Differential Expression of Growth Factors in Squamous Cell Carcinoma and Precancerous Lesions of the Lung


ABSTRACT

Purpose: This study was conducted to evaluate the clinical significance of the localization of epidermal growth factor receptor (EGF-r), transforming growth factor (TGF)-α, and erbB-2 in the development, progression and prognosis of squamous cell cancers (SCCs) of the lung.

Experimental Design: The localization of EGF-r, TGF-α, and erbB-2 was evaluated immunohistochemically in 60 archival specimens of SCC of the lung and in 60 lung specimens without cancer. To clarify the patterns of expression of EGF-r in these tumors, the patterns of expression of EGF-r in cells in culture were monitored after challenging normal human bronchial epithelial and SCC cell lines with either EGF or TGF-α at physiological concentrations.

Results and Conclusions: The expression of EGF-r, erbB-2, and TGF-α were significantly higher in SCC and associated precancerous lesions than in the normal bronchial epithelium and hyperplastic lesions of noncancer specimens. A statistically significant stepwise increase in expression from uninvolved bronchial epithelium to precancerous lesions to SCC was observed with EGF-r and TGF-α. The localization of EGF-r in the cytoplasm (P = 0.04), but not in the membrane (P = 0.20), of SCCs was significantly associated with poor overall survival of subjects. To demonstrate the biological relevance of cytoplasmic expression of EGF-r, we noted that there was a prompt reduction in the membrane expression and a concomitant increase in cytoplasmic expression of EGF-r after adding either EGF or TGF-α to the cell culture medium. Overall, the study identified an involvement of EGF-r and TGF-α in the development of SCCs. The prognostic importance of EGF-r expression in the cytoplasm of lung cancer probably is an indication of the prognostic importance of trafficking of the EGF-r receptor between the Golgi apparatus and cell membranes and of internalization of EGF-r after an interaction with one of the EGF-r ligands at the cellular membrane surface.

INTRODUCTION

Despite recent advances in the treatments for lung cancer, improvement in survival has only been modest, indicating that effective therapeutic approaches are still lacking. Because growth factors and their receptors play a central role in regulating neoplastic processes (1), the expression of various growth factor receptors and their ligands in human neoplasia has been investigated extensively as potential targets of receptor-directed therapies. Many studies have identified EGF-r3 and two of its ligands, EGF and TGF-α, and a closely related receptor, erbB-2 (p185HER-2), as some of the major growth factors and growth factor receptors involved in neoplastic transformation (2). However, a great heterogeneity in the incidence of overexpression and in the prognostic significance of these growth factors and receptors has been observed both within and between various tumor types, including cancers of the lung.

Most studies of lung cancer in which growth factors have been investigated have grouped together SCC and adenocarcinomas of the lung as “non-small cell lung cancer (NSCLC),” despite the likely biological differences between the two histological subtypes. EGF-r has been shown to be overexpressed in both squamous and adenocarcinoma of the lung, but a stronger correlation between EGF-r overexpression and an adverse outcome was noted for squamous carcinomas than for adenocarcinomas (3, 4). Therefore, a combination of varying proportions of SCCs and adenocarcinomas may result in mixed observations. Another major limitation in most studies relates to the lack of data or inconsistency in reporting data on the intensity and localization of the receptor in cells of interest and the relation of growth factor expression to survival of patients. For example, some investigators have reported only the cytoplasmic expression of EGF-r (5) and erbB-2 (6), whereas others considered a cell positive for EGF-r expression only if membrane staining was present (7). However, most investigators have failed to report the cellular localization of EGF-r expression (8, 9). A knowledge of the cellular localization of growth factor receptors and its relation to cancer development and prognosis will aid in understanding the future applications of growth factor receptor-directed therapies. Also, in targeted therapy, knowledge of the relative intensity of expression of target antigen in cancer and

3 The abbreviations used are: EGF-r, epidermal growth factor receptor; TGF, transforming growth factor; SCC, squamous cell carcinoma; CSCS, charcoal-stripped calf serum; PCNA, proliferating cell nuclear antigen.

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associated precancerous lesions and normal tissues is important, but reports of such nature on growth factor and growth factor receptor expression in the lung are rare.

