesions. A CR of the primary tumor represented disappearance of the tumor on CT scan and/or PET scan and endoscopy. A partial response (PR) was defined as >50% reduction in primary tumor size and lymph node metastasis, as confirmed by CT scan. Progressive disease (PD) was defined as >25% increase in the primary tumor or the appearance of new lesions. Stable disease (SD) was defined as neither sufficient decrease to qualify for PR nor sufficient increase to qualify for PD.

Based on the percentage of viable residual tumor cells at the primary site after neoadjuvant chemotherapy, curative effect was classified into five categories. Briefly, the percentage of viable residual tumor cells within the entire cancer tissue was assessed as follows: grade 3, no viable residual tumor cells are evident; grade 2, viable residual tumor cells account for less than one-third of tumor tissue; grade 1b, viable residual tumor cells account for less than one-third or more but less than two-thirds of tumor tissue; grade 1a, viable residual tumor cells account for two-thirds or more tumor tissue; and grade 0, no recognizable histlogical chemotherapy effect (6,25).

Statistical analysis. Statistical analysis of group differences was performed using the χ^2 test, Fisher's exact test or Mann-Whitney U test. For survival analysis, the Kaplan-Meier method was used to assess survival distribution according to EMT-marker expression and differences in survival were estimated using the log-rank test. The Cox proportional hazards regression model was used to analyze the simultaneous influence of prognostic factors. Wilcoxon signed-ranks test was used to assess the change in E-cadherin and Snail expression

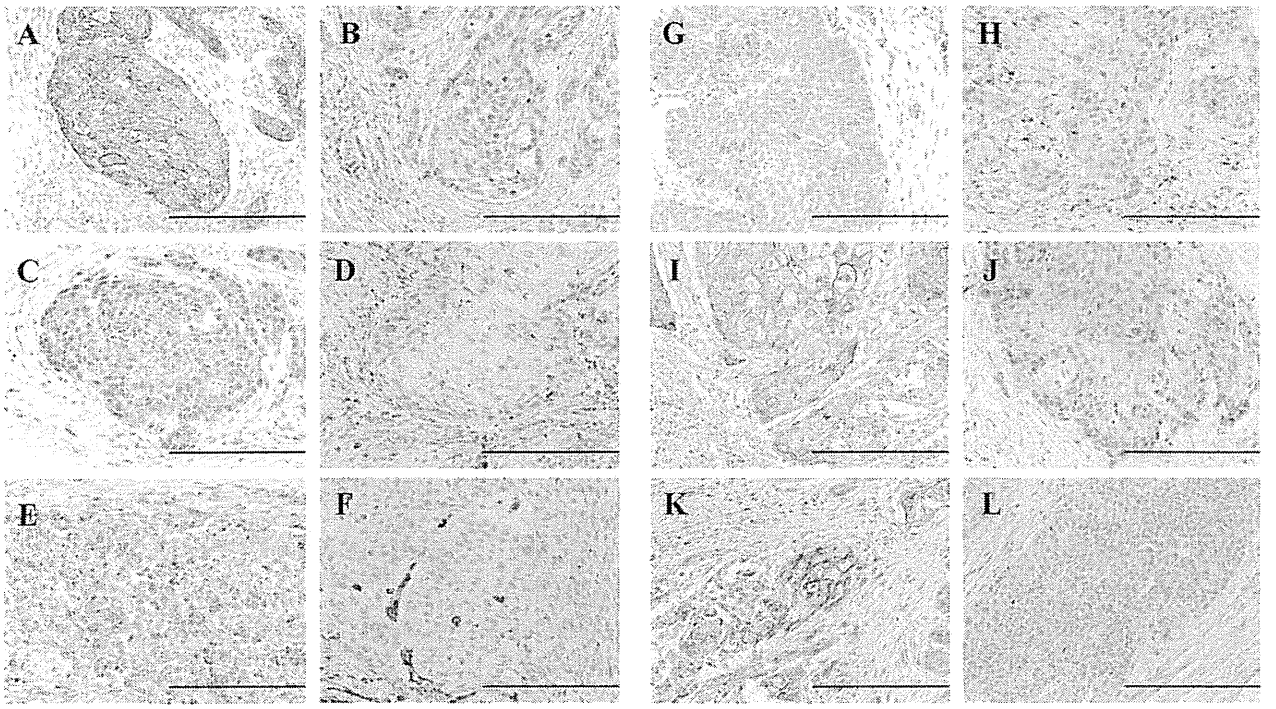

Figure 1. Immunohistochemical expression of E-cadherin, Snail, vimentin, ZEB1, β-catenin and N-cadherin in human esophageal squamous cell carcinoma. We examined the deepest invading area, known as the invasive front. (A) Membranous expression of E-cadherin. (B) Cytoplasmic and negative expression of E-cadherin. (C) Nuclear expression of Snail. (D) Negative nuclear expression of Snail. (E) Cytoplasmic expression of vimentin. (F) Lack of expression of vimentin. (G) Cytoplasmic expression of ZEB1. (H) Negative expression of ZEB1. (I) Membranous expression of β-catenin. (J) Cytoplasmic and negative expression of β-catenin. (K) Membranous or cytoplasmic expression of N-cadherin. (L) Lack of expression of N-cadherin. Magnification, x200.

Table I. Characteristics of 185 patients with esophageal cancer.

