Nuclear Expression of Phosphorylated EGFR Is Associated with Poor Prognosis of Patients with Esophageal Squamous Cell Carcinoma

Mina Hoshino\textsuperscript{a, b}, Hirokazu Fukui\textsuperscript{a}, Yuko Ono\textsuperscript{a}, Akira Sekikawa\textsuperscript{a}, Kazuhiro Ichikawa\textsuperscript{a}, Shigeki Tomita\textsuperscript{a}, Yasuo Imai\textsuperscript{a}, Johji Imura\textsuperscript{a}, Hideyuki Hiraishi\textsuperscript{b}, Takahiro Fujimori\textsuperscript{a}

Departments of \textsuperscript{a}Surgical and Molecular Pathology and \textsuperscript{b}Gastroenterology, Dokkyo University School of Medicine, Tochigi, Japan

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Esophagus · Squamous cell carcinoma · Epidermal growth factor receptor · Phosphorylation · Prognosis

Abstract
Objectives: Although it has been reported that epidermal growth factor receptor (EGFR) is able to translocate from the plasma membrane to the nucleus, the pathophysiological role of this translocation in tumorigenicity is still unclear. In the present study, to elucidate the pathophysiological significance of EGFR translocation, we investigated the expression not only of conventional EGFR but also its phosphorylated form (pEGFR), focusing on its cellular localization in esophageal cancer tissues. Methods: Fifty-two specimens of esophageal squamous cell carcinoma (SCC) obtained by surgery were examined immunohistochemically for their EGFR and pEGFR immunostaining patterns. The relationships between clinicopathological parameters and EGFR or pEGFR immunostaining patterns were then analyzed. Results: In 37 (71.2\%) of the 52 esophageal SCCs, EGFR immunoreactivity was clearly localized at the plasma membrane of the cancer cells, whereas pEGFR immunoreactivity was clearly localized in the nucleus in 19 (36.5\%) cases. Nuclear expression of pEGFR significantly correlated with TNM stage and lymph node metastasis, and moreover was associated with a poor outcome of esophageal SCC. Conclusions: Nuclear translocation of pEGFR is associated with an increase in the malignant potential of esophageal SCC and may affect prognosis in patients with esophageal SCC.

Introduction

Epidermal growth factor receptor (EGFR), a single transmembrane glycoprotein, possesses intrinsic tyrosine kinase activity \cite{1} and transmits extracellular information to the nucleus via an intracellular signaling network following its autophosphorylation \cite{2, 3}. In normal tissues, EGFR signaling is finely tuned for the precise control of cell proliferation, whereas in tumor tissues it is frequently dysregulated, thus contributing to the disordered proliferation of tumor cells \cite{4, 5}. EGFR overexpression has been identified as a common feature of many types of cancer, and its association with clinical outcome in patients with malignant diseases has been studied because of the roles of EGFR signaling not only in cell proliferation but also antiapoptosis, metastasis and angiogenesis \cite{6}. Indeed, several immunohistochemical studies have revealed that EGFR overexpression is a strong prognostic marker for patients with esophageal \cite{7} or head and neck cancer \cite{8}, and furthermore a modest one for patients with breast \cite{9}, colorectal \cite{10} or gastric can-
cer [11]. However, although such studies have addressed the total level of EGFR expression, the cellular localization of either total EGFR or phosphorylated EGFR (pEGFR; the active form of EGFR) expression has not been fully examined.

Although EGFR expression is detectable at the plasma membrane immunohistochemically, it is also reportedly detectable in the nucleus in various types of cancer cells [3]. Recently, accumulated evidence has begun to suggest that EGFR may be capable of translocating from the plasma membrane to the nucleus [12], and that this translocation may play a pathophysiological role in tumorigenicity. Therefore in the present study, to elucidate the pathophysiological significance of EGFR translocation, we investigated the expression not only of total EGFR but also pEGFR, focusing on its cellular localization in esophageal cancer tissues.

