Epidermal growth factor receptor protein overexpression and gene amplification are associated with aggressive biological behaviors of esophageal squamous cell carcinoma

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Abstract. Alterations of the epidermal growth factor receptor (EGFR), including overexpression or gene mutations, contribute to the malignant transformation of human epithelial cells. The aim of this study was to assess EGFR overexpression or gene amplification in esophageal squamous cell carcinoma (ESCC) tissue samples and investigate their correlations with biological behaviors. Tissue specimens from 56 patients with surgically resected ESCC were obtained for immunohistochemical analysis of EGFR expression and fluorescence *in situ* hybridization analysis of EGFR amplification. The data were statistically analyzed to determine the associations with patient clinicopathological and survival data. EGFR was overexpressed in 30 of the 56 (53.6%) ESCC samples and was associated with poor tumor differentiation (P=0.047). EGFR amplification was detected in 13 cases (23.2%) and was associated with advanced pathological stage (P=0.042) and tumor lymph node metastasis (P=0.002). The univariate analysis identified no association between EGFR overexpression and the overall survival (OS) of the patients. By contrast, EGFR amplification predicted ESCC prognosis (P=0.031), while the multivariate analysis revealed a marginal statistical significance for the association between EGFR amplification and OS (P=0.056). EGFR overexpression and increased EGFR copy number were common events in ESCC and contributed to malignant biological behaviors, including tumor dedifferentiation and lymph node metastasis. EGFR amplification may therefore be useful in predicting OS in patients with ESCC.

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Abbreviations: ESCC, esophageal squamous cell carcinoma; EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; OS, overall survival

Key words: epidermal growth factor receptor, esophageal squamous cell carcinoma, prognosis, immunohistochemistry, fluorescence *in situ* hybridization

Introduction

Esophageal cancer represents the sixth most frequent cause of cancer-related mortality worldwide (1). Histologically, esophageal cancer is classified primarily as either squamous cell carcinoma (SCC) or adenocarcinoma. Esophageal SCC (ESCC) accounts for approximately one-third of esophageal cancer cases in the United States and >90% of esophageal cancer cases worldwide (2,3). The risk factors for ESCC include tobacco smoking, heavy alcohol consumption and a diet lacking fresh fruits and vegetables (2,3). To date, the prognosis of esophageal SCC remains poor, despite improvements in surgical techniques, perioperative management, chemotherapy and/or radiotherapy (4-6). Thus, studies on early detection, novel treatment options, prevention and predictive tumor markers for ESCC treatment and prognosis are urgently required.

Epidermal growth factor receptor (EGFR) is a part of an important transmembrane signal transduction pathway in human epithelial cells and altered EGFR protein expression or gene amplification occurs in a number of solid tumors, including esophageal cancer (7-13). EGFR-mediated signaling is crucial for cell proliferation, as well as for cancer progression, including tumor angiogenesis, metastasis and cancer cell resistance to apoptosis. EGFR overexpression is mostly due to EGFR gene amplification or mutations, with the latter occurring frequently in EGFR exons 18-21, which encode the tyrosine kinase section of the EGFR protein. Previous studies have demonstrated an association between EGFR alterations and aggressive biological behaviors (e.g., tumor cell dedifferentiation, advanced tumor stage or cancer metastasis) of different human cancers of epithelial origin, including head and neck, breast, colon, stomach and lung cancer (7-19). It was also demonstrated that EGFR overexpression and gene

amplification are correlated with treatment response or survival rates among patients with breast and lung cancer (20-22). EGFR amplification and protein overexpression have been reported in ESCC and premalignant lesions (3,9,19) and the overexpression of EGFR was found to be significantly associated with the depth of invasion of the tumor (9). EGFR gene amplification may be a useful biological marker for the prediction of lymph node metastasis and poor prognosis for ESCC (19). In addition, EGFR gene mutations are rare in ESCC, although they have been reported (23).

The aim of the present study was to evaluate EGFR overexpression using immunohistochemistry and EGFR gene amplification using fluorescence *in situ* hybridization (FISH) in ESCC tissue specimens, in order to determine the associations between patient clinicopathological characteristics and survival rates and the association between EGFR gene amplification and overexpression in ESCC tissues.