To overcome these limitations, we examined the cytoplasmic and/or membrane expression of EGF-r, erbB-2, and TGF-α in archival specimens of SCC, histologically normal bronchial epithelium (uninvolved epithelium), epithelial hyperplasia, metaplasia, and dysplasia associated with SCC and in the normal bronchial epithelium and epithelial hyperplasia in archival lung specimens without cancer (noncancer). We also correlated the cytoplasmic and membrane expression of growth factors in SCCs individually and as an average of the two patterns of expression with indicators of tumor progression or aggressiveness (i.e., size of the tumor, nodal status, metastases, stage of the disease, and differentiation of SCCs) and overall survival from the disease. Cell culture studies were conducted to evaluate the pattern of expression of EGF-r in cells after challenging normal human bronchial epithelial and SCC cell lines with physiological concentrations of EGF and TGF-α.

**MATERIALS AND METHODS**

**Patients and Tissue Specimens.** Formalin-fixed, paraffin-embedded sections of the lung were obtained from the archival collections of the Veterans Affairs Medical Center and University Hospital in Birmingham, AL. Sixty noncancer specimens of the lung were selected at random from a list of lung surgeries performed between 1988 and 1998 for lung diseases or conditions other than lung cancer. From a list of patients with SCC of the lung who had undergone surgery between 1989 and 1996, sixty cases were selected at random. We obtained the surgical pathology report, demographic information, clinical follow-up, and cause of death from the respective hospital medical records or hospital tumor registries. The patients were followed-up by the respective tumor registries until their date of death or the date of last documented contact.

One tissue block from each noncancer patient was selected to provide a section that contained adequate normal and uninvolved bronchial epithelium. One or more tissue blocks from each patient with SCC were selected to provide sections that contained samples of uninvolved bronchial epithelium, epithelial hyperplasia, metaplasia, and dysplasia, when present, in addition to the invasive carcinomas.

**Histological Criteria and Classification of Lesions.** Bronchial epithelium was classified histologically as normal (in noncancer specimens), uninvolved (SCC associated, but histopathologically normal), hyperplastic, metaplastic, or dysplastic by the pathologists involved in the study (W. C. B. and A. R. F.). The normal and uninvolved bronchial epithelium was defined as pseudostatified ciliated columnar epithelium to ciliated cuboidal columnar epithelium, depending on the size of the bronchus assessed. The bronchial epithelium was considered hyperplastic if the number of layers of cells was increased in the epithelium (i.e., more than four cell layers present without an obvious tangential cut). When the normal ciliated columnar epithelium was completely replaced by squamous epithelium without significant cytological atypia, the epithelium was considered to be metaplastic. If the metaplastic squamous epithelium demonstrated nuclear atypia, it was deemed dysplastic. All SCCs of the lung exhibited squamous differentiation with various degrees of keratinization and were classified as well, moderately, or poorly differentiated. No mixed cases with well-defined components of adenocarcinoma or small cell carcinoma were included. SCC of the lung was classified into well-differentiated, moderately differentiated, and poorly differentiated subtypes, depending on the degree of squamous differentiation present (10). Tumor staging was evaluated by using the Tumor-Node-Metastasis system (11).

**Immunohistochemical Analysis.** Our methods of immunohistochemical analysis have been reported previously (12). Briefly, 5-μm sections were deparaffinized and treated with 3.0% H2O2 for 4 min to quench endogenous peroxidase activity. Sections were incubated with preimmune goat serum (1%) for 20 min at room temperature to suppress nonspecific staining and then subsequently incubated with monoclonal antibodies to EGF-r (Zymed Laboratories, Inc., San Francisco, CA), erbB-2, and TGF-α (Oncogene Research Products, Cambridge, MA) at a concentration of 1.0, 0.25, and 0.5 μg/ml, respectively, for 1 h at room temperature. Companion matching slides stained with no primary antibody served as controls (deletes). The remainder of the staining procedure was performed using a biotin-streptavidin detection system (BioGenex, San Ramon, CA). The substrate diaminobenzidine tetrahydrochloride was used for visualization of the antigen-antibody complex (a brown reaction product), and sections were counterstained lightly with hematoxylin.

