Overexpression of EGFR as an Independent Prognostic Factor in Adenocarcinoma of the Esophagogastric Junction

KENICHI ARATANI1*, SHUHEI KOMATSU1*, DAISUKE ICHIKAWA1, TAKUMA OHASHI1, MAHITO MIYAMAE1, WATARU OKAJIMA1, TAISUKE IMAMURA1, JUN KIUCHI1, KEJI NISHIBEPPE1, TOSHIYUKI KOSUGA1, HIROTAKA KONISHI1, ATSUSHI SHIOZAKI1, HITOSHI FUJWARA1, KAZUMA OKAMOTO1, HITOSHI TSUDA2,3 and EIGO OTSUJI1

1Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan; 2Department of Pathology, National Cancer Center Hospital, Tokyo, Japan; 3Department of Basic Pathology, National Defense Medical College, Saitama, Japan

Abstract. Background: Adenocarcinoma of the esophagogastric junction (AEG) has increased in Western and Eastern countries, and its prognosis remains poor. We tested whether epidermal growth factor receptor (EGFR), that is overexpressed in various tumors, acts as a cancer-promoting gene through overexpression in AEG. Materials and Methods: We analyzed 104 primary AEG tumors which were curatively resected in our hospital between 2000 and 2010. Results: Overexpression of EGFR protein was detected in 47% primary AEG tumor samples, and significantly associated with venous and lymphatic invasion, tumor depth and lymph node metastasis. The high-expression group had a significantly poorer prognosis than the low-expression group for overall and disease-free survival. EGFR positivity was independently associated with a worse outcome in the multivariate analysis (p=0.0397, hazard ratio(HR)=2.048). Conclusion: EGFR plays a pivotal role in AEG through its overexpression, which highlights its usefulness as a prognosticator and potential therapeutic target in AEG.

Over the past few decades, adenocarcinoma of the esophagogastric junction (AEG) has markedly increased in Western and Eastern countries (1-5). Despite the improvement of diagnosis and treatment technologies such as extended radical resection and chemoradiotherapy, many patients with AEG frequently develop metastasis and experience recurrence, and long-term survival remains poor because of the aggressive and systemic nature of the disease (6).

The epidermal growth factor receptor family of receptors, also called the human epidermal growth factor receptor (HER) family of tyrosine kinases, include ERBB1 (EGFR), ERBB2 (HER2), ERBB3, and ERBB4, which are encoded by ERB oncogenes (7) and have been implicated in tumor cell growth and differentiation. All members share a common structure, with an extracellular ligand-binding domain, a transmembrane domain, and an intracytoplasmic tyrosine kinase domain. Ligand binding to these receptors induces the formation of receptor homodimers and heterodimers, and the activation of downstream signaling pathways. The HER family might, therefore, contribute to malignant progression.

Recently, trastuzumab, a monoclonal antibody against human epidermal growth factor receptor type 2 (HER2), was shown to improve response rate, progression-free survival (PFS), and overall survival (OS) when added to cisplatin based chemotherapy in patients with HER2 overexpressing AEG and gastric adenocarcinomas (8). However, HER2 overexpressing esophagogastric tumors are in the minority, and the need for additional targeted agents is urgent. Protein overexpression or gene amplification of EGFR has been reported in several human tumors of epithelial origin, including of head and neck (9-11), thyroid (12), breast (13, 14), ovarian (15), colon (16-19), cervix, bladder (20) and lung (21). In a subset of these tumors, most notably breast cancer, colorectal cancer, and esophageal squamous cell carcinoma (22, 23), increased EGFR expression has been associated with advanced disease, development of metastases, and poor prognosis. Regarding gastric cancer,
several previous reports have identified the clinical and biological significance of EGFR overexpression (24-28). Specifically, Terashima et al. reported that EGFR overexpression is significantly associated with worse patient outcomes after curative gastrectomy for stage II/III gastric cancer (28). These findings prompted us to determine the clinicopathological and prognostic significance of EGFR overexpression/activation in primary AEG.

In this study, we tested whether EGFR acts as a cancer-promoting gene through its activation overexpression in AEG. Our results provide evidence that EGFR could be an important molecular marker for determining the malignant properties of AEG and also a target for molecular therapy.