Materials and methods

ESCC patients and tissue samples. In this retrospective study, a total of 56 tissue specimens from patients with surgically resected ESCC were obtained from Zhejiang Cancer Hospital (Hangzhou, China). The diagnosis of these patients was based on the primary tumor-regional lymph node-distant metastasis (TNM) staging system described in the American Joint Committee on Cancer Staging manual, seventh edition (24). The present study was approved by the Ethics Review Committee of the Zhejiang Cancer Hospital. The patients were followed up regularly following surgery for tumor recurrence and metastasis, vital status, mortality and cause of mortality. The last follow-up was conducted in June, 2012.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue blocks were retrieved from the Department of Pathology (Zhejiang Cancer Hospital) and cut into 4-mm sections for immunohistochemical staining. The sections were first deparaffinized in xylene (Chinasun Speciality Products Co., Ltd., Changshu, China) and rehydrated in ethanol (Shanghai Ling Feng Chemical Reagent Co., Ltd., Changshu, China), subjected to antigen retrieval in 10 mM citrate buffer (pH 9.0; Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) using microwave irradiation and treated with 3% hydrogen peroxide (Dako, Hamburg, Germany). The sections were subsequently incubated with a prediluted primary rabbit monoclonal anti-EGFR antibody (clone no. 5B7; cat no. 790-4347; Ventana Medical Systems, Inc., Tucson, AZ, USA), a monoclonal mouse anti-human Ki67 antibody (dilution, 1:400; clone no. MIB-1; cat. no. M7240; Dako) or a monoclonal mouse anti-cyclin-D1 antibody (dilution, 1:100; clone no. SP4; cat. no. RM-9104-S; Neomarker, Fremont, CA, USA) at 4˚C overnight. On the following day, the sections were further incubated with an EnVision kit indirect peroxidase system (Dako) and visualized using 3,3'-diaminobenzidine (Dako) as a chromogen. The sections were then counterstained with hematoxylin and viewed under a BX43 system microscope (Olympus Corporation, Tokyo, Japan) for the evaluation of the percentage and intensity of nuclear and non-nuclear staining in tumor cells, or background staining, by two independent observers in a blinded manner.

The intensity of the immunohistochemical staining was reviewed and scored using a four-tier system as follows: 0, no discernible staining or background staining; 1+, definitive cytoplasmic staining and/or equivocal discontinuous membrane staining; 2+, unequivocal membrane staining with moderate intensity; and 3+, strong and complete plasma membrane staining (7,8,25). Scores of 2+ and 3+ were classified as overexpression, whereas scores of 0 and 1 were classified as low expression (9).

FISH. FISH was used to assess EGFR gene amplification in tissue microarray sections of all ESCC cases using the Vysis EGFR/CEP7 FISH Probe kit (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. Chromosome 7 was considered to be amplified when the ratio of the mean copy number of chromosome 7 centromeres (EGFR/CEP7 genes) was >2.2, whereas a ≥two-fold increase in the EGFR signal relative to the CEP7 signal was considered as EGFR amplification.

Statistical analysis. All data were analyzed anonymously. The χ^2 or Fisher's exact test was used for independent data to identify the associations between EGFR alterations and clinicopathological factors. Overall survival (OS) was calculated according to a Kaplan-Meier curve and the log-rank test was used to evaluate the statistical significance of the differences. The multivariate analysis was performed using the Cox proportional hazard method. All the statistical analyses were performed using SPSS v.16 software for Windows (SPSS, Inc., Chicago, IL, USA). $P<0.05$ in a two-tailed test was considered to indicate statistically significant differences.

Results

Patient characteristics. The characteristics of the patients are summarized in Table I. In brief, there were 50 male and 6 female patients, with a median age of 61 years (mean, 59.6±7.6 years; range, 42-76 years). Of the 56 tumors, 23 (41.1%) were located in the lower and 33 (58.9%) in the upper and middle esophagus. A total of 8 tumors (14.3%) were well-differentiated, 40 (71.4%) were moderately differentiated and 8 (14.3%) were poorly differentiated SCCs, according to the World Health Organization criteria. In addition, 31 patients had stage II and 25 patients had stage III disease. A total of 51 patients underwent esophagectomy with a two-field technique and 5 patients with a three-field technique. Of the 56 patients, 21 received postoperative chemotherapy and/or radiotherapy, including 13 patients treated with combined chemotherapy and radiotherapy and 8 patients treated with chemotherapy alone.

