Further Evidence for Prognostic Significance of Epidermal Growth Factor Receptor Gene Amplification in Patients with Esophageal Squamous Cell Carcinoma

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ABSTRACT

To determine the value of epidermal growth factor receptor (EGFR) gene amplification as a biological prognostic indicator in patients with esophageal squamous cell carcinoma, the EGFR gene amplification was determined by slot-blot hybridization using DNA extracted from formalin-fixed and paraffin-embedded blocks of tissues from 107 patients with esophageal squamous cell carcinoma who had undergone curative surgery. Results were analyzed by both univariate and multivariate statistical analysis. EGFR gene amplification greater than 3-fold was detected in the primary tumors of 13 (12%) of the 107 cases. The cumulative survival rate for patients with EGFR gene amplification in the primary tumors was significantly lower than that for patients without amplification (P < 0.001). A significant correlation was observed between extensive lymph node involvement at the time of surgery and EGFR gene amplification (P < 0.05). In the multivariate analysis, EGFR gene amplification (P = 0.015) and vascular invasion (P = 0.003) proved to retain independent prognostic values. These results suggest that EGFR gene amplification may be a useful biological marker for the prediction of lymph node metastasis and a poorer prognosis of esophageal squamous cell carcinoma.

INTRODUCTION

Over the past few years, a number of human neoplastic cell types have been shown to express EGFR to various degrees (1–5). There is growing evidence that overexpression of EGFR is correlated with the biological behavior of neoplastic cells and that it is also organ specific.

In breast cancer, EGFR overexpression has been shown to be associated with increased risk of early recurrence and resistance to endocrinotherapy (6). EGFR overexpression in brain tumors is observed in the more aggressive tumors such as glioblastoma multiforme but is absent in the less aggressive astrocytoma (2). In cancer of the bladder, EGFR-positive tumors tend to be invasive and poorly differentiated (4).

On the other hand, in colorectal carcinoma, there appears to be no significant difference in the level of EGFR expression between carcinomatous and normal mucosa (7, 8). Although elevated levels of EGFR were detected in non-small cell lung carcinomas, no significant correlation was found between the EGF-binding activities and clinicopathological factors of non-small cell lung carcinomas (9).

In esophageal squamous cell carcinoma, the correlation between EGFR overexpression in the primary tumor and poorer prognosis was determined using receptor radioassay of fresh frozen specimens (1). Generally, EGFR overexpression has been recognized as an indicator of poor prognosis in human esophageal squamous cell carcinoma.

Hyperproduction of EGFR may be caused by any of several mechanisms: (a) gene amplification; (b) changes in transcriptional or translational control; (c) changes in mRNA; and/or (d) protein turnover (10). Although EGFR gene amplification is not common in human tumors overexpressing EGFR, it was reported previously that EGFR hyperproduction in several human tumors, including esophageal squamous cell carcinoma, was associated with EGFR gene amplification, at least in part (10–13). Although the number of patients studied was limited, the cases with esophageal squamous cell carcinoma with EGFR gene amplification in addition to the increased EGF binding capacity showed a poorer clinical course in previous preliminary observations (1). Basically, the present study was designed to test the hypothesis that EGFR gene amplification is a significant and clear prognostic marker for patients with esophageal squamous cell carcinoma.

A large number of cases would be necessary to evaluate the prognostic significance of EGFR gene amplification because of the relatively low incidence of significant amplification of this gene. In this respect, the use of paraffin-embedded tissues, as compared with that of fresh frozen tissues, has enabled us to analyze retrospectively many cases and investigate the correlation between the long-term clinical course and the presence of gene amplification. The present study investigated retrospectively the significance of EGFR gene amplification as a prognostic indicator using DNA extracted from formalin-fixed and paraffin-embedded tissues.

MATERIALS AND METHODS

Patients and Specimens. This study was approved by the Human Subjects Committee of the Keio University Hospital, and informed consent for any treatment was obtained from the patients or their nearest relatives. Permission for the use of resected tissues was obtained before the surgery for the purpose...
of pathological diagnosis and the advancement of medical science and education. This study covers a total of 107 patients with esophageal squamous cell carcinoma who underwent curative surgery at Keio University Hospital (Tokyo, Japan) between 1980 and 1986. Specimens for DNA extraction were obtained from formalin-fixed, paraffin-embedded blocks of primary tumor tissue and adjacent normal tissue as a control. The underlying clinicopathological factors of the cases at the time of curative surgery were assessed according to the classification of the Japanese Society for Esophageal Diseases (14). According to the protocol of a multi-institutional prospective study, the patients who fit in the protocol were divided into a preoperative radiation group (30 Gy before surgery and 24 Gy after surgery) and a postoperative radiation group (50 Gy after surgery) at random during the period from 1980 to 1983. During the period from 1984 to 1986, the patients were divided into a radiation group (50 Gy after surgery) and a chemotherapy group (cisplatin 140 mg/m², Vindesine 6 mg/m² after surgery) at random. The patients were followed by checkups every 3 months at the outpatient clinic. The diagnosis of recurrence was made on the basis of the findings at autopsy and/or diagnostic imaging, including routine chest X-rays, body computed tomography scans, and ultrasonography. The median observation period of the patients was 18 months (1–119 months).