	Chemotherapy		
	Residual (n=93)	Naive (n=92)	P-value
Gender (male/female)	79/14	83/9	0.276
Age (mean)	64.0	63.7	0.512
Tumor location (upper/middle/lower)	22/36/35	12/47/33	0.236
Differentiation (G1,2/G3,4)	65/28	75/17	0.418
Depth of invasion (pT1-2/3-4)	32/61	41/51	0.157
Lymph node metastasis (pN0/1)	27/65	33/59	0.345
Lymphatic permeation (positive/negative)	77/16	70/22	0.258
Venous permeation (positive/negative)	52/41	43/49	0.212

after chemotherapy. A P-value of <0.05 denoted the presence of statistically significant difference between groups. All statistical analyses were performed using the software package JMP 8 for Windows (SAS Institute, Inc., Cary, NC).

Results

Expression of EMT makers in residual and chemo-naive tumors. Of the 195 tumors, 93 tumors were residual tumors after preoperative chemotherapy and 92 tumors were chemo-naive tumors without preoperative therapy. There was no significant difference between residual tumors and chemo-naive tumors in differentiation, tumor depth and lymph node metastasis (Table I).

We quantitated the expression of the epithelial marker E-cadherin and mesenchymal markers snail, vimentin, ZEB1, and N-cadherin in residual tumors and chemo-naive tumors (Table II). Fifty percent (46/92) of chemo-naive tumors stained strongly for E-cadherin, while 71% of residual tumors stained weakly for E-cadherin. Statistical analysis indicated significant underexpression of E-cadherin, as a marker of epithelial cells, in residual tumors compared with chemo-naive tumors (P=0.003). Snail expression was significantly higher in residual tumors than in chemo-naive tumors (P=0.028). Similarly, the expression levels of ZEB1 and N-cadherin were significantly higher in residual tumors than in chemo-naive tumors (P<0.001 and P=0.001, respectively). However, there were no significant differences in the expression levels of vimentin and β-catenin

Table II. Expression of mesenchymal and epithelial markers in residual tumors after chemotherapy and chemo-naive tumors.

	Chemotherapy			
	Residual (n=93)	Naive (n=92)	Total (n=185)	P-value
E-cadherin				
Preserved	27 (29.0)	46 (50.0)	73 (39.5)	0.003
Reduced	66 (71.0)	46 (50.0)	112 (60.1)	
Snail				
Positive	66 (71.0)	51 (55.4)	117 (63.4)	0.028
Negative	27 (29.0)	41 (44.6)	68 (36.6)	
Vimentin				
Positive	11 (11.8)	8 (8.7)	19 (10.3)	0.482
Negative	82 (88.2)	84 (91.3)	166 (89.7)	
ZEB1				
Positive	36 (38.7)	14 (15.2)	50 (27.0)	<0.001
Negative	57 (61.3)	78 (84.8)	135 (73.0)	
β-catenin				
Preserved	32 (34.4)	27 (29.3)	59 (31.9)	0.460
Reduced	61 (65.6)	65 (70.1)	126 (68.1)	
N-cadherin				
Positive	51 (54.8)	29 (31.5)	80 (43.2)	0.001
Negative	42 (45.2)	63 (68.5)	105 (66.8)	

Data are numbers (percentages) of patients.

Table III. Relationship between expression of E-cadherin and EMT markers in the residual group.

	E-cadherin			
	Preserved (n=27)	Reduced (n=66)	Total (n=93)	P-value
Snail				
Positive	10 (37.0)	56 (84.8)	66 (71.0)	<0.001
Negative	17 (63.0)	10 (15.2)	27 (29.0)	
Vimentin				
Positive	2 (3.7)	9 (13.6)	11 (11.8)	0.379
Negative	25 (96.3)	57 (86.4)	82 (88.1)	
ZEB1				
Positive	8 (29.6)	28 (42.4)	36 (38.7)	0.245
Negative	19 (70.4)	38 (57.6)	57 (61.3)	
β-catenin				
Preserved	12 (44.4)	20 (30.3)	32 (34.4)	0.197
Reduced	15 (55.6)	46 (69.7)	61 (65.6)	
N-cadherin				
Positive	17 (63.0)	34 (51.5)	51 (54.8)	0.311
Negative	10 (27.0)	32 (48.5)	42 (45.2)	

Data are numbers (percentages) of patients.

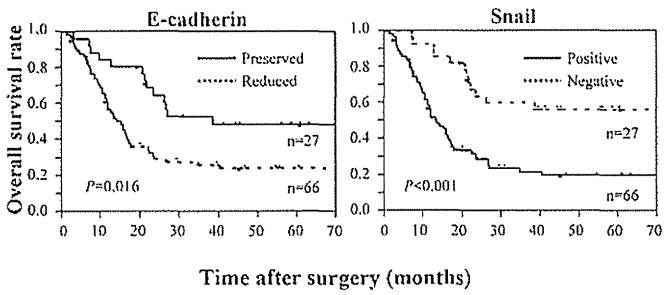

Figure 2. Postoperative overall survival curves according to the immunohistochemical expression of E-cadherin and Snail in the residual group. Left: reduced expression of E-cadherin correlated significantly with short survival of patients of the residual group. Right: high expression of Snail correlated significantly with short survival of patients of the residual group.

between the two types of tumors. Taken together, higher expression of mesenchymal markers and lower expression of epithelial markers characterize residual tumors after chemotherapy.

We examined the relationship between E-cadherin expression, as an epithelial marker, and the expression of several mesenchymal markers (N-cadherin, vimentin, Snail and ZEB1) in the residual group. E-cadherin expression correlated inversely with Snail expression (Table III).

Relationship between EMT markers and response to chemotherapy. Next, we examined the relationship between the expression of EMT markers and the response to chemotherapy in the residual tumors. With regard to the clinical response, weak E-cadherin expression correlated significantly with clinically poor response (SD/PD), but not with clinically good response (PR) (P=0.009, Table IV). On the other hand, positive staining for Snail expression in tumors correlated significantly with SD/PD, but not PR (P=0.009).