Expression of EGFR protein in ESCC tissues. The EGFR protein was expressed in 55 (98.2%) of the 56 tissue specimens, among which 30 cases (53.6%) were found to overexpress the EGFR protein [10 cases (17.9%) were scored as 3+ and 20 cases (35.7%) as 2+]; the remaining 26 ESCC cases (46.4%) exhibited low EGFR protein expression [25 cases (44.6%) were scored as 1+ and 1 case (1.8%) as 0]. The EGFR protein was expressed differentially in normal vs. abnormal tissues. For example, a tissue with atypical epithelial hyperplasia had a score of 2+

Table I. Associations of EGFR overexpression and gene amplification with the clinicopathological characteristics of patients with esophageal squamous cell carcinoma.

Characteristics	Total no.	Patient no. (%)					
		$EGFR^-$ (n=26)	$EGFR^+$ (n=30)	P-value	$FISH^-$ (n=43)	$FISH^+$ (n=13)	P-value
Age (years)				0.757			0.480
<65	42	20 (47.6)	22 (52.4)		31 (73.8)	11 (26.2)	
≥65	14	6 (42.9)	8 (57.1)		12 (85.7)	2 (14.3)	
Gender				0.401			0.615
Male	50	22 (44.0)	28 (56.0)		39 (78.0)	11 (22.0)	
Female	6	4 (66.7)	2 (33.3)		4 (66.7)	2 (33.3)	
Tumor differentiation				0.047			0.241
High	8	6 (75.0)	2 (25.0)		8 (100.0)	0 (0.0)	
Moderate	40	18 (45.0)	22 (55.0)		29 (72.5)	11 (27.5)	
Poor	8	2 (25.0)	6 (75.0)		6 (75.0)	2 (25.0)	
Vascular invasion				0.693			0.553
Yes	7	4 (57.1)	3 (42.9)		6 (85.7)	1 (14.3)	
No	49	22 (44.9)	27 (55.1)		37 (75.5)	12 (24.5)	
Tumor location				0.145			0.827
Upper/middle	33	18 (54.5)	15 (45.5)		25 (75.8)	8 (24.2)	
Lower	23	8 (34.8)	15 (65.2)		18 (78.3)	5 (21.7)	
pT stage				0.305			0.870
T2	12	4 (33.3)	8 (66.7)		9 (75.0)	3 (25.0)	
T3	44	22 (50.0)	22 (50.0)		34 (77.3)	10 (22.7)	
pN stage				0.972			0.002
N0	27	14 (51.9)	13 (48.1)		26 (96.3)	1 (3.7)	
N1	21	8 (38.1)	13 (61.9)		13 (61.9)	8 (38.1)	
N2	6	2 (33.3)	4 (66.7)		3 (50.0)	3 (50.0)	
N3	2	2 (100.0)	0 (0.0)		1 (50.0)	1 (50.0)	
pTNM stage				0.832			0.042
II	31	14 (45.2)	17 (54.8)		27 (87.1)	4 (12.9)	
III	25	12 (48.0)	13 (52.0)		16 (64.0)	9 (36.0)	

EGFR, epidermal growth factor receptor; TNM, tumor-node-metastasis; FISH, fluorescence *in situ* hybridization.

for EGFR staining, whereas normal esophageal tissue was negative for EGFR immunostaining (Fig. 1). Furthermore, the expression of the EGFR protein also differed according to differentiation; i.e., poorly differentiated ESCC tissues expressed high levels of the EGFR protein (score 3+), while moderately differentiated ESCC tissues moderately expressed the EGFR protein (score 2+). By contrast, well-differentiated ESCC tissues expressed low levels of the EGFR protein (score 1+) (P=0.047, Fig. 1). However, there were no statistically significant correlations between EGFR expression and age, gender, presence of vascular invasion, tumor location, T stage, N stage, distant metastasis, or pathological TNM stage (P>0.05, Table I).

EGFR amplification in ESCC tissues. The FISH analysis demonstrated that the EGFR gene was amplified in 13 (23.2%) of the 56 tumor samples. On immunohistochemical staining, 11 of these samples exhibited a high EGFR protein expression, while the remaining 2 samples exhibited low EGFR protein expression. Clinically, 12 (92%) of these 13 patients received postoperative adjuvant chemotherapy. EGFR gene amplification was associated with tumor lymph node metastasis (P=0.002) and advanced pathological TNM stage (P=0.042). However, no statistically significant correlations were identified between EGFR gene amplification and other clinicopathological parameters (Table I). EGFR protein expression was statistically significantly associated with EGFR gene amplification (P<0.05; Table II).

