Increased Epidermal Growth Factor Receptor Expression in Metaplastic Bronchial Epithelium


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ABSTRACT

Epidermal growth factor receptor (EGFr) is expressed in human bronchial epithelial cells, and non-small cell lung cancers express increased EGFr. Squamous metaplasia of the bronchial epithelium occurs in chronic smokers and is considered an early premalignant change. In this study, EGFr expression was examined in biopsies of histologically normal and metaplastic bronchial tissues obtained from 69 smokers who were enrolled in a randomized placebo-controlled chemoprevention trial. This trial tested the effects of 6 months of treatment with 13-cis retinoic acid (13cRA) on bronchial metaplasia. EGFr expression was examined as a marker of bronchial metaplasia and response to 13cRA treatment. In bronchial biopsies obtained from patients in this study, EGFr expression was higher in metaplastic biopsies than in normal biopsies (P = 0.02). Smoking cessation during treatment correlated with reduced metaplasia (P < 0.001) and EGFr expression (P = 0.02), but multivariate analysis suggested that this effect of smoking cessation on EGFr expression was dependent upon reversal of bronchial metaplasia. 13cRA treatment did not alter EGFr expression (P = 0.23). Baseline EGFr expression levels in metaplastic biopsies did not predict metaplasia reversal. This study demonstrated that increased EGFr expression is a biomarker of bronchial metaplasia, but it did not support the hypothesis that EGFr is a biomarker of retinoid response in lung cancer chemoprevention trials.

INTRODUCTION

Despite three decades of advancements in cancer treatment modalities, the rate of mortality from NSCLC has not decreased. Surgical resection remains the only means of cure, but the long-term survival rate of patients who have undergone surgical resection is only 50% (1). A major cause of death in resected NSCLC patients is second primary tumors, which occur at the rate of 2–3% per year (2). Retinoids have demonstrated activity in the prevention of second primary tumors and thus have the potential to reduce the mortality from NSCLC. For example, after resection of early stage head and neck cancer, 13cRA treatment reduces the incidence of second primary tumors (3). Similarly, retinyl palmitate reduces the incidence of second primary tumors in resected NSCLC patients (2). To improve these results, we must better understand lung carcinogenesis and mechanisms by which retinoids inhibit the carcinogenic process.

Epithelial carcinogenesis is a multistep process. Metaplastic and dysplastic epithelial changes have been frequently observed in contiguity with epithelial cancers and are considered premalignant (4). Auerbach et al. demonstrated that, in pathological examination of autopsy specimens, the extent of metaplastic bronchial changes correlated with tobacco exposure (4, 5). Furthermore, supporting the hypothesis that bronchial metaplasia is premalignant, analysis of microdissected regions of metaplastic bronchial epithelia has revealed clonal genetic alterations (6–8). Based on the premise that bronchial metaplasia is an early change leading to invasive lung cancer, bronchial metaplasia has been used as a marker of therapeutic response in lung cancer chemoprevention trials (9–11). However, randomized placebo-controlled trials have shown chemopreventive treatment to have minimal effects on the reversal of bronchial metaplasia. The strongest predictor of metaplasia reversal was smoking cessation (10). Obviously, there is a growing need to develop objective biological markers to complement histological assessment of these putative premalignant lesions. Useful biomarkers would predict individual risk of lung carcinogenesis and likelihood of response to chemopreventive intervention.

Among the multiple biomarkers that have been implicated in multistep carcinogenesis (12), we were particularly interested in EGFr expression as a potential intermediate biomarker. EGFr expression is increased in many lung carcinomas, including squamous cell as well as other NSCLCs, suggesting that the EGFr signaling pathway may be involved in lung carcinogenesis (13–19). Increased EGFr expression has also been found in metaplastic lung tissue adjacent to malignant tumors and in apparently normal bronchial epithelium of patients with lung cancer (14, 17, 18). Furthermore, activation of EGFr expression has been observed during experimental carcinogenesis in the hamster cheek pouch model, and retinoids retarded the development of squamous cell carcinoma in this model (20, 21), raising the possibility that chemoprotective effects of retinoids may be due in part to suppression of EGFr gene expression. In addition, we observed an approximately 8-fold reduction in EGFr mRNA levels after treatment of retinoid-sensitive 1483 squamous carcinoma cells with t-RA (22). There was a substantial decrease in EGFr kinase activity in this cell line after.