**Assessment of Expression of EGF-r, erbB-2, and TGF-α.** The expression of EGF-r, erbB-2, and TGF-α was scored by three observers (C. J. P., A. R. F., U. M.). Because the intensity of expression of EGF-r and erbB-2 was often different in basal and luminal cells, a separate score was given for basal and luminal cells in the normal, uninvolved, and hyperplastic bronchial epithelium. Also, EGF-r and erbB-2 exhibited both cytoplasmic and membrane staining in both basal and luminal cells. However, it was difficult to separate membrane staining from cytoplasmic staining in the basal layer of normal, uninvolved, and hyperplastic epithelium, and thus the basal layer was assigned a single score that combined the membrane and cytoplasmic immunostaining. In the luminal cells, we were able to separate membrane staining from cytoplasmic staining, and thus luminal cells were given separate scores for membrane and cytoplasmic staining of EGF-r and erbB-2. TGF-α expression was primarily cytoplasmic, and only cytoplasmic staining was scored. Because the intensity of expression of TGF-α was often different in basal and luminal cells, a separate score was given for basal and luminal cells in the normal, uninvolved bronchial epithelium and epithelial hyperplasia. The basal and luminal scores were averaged in specific analyses. Membrane and cytoplasmic staining for EGF-r and erbB-2 was scored separately in metaplasia, dysplasia, and SCC, and these scores were averaged in specific analyses. Each observer graded the intensity of immunostaining based on a visual assessment of the intensity of brown reaction product within the cell cytoplasm or on the cell membrane on a scale of 0 (no staining) to + (intense staining) in each of the epithelial components. In addition, the percentage of cells at each intensity was estimated (by microscopic examination of the entire tissue section) and multiplied by the appropriate intensity score to obtain a weighted average of the inten-
Cell Culture Experiment. The NCI-H226 cell line (derived from SCC of the lung) were grown on glass coverslips in medium with 10% calf serum (13). One day before testing, this medium was replaced with either medium plus 10% charcoal stripped calf serum (CSCS), or 10% calf serum. Similarly, an immortalized human bronchial epithelial cell line from American Type Culture Collection (BEAS-2B) was grown in specified medium plus 10% calf serum, and 1 day before testing, this was replaced by medium plus either 10% CSCS or 10% CS. On the test day, either vehicle, EGF at 10 ng/ml, or TGF-α at 10 ng/ml was added to the medium, and the effects of the addition of the ligands to EGF-α on the pattern of EGF-α expression in cells was monitored at 0, 0.5, 1, and 24 h. Specifically, after exposure to the ligands for the specified times, the cells were fixed in neutral buffered 10% formalin and stained for EGF-α by immunohistochemistry using the EGF-α antibody at 3 μg/ml final dilution.

Statistical Analysis. Descriptive statistics such as mean, median, and SE were calculated for the expression of EGF-α, erbB-2, and TGF-α for each type of tissue. The difference in expression was calculated between types of epithelial tissues and either the Wilcoxon rank sum or Wilcoxon sign rank test was used to determine whether there is differential expression between different types of tissues. The expression of EGF-α, erbB-2, and TGF-α in SCC was compared according to different histopathological characteristics (nodal status, tumor size, stage, and grade of differentiation). The Wilcoxon rank sum was used to evaluate significant differences in expression of EGF-α, erbB-2, and TGF-α for each histopathological parameter. Finally, the relationship between patient survival and the expression of EGF-α, erbB-2, and TGF-α in the SCCs, either separately or in combinations, was evaluated using the log-rank test.

RESULTS

Sixty noncancer subjects included in the study were operated upon secondary to the diagnosis of emphysema and/or fibrosis of the lung. All specimens from these subjects contained adequate normal bronchial epithelium for the evaluation of expression of EGF-α, erbB-2, and TGF-α. Fifty-eight of the 60 specimens examined contained SCC. The majority of subjects had early stage SCCs (79%, stages 1 and 2; 21%, stages 3 and 4), smaller tumors (81%, T₁ and T₂; 19%, T₃ and T₄); 69% of subjects had no nodal involvement (N₀), and 31% of subjects were N₁ and N₂.