Patients and Methods

Tissue Samples and Patients

Fifty-two patients with esophageal squamous cell carcinoma (SCC; 42 males, 10 females; mean age 65.4 ± 1.1 years, range 50–84 years) who underwent surgery at the Dokkyo University School of Medicine from 1987 to 2002 were enrolled. Patients with other malignant diseases were excluded, as were patients who had received preoperative treatment such as chemotherapy and radiation therapy. The pathologic staging was determined for each specimen according to the TNM classification of esophageal cancer [13]. This study was done with the approval of the Dokkyo University Surgical Pathology Committee. The clinicopathological features of the patients are summarized in table 1.

### Immunohistochemistry

The surgically resected specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemical staining was performed as described previously [14]. In brief, 4-μm-thick sections were placed on slides, deparaffinized and dehydrated. They were then placed in 0.01 mol/l citrate buffer (pH 6.0) and treated by microwave heating (MI-77; Azumaya, Tokyo, Japan) at 400 W and 95 °C for 10 min to facilitate antigen retrieval, followed by pretreatment with 0.3% H2O2 in methanol for 20 min at room temperature to quench endogenous peroxidase activity. The sections were then allowed to immunoreact using an Ultra Tech kit (Immunotech, Marseille, France) in accordance with the supplied protocols. The sections were incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min, and then with anti-EGFR antibody (clone 31G7; Upstate Biotechnology, Lake Placid, N.Y., USA; 1:30) and anti-pEGFR antibody Tyr845 (Cell Signaling Technology, Danvers, Mass., USA; 1:50) for 1 h. Thereafter, the sections were incubated with biotinylated secondary antibody for 15 min, washed with PBS and treated with peroxide-conjugated streptavidin for 20 min. Finally, the sections were incubated in 3,3′-diaminobenzidine tetrahydrochloride with 0.05% H2O2 in PBS for 3 min and then counterstained with Carazzi’s hematoxylin.

### Evaluation of EGFR and pEGFR Expression

We assessed the immunoreactivity of EGFR and pEGFR in the invasive front of esophageal squamous cell carcinoma (magnification ×200).
As described in the Results section, some esophageal SCCs showed clear EGFR immunoreactivity at the plasma membrane (membrane type), while others showed diffuse immunoreactivity in the cytoplasm and nucleus but not at the plasma membrane (nonmembrane type). Every lesion was classified as either membrane or nonmembrane type according to its dominant immunostaining pattern.

With regard to pEGFR, some esophageal SCCs showed clear immunoreactivity in the nucleus and a weak signal in the cytoplasm and at the plasma membrane (nuclear type), whereas others showed no nuclear immunoreactivity but a diffuse signal in the cytoplasm and at the plasma membrane (nonnuclear type). Every lesion was classified as either nuclear or nonnuclear type according to its dominant immunostaining pattern.

Western Blot Analysis

A human esophageal SCC cell line, T.Tn, was maintained in a 1:1 mixture of Dulbecco’s modified medium (Nissui, Tokyo, Japan) and F-12 (Life Technologies Inc., Gaithersburg, Md., USA) supplemented with 10% fetal calf serum (Sigma, St. Louis, Mo. USA) [15]. Proteins were extracted from T.Tn cells and separated to the nuclear and membrane-cytoplasmic fractions as previously described [16]. In brief, the cells were mixed with lysis buffer containing 10 mM Tris-HCl (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 1% Nonidet P-40 and 1× proteinase inhibitor (Complete Mini; Roche). Proteins from membrane and cytoplasm were extracted from the supernatants and the precipitation was additionally treated with nuclear lysis buffer containing 20 mM Tris-HCl (pH 7.9), 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 5% glycerol and 1× proteinase inhibitor (Complete Mini; Roche). After centrifugation, nuclear protein was extracted from the treated supernatants.