Similar to the clinical response, negative E-cadherin expression and positive staining for Snail expression correlated with histopathologically minor response (Grade 0/1a), but not with major response Grade 1b/2 (P=0.001 and P=0.027, respectively) (Table V).

Relationship between EMT markers and survival. We also examined relationship between the expression of EMT markers and prognosis of patients who underwent preoperative chemotherapy for esophageal cancer. Low expression of E-cadherin correlated significantly with short survival time (Fig. 2). In contrast, high expression of Snail correlated significantly with short survival time (Fig. 2). Multivariate analysis identified Snail expression as an independent prognostic factor, together with tumor depth, in patients who received preoperative chemotherapy for esophageal cancer (Table V).

Changes in E-cadherin and Snail expression after chemotherapy and survival. In 65 of 93 patients with esophageal cancer who underwent preoperative chemotherapy followed by surgery, we used immunohistochemistry to compare biopsy samples obtained before chemotherapy with the surgical

Table IV. Relationship between response to chemotherapy and immunohistochemical expression of E-cadherin, Snail, vimentin, ZEB1, β-catenin and N-cadherin in residual tumors.

		Clinical response			Pathological response		
	Total (n=93)	PD/SD (n=47)	PR (n=46)	P-value	Grade 0/1a (n=67)	Grade 1b/2 (n=26)	P-value
E-cadherin							
Preserved	27 (29)	8 (17)	19 (41)	0.009	13 (19)	14 (54)	0.001
Reduced	66 (71)	39 (83)	27 (59)		54 (81)	12 (46)	
Snail							
Positive	66 (71)	39 (83)	27 (59)	0.009	52 (72)	14 (54)	0.027
Negative	27 (29)	8 (17)	19 (41)		15 (22)	12 (46)	
Vimentin							
Positive	11 (12)	5 (11)	6 (13)	0.719	8 (12)	3 (12)	0.957
Negative	82 (88)	42 (89)	40 (87)		59 (88)	23 (88)	
ZEB1							
Positive	36 (39)	15 (31)	21 (46)	0.173	26 (39)	10 (38)	0.976
Negative	57 (61)	32 (68)	25 (54)		41 (41)	16 (62)	
β-catenin							
Preserved	32 (34)	17 (36)	15 (33)	0.717	23 (34)	9 (35)	0.979
Reduced	61 (66)	30 (64)	31 (67)		44 (66)	17 (65)	
N-cadherin							
Positive	51 (55)	28 (60)	23 (50)	0.353	40 (60)	11 (42)	0.131
Negative	42 (45)	19 (40)	23 (50)		27 (40)	15 (58)	

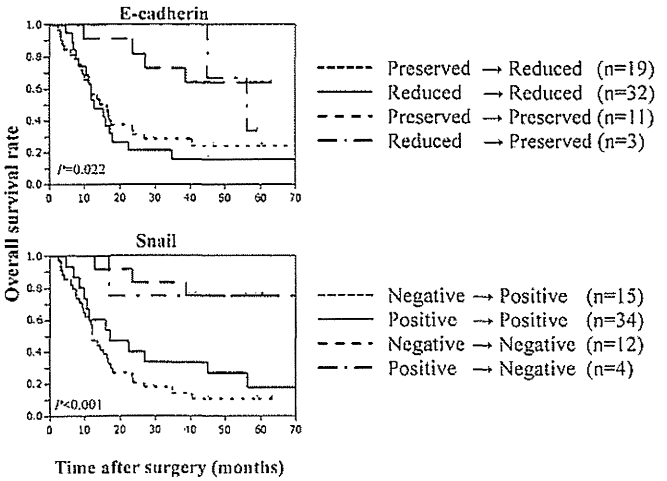

Figure 3. Postoperative overall survival curves according to the immunohistochemical expression of E-cadherin and Snail before and after chemotherapy. Top: short survival of patients (n=51) with decreased expression of E-cadherin after chemotherapy and unchanged low expression of E-cadherin after chemotherapy. Bottom: short survival of patients (n=49) with increased expression of Snail after chemotherapy and unchanged positive expression of Snail after chemotherapy.

specimens after chemotherapy. Among these 65 patients, chemotherapy decreased the expression of E-cadherin in 19 (preserved→reduced) (Table VI). The survival time was significantly shorter in 51 patients with low E-cadherin expression [including the above 19 patients and 32 patients who showed no change in their low E-cadherin expression after chemotherapy (reduced→reduced)], compared with 11 patients with preserved expression of E-cadherin throughout chemotherapy (Fig. 2). With regard to Snail expression, chemotherapy increased Snail expression in 15 of the 65 patients (negative to positive). The survival time was significantly shorter in 49 patients with positive Snail expression [including the above 15 patients and 34 patients who showed no change in positive Snail expression after chemotherapy (negative to positive)], compared with 12 patients with Snail-negative tumors throughout chemotherapy (Fig. 3).

Discussion

Although recent evidence indicates that EMT does not only cause increased metastasis but also contributes to chemoresistance, there is no direct clinical evidence for a link between mesenchymal phenotype and chemoresistance in human malignancies. In this study, we examined the expression of EMT-related markers in residual tumors after chemotherapy using samples obtained from patients who underwent preoperative chemotherapy for esophageal cancer. The results showed reduced expression of E-cadherin (a marker of epithelial cells) and increased expression of Snail, ZEB1 and N-cadherin (markers of mesenchymal cells) in residual tumors after chemotherapy, compared with chemo-naive tumors. Moreover, the reduced expression of E-cadherin and increased expression of snail in residual tumors were significantly asso-

Table V. Univariate and multivariate analyses of prognostic factors.