Fifty-eight specimens contained adequate uninvolved bronchial epithelium, and 46 specimens also contained adequate epithelial hyperplasia for evaluation. We identified eight dysplasias and four metaplasias in 60 cancer specimens. Because of the small number of dysplastic and metaplastic tissues identified, the immunohistochemical staining scores of EGF-α, erbB-2, and TGF-α in these lesions were not statistically significant compared with EGF-α, erbB-2, and TGF-α scores in the other types of tissues. Because the average expression of EGF-α, erbB-2, and TGF-α in normal bronchial epithelium from specimens with diagnoses of emphysema or fibrosis was not significantly different, the two groups were combined.

The expression of EGF-α, erbB-2 (a combination of cytoplasmic and membrane expression in basal cells and cytoplasmic and membrane expression separately in luminal cells), and TGF-α (cytoplasmic expression in basal and luminal cells) in histologically normal bronchial epithelial cells and in epithelial hyperplasia associated with SCC was significantly higher compared with similar lesions in noncancer specimens (P < 0.05 for all comparisons). In histologically normal bronchial epithelium and epithelial hyperplasia of noncancer and cancer-associated lesions, higher expression of EGF-α in the basal cells than in luminal cells was a consistent observation. Similarly, lower membrane expression of EGF-α in the luminal cells of these lesions was a consistent finding. However, we noted that the difference between cytoplasmic and luminal expression is larger in normal and hyperplastic lesions of noncancer specimens compared with similar lesions associated with cancer. Metaplastic lesions displayed a similar pattern of membrane:cytoplasmic staining ratio with that of hyperplastic lesions associated with invasive cancer. Interestingly, in dysplastic lesions and in invasive cancer, expression of EGF-α in the membrane was higher than its expression in the cytoplasm, suggesting that this switch from greater cytoplasmic to greater membrane expression may occur at the stage of dysplasia. The expression of erbB-2 was consistently lower in the basal cells compared with cytoplasmic expression of erbB-2 in the luminal cells but was higher compared with its membrane expression in the luminal cells of normal and hyperplasia of noncancer and cancer specimens. The cytoplasmic expression of erbB-2 was higher than its membrane expression in the luminal cells of normal and hyperplastic epithelium, similar to EGF-α. In contrast to EGF-α, this pattern did not change in dysplastic lesions or invasive cancer. The expression of TGF-α was mainly cytoplasmic, and the degree of staining was higher in the luminal cells compared with basal cells in histologically normal bronchial epithelium and epithelial hyperplasia of noncancer and cancer.

As shown in Fig. 1, when the expression of EGF-α (Fig. 1A) and TGF-α (Fig. 1B) in the cytoplasm and membrane of basal and luminal cells was averaged, there was a statistically significant stepwise increase in their expression from uninvolved bronchial mucosa to epithelial hyperplasia to cancer, suggesting their involvement in lung carcinogenesis. In contrast, a statistically significant stepwise increase was not observed with erbB-2, suggesting that this growth factor may not be involved in lung carcinogenesis (Fig. 1C). Because of the small number of samples, metaplastic and dysplastic lesions were not included in this analysis. The average expression of EGF-α, TGF-α, and also erbB-2, however, was significantly higher in uninvolved bronchial mucosa and epithelial hyperplasia associated with SCC compared with their expression in the normal bronchial epithelium and hyperplastic lesions of noncancer specimens, suggesting their early expression in lung carcinogenesis (Fig. 2, A–C). Figs. 2–4 shows the immunohistochemical staining for EGF-α (Fig. 2, A–D), erbB-2 (Fig. 3, A–D), and TGF-α (Fig. 4, A–D) in histologically normal bronchial epithelium in noncancer specimens (A) uninvolved bronchial epithelium associated with SCC (B), epithelial hyperplasia associated with SCC (C) and SCC (D) of the lung, respectively.

We observed a statistically significant positive Spearman correlation coefficient between cytoplasmic expression of EGF-α and TGF-α (r = 0.27, P = 0.04) and a nonsignificant
positive correlation between membrane expression of EGF-r and cytoplasmic expression of TGF-α ($r = 0.17, P = 0.22$) in SCCs. erbB-2 expression, either cytoplasmic ($r = 0.22, P = 0.09$) or membrane ($r = 0.09, P = 0.47$), was not significantly associated with expression of TGF-α. There was also no statistically significant association between expression of EGF-r and erbB-2, either cytoplasmic or membrane ($r = 0.26, P = 0.05; r = 0.22, P = 0.10$, respectively). The correlation between the degree of cell proliferation as measured by immunohistochemical assessment of PCNA, and expression of EGF-r in the cytoplasm and membrane was positive and statistically significant ($r = 0.40, P = 0.002$ for both comparisons). The correlation between PCNA and TGF-α was also positive and significant ($r = 0.41, P = 0.002$). In contrast, there was no statistically significant correlation between PCNA and expression of erbB-2, cytoplasmic or membrane ($r = 0.02, P = 0.90; r = 0.12, P = 0.12$, respectively).

