Overexpression of the Epidermal Growth Factor Receptor and Its Ligand Transforming Growth Factor α Is Frequent in Resectable Non-Small Cell Lung Cancer but Does Not Predict Tumor Progression

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ABSTRACT

The epidermal growth factor receptor (EGFR) and its ligand transforming growth factor (TGF) α are hypothesized to form an autocrine growth loop in non-small cell lung cancer (NSCLC) and to play an important role in tumor formation and progression. We studied the association between overexpression of EGFR, TGF-α, or both, and overall survival of patients with resectable NSCLC. Overexpression, defined as >20% of tumor cells staining on immunohistochemistry, was examined in 96 tumor samples from consecutive patients having resection of previously untreated, well-staged NSCLC who were then followed prospectively (median follow-up, 20.7 months). The expression of three other ligands for EGFR (epidermal growth factor, cripto, and amphiregulin) was examined by Northern analysis in tumor or benign lung, suggesting that in NSCLC these ligands may not participate in an autocrine growth stimulatory loop with EGFR. Differential overexpression of amphiregulin in malignant versus normal lung was observed, but this expression pattern did not have a prognostic impact. Thus, EGFR and TGF-α overexpression is frequent in early-stage NSCLC but is not associated with a survival difference. These findings suggest that this growth factor/receptor loop is more important for lung tumor formation than for tumor progression.

INTRODUCTION

Despite advances in clinical care, lung cancer remains the most common cause of cancer-related deaths in the United States in both men and women. A better understanding of lung cancer biology is urgently needed to develop novel treatment strategies that could improve the dismal survival rates of most lung cancer patients. Current information suggests that lung cancers, like other solid tumors, result from activation of oncogenes and inactivation of tumor suppressor genes. Autocrine loops involving specific growth factors and their receptors are also thought to play an important role in the formation and progression of lung cancers (1-3). In NSCLC, EGFR and one of its ligands, TGF-α, are hypothesized to function in this manner (4). Previously, we reported that EGFR overexpression, as detected by immunohistochemistry, occurs in approximately half of all lesions of the bronchial epithelium, including metaplasia and dysplasia, and may be an important step linked to lung tumor formation (5). In another study, we reported that overexpression of EGFR and TGF-α, as assessed by total cellular RNA and immunohistochemistry, occurred in approximately half of all resectable NSCLC independent of histological subtype. Correlation with early clinical outcome of the patients from whom the primary tumors were examined showed that neither EGFR nor TGF-α overexpression was associated with a statistically worse overall survival (6). However, other published studies suggest that EGFR and TGF-α overexpression plays a key role in tumor progression.

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4 The abbreviations used are: NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; SCLC, small cell lung cancer; EGF, epidermal growth factor.
progression, because these are associated with a significantly worse clinical survival (7–9).

This study sought to determine definitively whether overexpression of EGFR and TGF-α is associated with an unfavorable overall survival. It is based on larger numbers of patients prospectively followed for a much longer time than in our initial study. We reported previously that overexpression (as measured by total cellular RNA) correlated with, but underestimated, protein overexpression detected by immunohistochemistry (6). Therefore, the association made in this study between EGFR and TGF-α overexpression in NSCLC and survival was based on immunohistochemical analysis. This work was extended to Northern analysis expression of three additional ligands for EGFR: EGF, cripto, and amphiregulin. Through these studies, a comprehensive analysis of the expression of EGFR and its ligands in NSCLC versus benign lung tissues was accomplished. This permitted analysis of the impact of this growth factor/receptor loop on survival.

MATERIALS AND METHODS

Tumor Bank. Tissue specimens were obtained from previously untreated patients undergoing potentially curative operations for primary NSCLC. Pulmonary resection was accompanied by careful intraoperative staging with complete mediastinal lymph node dissection (10). Lymph nodes were labeled for the pathologist according to the American Thoracic Society Lymph Node Map (11).