DNA Extraction. DNA was extracted by a previously described method from specimens of formalin-fixed, paraffin-embedded blocks of each primary tumor and its adjacent normal tissue (15). The blocks were cut into sections 20 μm thick, and the specimens of neoplastic and normal tissues were obtained from the same sections. One section was stained with hematoxylin and eosin to identify the areas of neoplastic and normal tissue. Then the samples of neoplastic and normal tissues were obtained from the same sections. Total cellular DNA was prepared by the chloroform-isooamyl alcohol method after treatment with proteinase K [200 μg/ml; in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl, and 1% SDS] at 37°C.

Slot-Blot Analysis. DNA concentrations were determined spectrophotometrically and slot-blot hybridization analysis was performed according to the method described previously (16). Ten μg of DNA were applied to a polyvinylidene difluoride filter (Immobilon, Millipore). Hybridization was performed at 42°C for 12 h in 5× Denhardt’s solution, 50% formamide, 6× SSC [1× SSC: 150 mM NaCl and 15 mM sodium citrate (Na2HPO4·H2O, pH 7.0), 0.5% SDS, 10 mmol EDTA, and 150 mg denatured salmon sperm DNA per ml. Probes were made by multiprime radioactive labeling with [32P]deoxyctydine triphosphate using the oligo-labeling system (17). After hybridization, the filters were washed twice at 58°C for 2 h in 0.5× SSC and 1% SDS. Radioactivity of the autoradiograms was determined quantitatively by using serially diluted samples as described previously (16). A degree of amplification greater than 3-fold compared with the normal adjacent tissue from the same patient was defined as a case with amplification. The probes were removed and the filters hybridized repeatedly.

The probes used were: pE 7, a 2.4-kb BamHI fragment of the EGFR gene, c-erbB (18, 19); and a 5.1-kb EcoRI fragment of D7S18 (20). D7S18 was provided by the Japanese Cancer Research Resources Bank.

Statistical Analysis. The various groups of patients were compared by the χ² test or Mann-Whitney U test. The cumulative survival curves for the various groups of patients were calculated by the Kaplan-Meier method and compared by the generalized Wilcoxon test. The parameters, including sex, age, depth of tumor invasion, lymph node metastasis, lymphatic invasion, vascular invasion, intramural metastasis, preoperative radiation, postoperative radiation, preoperative chemotherapy, postoperative chemotherapy, histological type, and EGFR gene amplification, were analyzed by Cox multivariate regression analysis (21) to assess the relative order of prognostic significance to survival.

RESULTS

EGFR Gene Amplification. EGFR gene amplification was determined by slot-blot hybridization (Fig. 1). The degree of amplification ranged from 3-fold to 16-fold compared with normal adjacent tissue from the same patient. EGFR gene amplification greater than 3-fold was observed in primary tumors in 13 (12%) of the 107 cases. The D7S18 fragment derived from the q31.1–q31.2 region of chromosome 7 was used as an internal control, and no amplification was detected in these tissues.

Prognostic Value of EGFR Gene Amplification. The survival rate for patients with amplification was significantly lower than that for patients without amplification (P < 0.001; generalized Wilcoxon test). The cumulative survival curves for patients with and without EGFR gene amplification as calculated by the Kaplan-Meier method are shown in Fig. 2a. The 50% survival period did not exceed 9 months in patients with EGFR gene amplification cases, whereas it reached 42 months in patients without amplification. The 5-year survival rate was 7% for patients with amplification and 43% for those without amplification. The treatments given the two groups with and without EGFR gene amplification were not statistically different (χ² test). Pre- and postoperative management of these patients is summarized in Table 1. The clinicopathological backgrounds of the patients are shown in Table 2. Although 35 of 94 cases without EGFR gene amplification were negative for lymph node metastasis, metastatic lymph nodes beyond group I were detected in all patients with EGFR gene amplification, even in those with no invasion reaching the adventitia at the time of surgery. A statistically significant correlation was observed between extensive lymph node involvement at the time of surgery and EGFR gene amplification (P < 0.05; Mann-Whitney U test). Other background factors, such as sex, age, location of the primary tumor, histological type, and depth of the adventitial invasion, were not significantly different between the groups with and those without EGFR gene amplification (Mann-Whitney U test and χ² test). However, all the tumors with EGFR gene amplification were categorized as stage III or IV because of the lymph node involvement (P < 0.01, Mann-Whitney U test). In the patients categorized as stage III or IV, the survival rate for patients with amplification was significantly lower than that for patients without amplification (P < 0.001; generalized Wilcoxon test). The cumulative survival curves for the stage III and IV patients of each group are shown in Fig. 2b.
Multivariate Analysis of Clinicopathological Parameters and EGFR Gene Amplification as Prognostic Parameters. Multivariate analysis using the Cox proportional hazards model showed that EGFR gene amplification (P = 0.015) and vascular invasion (P = 0.003) were significant and independent prognostic factors for esophageal squamous cell carcinoma.