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2 To whom requests for reprints should be addressed, at M. D. Anderson Cancer Center, Box 80, 1515 Holcombe Boulevard, Houston, TX, 77030. Phone: (713) 792-6363; Fax: (713) 796-8655.
3 The abbreviations used are: NSCLC, non-small cell lung cancer; 13cRA, 13-cis retinoic acid; EGFr, epidermal growth factor receptor; HBE, human bronchial epithelial; t-RA, all-trans retinoic acid.

Clinical Cancer Research 1787
retinoid treatment. Based on these findings, we postulated that EGFr levels are modulated in vivo by 13cRA treatment.

In this study, we examined EGFr expression in bronchial biopsies obtained from participants enrolled in a randomized placebo-controlled trial testing the chemopreventive effects of 13cRA on bronchial metaplasia (10). We found that EGFr expression was increased in metaplastic biopsies compared to its expression in histologically normal biopsies. However, 13cRA treatment did not alter EGFr expression.

PATIENTS AND METHODS

Selection of Tissue Samples. A total of 152 patients (87 men and 65 women) with a smoking history of at least 15 pack-years were registered in the study and underwent initial bronchoscopy with biopsies. Biopsies were obtained from six predetermined sites, including the main carina, right upper lobe, right middle lobe, right lower lobe, left upper lobe, and left lower lobe. H&E-stained histological slides were reviewed, and the diagnoses were confirmed by a pathologist. Metaplastic lesions were identified according to criteria described previously (10). Individuals who were found to have dysplasia or a metaplasia index ≥15% were randomized to receive treatment. Of these 152 subjects, 86 were randomized to receive treatment (1 mg/kg/day 13cRA or placebo). Of the 86 randomized participants, 69 had repeat bronchoscopy, and the biopsies obtained from these 69 subjects are the subject of this analysis.

Immunohistochemical Analysis. Four-μm sections were mounted on aminoalkylsilane-coated slides (Histology Control System, Glen Head, NY). Anti-EGFr monoclonal antibody (clone E30) was obtained from BioGenex, Inc. (San Ramon, CA). Immunohistochemical analysis used a modification of the avidin-biotin immunoperoxidase method described previously (23). Briefly, after deparaffinization by xylene and rehydration with graded alcohols, endogenous peroxidase activity was blocked by incubating the slides in 3% H2O2 with methanol for 10 min. After washing in PBS, the slides were incubated with nonimmune horse serum to decrease the background signal, rinsed in PBS, and incubated with a 1:1 dilution of prediluted anti-EGFr monoclonal antibody for 2 h at 37°C according to the manufacturer’s recommendation. The slides were subsequently washed with PBS, incubated with biotinylated secondary antibody for 45 min at room temperature, and then incubated with avidin-biotin peroxidase conjugate (ABC kit; Vector Laboratories, Burlingame, CA) at a dilution of 1:500 for 30 min at room temperature. After washing in PBS, the EGFr antigen was visualized with a 0.1% 3,3’-diaminobenzidine solution (Sigma Chemical Co., St. Louis, MO) in 1 × solution of PBS and hydrogen peroxide (0.01%). The slides were then counterstained in Mayer’s hematoxylin solution. Under light microscopy, EGFr expression in the bronchial biopsies was graded by visual comparison of staining intensity with photographs of stained bronchial epithelium that represented grades 0–4. If EGFr staining was not homogeneous, the grading was based on the area of greatest staining intensity. The investigators were blinded to the treatment received at the time of scoring.

Statistical Analysis. Because both the site-specific EGFr expression and metaplasia status were measured, the biopsy site was used as the analysis unit. The Pearson χ2 test was applied to reveal the association between EGFr expression and biopsy site. The χ2 test was used for contingency table analyses. For the analysis of changes in EGFr expression over time, both repeated-measures ANOVA and paired t test were used. To compare changes in mean EGFr expression over time between groups, the two-sample t test was applied. Logistic regression analysis was used to incorporate covariates into modeling the change in EGFr staining intensity (24).