Within 10 min of completion of the pulmonary resection, a specimen of the primary tumor trimmed of the surrounding lung tissue and of any grossly necrotic material was snap frozen in liquid nitrogen. A separate specimen of uninvolved lung tissue was harvested from an area distant from the primary tumor and also frozen in liquid nitrogen. Immediately adjacent specimens of tumor and uninvolved lung were submitted to the Department of Pathology for histological examination. One pathologist (D.K.) reviewed all of the submitted specimens to confirm tumor histology and stage in order to determine that viable tumor was present and to exclude concurrent lung pathology. The tumor-node-metastasis stage of the primary tumor was determined according to the new International Staging System for NSCLC (12).

Isolation of Total Cellular RNA. Stored tissues were removed from liquid nitrogen, minced with a sterile razor blade, placed in ice-cold guanidine isothiocyanate solution, and finely ground before being placed on a cesium chloride cushion and ultracentrifuged to purify RNA as described previously (13). To prevent RNA degradation, all solutions that came in contact with the specimens were autoclaved and washed with diethylpyrocarbonate-treated H2O. After ultracentrifugation, RNA pellets were resuspended in H2O, ethanol precipitated with 0.3 M sodium acetate on dry ice, washed once with dry, ice-cold 70% ethanol, lyophilized, and resuspended in H2O as a 1 μg/μl stock stored at −70°C.

Nucleic Acid Probes. Probes for EGF, cripto, amphiregulin, and β-actin were obtained as inserts contained within plasmid vectors. Inserts were freed from vectors as specified by the unique flanking restriction sites for each probe. The fragments were gel purified, electroluted, and purified further through passage over an elutrap-D (Schleicher & Schuell, Keene, NH) column. The probes included: a 3.8-kb XhoI cut human EGF cDNA (a gift of Dr. Graeme Bell, Howard Hughes Medical Institute Research Laboratories, University of Chicago, Chicago, IL); a 1.1-kb EcoRI cut human amphiregulin cDNA (a gift of Dr. Greg Plowman, Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA); a 0.9-kb EcoRI cut human cripto cDNA (a gift of Dr. Fortunato Ciardiello, University of Naples, Naples, Italy); and a 1.9-kb BamHI cut human β-actin cDNA (14). Probes were radiolabeled with [32P]dCTP using random-priming techniques.

Northern Analysis. Ten μg of total cellular RNA from each of the paired samples of primary tumor and benign lung were used for Northern analysis. This was performed by size fractionation on a 1% agarose-formaldehyde gel in a 0.2 M 3-N-morpholino-propanesulfonic acid/0.05 M sodium acetate/0.01 M EDTA buffer. Transfer to nitrocellulose filters was accomplished with Kodak XAR film and exposure to an intensifying screen at −70°C. The appropriate exposure time was determined for each probe.

Scoring of Hybridization Results for EGF, Cripto, Amphiregulin, and β-Actin. Scoring of the results of hybridizations performed on the paired samples of primary tumor and benign lung was done independently by two individuals who were not aware of the clinical data (J. L. and E. D.). The hybridization to β-actin was used as a control for the integrity and amount of RNA loaded for Northern analysis, and positive controls were used for each of the nucleic acid probes. Results of the hybridizations performed on each of the primary tumors were compared with the paired samples of normal lung.

Methods of Tissue Preparation and Immunohistochemical Staining for EGFR and TGF-α. Five-μm sections were cut from the archival paraffin blocks of the paired samples of invasive carcinoma and benign lung and were placed on superfrost/plus microscope slides (Fischer-Grand). Immunohistochemical staining was then performed using standard tech-

### Table 1 Stage and histopathology of the 96 tumors harvested

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>Squamous cell carcinoma</th>
<th>Adenocarcinoma</th>
<th>Large cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>(n = 22)</td>
<td>(n = 28)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>T,N,M0</td>
<td>5</td>
<td>11</td>
<td>0</td>
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<tr>
<td>T,N,M1</td>
<td>17</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Stage II</td>
<td>(n = 4)</td>
<td>(n = 11)</td>
<td>(n = 1)</td>
</tr>
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<td>T,N,M0</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>T,N,M1</td>
<td>3</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Stage III</td>
<td>(n = 10)</td>
<td>(n = 13)</td>
<td>(n = 2)</td>
</tr>
<tr>
<td>T,N,M0</td>
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<td>0</td>
</tr>
<tr>
<td>T,N,M1</td>
<td>1</td>
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<td>0</td>
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* TNM, tumor-node-metastasis.
Fig. 1 Overall patient survival by stage. The numbers of patients at risk within stages I, II, and III are noted at each time point.