Western blot analysis was carried out as previously described [17]. Briefly, protein extract (12.5 μg) was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with primary antibodies and then with a peroxidase-conjugated secondary antibody. Proteins were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Statistical Analysis

Statview 5.0J statistical software (Abacus Concepts Inc., Berkeley, Calif., USA) was used for all analyses. Overall survival rate was calculated by using the Kaplan-Meier method and analyzed by the log-rank test. χ² analyses were performed to investigate the relationship between conventional EGFR and pEGFR expression. Differences at p < 0.05 were considered to be statistically significant.

Results

Expression of EGFR and pEGFR in Esophageal SCC

Representative immunostaining patterns of EGFR and pEGFR are shown in figure 1. In 37 (71.2%) of the 52 esophageal SCCs, EGFR immunoreactivity was clearly localized at the plasma membrane of cancer cells (fig. 1a).

and in the remaining 15 cases (28.8%) it was detected diffusely in the cytoplasm (fig. 1b; table 1). pEGFR immunoreactivity was clearly localized in the nucleus (fig. 1c) and diffusely in the cytoplasm (fig. 1d) of cancer cells in 19 (36.5%) and 33 (63.5%) of the esophageal SCCs, respectively (table 1).

Localizations of EGFR and pEGFR were also examined using the esophageal SCC cell line (fig. 2). Western blots showed that EGFR was strongly expressed in the fraction of the membrane and cytoplasm, and also faintly in the fraction of the nucleus of esophageal SCC cells. The expression of pEGFR was detected in the fraction not only of the membrane and cytoplasm but also the nucleus. These findings, together with immunohistochemistry, support the specificity of pEGFR immunoreactivity in the nuclei of esophageal SCC cells.

Relationship between Clinicopathological Factors and EGFR or pEGFR Expression in Esophageal SCC

A membrane-type EGFR immunostaining pattern tended to be detected in early-stage esophageal SCCs (p = 0.071). However, none of the clinicopathological factors, including gender, age, histology, lymphatic invasion, venous invasion or lymph node metastasis, were significantly related to the EGFR immunostaining pattern (table 1).

A significant proportion of advanced-stage esophageal SCCs showed a nuclear pEGFR immunostaining pattern (p = 0.004). In addition, a higher proportion of SCCs showed a nuclear pEGFR staining pattern in patients with lymph node metastasis (60.0%) than in those without (21.9%). In contrast, none of the remaining parameters – gender, age, histology, lymphatic invasion or venous invasion – had a significant relationship to the pEGFR immunostaining pattern (table 1).

Relationship between EGFR and pEGFR

Immunostaining Patterns in Esophageal SCC

We next investigated the relationships between EGFR and pEGFR immunostaining patterns because, as shown in figure 3, we often observed that cancer cells showing a nonmembrane-type EGFR immunostaining pattern expressed nuclear immunoreactivity for pEGFR. It was noteworthy that SCC specimens showing a nonmembrane-type EGFR immunostaining pattern had a significantly higher incidence of nuclear pEGFR immunostaining, whereas those showing a membrane-type EGFR immunostaining pattern had a significantly higher incidence of nonnuclear pEGFR immunostaining (p = 0.0005; table 2).

Localization of pEGFR in Esophageal Squamous Cell Carcinoma
The log-rank test showed that disease stage and lymph node metastasis were significantly prognostic for overall patient survival ($p < 0.0001$ and $p = 0.0005$, respectively). In contrast, none of the clinicopathological parameters, including age, gender, histology, lymphatic invasion or venous invasion, were significantly prognostic for overall survival.

To evaluate the prognostic significance of EGFR and pEGFR immunostaining patterns, Kaplan-Meier curves were constructed (fig. 4). We found that patients whose esophageal SCCs showed a membrane-type EGFR immunostaining pattern tended to have a good outcome (fig. 4a). With regard to the relationship between prognosis and pEGFR immunostaining pattern, patients with a nuclear pattern had a significantly worse outcome than those with a nonnuclear pattern (fig. 4b).
We next examined whether staging and pEGFR expression pattern were independent for predicting overall survival. Multivariate Cox regression analysis revealed that staging was significantly predictive for overall survival ($p = 0.006$, hazard ratio = 5.56, 95% confidence interval 1.64–18.87), whereas pEGFR expression pattern was not statistically significant ($p = 0.162$, hazard ratio = 2.29, 95% confidence interval 0.72–7.30).