	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender (male/female)	0.84	0.46-1.71	0.619			
Age (≤65/>65)	1.22	0.74-2.02	0.422			
Tumor location (upper, middle/lower)	0.73	0.43-1.21	0.225			
Differentiation (G1,2/G3,4)	0.97	0.55-1.74	0.920			
Depth of invasion (pT1-2/pT3-4)	2.49	1.35-5.05	0.003	2.13	1.12-4.37	0.018
Lymph node metastasis (pN0/1)	3.12	2.32-4.21	<0.001	2.12	1.21-4.24	0.009
Lymphatic invasion (positive/negative)	2.09	0.81-3.99	0.181			
Venous invasion (positive/negative)	1.21	0.74-2.02	0.437			
E-cadherin (preserved/reduced)	0.56	0.30-0.98	0.043	1.21	0.63-2.21	0.551
Snail (positive/negative)	3.31	1.78-6.71	<0.001	3.83	1.96-8.11	<0.0001
Vimentin (positive/negative)	0.86	0.38-1.70	0.679			
ZEB1 (positive/negative)	0.88	0.51-1.45	0.617			
β-catenin (preserved/reduced)	1.41	0.85-2.33	0.179			
N-cadherin (positive/negative)	0.93	0.56-1.53	0.760			
Clinical response (PD-SD/PR)	2.29	1.38-3.87	0.001	1.68	0.99-2.92	0.052

HR, hazard ratio; 95% CI, 95% confidence interval; PD, progressive disease; SD, stable disease; PR, partial response.

Table VI. Changes in E-cadherin and Snail expression after chemotherapy.

	Pre-CT biopsy	Residual	n	P-value
E-cadherin	Preserved	Reduced	19	<0.001
	Reduced	Reduced	32	
	Preserved	Preserved	11	
	Reduced	Preserved	3	
Snail	Negative	Positive	15	0.019
	Positive	Positive	34	
	Negative	Negative	12	
	Positive	Negative	4	

ciated with poor response to chemotherapy and short survival time in patients who underwent preoperative chemotherapy. These results suggest that residual esophageal tumors after chemotherapy display mesenchymal features, resulting in chemoresistance and poor prognosis.

Reduced expression of E-cadherin, which is a central adhesion molecule located at cell-cell adhesion junctions, is one of the characteristics findings during progression of EMT (26). Previous studies demonstrated that the loss of E-cadherin is associated with tumor progression, tumor metastasis and poor clinical outcome in various human carcinomas (27-31). The association of E-cadherin expression and drug sensitivity has been examined in several types of human cancer. In colorectal cancer, E-cadherin was downregulated in oxaliplatin-resistant colorectal cancer (CRC) cells (28). In gemcitabine-resistance pancreatic cancer cells, E-cadherin expression was decreased and nuclear localization of total β-catenin was increased (30). While the above studies showed downregulation of E-cadherin in drug-resistant tumor cell lines, there is little or no evidence for the clinical importance of E-cadherin expression in drug-resistant human cancers. Using samples from patients who underwent preoperative chemotherapy for esophageal cancers, we demonstrated in this study the importance of E-cadherin underexpression in chemoresistance in human esophageal cancer.

Snail is recognized as a suppressor of E-cadherin expression. Snail represses the transcription of E-cadherin by binding to the E-box elements in the proximal E-cadherin promoter, thereby triggering a complete EMT and resulting in enhanced tumor invasiveness (30). Accumulating evidence suggests the contribution of Snail expression to therapeutic resistance in various cancers (28-30,33). Paclitaxel-resistant ovarian cancer cells showed upregulation of Snail expression, with marked enhancement of metastatic activity, compared with control cells (30). In head and neck cancer, Snail contributes to cisplatin resistance by upregulating excision repair cross complementation group 1 (ERCC1), which plays a key role in nucleotide excision repair and in platinum-induced DNA adducts (33). In the present study, upregulation of Snail was observed in residual tumors after chemotherapy for esophageal cancers and such high expression was significantly associated with poor response to chemotherapy. These results provide direct evidence for the important role of Snail expression in chemoresistance in human esophageal cancer.

In the present study, we examined the relationship between E-cadherin expression, as an epithelial marker, and the expression of several mesenchymal markers (N-cadherin, vimentin, Snail and ZEB1). In recent years, a switch from E-cadherin to N-cadherin has been often used to monitor the progress of EMT during embryonic development and cancer progression (34). In

our study, although N-cadherin expression was increased in residual tumors, compared with chemo-naive tumors, we could not find significant inverse relationship between E-cadherin and N-cadherin expression. Snail and ZEB1 are well known transcription repressors of E-cadherin (29,30,32,35), and our results showed inverse correlation between E-cadherin and Snail expression, although we could not find a significant correlation between E-cadherin and ZEB1 expression.

Recent studies have indicated that cancer cells undergoing EMT develop resistance to anticancer drugs. However, it has been difficult to establish the role of EMT in chemoresistance in human clinical samples. In the present study, we investigated whether EMT confers resistance to chemotherapy by comparing the expression of EMT-related markers in residual tumors after chemotherapy with that in chemo-naive tumors. A few studies have previously shown the presence of EMT in residual tumors after conventional anti-cancer therapy. One such study demonstrated recently mesenchymal features of tumor cells that had survived conventional treatment, such as chemotherapy and endocrine therapy, in human breast cancer (36). The results of the present study demonstrating mesenchymal features of tumor cells after chemotherapy in esophageal cancer provide further support to the above previous studies.