Fig. 2 Representative total cellular RNA Northern analysis demonstrating differential overexpression of EGFR and TGF-α in tumor (T) compared with paired benign lung (N) specimens and absent expression in both tumor and benign lung for EGF and cripto. Amphiregulin (AR) is differentially overexpressed in benign lung specimens versus primary lung tumors. The positions of the 18S and 28S ribosomal bands are indicated.
Fig. 3 Immunohistochemical staining for EGFR. Staining of nonneoplastic lung tissue (A) demonstrates intense positivity in the basal cell layer of the respiratory epithelium lining the airways. Some basal cells lining the subjacent glands are also stained (arrow). No significant staining is seen in the alveolar lining cells (lower right) or within the stromal elements of the lung. A squamous cell carcinoma showing 4+ positivity (B) demonstrates intense deposition of reaction product in nearly all of the tumor cells. The staining is cytoplasmic with cell membrane enhancement. In a 1+ positive case (C), there is faint cytoplasmic positivity in approximately 10% of the tumor cells (arrows). Note the intense positivity in the basal layer of the overlying nonneoplastic respiratory epithelium.

Fig. 4 Immunohistochemical staining for TGF-α. In the nonneoplastic lung (A), there is variable intense staining in the respiratory epithelium (arrowheads), with strong staining of the submucosal glands (long arrows). Scattered bundles of smooth muscle also show positivity (short arrows). In a squamous cell carcinoma showing 4+ positivity (B), there is intense cytoplasmic staining in the majority of tumor cells. In a tumor with negative staining (C), there is no positivity in tumor cells, although staining is present in a medium-sized peripheral nerve (top), another nonneoplastic cell type that consistently stained.

body. Positive controls consisted of pulmonary squamous cell carcinomas known to exhibit cytoplasmic overexpression of EGFR or TGF-α, respectively. The uniform presence of EGFR staining in the basal layer of the bronchial epithelium and its absence in other normal pulmonary tissues served as additional internal positive and negative controls, respectively. The uniform staining of nerves and submucosal salivary glands for TGF-α served as an internal positive control.

Criteria for Evaluation of Immunostaining Results. Scoring was performed by a pathologist who was unaware of the clinical data (D. K.). For both EGFR and TGF-α, microscopic examination for the cytoplasmic reaction product was
performed in the invasive carcinomas and scored as follows: negative, <5% of cells staining; 1+, 5–20% of cells staining; 2+, 20–50% of cells staining; 3+, 50–80% of cells staining; and 4+, >80% of cells staining. Only tumors exhibiting 2+–4+ staining were considered to show clear protein overexpression.

**Clinical Data Base.** After pulmonary resection, patients were seen in follow-up by a single surgeon (V. R.) every 3 months for the first 2 postoperative years, then every 6 months for 3 years, and annually thereafter. Follow-up included a history and physical exam, chest X-ray, CBC, and chemistry profile. Computerized tomography and radionuclide bone scans were used selectively to investigate new signs or symptoms, or to provide more accurate follow-up in patients whose potential area of recurrence could not be adequately evaluated by standard clinical means. Suspected areas of recurrent disease (e.g., adrenal metastases) were confirmed by biopsy whenever technically possible. The clinical and pathological parameters recorded included patient age and gender, tumor cell type and stage, and the length of overall survival as calculated from the date of the operation. The clinical information, Northern analysis, and immunohistochemical data were entered prospectively into a computerized data base.