Prognostic significance of EGFR alterations. All the patients were followed up until June, 2012. Among these patients, 26 succumbed to cancer-related ailments, with a median OS of 24 months (range, 1-53 months). Clinicopathological characteristics, including age, gender, pT stage, lymph node metastasis, tumor differentiation, vascular invasion and EGFR alterations were correlated with OS in patients

Table II. Association of EGFR protein expression with gene amplification in esophageal squamous cell carcinoma tissues.

EGFR protein expression level	EGFR amplification		P-value
	-	+	
0	1	0	
1+	25	2	
2+	19	3	
3+	11	8	<0.05

EGFR, epidermal growth factor receptor.

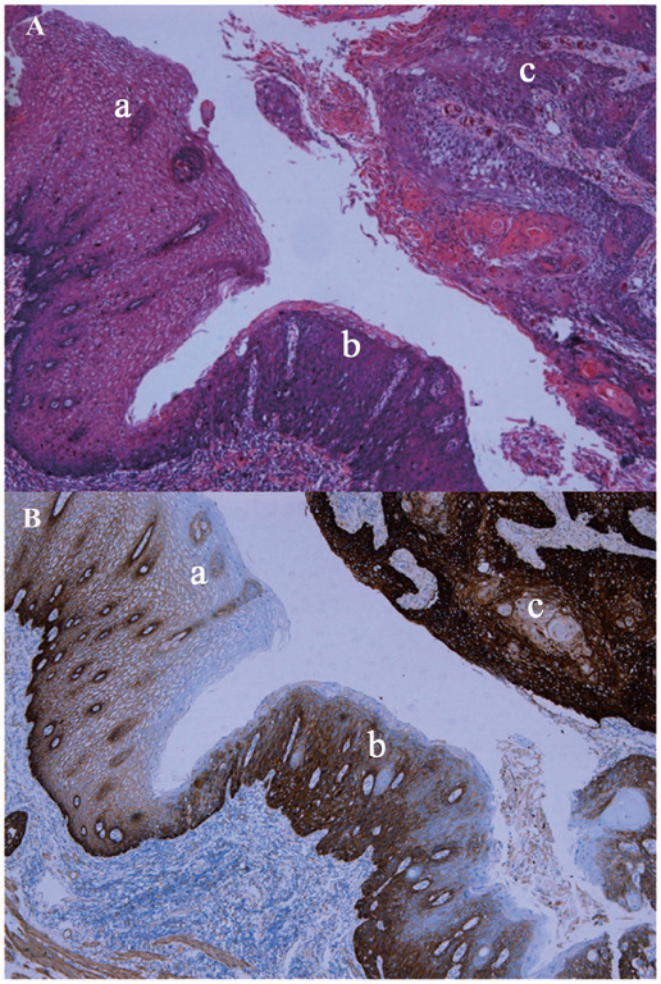

Figure 1. Esophageal squamous cell carcinoma morphology. (A) Hematoxylin and eosin staining and (B) EGFR immunostaining. a, normal esophageal epithelium; b, epithelial atypical hyperplasia; c, tumor tissues. EGFR, epidermal growth factor receptor.

with ESCC. The univariate analysis revealed no association between EGFR protein expression and OS (P=0.673; Table III, Fig. 2A), although EGFR gene amplification was able to predict OS in these patients (P=0.031; Table III, Fig. 2B). However, on multivariate analysis, there was only a marginal association between EGFR gene amplification and OS (P=0.056), indicating that a larger sample size is required.