One important problem in the present study is whether tumor cells with initial mesenchymal phenotype survive the chemotherapy or whether residual tumor cells acquire mesenchymal features during chemotherapy. In this study, we compared the expression of EMT-related markers such as E-cadherin and Snail before and after chemotherapy in the same case, and found in certain cases mesenchymal features in residual tumors after chemotherapy compared with epithelial features before treatment. This finding suggests that residual tumor cells seem to acquire mesenchymal features during chemotherapy. However, the value of immunohistochemistry in accurate assessment of gene expression in biopsy samples is limited, because biopsy samples do not allow accurate estimation of such events in the invasive front of tumors. Recent studies have pointed to link between EMT phenotype and development of cancer stem cells; cancer cells undergoing EMT exhibit characteristic markers of cancer stem cells and properties of cancer stem cells (17). However, other studies have suggested that cancer stem cells from solid tumors are not actually static entities but rather tumor cells that transiently acquire stemness properties depending on the tumor context (37), although the traditional concept of cancer stem cells is a unidirectional hierarchical model. These findings suggest that residual esophageal cancer cells may transiently acquire mesenchymal features to survive during chemotherapy. In support of this notion, one recent study showed that cancer cell populations employ a dynamic strategy in which individual cells transiently assume a reversibly drug-tolerant state to protect the remaining population from eradication by exposure to lethal anti-cancer drugs (38). Further studies are required to ascertain whether esophageal cancer cells transiently acquire mesenchymal features and stemness properties during chemotherapy in human esophageal cancers.

In conclusion, the present study demonstrated decreased expression of E-cadherin and increased expression of Snail, ZEB1 and N-cadherin in residual tumors after chemotherapy in human esophageal cancers, compared with chemo-naive tumors. Moreover, in patients who underwent preoperative chemotherapy, the reduced expression of E-cadherin and increased expression of Snail in residual tumors correlated significantly with poor response to chemotherapy and poor prognosis. These findings suggest that residual tumors after chemotherapy for esophageal cancer switch to mesenchymal phenotype, resulting in chemoresistance and poor clinical outcome.

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Prognostic Value of CEA and CK20 mRNA in the Peritoneal Lavage Fluid of Patients Undergoing Curative Surgery for Gastric Cancer

Akihiro Takata · Yukinori Kurokawa · Yoshiyuki Fujiwara · Yurika Nakamura · Tsuyoshi Takahashi · Makoto Yamasaki · Hiroshi Miyata · Kiyokazu Nakajima · Shuji Takiguchi · Masaki Mori · Yuichiro Doki

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Abstract

Background Peritoneal recurrence is the most common type of recurrence in gastric cancer. Although cytological examination of peritoneal lavage fluid has been used to predict peritoneal spread, peritoneal recurrences often occur even in patients with negative cytology. Our previous retrospective study suggested that reverse transcriptase-polymerase chain reaction (RT-PCR) using peritoneal lavage fluid may be useful for predicting peritoneal recurrence in patients with negative cytology. This prospective study was conducted to validate the clinical impact of this RT-PCR method.

Methods From July 2009 to June 2012, a total of 118 cT2-4 gastric cancer patients underwent surgery. Since 14 patients were ineligible because they had incurable factors, the remaining 104 eligible patients were evaluated for carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) messenger RNA (mRNA) using RT-PCR. If either CEA or CK20 mRNA was detected by RT-PCR, the patient was defined as PCR-positive as in our previous study. The association between recurrence-free survival (RFS) and background factors was analyzed using Cox proportional hazards models.

Results Of 104 patients, 16 (15.4 %) were positive for either CEA or CK20. PCR-positive patients had significantly worse RFS than PCR-negative patients (log-rank $p = 0.007$). Regarding the pattern of recurrence, 4 of 16 (25 %) PCR-positive patients and 2 of 88 (2 %) PCR-negative patients had peritoneal recurrence ($p < 0.001$), but there were no significant differences in recurrence at other sites. Cox multivariate analysis indicated only PCR-positivity as a significant predictor of poor RFS ($p = 0.029$).

Conclusion This prospective study demonstrated that CEA and CK20 PCR results could predict peritoneal recurrence after curative surgery.

A. Takata · Y. Kurokawa (✉) · Y. Nakamura · T. Takahashi · M. Yamasaki · H. Miyata · K. Nakajima · S. Takiguchi · M. Mori · Y. Doki
Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2-E2, Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: ykurokawa@gesurg.med.osaka-u.ac.jp

Y. Fujiwara
Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka, Japan

Introduction

The prognosis of advanced gastric cancer remains poor, even after curative surgery. Peritoneal dissemination, mainly caused by the seeding of free cancer cells from the primary lesion, is the most common type of recurrence [1]. Cytological examination of peritoneal lavage fluid collected during surgery is used to predict peritoneal spread since positive peritoneal cytology (CY1) has been found to be an independent predictor of disease recurrence and poor overall survival [2–4]. However, peritoneal recurrences often occur even in patients with negative cytology, which indicates that cytological examination is not sensitive enough for the detection of residual cancer cells in the peritoneum.

Molecular diagnosis using reverse transcriptase-polymerase chain reaction (RT-PCR) has been used to detect cancer micrometastases [5–7]. Carcinoembryonic antigen (CEA) and cytokeratine-20 (CK20) are the most common targets for detecting isolated tumor cells using PCR [8–10],

and we previously reported that, among 36 gastric cancer patients, PCR-positive patients had significantly worse survival than PCR-negative patients [11]. However, our previous study was retrospective in nature and included a small number of patients, so we conducted a prospective study to validate the prognostic value of molecular detection in over 100 patients undergoing curative surgery for gastric cancer.