The relationship between expression of EGF-r, erbB-2, and TGF-α and the pathological features of SCCs is shown in Table 1. Although the overall results were suggestive of higher expression of EGF-r and TGF-α with advancing disease, as indicated by nodal involvement, larger tumors, and higher stage,
these differences were statistically nonsignificant. A higher expression of both cytoplasmic and membrane EGF-r in tumors with nodal involvement compared with tumors with an absence of nodal involvement reached statistical significance. When cytoplasmic and membrane expression of EGF-r was averaged, the results remained the same (data not shown). The degree of expression of erbB-2, cytoplasmic or membrane, did not seem to be associated with pathological features of the tumors. Grade of differentiation of the tumors (well, moderate, and poor) was not associated with the degree of expression of EGF-r, erbB-2, or TGF-α (data not shown).

A higher level (>median) of cytoplasmic expression of EGF-r in the cancer was associated with a statistically significant poor survival of subjects (P = 0.02; Fig. 5A), whereas its membrane expression was not (P = 0.20; Fig. 5B). When the expression of EGF-r in the cytoplasm and membrane was averaged, it was not significantly associated with survival (P = 0.26). The association between higher expression of TGF-α and poor patient survival approached statistical significance (P = 0.08; Fig. 5C). The expression of erbB-2, cytoplasmic (Fig. 5D), membrane, or an average of the two, was not significantly associated with survival of subjects.

Cancers expressing higher levels of TGF-α and EGF-r, either cytoplasmic or membrane, demonstrated statistically significant poor survival (P = 0.03, Fig. 6A; P = 0.04, Fig. 6B, respectively). A higher expression of TGF-α along with a higher expression of an average of cytoplasmic and membrane expression of EGF-r also was associated with a significantly poor survival of subjects (P = 0.04). The expression of erbB-2 in combination with EGF-r or TGF-α was not associated with survival of patients (P > 0.05 for all comparisons). The analysis of survival data using receiver operating characteristic curves (14) to define cutoff levels provided very similar results as when the median values were used in the analysis.

The pattern of EGF-r expression when NCI-H226 and BEAS-2B cells were grown in medium with CSCS versus intact calf serum is similar; in both cases, EGF-r is expressed strongly on cell membranes (Figs. 7 and 8, A, solid, notched arrows) and to a lesser extent in the cytoplasm. After addition of 10 ng/ml of either EGF or TGF-α to the medium, there is a prompt reduction in the membrane expression of EGF-r within 30 min (Figs. 7 and 8, B, solid, notched arrows) and a marked accentuation of perinuclear expression of EGF-r (open arrows). The loss of EGF-r expression on cellular membranes is almost complete after 1 h of exposure to EGF; however, the loss of EGF-r expression on cellular membranes required 4 h of exposure to TGF-α in the NCI H226 cells. In contrast to our observations in DU145 cells (prostate cancer; Ref. 15), EGF-r expression was not down-regulated at 24 h, where in both cell lines there was the return of some membrane staining as well as the presence of...
perinuclear staining (Figs. 7 and 8, D). When EGF or TGF-α was withdrawn after 4 h and replaced with CSCS, the pattern of EGF-r expression returned to the original pattern of expression after 20 h (data not shown).

**DISCUSSION**

Understanding the role of growth factor biochemical signaling networks in cancer is pivotal to improvements not only in diagnosis and prognosis of this disease but also in the development of targeted therapies. It is likely that knowledge of the relationship between differential expression and cellular localization of growth factor receptors and their ligands in preneoplastic and neoplastic lesions and patient survival will prove to be beneficial to the development of novel pharmacological approaches to therapy. However, studies that address these issues are scarce. We have examined the localization of immunostaining scores of EGF-r, erbB-2, and TGF-α in SCCs by pathological diagnosis: N₀ (absence of nodal involvement), N₁ + N₂ (presence of nodal involvement), tumor size (T₁ + T₂ and T₃ + T₄), and stage (stages 1 + 2 and stages 3 + 4).