**Statistical Methodology.** Overall survival was calculated from the date of thoracotomy and described using the product limit method of Kaplan and Meier (16). Overall survival was compared across the levels of prognostic factors using the log-rank test (17). All statistical tests were conducted at the two-sided 0.05 level.

**RESULTS**

Tissue specimens were harvested from 96 consecutive patients with previously untreated primary NSCLC who underwent pulmonary resection between January 1990 and December 1994. There were 50 men and 46 women with a mean age of 67 years (range, 38–86 years). The stage and histopathology of these tumors are shown in Table 1. Fifty-five tumors were stage I, 16 were stage II, and 25 were stage IIa. There were 36 squamous cell carcinomas, 52 adenocarcinomas, and 8 large cell carcinomas. The median follow-up in all 96 patients was 20.7 months. The overall survival by stage is shown in Fig. 1. As expected, the difference in survival between stage I and stages II and III was significant (P = 0.003). There is no significant difference in survival by histology (squamous cell versus adenocarcinoma versus large cell carcinoma, P = 0.076) or by gender (men versus women, P = 0.696). Twenty-three paired samples of tumor and adjacent benign lung were examined for expression of EGF and 21 paired samples were examined for expression of cripto by Northern analysis (Fig. 2 and data not shown). No detectable total cellular RNA expression of these ligands was seen in either tumor or benign lung tissue.

The characteristic patterns of immunohistochemical staining for EGFR and for TGF-α are shown in Figs. 3 and 4. Benign lung tissue normally stains positively for EGFR only in the basal cell layer of the respiratory epithelium lining the airways, and for TGF-α in the submucosal glands and peripheral nerves. However, other tissues may show occasional staining for TGF-α in a less predictable manner. The frequency of overexpression of EGFR and TGF-α in the tumor is shown in Fig. 5. Thirty-seven tumors (38.5%) had both EGFR and TGF-α overexpression, 18 tumors (19%) did not overexpress either EGFR or TGF-α, 31 tumors (32%) showed only EGFR overexpression, and 10 tumors (10.4%) showed only TGF-α overexpression. Although EGFR and TGF-α overexpression was seen in more than half of non-squamous cell tumors, it occurred more frequently in squamous cell carcinomas. Thirty-three squamous cell carcinomas (91.6%) showed EGFR overexpression, and 23 (63.8%) of these tumors exhibited both EGFR and TGF-α overexpression (Fig. 6). There was no significant difference in the number of men versus women whose tumors showed EGFR overexpression (P = 0.108) or TGF-α overexpression (P = 0.107).

The relationship between overall survival and overexpression of EGFR, TGF-α, or both is shown in Figs. 7 and 8. By univariate analysis, no significant difference in survival was observed according to the expression of TGF-α (P = 0.501). A significant difference in survival in favor of tumors overexpressing EGFR was found by univariate analysis. However, by multivariate analysis, only stage, not histology, gender, or EGFR or TGF-α expression, had a significant impact on survival (stage I versus stages II and III, P = 0.003). Thus, the apparent survival benefit associated with EGFR overexpression by univariate
DISCUSSION

Most lung cancers develop as a result of tobacco carcinogen-induced activation of oncogenes and inactivation of tumor suppressor genes (1, 18). Autocrine growth loops are also viewed as having an important role in lung tumorigenesis (2, 3, 9). In NSCLC, EGFR and its ligand TGF-α are thought to function in this manner (4). Previous studies suggested that EGFR and TGF-α are frequently overexpressed in NSCLC at both the RNA and protein levels and that EGFR overexpression predicted a poor outcome. These previous studies were limited because they were performed in cell lines or in specimens from small numbers of patients with incomplete staging and follow-up (8, 9, 18–36). However, more recent studies using specimens from larger numbers of patients suggest that EGFR overexpression is not associated with a worse survival (37–39). In our previous study performed in specimens obtained from 57 patients, we reported that overexpression of EGFR and TGF-α, compared with paired samples of benign lung tissue,
occurred in 45 and 61% of primary tumors, respectively. Simulta-
neous overexpression of EGFR and TGF-α was found in 38% of
tumors. A trend toward worse overall survival was seen with
overexpression of either EGFR or TGF-α, but was not statistically
significant in this relatively small patient cohort with short fol-
low-up (6).