Patients and methods

Patients

Peritoneal lavage fluid was prospectively collected during surgery from 118 consecutive patients with cT2-4 gastric cancer at Osaka University Hospital between July 2009 and June 2012. All patients were histologically diagnosed with adenocarcinoma of the stomach. Patients with incurable factors, such as peritoneal metastases (P1), CY1, or other distant metastases (M1) were excluded from this study.

Peritoneal lavage fluid was collected as described in our previous report [12]. In brief, peritoneal lavage fluid was immediately obtained from the pouch of Douglas and the left subdiaphragmatic space after laparotomy or the insertion of trocars. We injected 100 mL of normal saline and suctioned again. Approximately half of the sample was examined cytologically and the remainder was centrifuged at $300\times g$ for 5 min. Cells were then suspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C. Pathological staging of the tumor was based on the seventh edition of the International Union Against Cancer (UICC) tumor–node–metastasis classification guidelines [13]. The study protocol was approved by the institutional review board of Osaka University Hospital. All patients provided written informed consent for their samples to be used in research.

Quantitative RT-PCR

RNA isolation and RT-PCR were performed using a method similar to those in our previous studies [11, 12]. Frozen samples in TRIzol reagent were thawed and total RNA was extracted using the acid guanidinium thiocyanate-phenol–chloroform method [14]. Its concentration was determined spectrophotometrically by measuring the absorbance of RNA at 260 nm. First strand complementary DNA (cDNA) was synthesized from total RNA (1 μg), mixed with RT reaction reagents, including oligo-(dT)15 primer, using the protocol recommended by the manufacturer (Promega, Madison, WI, USA). CEA-specific oligonucleotide primers for RT-PCR were 5′-TCTGGAACTTCTCCTGGTCTCTC AGCTGG-3′ (forward) and 5′-TGTAGCTGTTGCAAATG CTTTAAGGAAGAAGC-3′ (reverse) to amplify a 160 bp PCR product. CK20-specific oligonucleotide primers for RT-PCR were 5′-GGTCGCGACTACAGTGCATATTACA -3′ (forward) and 5′-CCTCAGCAGCCAGTTTAGCAT TATC-3′ (reverse) to amplify a 121 bp PCR product. The integrity of extracted RNA was confirmed by RT-PCR analysis of a housekeeping gene, porphobilinogen deaminase (*PBGD*). Primer sequences for *PBGD* were 5′-TGTCT GGTAACGGCAATGCGGCTGCAAC-3′ (forward) and 5′-TCAATGTTGCCACCACACTGTCCGTCT-3′ (reverse). The integrity of all RNA samples was verified by quantitative RT-PCR for *PBGD* in each sample. The emission intensity of SYBR Green was detected in real time with the LightCycler 3.5 instrument (Roche Diagnostics, Mannheim, Germany). The external standards were prepared by serial dilution (1:1–1:10,000) of cDNA from the MKN45 cell line. CEA messenger RNA (mRNA) was detected to 10,000 times attenuation (1:10,000), and CK20 mRNA was detected to 500 times attenuation (1:500). If either CEA or CK20 mRNA was detected by RT-PCR analysis, the patient was defined as PCR-positive, similar to our previous study [11].

Statistical analysis

Patient clinicopathological data were prospectively recorded. The relationship between RT-PCR results and various background factors was assessed using the χ^2 test. Recurrence-free survival (RFS) was defined as the time from surgery to first recurrence. RFS was censored at the time of the last follow-up or death without recurrence. Survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. The impact of background factors (age, sex, histology, neoadjuvant chemotherapy, and pathological T and N stages) on survival was analyzed with univariate and multivariate Cox proportional hazards models. p values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics software, version 20 (IBM Corp., Armonk, NY, USA).

Results

PCR results

From July 2009 to June 2012, a total of 118 patients with cT2-4 gastric cancer underwent surgery; 14 were ineligible due to incurable factors such as P1, CY1, or M1. The remaining 104 eligible patients were evaluated for CEA and CK20 mRNA using RT-PCR. Among the 104 patients, 11 patients (10.6 %) were positive for CEA and ten patients (9.6 %) were positive for CK20 (Table 1). In total,

Table 1 RT-PCR positive rate for each marker

CK20	CEA, *n* (%)	
	Positive	Negative
Positive	5 (4.8 %)	5 (4.8 %)
Negative	6 (5.8 %)	88 (84.6 %)

CEA carcinoembryonic antigen, *CK* cytokeratin, *RT-PCR* reverse transcriptase-polymerase chain reaction

16 patients (15.4 %) were positive for either CEA or CK20, and we defined these patients as PCR-positive.

We examined the relationship between the PCR results and background factors (Table 2). The PCR-positive group included more female patients and higher pathological N-stage patients than the PCR-negative group ($p = 0.032$, $p = 0.029$). No significant relationship was observed with other background factors, including age, histology, surgical approach, neoadjuvant chemotherapy, clinical T or N stage, and pathological T stage. Regarding the neoadjuvant chemotherapy, we used three types of regimens; S-1 plus cisplatin ($n = 6$), S-1 plus docetaxel ($n = 2$), and S-1 plus cisplatin plus docetaxel ($n = 12$). There was no significant difference in neoadjuvant regimens between PCR-positive and negative patients ($p = 0.25$).

Prognostic value of CEA and CK20 mRNA

The median follow-up in this prospective study was 18.2 months, during which 7 of 16 (44 %) PCR-positive patients and 13 of 88 (15 %) PCR-negative patients had recurrences ($p = 0.007$). PCR-positive patients had significantly worse RFS than PCR-negative patients (log-rank $p = 0.007$) (Fig. 1), and the hazard ratio for recurrence in PCR-positive patients was 3.28 (95 % confidence interval [CI] 1.31–8.24). The 2-year RFS rate in PCR-positive patients was 50.3 %, while that of PCR-negative patients was 83.0 %. Regarding the pattern of recurrence, 4 of 16 (25 %) PCR-positive patients and 2 of 88 (2 %) PCR-negative patients had peritoneal recurrence ($p < 0.001$), while PCR-positive and -negative patients were similar with respect to other sites of recurrence (Table 3).