RESULTS

In this study, 728 bronchial biopsies from 69 subjects were evaluated for EGFr expression before and after treatment with 13cRA or placebo. Of these 728 biopsies, 359 were available from baseline examinations and 369 were posttreatment biopsies. Paired pre- and posttreatment biopsies were available for 324 biopsy sites (648 total biopsies). The characteristics of the 359 baseline biopsies are described in Table 1. On the basis of standard pathological criteria, 44% of the evaluable baseline biopsy sites exhibited metaplasia and 56% were without metaplasia. EGFr expression was detected in all of the biopsies. EGFr staining intensity in the biopsies was considered grade 1 in 6%, grade 2 in 20%, grade 3 in 35%, and grade 4 in 39%.

Comparison of EGFr Expression among Six Biopsy Sites. We examined whether EGFr expression was dependent upon biopsy site within the bronchial tree by analysis of the six predetermined biopsy sites (the main carina, right upper lobe, right lower lobe, right middle lobe, left upper lobe, or left lower lobe). Among the 359 baseline biopsies, χ2 testing revealed no correlation between biopsy site and EGFr staining intensity (P = 0.10), demonstrating that there were no statistically significant differences in EGFr intensity among the six biopsy sites. In addition, the variance component analysis indicated that the variability in EGFr intensity between the sites was much larger than between the participants in this trial. Therefore, the effects of treatment, smoking cessation, and change in metaplasia status on EGFr expression were analyzed according to biopsy site.

Distribution of EGFr Expression in Bronchial Epithelium. Examples of EGFr expression in normal and metaplastic bronchial epithelium are illustrated in Fig. 1. Positive immunohistochemical staining was located in the basal layer of normal bronchial epithelium. In contrast, EGFr expression was distributed throughout the full thickness of metaplastic bronchial epithelium. Distribution of immunoreactive cells was either diffuse or patchy in pattern. The diffuse pattern demonstrated positivity throughout the epithelium (Fig. 1), either normal or
metaplastic, with similar intensity of staining and without skipped areas. This pattern was found in over 95% of the biopsies. The patchy pattern had areas of immunoreactive cells interspersed with focal areas that did not stain or stained less intensely (Fig. 2). Of 18 biopsy sites demonstrating a patchy pattern, 11 were baseline samples and 7 were posttreatment samples. The patchy pattern occurred predominately in histologically normal epithelium. Of five biopsies that contained both metaplastic and nonmetaplastic areas, three demonstrated the patchy distribution of EGFr staining, and patchiness was confined to the histologically normal areas. In many of the biopsies with patchy staining, basal cells appeared to be missing from the areas that lacked staining. The significance of the patchy pattern of EGFr staining is not known.

EGFr Expression in Bronchial Metaplasia. Low intensity EGFr staining (grade 1 or 2) was observed more frequently in normal biopsies (33% of normal and 19% of metaplastic biopsies), and high intensity staining (grade 3 or 4) was observed more frequently in metaplastic biopsies (67% of normal and 81% of metaplastic biopsies; Table 1). This demonstrated that EGFr expression was higher in metaplastic biopsies than in normal bronchial epithelium (P = 0.02).

To further examine the effect of changes in metaplasia on EGFr expression in bronchial epithelium, EGFr expression and metaplasia status were examined before and after treatment in 324 paired biopsies. Biopsy sites were grouped into one of four categories, including sites that reverted from metaplastic to normal (M–N), began and remained normal (N–N), began and remained metaplastic (M–M), or began normal and became metaplastic (N–M; Table 2). EGFr expression decreased in 50% of the biopsy sites that underwent a reversal of metaplasia (M–N). The decrease in EGFr expression in sites with no metaplasia reversal (M–M) was significantly less (P = 0.004). The proportion of sites with increased EGFr expression was greatest in biopsy sites that developed metaplasia during treatment (N–M). These data demonstrate that EGFr expression was increased in metaplastic bronchial epithelium, and reversal of bronchial metaplasia was associated with decreased EGFr expression.

Effects of Smoking Cessation on Metaplasia. Metaplasia was observed in 11% and 41% of the posttreatment biopsies from quitters and nonquitters, respectively (Table 3). Metaplastic biopsies at baseline reverted to normal in 82% (27 of 33) and 43% (49 of 113) of the biopsies from quitters and nonquitters, respectively, supporting the previous observation (10) that smoking cessation was associated with metaplasia reversal (P < 0.001).