DISCUSSION

The mapping of the EGFR gene on human chromosome 7 has been reported on several occasions since the first report using the somatic cell hybridization technique by Shimizu et al. (22). Recently, it has been localized precisely to p12 of human chromosome 7 using a novel method in which fluorescence images from in situ hybridization and Q-banding are merged with the use of compute graphics (23). The structure of the EGFR-containing amplicon was unique and relatively large (24). In this study, EGFR gene amplification in esophageal squamous cell carcinoma was revealed by slot-blot analysis using an internal control probe on chromosome 7. The compatibility of the results from slot-blot analysis using paraffin-embedded specimens and Southern blot analysis using fresh frozen specimens were confirmed in several specimens (data not shown). The presence of EGFR gene amplification correlated with EGFR expression as determined by receptor radio assay (1).

The 12% incidence (13 of 107 cases) of EGFR gene amplification in the primary tumors of patients with esophageal squamous cell carcinoma observed in the present study correlates well with the results of previous Southern blot analysis, which used fresh frozen specimens (10, 25). This study analyzing more than 100 cases clarified the incidence and the clinical significance of EGFR gene amplification in esophageal squamous cell carcinoma, giving evidence that EGFR gene amplification is an excellent indicator of cases of poor prognosis. The relationship observed here seems analogous to that seen between N-myc gene amplification and the prognosis of advanced neuroblastoma (26). The degree of gene amplification may reflect a biological behavior of cancer cells in the patients with amplification. Additional studies are necessary to address this question.

A retrospective analysis such as the present study poses one obvious limitation that should be eliminated by a future prospective study. The effect of chemotheraphy or radiation therapy on EGFR gene amplification or EGFR status is still controversial and inconclusive. In squamous cell carcinoma of the tongue, Sauter et al. (27) reported that radiation did not influence EGFR or p53 expression, whereas transforming growth factor α expression was decreased. In this regard, it would be necessary to confirm our observation by a prospective study in which the treatments for the patients are matched.

In previous studies, pathophysiological relevance of the correlation between the EGF-EGFR system and biological behaviors of cells has been investigated extensively in clinical cases, animal models, and in vitro systems. One of the early studies demonstrated that a high degree of EGFR expression in the EGF cDNA-transfected cells conferred a transformed phenotype in the presence of a ligand (28). We have shown that EGF promotes the growth of EGFR-hyperproducing squamous carcinoma cells in vivo (29). Moreover, EGF expression was detected in several human cancer cells, including esophageal squamous cell carcinoma, and it was suggested that an autocrine system of EGF-EGFR may be involved in the aggressive biological behavior of cancer cells (30).

A number of activated proto-oncogenes have been demonstrated in various human neoplastic cells. A relatively high incidence of EGFR gene and int-2/hst-1 amplification has been observed in esophageal squamous cell carcinoma (16, 25, 31). These genes, EGFR gene and int-2/hst-1, were associated with an increased risk of early recurrence and lower survival rate. Whereas a close relation between int-2/hst-1 coamplification
EGFR Gene Amplification in Esophageal Cancer

Fig. 2 Cumulative survival curves for patients with and without EGFR gene amplification as calculated by the Kaplan-Meier method. a, curves for all patients, including 13 with EGFR gene amplification (A) and 94 without EGFR gene amplification (B). b, curves for stage III and IV patients, including 13 with EGFR gene amplification (A) and 66 without EGFR gene amplification (B). In both a and b, P < 0.001 by the generalized Wilcoxon test.