Materials and Methods

Primary AEG tissue samples. Primary tumor samples of AEG were obtained from 104 consecutive patients with AEG who had undergone curative resection at the Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine (Kyoto, Japan) between 2000 and 2010. The samples were embedded in paraffin after 24 h of formalin fixation. Relevant clinic and survival data were available for all patients. Written consent was always obtained in the formal style and after approval by the local Ethics Committee. None of these patients underwent endoscopic mucosal resection, palliative resection, preoperative chemotherapy, or radiotherapy, and none of them had synchronous or metachronous multiple cancer in other organs. Disease stage was defined in accordance with the International Union Against Cancer Tumor-Lymph Node-Metastases (TNM) classification (seventh edition) (29). The mean follow-up period for surviving patients was 51.2 months.

Immunohistochemistry. The protein expression of EGFR was quantified by immunohistochemical analysis. The tissues were reacted with primary antibody EGFR(sc-03), which was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Primary tumor samples were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) and routinely embedded in paraffin. Horseradish peroxidase (HRP) staining method was used. In brief, after deparaffinization, antigen retrieval was performed by heating the samples in 10 mmol/l citrate buffer (pH 6.0) at 95°C for 60 min. Endogenous peroxidases were quenched by incubating the sections for 20 min in 3% H₂O₂. After treatment with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature, sections were incubated overnight at 4°C with an anti-EGFR (1:500). PBS was used for all dilutions and washings. Bound primary antibody was detected with EnVision™+ Horse Radish Peroxidase Systems (EnVision + dual link System-HRP; Dako North America, Inc., Carpenteria, CA, USA). HRP labeling was visualized using color development with diaminobenzidine tetrahydrochloride. Slides were counterstained with Mayer's hematoxylin.

To evaluate EGFR expression, primary tumors were judged positive if at least 10% or more of the total cell population showed EGFR immunopositivity and negative if fewer than 10% showed EGFR immunopositivity. For the intensity of EGFR expression, the intensity score (0=negative, 1=weak, 2=moderate, 3=strong) was recorded. Namely, primary tumors with non-detectable EGFR expression, that were similar to non-tumorous gastric mucosa and stroma, were given an intensity score of 0, whereas those with the greatest EGFR abundance were given an intensity score of 3. The remaining tumors were categorized with intensity scores of 1 or 2 according to the intensity of immunohistochemical staining for EGFR. The expression of EGFR was regarded as high expression for both an intensity score of 3 and ≥10% of tumor cells showing immunopositivity, or low expression for an intensity score of or less 2 or <10% using high-powered (×200) microscopy (30, 31).

Statistical analysis. Clinicopathological variables pertaining to the corresponding patients were analyzed for statistical significance using the Chi-squared test or Fisher’s exact test. For the analysis of survival, Kaplan–Meier survival curves were constructed for groups based on univariate predictors, and differences between the groups were analyzed with the log-rank test (Figure 1B and C). Univariate and multivariate survival analyses were performed using the likelihood ratio test of the stratified Cox proportional hazards model. The data were stratified for multivariate analysis using both forward and backward stepwise Cox regression procedures. Differences were assessed with a two-sided test and were considered statistically significant at p<0.05.

Results

Clinicopathological characteristics of patients with AEG. The clinical characteristics in 104 consecutive patients with AEG were as follows. Of 104 patients, 12 patients were defined as having tumor Siewert type I, 60 patients as Siewert type II and 32 patients as Siewert type III based on the tumor location. The study group consisted of 82 male and 22 female patients, with a median age of 66 years (range=28-85 years). Of 104 patients, 37 had disease staged as pStage I, 17 as pStage II, and 50 as pStage III.

Immunohistochemical analysis of EGFR expression in primary AEG tumors. The clinicopathological significance of EGFR expression in primary tumor samples of AEG based on the immunohistochemical staining pattern of this protein was examined. EGFR expression was detected in the surface membrane of AEG cells. We classified 104 AEG tumors into positive and negative groups according to the intensity of EGFR staining of tumor cells as described in the Materials and Methods Section. In primary cases, normal esophageal squamous mucosa show complete membranous staining EGFR. In contrast, the nonneoplastic gastric mucosa show no EGFR immunoreactivity. The underlying cardiac glands show incomplete membranous staining. We divided 104 AEG tumors into a high expression group (n=49, 47%) and a low expression group (n=55, 53%) according to the intensity of EGFR staining of tumor cells (Figure 1A). The high-expression group had a significantly poorer prognosis than the low expression group for overall survival (p=0.0035, log-rank test; Figure 1B) and disease-free survival (p=0.015, log-rank test; Figure 1C).
Figure 1. Immunohistochemical-staining analyses and postoperative overall and disease-free survival curves according to the expression of epidermal growth factor receptor (EGFR). A: Specific immunostaining of the EGFR protein in primary samples was confirmed. Expression of EGFR protein was observed in the cytoplasm of cancer cells. For scoring of EGFR expression, the intensity score was defined as 0=negative, 1=weak, 2=moderate, 3=strong. B, C: The group with high EGFR expression had a significantly poorer prognosis than that with low expression in both overall (p<0.0001, log-rank test) and recurrence-free (p=0.0013, log-rank test) survival.
The protein expression of EGFR was significantly associated with more aggressive venous (\( p=0.0003 \)) and lymphatic invasion (\( p=0.0056 \)), deeper depth of invasion (\( p=0.0023 \)), and higher rates of lymph node metastasis (\( p=0.0001 \)), and  lymphatic invasion (\( p=0.0003 \)), deeper depth of invasion (\( p=0.0023 \)), and higher rates of lymph node metastasis (\( p=0.0001 \)).
whereas other characteristics, including the Siewert classification and histological grade, were not.