**Table 1** Mean ± SE and median immunostaining scores of EGF-r, erbB-2, and TGF-α in SCCs by pathological diagnosis: N₀ (absence of nodal involvement), N₁ + N₂ (presence of nodal involvement), tumor size (T₁ + T₂ and T₃ + T₄), and stage (stages 1 + 2 and stages 3 + 4).

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<th>Growth factor</th>
<th>N₀</th>
<th>N₁ + N₂</th>
<th>P</th>
<th>T₁ + T₂</th>
<th>T₃ + T₄</th>
<th>Stages 1 + 2</th>
<th>Stages 3 + 4</th>
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<tr>
<td>EGF-r cytoplasmic</td>
<td>1.3 ± 0.1 1.5 ± 0.1 0.09</td>
<td>1.3 ± 0.1 1.5 ± 0.1 0.50</td>
<td>1.3 ± 0.1 1.5 ± 0.1 0.30</td>
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<tr>
<td>EGF-r membrane</td>
<td>1.6 ± 0.1 1.9 ± 0.1 0.07</td>
<td>1.6 ± 0.1 2.0 ± 0.1 0.30</td>
<td>1.6 ± 0.1 2.0 ± 0.1 0.10</td>
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<tr>
<td>erbB-2 cytoplasmic</td>
<td>1.3 ± 0.1 1.3 ± 0.1 0.70</td>
<td>1.3 ± 0.1 1.3 ± 0.1 0.70</td>
<td>1.3 ± 0.1 1.3 ± 0.1 0.90</td>
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<tr>
<td>erbB-2 membrane</td>
<td>1.3 ± 0.1 1.2 ± 0.1 0.40</td>
<td>1.0 ± 0.1 1.0 ± 0.2 0.80</td>
<td>1.0 ± 0.1 1.0 ± 0.2 0.80</td>
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<tr>
<td>TGF-α cytoplasmic</td>
<td>1.2 ± 0.1 1.3 ± 0.1 0.60</td>
<td>1.2 ± 0.1 1.4 ± 0.1 0.20</td>
<td>1.2 ± 0.1 1.4 ± 0.1 0.10</td>
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**Fig. 5** Overall survival after resection of SCCs in two groups defined by expression of EGF-r, TGF-α, and erbB-2, ≤median and >median of immunostaining score.
nohistochemically demonstrable EGR-r, erbB-2, and TGF-α expression in histologically normal bronchial epithelial cells and hyperplastic epithelial lesions of smokers who have not developed lung cancer and in SCCs and in histologically normal bronchial epithelium, epithelial hyperplasia, metaplasia, and dysplasia associated with SCC.

The noncancer subjects identified in our study are likely to have a higher risk of developing smoking-related lung cancer because of their exposures to carcinogens from cigarette smoke similar to SCC subjects. This is supported by their reported long-term smoking habits and by the presence of emphysematous changes of the lung, which are associated with cigarette smoking through the activation of elastase leading to elastic tissue damage (16). The differential localization of all three markers was significantly higher in histologically normal bronchial mucosa and epithelial hyperplasia of subjects who had developed lung cancer compared with those who had not, identifying these as susceptibility markers for the development of SCCs. To our knowledge, this is the first report to include a subset of smokers who have been resistant to the development of lung cancer enabling us to document such an observation.

The expression of EGF-r was higher in basal cells compared with luminal cells of normal and hyperplastic lesions, suggesting its association with cell proliferation. In contrast, the expression of erbB-2 and TGF-α was lower in basal cells compared with luminal cells in the same lesions, suggesting a lack of association with cell proliferation. To our knowledge, the clinical significance of cellular localization (membrane versus cytoplasmic) of growth factor receptors in the lung has not been addressed previously. Receptor localization may signify adaptations that permit growth factor responsiveness in a milieu of available ligand. The down-regulation of growth factor receptors in the cytoplasm may reflect receptor-ligand internalization, a rapid process that occurs after ligand binding (17). Although internalized receptors in most cell culture systems have been shown to be degraded rapidly (17), a proportion of internalized receptor in EGF-treated hepatocytes was thought to recycle to the cell surface (18). Intracellular localization of receptor also may represent newly synthesized molecules within the Golgi that have yet to be processed and inserted into the membranes. Lack of detection of the receptor on the membrane by immunohistochemical means, however, cannot be interpreted as its total absence but may reflect a limited number of receptors on the surface.