This study was performed to extend our initial work. The
quality of the material used is unusual because paired samples of
tumor and benign lung were harvested at thoracotomy in all
cases and because patients had complete pathological staging
and careful follow-up recorded in a prospective clinical data
base. This permits an accurate correlation between EGFR and
TGF-α overexpression and clinical outcome. The importance of
careful staging is illustrated by the results of the multivariate
analysis in this study; an apparently significant difference in
overall survival according to EGFR expression observed in a
univariate analysis is not significant in a multivariate analysis
that controls for stage. This finding may explain the discrepancy
between our results and some other studies in which patients
were not as carefully staged or followed for as long.

In our initial study, we examined specimens by Northern
analysis and by immunohistochemistry performed on snap-fro-
zen specimens. Increased expression at the total cellular RNA
level correlated with immunohistochemical staining, but only
when this was scored as ++ intensity in 100% of cells (6).
Subsequently, we developed reliable techniques for performing
immunohistochemical staining using paraffin-embedded speci-
mens that allowed us to reexamine all of the specimens used in
the initial study and to extend the analysis to specimens obtained
from an additional 39 patients. The immunohistochemical scor-
ing for the current study is based on the percentage of tumor
cells staining positively rather than on the intensity of staining,
because our experience in studying NSCLC indicates that this is
the more reliable way to score the assay (1, 5). The use of
paraffin-embedded material in this study permitted precise se-
lection of tumor cells for staining. We believe that these tech-
nical differences between our initial study and the current one
account for the higher percentage of squamous cell tumors
(91.6% versus 57.8%) found to overexpress EGFR. These
immunohistochemical results otherwise confirm the findings of
our initial study regarding the frequency of TGF-α overexpres-
sion and dual overexpression of EGFR and TGF-α. Our results
also show that EGF and cripto are not expressed in NSCLC or
adjacent benign lung tissue at the level of total cellular RNA
Northern analysis. Of course, this study does not exclude lower
levels of EGF or cripto expression in these tissues. With the
longer follow-up provided by this study, the differential expres-
sion of amphiregulin in benign versus malignant lung still did
not provide a statistically significant association with survival,
and therefore is not thought to contribute to a potential autocrine
growth loop.

Our results confirm that overexpression of EGFR and
TGF-α, either individually or simultaneously, does not correlate
with overall survival. We recently reported that overexpression
of EGFR is frequent in early bronchial neoplasia, occurring not
only in dysplasia and carcinoma *in situ*, but also in areas of
squamous metaplasia. Overexpression of TGF-α is not uni-
formly seen in early bronchial neoplasia and appears to be a
later event in lung tumorigenesis (5). When combined with our
current results, these findings suggest that EGFR and TGF-α
overexpression may be more important steps in tumor formation
than in progression.

Although EGFR and TGF-α overexpression is not a useful
prognostic indicator, it has other potential clinical implications.
Immunotoxins based on murine monoclonal antibodies are cy-
totoxic to tumor cell lines overexpressing EGFR (40–42). In
clinical trials, radiolabeled monoclonal antibodies to EGFR
have successfully imaged NSCLC (43). Anti-EGFR antibodies
enhance the effect of chemotherapeutic agents in animal models
of breast cancer, and this treatment approach is currently being
tested in clinical trials (44). A similar therapeutic strategy could
be applied to NSCLC. The recent development of EGFR-selec-
tive tyrosine kinase antagonists could also provide useful phar-
macological agents for testing in clinical trials (45). The fre-
quency of EGFR and TGF-α overexpression suggests that
similar therapeutic strategies should be explored in NSCLC, a
solid tumor that is rarely cured by current standard approaches
to cancer treatment.

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Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor alpha is frequent in resectable non-small cell lung cancer but does not predict tumor progression.

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