We conducted Cox univariate and multivariate analyses to find independent prognostic factors of RFS. The multivariate analysis indicated that PCR-positivity was a significant predictor of poor RFS ($p = 0.029$) (Table 4).

Table 2 Relationship between PCR results and background factors

Factors	PCR-positive ($n = 16$)	PCR-negative ($n = 88$)	p value*
Age, years			0.30
≤65	4 (25 %)	34 (39 %)	
>65	12 (75 %)	54 (61 %)	
Sex			0.032
Male	8 (50 %)	67 (76 %)	
Female	8 (50 %)	21 (24 %)	
Histology			0.74
Differentiated	8 (50 %)	48 (55 %)	
Undifferentiated	8 (50 %)	40 (45 %)	
Surgical approach			0.71
Open	15 (94 %)	80 (91 %)	
Laparoscopic	1 (6 %)	8 (9 %)	
Neoadjuvant chemotherapy			0.52
Yes	4 (25 %)	16 (18 %)	
No	12 (75 %)	72 (82 %)	
cT			0.86
T2	3 (19 %)	18 (20 %)	
T3	6 (38 %)	27 (31 %)	
T4	7 (44 %)	43 (49 %)	
cN			0.50
N0	6 (38 %)	47 (53 %)	
N1	4 (25 %)	17 (19 %)	
N2–3	6 (38 %)	24 (27 %)	
pT			0.14
T1–2	2 (13 %)	33 (38 %)	
T3	10(63 %)	42 (48 %)	
T4	4 (25 %)	13 (15 %)	
pN			0.029
N0	5 (31 %)	52 (59 %)	
N1	4 (25 %)	22 (25 %)	
N2–3	7 (44 %)	14 (16 %)	

Data are presented as *n* (%)

PCR polymerase chain reaction

* χ^2 test

Discussion

Peritoneal recurrence of gastric cancer occurs often, even in patients who have undergone curative resection. Although peritoneal lavage cytology has been widely used for the detection of isolated tumor cells and prediction of peritoneal recurrence, the sensitivity is relatively low. Our previous retrospective study involving 36 gastric cancer patients suggested that RT-PCR of peritoneal lavage fluid may be useful in predicting peritoneal recurrence in patients with negative cytology (CY0) [11]. This prospective study involving over 100 patients undergoing curative surgery for cT2-4 gastric cancer revealed that PCR results were a significant and independent prognostic factor of RFS. Indeed, 25 % of PCR-positive patients experienced peritoneal recurrence, compared with only 2 % of PCR-

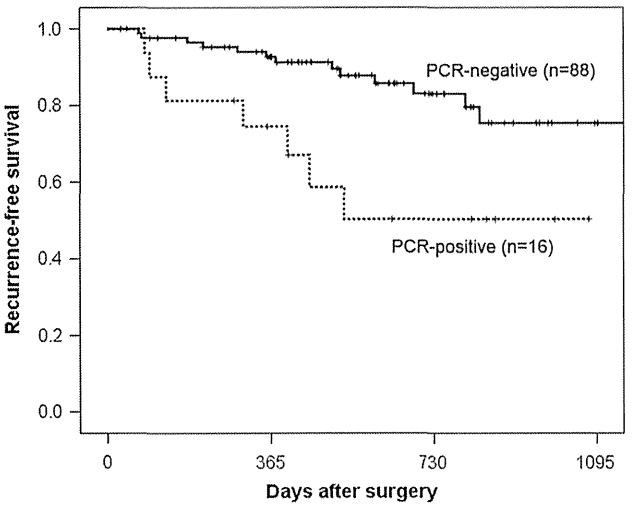

Fig. 1 Recurrence-free survival of PCR-positive patients ($n = 16$) versus PCR-negative patients ($n = 88$). *PCR* polymerase chain reaction

Table 3 Sites of tumor recurrence

Site	PCR-positive ($n = 16$)	PCR-negative ($n = 88$)	p value*
Peritoneum	4 (25 %)	2 (2 %)	<0.001
Liver	2 (13 %)	8 (9 %)	0.67
Lymph nodes	1 (6 %)	2 (2 %)	0.38
Others	1 (6 %)	2 (2 %)	0.38

Data are presented as n (%). Both groups had one duplicate site of recurrence

PCR polymerase chain reaction

* χ^2 test

negative patients. Therefore, this study demonstrated the clinical usefulness of PCR of peritoneal lavage fluid.

The RT-PCR technique has become popular as a highly sensitive method for detecting cancer cells. CEA is the most common tumor marker, and has been reported to be a reliable target for the detection of isolated tumor cells [10, 15, 16]. Ito et al. [17] reported that survival in patients with positive CEA mRNA was significantly worse than in patients with negative CEA mRNA in their retrospective study. However, another study reported that CEA frequently resulted in false positives [18], because the expression level of CEA mRNA was heterogeneous in gastric tumors [16] and there is weak expression in non-cancerous cells, such as mesothelial cells [10]. Thus, in order to more precisely predict recurrence, it may be necessary to use multiple markers [16, 19, 20]. Since CK20 is usually expressed in adenocarcinomas, it is one of the candidates for improving the sensitivity of gastric cancer cell detection [21]. Tamura et al. [22] reported that detection of CEA and CK20 mRNA by RT-PCR with peritoneal lavage fluid was useful for identifying patients at high risk of peritoneal recurrence. However, their study included many patients with incurable factors such as P1, CY1, or M1. Such incurable patients should be treated with intensive chemotherapy, regardless of PCR results. Therefore, we only included patients without incurable factors in this study in order to identify patients who need intensive adjuvant chemotherapy.