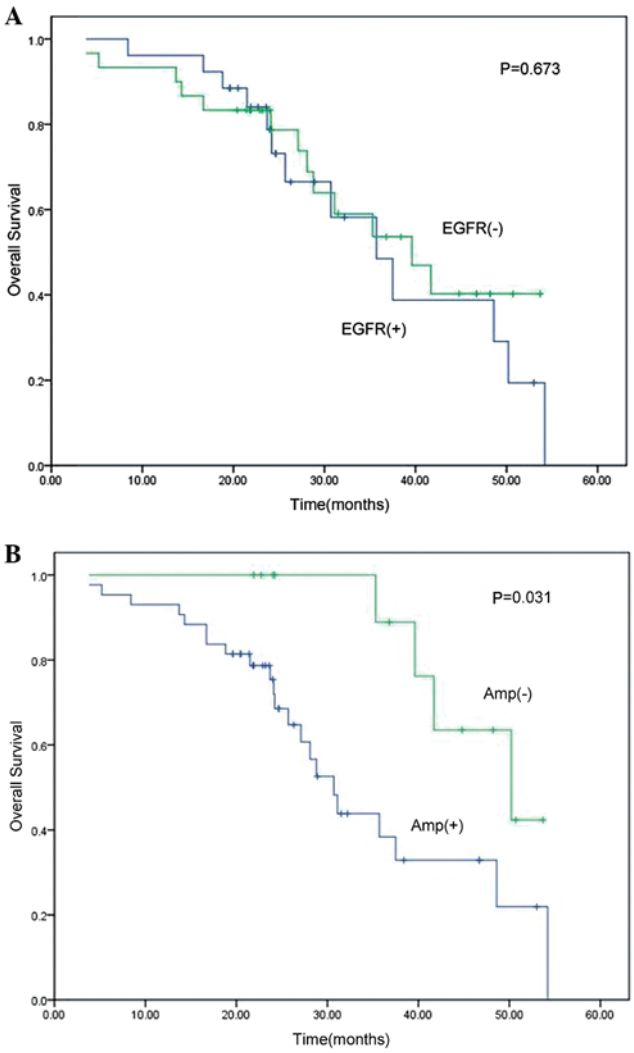

Figure 2. Kaplan-Meier curves for OS in patients with ESCC. (A) OS of ESCC patients stratified by EGFR expression (log-rank test: P>0.05). (B) OS of ESCC patients stratified by EGFR amplification (log-rank test: P=0.031). OS, overall survival; ESCC, esophageal squamous cell carcinoma; EGFR, epidermal growth factor receptor.

Discussion

EGFR and its signaling play important roles in tumor cell proliferation, migration, apoptosis resistance and angiogenesis. Thus, the role of EGFR overexpression and gene amplification in cancer development, progression and aggressiveness has been extensively investigated (10-12). Upon binding of the EGFR extracellular domain to several different ligands (including EGF and transforming growth factor-α), the EGFR protein forms a dimerized receptor to activate the EGFR intracellular tyrosine kinase domain, triggering a cascade of phosphorylation events in the cytoplasm, which result in the activation of target gene transcription and expression and a subsequent change in cell behavior. Mitogen-activated protein kinases (MAPKs), activator protein 1 and protein kinase B are all downstream cascade genes in the EGFR signaling pathway (26). For example, EGFR activates MAPKs and MAPK/extracellular signal-regulated kinase (ERK) kinase through Ras and then activates ERK1/2, which, in turn, translocates into the nucleus and promotes the expression

Table III. Univariate and multivariate regression analyses of overall survival in patients with esophageal squamous cell carcinoma.

	Univariate analysis			Multivariate analysis		
Variables	HR	95%CI	P-value[a]	HR	95%CI	P-value[a]
Age[b]	1.358	0.533-3.462	0.520	1.301	0.607-3.011	0.490
Gender[c]	1.574	0.461-5.380	0.460	1.427	0.433-4.557	0.570
Location[d]	0.927	0.408-2.106	0.850	1.020	0.481-2.230	0.880
Differentiation[e]	0.670	0.305-1.474	0.320	0.737	0.371-1.487	0.430
Vascular invasion[f]	1.970	0.652-5.953	0.220	1.332	0.587-4.026	0.610
pT status[g]	1.715	0.586-5.016	0.320	1.492	0.673-4.542	0.430
pN status[h]	1.013	0.456-2.252	0.970	1.162	0.531-2.103	0.760
pTNM stage[i]	1.075	0.489-2.362	0.850	0.983	0.565-1.967	0.960
EGFR expression[j]	0.793	0.233-2.341	0.673	0.710	0.167-2.015	0.640
EGFR gene amplification[k]	0.392	0.146-0.896	0.031	0.392	0.197-1.062	0.056
Postoperative treatment[l]	1.320	0.967-1.802	0.080	1.296	0.985-1.874	0.062