**Discussion**

In the present study, we confirmed that esophageal SCC cells show 2 distinct immunostaining patterns, not only for EGFR but also for pEGFR: a membrane- or nonmembrane-type pattern, and a nuclear- or nonnuclear-type pattern, respectively. Since EGFR is a transmembrane protein, it was originally believed that its expression would be detectable at the plasma membrane and in the cytoplasm, or that nuclear EGFR expression would be detected nonspecifically by immunohistochemistry. However, recent studies have suggested that EGFR may translocate from the plasma membrane to the nucleus [12], and moreover that translocated EGFR may function as a transcriptional regulator for oncogenic cyclin D1 or B-Myb, and subsequently play a role in tumorigenesis [12, 18]. Thus, an evaluation of immunoreactive localization for EGFR might yield some useful information on the association between the malignant potential and EGFR expression of tumors. Indeed, in the present study, we showed that diffuse cytoplasmic expression of EGFR, in parallel with the disappearance of its expression at the plasma membrane, was significantly prognostic for overall survival in patients with esophageal SCC.

In the present study we examined the expression not only of EGFR but also pEGFR, because pEGFR is the active form of EGFR and its intracellular translocation may reflect biological changes in tumors more clearly than conventional EGFR. Interestingly, nuclear translocation of pEGFR was significantly related not only to tumor stage but also lymph node metastasis, and moreover was a significantly better predictor of poor outcome than nonmembrane-type EGFR immunostaining in patients with esophageal SCC. Since the number of patients examined was relatively small, these findings will need to be verified by larger-scale studies. However, our data clearly suggest that nuclear translocation of pEGFR is associated with the malignant potential of esophageal SCC.

There are a number of commercially available antibodies against EGFR, and the selection of the most ap-

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**Table 2. Relationship between EGFR and pEGFR immunostaining patterns in esophageal SCC**

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<tr>
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<td>nonnuclear</td>
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<td>29</td>
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[Fig. 4. Overall survival according to EGFR (a) and pEGFR (b) expression patterns in patients with esophageal SCC (n = 44). Kaplan-Meier curves were constructed and pairwise differences were analyzed by log-rank test.]
propriate one is important for examining the pathophysiological significance of EGFR expression in human diseases. In this study, we utilized a pEGFR(Tyr845) antibody because Tyr845 phosphorylation in the activation loop of the EGFR kinase domain is considered to participate in the general regulation of its function by maintaining enzymatic activity [19–21]. Regarding the mechanism of translocation of the EGFR family, it is considered that the intracellular or transmembrane domain may be transported by importins [22, 23]. Recently, Lo et al. [24] have examined the immunostaining of breast cancer samples by various kinds of anti-EGFR antibody and shown that such antibodies recognizing the intracellular domain can detect the signal not only at the plasma membrane but also in the cytoplasm and nucleus, whereas those recognizing the extracellular domain detect the membrane signal alone. Indeed, both the anti-EGFR and anti-pEGFR antibodies used in this study were raised against the intracellular domain of EGFR, and therefore the pathological significance of this phenomenon needs to be investigated in future studies.

In summary, we have observed that EGFR is detectable not only at the plasma membrane but also in the cytoplasm or nucleus, although the dominant signal localization may be changed by the biological condition of the tumor cells. Interestingly, in the present study, SCC showing nuclear immunostaining of pEGFR mostly showed a nonmembrane-type EGFR immunostaining pattern. This finding suggests that the EGFR activated by phosphorylation is actively translocated to the nucleus in such SCC cells. Importantly, however, patients with this type of SCC had a significantly poor outcome. Accordingly, although the molecular mechanism involved still remains unclear, nuclear translocation of pEGFR may be closely associated with higher malignancy of esophageal SCC.

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References


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