Effects of 13cRA Treatment on EGFr Expression. Correlations between treatment and EGFr expression were examined within each histological category to control for changes...
EGF\(\text{r}\) Expression in Bronchial Epithelium

Table 2  Percentage of bronchial biopsies with EGF\(\text{r}\) staining intensity that decreased, increased, or did not change during treatment, stratified by histologic change

<table>
<thead>
<tr>
<th>EGF(\text{r}) change</th>
<th>M–N (%)</th>
<th>N–N (%)</th>
<th>M–M (%)</th>
<th>N–M (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease</td>
<td>38 (30%)</td>
<td>49 (36%)</td>
<td>20 (29%)</td>
<td>8 (20%)</td>
<td>115 (35%)</td>
</tr>
<tr>
<td>No change</td>
<td>22 (29%)</td>
<td>48 (35%)</td>
<td>29 (41%)</td>
<td>18 (44%)</td>
<td>117 (36%)</td>
</tr>
<tr>
<td>Increase</td>
<td>16 (21%)</td>
<td>40 (29%)</td>
<td>21 (30%)</td>
<td>15 (37%)</td>
<td>92 (29%)</td>
</tr>
<tr>
<td>Total</td>
<td>76 (100%)</td>
<td>137 (100%)</td>
<td>70 (100%)</td>
<td>41 (100%)</td>
<td>324 (100%)</td>
</tr>
</tbody>
</table>

* M, metaplastic; N, normal.

Table 3  Percentage of bronchial biopsies in each histologic change category in quitters and nonquitters

<table>
<thead>
<tr>
<th>Histologic change</th>
<th>M–N (%)</th>
<th>N–N (%)</th>
<th>M–M (%)</th>
<th>N–M (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M–N</td>
<td>27 (37%)</td>
<td>49 (20%)</td>
<td>76 (23%)</td>
<td>38 (11%)</td>
<td>157 (24%)</td>
</tr>
<tr>
<td>N–N</td>
<td>38 (52%)</td>
<td>99 (39%)</td>
<td>137 (42%)</td>
<td>8 (2%)</td>
<td>264 (40%)</td>
</tr>
<tr>
<td>M–M</td>
<td>8 (8%)</td>
<td>64 (25%)</td>
<td>70 (22%)</td>
<td>2 (1%)</td>
<td>149 (23%)</td>
</tr>
<tr>
<td>N–M</td>
<td>2 (3%)</td>
<td>39 (16%)</td>
<td>41 (13%)</td>
<td>1 (1%)</td>
<td>83 (13%)</td>
</tr>
<tr>
<td>Total</td>
<td>73 (100%)</td>
<td>251 (100%)</td>
<td>324 (100%)</td>
<td>32 (10%)</td>
<td>615 (100%)</td>
</tr>
</tbody>
</table>

* M, metaplastic; N, normal.

in EGF\(\text{r}\) expression associated with metaplasia change (Table 4). Compared to biopsies from placebo-treated subjects, biopsies from subjects treated with 13cRA had greater reductions in EGF\(\text{r}\) expression, but this did not reach statistical significance (P = 0.23). To further investigate the effects of 13cRA treatment on EGF\(\text{r}\) expression without confounding effects of histological change, mean EGF\(\text{r}\) expression levels were determined in biopsies that underwent no histological change (N–N and M–M) during treatment (Fig. 3). Relative to changes in mean EGF\(\text{r}\) expression in biopsies from the placebo-treated group, changes in mean EGF\(\text{r}\) expression in biopsies from the 13cRA-treated group were not significantly different in either the N–N (P = 0.76) or the M–M (P = 0.64) category.

**Baseline EGF\(\text{r}\) Expression as a Predictor of Metaplasia Reversal.** A biomarker should identify individuals who are likely to benefit from chemopreventive intervention. After stratifying by treatment group, we examined whether baseline EGF\(\text{r}\) levels could predict metaplasia reversal after 13cRA treatment.
For each biopsy, EGFr expression was graded from 1–4 by comparison to an internal standard as described in "Patients and Methods." Mean EGFr expression (± SE) was determined for pretreatment and posttreatment biopsies in subjects treated with placebo or 13cRA. This figure includes biopsy sites that began and remained normal (N-N; A) or began and remained metaplastic (M-M; B).