We observed that the expression of EGF-r in the cytoplasm is higher than its membrane expression in early lesions of lung cancer, including metaplastic lesions. In dysplastic lesions and SCCs, however, the membrane expression is higher compared with cytoplasmic expression. Such a pattern was not observed with erbB-2. Because it is thought that for the EGF-r to bind ligand it must be expressed at the cell surface, the observed results may suggest an important role for EGF-r in lung carcinogenesis. We also observed a stepwise increase in average EGF-r and TGF-α expression from SCC-associated uninvolved

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**Fig. 6** Overall survival after resection of SCCs in two groups defined by expression of > and ≤ median immunostaining scores of both EGF-r cytoplasmic and TGF-α (A) and EGF-r membrane and TGF-α (B).
Fig. 7  A (×600) demonstrates that cancer cells grown on coverslips and exposed for 24 h to medium with CSCS have a strong expression of EGF-\( \alpha \) on cellular membranes (solid notched arrow); however, there is some diffuse cytoplasmic expression as well as perinuclear staining (open arrow). After EGF (10 ng/ml) was added to the medium, the pattern changed within 30 min (B, ×600), but the expression of EGF-\( \alpha \) on membranes was still present (solid notched arrows), although there is marked accentuation of perinuclear staining (open arrows). The loss of expression of EGF-\( \alpha \) on membranes with a shift to perinuclear accumulation of EGF-\( \alpha \) is almost complete at 1 h of exposure (C, ×600). The staining remains unchanged even after 24 h of exposure (D, ×600), except for some slight membrane staining (solid notched arrow) as well as some membrane staining in small circular areas of cells (line arrows).

bronchial mucosa to epithelial hyperplasia to SCC, suggesting their involvement in lung carcinogenesis. The absence of such a stepwise increase with expression of erbB-2 may suggest its lack of importance in lung carcinogenesis. We emphasize that this stepwise alteration was present with an averaged expression, which combines marker expression in basal cells and membrane and cytoplasmic expression in luminal cells. The same pattern is not observed with some markers if the results are summarized differently, e.g., the degree of expression of TGF-\( \alpha \) in the luminal cells of epithelial hyperplasia is similar to that of invasive cancer. These observations indicate the importance of stating precisely the manner in which the evaluation of markers was performed so that results can be compared among studies.

Although the overexpression of EGF-\( \alpha \) and its ligand, TGF-\( \alpha \), is shown to be frequent in resectable non-small cell lung cancer (19), its relation to tumor progression is inconsistent; some studies report no relationship to tumor progression (19), whereas others report a relationship between the expression of EGF-\( \alpha \) and TGF-\( \alpha \) and aggressiveness of SCCs of the lung (20). The study of Pavelic et al. (20) is also suggestive of an association between expression of EGF-\( \alpha \) and progression of adenocarcinomas of the lung. The expression of TGF-\( \alpha \) was not identified as an independent tumor maker in several studies of non-small cell lung cancer, which includes both squamous and adenocarcinomas (21). Our results are suggestive of an association between higher expression of EGF-\( \alpha \), both cytoplasmic and membrane, and nodal involvement, but not the other features of tumor aggressiveness. Similar associations have been reported previously by Gorgoulis et al. (9). The overexpression of EGF-\( \alpha \) in breast cancer has been correlated with both a poor prognosis (22), a lack of response to endocrine therapy (23), and increased metastatic potential (24). An increased expression of EGF-\( \alpha \) has also been associated with adverse outcome of patients with cancers of the cervix (25), ovary (26), and endometrium (27). Also, EGF-\( \alpha \) overexpression in primary laryngeal cancer was shown to be an independent prognostic factor of neck node relapse (28). Increased EGF-\( \alpha \) expression was shown to be a biomarker of bronchial metaplasia of smokers but not a biomarker of retinoid response in lung cancer chemoprevention trials (29). These studies, however, have not distinguished cytoplasmic expression from membrane expression.