Although we successfully demonstrated associations between peritoneal recurrence and CEA and CK20 PCR results in our preliminary reports, one limitation of this study was the relatively small number of patients and the short follow-up period. Although our study could not evaluate overall survival due to the low number of events, RFS could be evaluated. We think a multicenter study with a larger cohort and a longer follow-up period is required to evaluate the generalizability of this method.

In conclusion, our prospective study confirmed our preliminary findings that CEA and CK20 RT-PCR results could predict peritoneal recurrence after curative surgery. This sensitive system can be used to identify high-risk patients who require intensive adjuvant chemotherapy and close follow-up. When this system is used as a preoperative screening tool, with peritoneal lavage fluid collected by staging laparoscopy, we can also do neoadjuvant chemotherapy for PCR-positive patients before surgery.

Table 4 Univariate and multivariate Cox analysis of recurrence-free survivals

	Univariate		Multivariate	
	HR(95 % CI)	p value	HR (95 % CI)	p value
Age (≤65 years)	1.06 (0.43–2.59)	0.90	1.07 (0.37–3.11)	0.90
Sex (male)	1.72 (0.57–5.13)	0.33	3.02 (0.89–10.3)	0.077
Histology (undifferentiated)	1.95 (0.75–5.08)	0.17	1.95 (0.71–5.38)	0.20
Neoadjuvant chemotherapy (yes)	2.27 (0.87–5.94)	0.094	2.02 (0.72–5.63)	0.18
pT (T3–4)	3.51 (1.02–12.0)	0.046	2.17 (0.59–8.01)	0.24
pN (N1–3)	1.88 (0.78–4.55)	0.16	1.44 (0.55–3.73)	0.46
PCR (positive)	3.28 (1.31–8.24)	0.011	3.49 (1.14–10.7)	0.029

CI confidence interval, *HR* hazard ratio, *PCR* polymerase chain reaction

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Conflicts of interest The authors have no conflicts of interest to declare.

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ORIGINAL ARTICLE – THORACIC ONCOLOGY

Evaluation of the Nodal Status in the 7th Edition of the UICC-TNM Classification for Esophageal Squamous Cell Carcinoma: Proposed Modifications for Improved Survival Stratification

Impact of Lymph Node Metastases on Overall Survival after Esophagectomy

Makoto Yamasaki, MD, Hiroshi Miyata, MD, Yasuhiro Miyazaki, MD, Tsuyoshi Takahashi, MD, Yukinori Kurokawa, MD, Kiyokazu Nakajima, MD, Shuji Takiguchi, MD, Masaki Mori, MD, and Yuichiro Doki, MD

Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

ABSTRACT

Background. The 7th edition of the Union for International Cancer Control-TNM (UICC-TNM) classification for esophageal carcinoma made considerable modifications to the definition of N-staging by the number of involved lymph nodes and the regional node boundary. There were few validations of the regional boundary. We evaluated the nodal status of this classification for esophageal squamous cell carcinoma (ESCC).

Methods. There were 665 patients reviewed who had ESCC and underwent esophagectomy between 1997 and 2012. We evaluated the impact of the location of lymph node metastasis on overall survival.

Results. There were 414 patients (61.7 %) who had lymph node metastases. The overall 5-year survival rate was 54.7 %. There were no significant differences in survival among N2, N3, and M1 patients. Cox regression analysis revealed that common hepatic or splenic node involvements ($P = 0.001$), pT stage ($P = 0.0002$), and pN stage ($P < 0.0001$) were independent predictors of survival, but supraclavicular node involvement ($P = 0.29$) was not. We propose a modified nodal status that designates supraclavicular node as regional: m-N0 (5-year survival = 79 %; $n = 251$); m-N1 (5-year = 56 %; $n = 212$); m-N2 (5-year = 30 %; $n = 114$); m-N3 (5-year = 18 %; $n = 52$); m-M1 (5-year = 6.2 %; $n = 36$). This modified nodal staging predicts survival better than the current staging system.

Conclusions. The modification of supraclavicular lymph node from nonregional to regional in the 7th UICC classification of ESCC may allow for better stratification of overall survival.

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M. Yamasaki, MD
e-mail: myamasaki@gesurg.med.osaka-u.ac.jp

Esophageal carcinoma is the sixth most common cause of cancer deaths worldwide, and it is generally regarded as an aggressive disease with poor prognosis.[1] A cancer staging system that is based on the accurate prediction of survival in patients with esophageal carcinoma helps clinicians to plan the best treatment. The International Union Against Cancer (UICC) tumor node metastasis (TNM) cancer staging system has been widely used to stratify patients and select treatment strategies.

Recently, the 7th edition of the TNM staging system was published;[2] this new edition drastically revised N-staging in esophageal carcinoma. Among these modifications, there were two great changes in the definition of lymph node metastasis. One change was the modification of N status from the presence or absence to the number of involved lymph nodes. This change was made on the basis of some reports that showed the number of positive lymph nodes provided a better stratification of survival than the presence or absence of positive lymph nodes.[3–8] The revised staging by N status is a numerically based classification from N0 to N3 (N0: those without lymph node metastasis, N1: those with 1–2 positive nodes, N2: those with 3–6 positive nodes, and N3: those with ≥7 positive nodes).

The other change in ESCC N-staging was the modification of the boundary between the regional (N) and nonregional (M) lymph nodes. The 7th edition of TNM staging defined celiac axis nodes and cervical