In the Cox proportional hazard regression model (Table II), univariate analyses demonstrated that EGFR protein expression, venous invasion, lymphatic invasion, pT stage, and pN stage were significantly associated with overall survival. When the data were stratified for multivariate analysis using the Cox proportional hazards analysis procedures, EGFR immunoreactivity in tumor cells remained significant (hazard ratio (HR)=2.048, 95% CI=1.034-4.254; \( p=0.0397 \)) for overall survival for the whole patient cohort, suggesting that EGFR immunoreactivity can be an independent predictor of overall survival.

**Discussion**

The present study retrospectively evaluated the influence of EGFR overexpression on the outcomes of patients with AEG. Our data show that EGFR expression is strongly correlated with poor overall and disease-free survival in univariate analyses and an independent predictor of overall survival in multivariate analyses. As far as we are aware of, this is the first study to show that EGFR status correlates significantly with poor outcome in patients with AEG. These results suggest that EGFR could be an important molecular marker for predicting the malignant properties of AEG and also a target for molecular therapy.

In patients with head and neck, ovarian, cervical, bladder, and esophageal squamous cell carcinomas, EGFR expression is a strong prognostic indicator that correlates with both higher recurrence rates and shorter survival. Regarding gastric cancer, EGFR overexpression is also significantly associated with a worse outcome after curative resection of stage II/III gastric cancer (28); however, previous studies of the prognostic role of EGFR in esophageal adenocarcinomas or AEG have been limited (32-36). Wang et al. reported that overexpression of EGFR in esophageal adenocarcinoma and AEG relates to poor outcomes (36). However, EGFR overexpression was not an independent prognostic factor. In the current study, we investigated only patients with AEG restricted to the Siewert definition and, thereby, demonstrated that EGFR positivity was independently associated with a worse outcome in the multivariate analysis.

EGFR is a trans-membrane glycoprotein receptor for the EGF family of extracellular protein ligands (37) and is overexpressed in several malignancies. Ligand binding to the extracellular domain leads to EGFR activation and phosphorylation of the intracellular tyrosine kinase, which then directs activation of the rat sarcoma (RAS)/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase (MAPK) or the protein kinase B (AKT)/mammalian target of rapamycin (MTOR) pathway (38-40). EGFR overexpression occurred in 30-50% of AEG in previous reports (36), and the frequency in our present study is similar to this value.

Regarding the effect of EGFR inhibitors in gastric cancer or AEG, the phase III trial EXPAND (erbitux in combination with xeloda and cisplatin in advanced gastric or esophagogastric junction cancer) randomized 904 patients to cisplatin with capecitabine with or without cetuximab. No PFS or OS benefit for the cetuximab-treated group was found (41). Panitumumab is the first fully human IgG2 monoclonal antibody targeting EGFR. The REAL-3 study did not show any benefit at preplanned interim analysis and was stopped early (42). Gefitinib is an oral EGFR tyrosine kinase inhibitor (TKI). A phase III trial (NCT01243398) randomized patients with advanced AEG to gefitinib versus
placebo after progression on chemotherapy. The study was completed and the pending results will help better delineate the activity of gefitinib (43). Erlotinib is another oral EGFR TKI, which was found to be active in patients with AEG cancer with a response rate of 9%, but with no responses in gastric cancer (44). Thus, EGFR-targeted agents have produced only modest success in AEG and gastric cancer. However, our present study indicates that EGFR overexpression could be a strong indicator for prognosis in AEG. Further studies may be warranted to better understand the role of EGFR receptor signaling in AEG and to develop novel EGFR-targeted agents for AEG.

Conflicts of Interest

All Authors have no conflict of interest to declare in regard to this study.

References


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