We report that cytoplasmic expression of EGF-\( \alpha \), but not its membrane expression, is significantly associated with poor patient survival. Higher expression of EGF-\( \alpha \), either cytoplasmic or membrane, together with higher expression of TGF-\( \alpha \), however, was significantly associated with poor survival of patients, suggesting the significance of coexpression of these markers. Because most previous reports have failed to indicate the cellular localization of EGF-\( \alpha \), it is not possible to compare our results with others. Higher expression of EGF-\( \alpha \) and TGF-\( \alpha \) together, however, was shown to predict poor survival rate of subjects diagnosed with primary adenocarcinomas of the lung. EGF-\( \alpha \) and TGF-\( \alpha \) have been shown to be up-regulated in a variety of tumors other than lung, including esophagus (30) and head and neck (31, 32). Grandis et al. (33), Ozanne et al. (34), and Cerny et al. (35) reported that the elevated expression of both EGF-\( \alpha \) and TGF-\( \alpha \) in primary tumors was the most important prognostic factor identified in cancers of the head and neck.

erbB-2 expression in lung adenocarcinomas has been shown to predict shortened survival in some studies (36, 37),
whereas others have reported no significant association with survival (6). Our results clearly indicated that erbB-2 expression is very unlikely to be associated with progression of SCCs or in the modification of patient survival. Overexpression of c-erbB-2, however, has been shown to be a frequent event in oral SCC and was also correlated with poor survival (38). In contrast to our results of SCC of the lung, erbB-2 expression in oral SCCs has also been shown to correlate significantly with expression of EGF-r, and coexpression of erbB-2 and EGF-r was shown to predict a significantly poor survival of these patients (39). These observations suggest that the relationship between clinical outcome and expression of erbB-2 in SCCs may vary with the site of origin of the SCCs. We also noted that the expression of EGR-r and TGF-α, but not erbB-2, was positively associated with PCNA (data not shown), suggesting that in SCCs of the lung, EGF-r and TGF-α may form an autocrine stimulatory loop and hence be involved in the control of cell proliferation leading to poor outcome of patients.

The development of novel therapies for neoplastic processes including immunotherapy, ligand receptor therapy, and gene therapy has been a high priority goal in the 1990s (40). The new targets for these therapies include oncogenes, growth factors and their receptors, signal transduction pathways, and cell differentiation signals. For each of these therapies, knowledge of the expression of specific biomarkers in preneoplastic lesions and neoplastic lesions and their localization is important. Although the documentation of biomarker expression in cancers has advanced over the past decade, a large majority of studies have been conducted with a heavy focus on biomarker expression in invasive cancers rather than in corresponding preneoplastic lesions. The knowledge of the relative expression of these markers in adjacent normal and preneoplastic lesions is of great value, especially when toxins are delivered to cells expressing some of these markers (for example, EGF-diphtheria toxin (15)). We observed that the relative distribution of growth factors and their ligands in preneoplastic tissues may vary with the specific cellular localization and protocols used to document these patterns. Although the effectiveness of targeted therapy may vary with specific cellular localization (membrane versus cytoplasmic) of growth factors and receptors in the tumor, there are no reports addressing this issue. We documented that the association between expression of growth factor receptors and survival is dependent on the specific cellular localization of the growth factor receptors, suggesting the importance of detailed evaluation of their expression.

The pattern of phenotypic expression of EGF-r varies in all tissues with the strongest membrane and cytoplasmic expression in squamous epithelia and in tumors and in cell lines derived from squamous epithelia. Because EGF-r is known to be internalized after interaction with its ligands including EGF and TGF-α and to traffic from the Golgi apparatus to cell membranes, the localization of EGF-r within cells varies based on the extent of interaction of its ligands with EGF-r and with the synthesis of EGF-r. The changes in the phenotypic pattern of EGF-r expression are demonstrated in both cell lines from the lung (cancer and normal). Thus, our finding of the prognostic importance of EGF-r expression in the cytoplasm of lung cancer probably is an indication of the prognostic importance of trafficking of the EGF-r receptor between the Golgi apparatus and the plasma membrane.
cell membranes and of internalization of EGF-r after an interaction with one of the EGF-r ligands.

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