Table 1 Pre- and postoperative treatments for the patients with and without EGFR gene amplification

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<th>Treatment</th>
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<td>Radiation (+)</td>
<td>22 (23%)</td>
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<td>72 (77%)</td>
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* NS, not significant (χ² test).

expression and low metastatic potential promotes matrix protein adhesion and lung colonization (33). To date, the possible mechanisms of enhanced metastatic capacity induced by the EGF-EGFR system have been studied extensively in each aspect of the metastatic process of cancer cells, including invasion, lodgment, extravasation, and cell locomotion (reviewed in Ref. 34). Recently, Yudoh et al. reported that EGF stimulated three actions: (a) invasiveness through Matrigel; (b) attachment to the extracellular matrix components; and (c) the degradation of type IV collagen through high-affinity EGF in high-metastatic murine RCT sarcoma cells with high laminin receptor expression (35). In their study, low-metastatic RCT sarcoma cells were insensitive to EGF stimulation in the invasion assay. To determine the mechanisms of lymph node metastasis in the patients with EGFR gene amplification, the components of cell attachment and matrix degradation should be investigated in additional studies. Despite accumulating data on examples where stimulation of EGFR increased the potency of cancer cells to succeed in steps of the metastatic cascade (34), evidence that specifically links the EGF-EGFR system to lymph node involvement (not to distant organ metastasis) is still missing.

In the multivariate analysis, EGFR gene amplification (P = 0.015) and vascular invasion (P = 0.003) proved to retain an independent prognostic value in this study. The vascular invasion has been described as one of the most important histopathological prognostic parameters of esophageal squamous cell carcinoma (36). However, preoperative determination of the vascular invasion using small biopsy specimens is difficult and impractical. In contrast, gene amplification can be determined preoperatively in small biopsy specimens obtained from endoscopic examination. This information makes it possible to determine which cases have a high malignant potential and to decide necessary multidisciplinary management preoperatively. In this respect, quantitative assessment of gene amplification is of great prognostic value. Recently, rapid and quantitative DNA diagnosis of the EGFR gene amplification in individual cells of tumor specimens using fluorescence in situ hybridization has been established, and this novel method would provide more quantitative and specific information in these aspects (37).

Preoperative detection of EGFR gene amplification using endoscopic biopsy specimens can be one of the useful methods...

and distant organ metastasis after curative surgery have been demonstrated (14), the present study demonstrates that EGFR gene amplification was detected in all of the advanced cases with lymph node metastasis at the time of surgery. It is interesting that a certain gene alteration correlates with a specific metastatic mode in patients with esophageal squamous carcinoma.

It is unclear whether the growth-promoting effect of the EGF-EGFR system is the sole mechanism for lymph node involvement in patients with EGFR gene amplification. However, the enhancement of the metastatic capacity of tumor cells by the EGF-EGFR system has been demonstrated in animal models. In rat rhabdomyosarcoma, spontaneous lung metastasis and axillary lymph node and mediastinal metastases were enhanced by EGF treatment (32). Lichtner et al. reported that introduction of a full-length cDNA of the human EGFR to rat mammary adenocarcinoma cells with a low level of EGFR

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for preoperative prediction of lymph node involvement. Additional studies using fresh specimens of early-stage lesions are necessary to determine the correlation between gene amplification and tumor progression. Vogelstein et al. have described a model of colorectal tumorigenesis in which the steps required for the development of cancer often involve the mutational activation of an oncogene coupled with the loss of several genes that normally suppress tumorigenesis (38). EGF gene amplification may be one of the many genetic alterations that orchestrate the behavior of malignant cells (4). Additional studies may reveal many other unknown genetic alterations affecting the biological behavior of esophageal squamous cell carcinoma. It will then be possible with such new biological information to identify and categorize the groups at high risk of early recurrence and thereby improve the clinical management of such patients.

In our observation, the effect of conventional anticancer therapy, including surgical resection, chemotherapy, and radiotherapy is still merely palliative for the patients with high malignant potential such as the patients with EGF gene amplification. Therefore, it is necessary to develop a novel therapeutic approach based on the knowledge of molecular mechanisms of carcinogenesis and progression. On the basis of such a concept, a number of studies designed to target the EGF-EGFR system in various cancers have been reported (39-44). Hirota et al. have demonstrated the growth-inhibitory effect of immunotoxin, which is the conjugated form of anti-EGFR monoclonal antibody and plant toxin, to the EGFR-hyperproducing A431 tumors transplanted in athymic mice as a model of the anti-EGF-EGFR system treatment (44). Recently, Chen et al. have established a novel gene delivery system using EGF-R-mediated endocytosis (45). This novel method would be especially suitable for gene therapy for EGFR-hyperproducing squamous cell carcinomas. We would like to propose that the combination of these novel therapeutic approaches and the diagnostic strategy demonstrated in this study would be instrumental in improving the management of patients with esophageal carcinoma.

### REFERENCES


Further evidence for prognostic significance of epidermal growth factor receptor gene amplification in patients with esophageal squamous cell carcinoma.

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