[a]Cox proportional hazards model. [b]<65 vs. ≥65 years; [c]male vs. female; [d]upper/middle vs. lower esophagus; [e]poor vs. moderate vs. high; [f]with vs. without; [g]T2 vs. T3; [h]N0 vs. N1 vs. N2 vs. N3; [i]II vs. III; [j]-/1+/2+ vs. 3+; [k]positive vs. negative; [l]none vs. chemotherapy or chemoradiotherapy. HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis; EGFR, epidermal growth factor receptor.

of genes, including c-Jun, c-Fos and cyclooxygenase-2, thus promoting cell growth and angiogenesis (27). It was previously demonstrated that EGFR overexpression occurs in ≤65% of ESCC cases (13,28), whereas EGFR gene amplification occurs in 2-19% of ESCC cases (14,15,29,30). The present study demonstrated overexpression of the EGFR protein in 53.6% of the ESCC cases and gene amplification in 23.2% of the cases. These data suggest that overexpression of the EGFR protein and increased EGFR gene copy number are frequent events in ESCC; thus, targeted therapy using anti-EGFR inhibitors may be effective in treating ESCC.

Previous studies also demonstrated that EGFR overexpression/gene amplification may be indicative of unfavorable parameters for ESCC (16,17). For example, Delektorskaya *et al* (18) reported that overexpression of the EGFR protein is significantly correlated with tumor intravascular invasion and depth of invasion, whereas EGFR gene amplification is associated with tumor dedifferentiation. Kitagawa *et al* (19) demonstrated a significant correlation between EGFR gene amplification and ESCC lymph node metastasis. The present study revealed that EGFR protein expression was associated with ESCC dedifferentiation, whereas EGFR gene amplification was associated with advanced stage and lymph node metastasis. These data are consistent with the results of previous studies (14-19). Furthermore, Nicholson *et al* (31) demonstrated that EGFR overexpression is correlated with prognosis in esophageal, head and neck, ovarian, cervical and bladder cancer. In these types of cancer, increased EGFR expression was found to be associated with reduced recurrence-free survival or OS rates. Other previous studies also demonstrated that EGFR overexpression/ gene amplification is associated with poor postoperative prognosis, reduced OS and an increased risk of local recurrence in patients with ESCC (15,32-34). However, our data did not demonstrate that EGFR overexpression is of prognostic value for ESCC; however, EGFR gene amplification may predict a poorer prognosis. The reason for this discrepancy is unknown, although it may be due to the antibody used to detect EGFR protein expression or the quality of the tissue specimens. In addition, the present data revealed that patients in whom the EGFR protein was overexpressed exhibited a higher EGFR gene amplification rate compared with those with low EGFR protein expression. Further studies, including larger sample sizes, are required to confirm our data.

The present findings suggest that the frequent alterations of EGFR in patients with ESCC may indicate that EGFR is a candidate for targeted therapy using anti-EGFR inhibitors, such as nimotuzumab, cetuximab and gefinitib. Indeed, a previous *in vitro* study demonstrated that nimotuzumab, an anti-EGFR monoclonal antibody, promotes the radiosensitivity of EGFR-overexpressing ESCC cells (35). A phase II clinical trial demonstrated that cetuximab is an effective and safe adjuvant to chemotherapy and radiotherapy for esophageal cancer patients with a clinical complete response rate of 70% (40/57) (36). Another phase II study, which used gefinitib as second-line treatment for advanced esophageal cancer, reported a significantly higher disease control rate (overall response and stable disease) in patients with EGFR-overexpressing ESCC (37). However, there are currently no established eligibility criteria for targeted therapy in patients with ESCC; for example, it is not clear whether EGFR overexpression or gene amplification should be used as an indicator. Thus, there is a requirement for predictive biomarkers that identify the ESCC patients most likely to respond to EGFR-targeted therapy. Evaluation of EGFR overexpression detected by immunohistochemistry and EGFR gene amplification detected by FISH may aid the selection of patients and prediction of sensitivity to adjuvant EGFR-targeted therapy for ESCC.

There were certain limitations to this study, including the small sample size and fact that only patients with ESCC were

recruited. The results of the present study demonstrated that EGFR overexpression and increased EGFR gene copy number are common events in ESCC and contribute to ESCC malignant biological behaviors, including tumor dedifferentiation and lymph node metastasis. Therefore, EGFR gene amplification may be useful in predicting the OS of patients with ESCC.

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