**Table 5** Percentage of bronchial biopsies that underwent histologic reversal (M–N), stratified by baseline EGFr intensity and treatment group

<table>
<thead>
<tr>
<th>Baseline EGFr staining intensity</th>
<th>Treatment</th>
<th>Placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–2</td>
<td>8/12 (67%)</td>
<td>5/15 (33%)</td>
<td>0.18</td>
</tr>
<tr>
<td>3–4</td>
<td>29/50 (58%)</td>
<td>34/69 (49%)</td>
<td>0.45</td>
</tr>
</tbody>
</table>

**Table 6** Logistic regression model for EGFr reduction

<table>
<thead>
<tr>
<th>Model</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate model</td>
<td></td>
</tr>
<tr>
<td>Change of metaplasia status</td>
<td>0.004</td>
</tr>
<tr>
<td>Smoking cessation</td>
<td>0.01</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.22</td>
</tr>
<tr>
<td>Multivariate model</td>
<td></td>
</tr>
<tr>
<td>Change of metaplasia status</td>
<td>0.02</td>
</tr>
<tr>
<td>Smoking cessation</td>
<td>0.13</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.24</td>
</tr>
</tbody>
</table>

The percentage of metaplastic biopsies that reverted to normal was not significantly different in biopsies with low (1–2) or high (3–4) baseline EGFr expression levels, demonstrating that baseline EGFr expression levels did not predict metaplasia reversal in response to 13cRA treatment.

**Logistic Regression Analysis of EGFr Expression Change Adjusted by Covariates.** Logistic regression analysis was performed using reduction in EGFr expression over 6 months as an outcome variable (Table 6). The covariates measured during the 6-month period include smoking status (quitter or nonquitter), histological change (M–N, N–N, N–M, and M–M), and treatment (13cRA or placebo). In the univariate analysis, both smoking cessation and histological change were statistically significant predictors of reduced EGFr expression (P = 0.01 and 0.04, respectively). Treatment arm did not correlate with decreased EGFr expression (P = 0.22). In the multivariate analysis, smoking cessation did not correlate with reduced EGFr expression after adjusting for histological change (P = 0.13). Histological change (particularly metaplasia reversal) remained a significant predictor of reduced EGFr expression after adjusting for smoking cessation and treatment (P = 0.02). Treatment did not correlate with reduced EGFr expression after adjusting for the other covariates (P = 0.24), indicating that 13cRA had no discernible association with in vivo expression of EGFr in bronchial epithelium.

**DISCUSSION**

This study examined the effects of 13cRA treatment on bronchial metaplasia in a randomized placebo-controlled trial with chronic smokers. EGFr expression was examined as a biomarker of bronchial metaplasia and retinoid response. The findings from this study revealed that smoking cessation correlated with reduced bronchial metaplasia, but treatment did not affect bronchial metaplasia (10). Our analysis of bronchial biopsies from this study revealed that EGFr expression was increased in metaplastic lesions, and subsequent reversal of bronchial metaplasia was associated with reduced EGFr expression. Treatment did not significantly alter EGFr expression. This study revealed the importance of a placebo-controlled trial design. The placebo arm demonstrated the relationships between smoking cessation, metaplasia, and EGFr expression.

Findings reported here demonstrated that increased EGFr expression is associated with bronchial metaplasia. Other studies have linked EGFr to the squamous phenotype. In NSCLC, EGFr expression is higher in squamous than in nonsquamous tumors (25). Squamous cell carcinomas of the head and neck and skin also express high levels of EGFr (26, 27). Although it is possible that EGFr plays a role in the development or maintenance of the squamous phenotype, increased EGFr expression...
in squamous cells may instead control the proliferative activity of these cells. Supporting the latter hypothesis, EGFr is mitogenic when added to normal HBE cells in tissue culture, and normal HBE cells activate EGFr through autocrine mechanisms, stimulating their growth (28). In addition, expression of EGFr and transforming growth factor α increases during epithelial carcinogenesis of the head and neck and skin (26, 27), suggesting that EGFr autocrine signaling may contribute to the dysregulated growth observed in premalignant epithelium. Compared to histologically normal oral epithelium, hyperplastic and dysplastic oral epithelial cells are hyperproliferative, as shown by in situ examination of the proportion of cells expressing proliferating cell nuclear antigen (29). Similarly, the proportion of bronchial epithelial cells that proliferate is increased in regions of metaplasia, as shown by proliferating cell nuclear antigen and ki-67 labeling studies (30, 31).

Data presented here revealed that retinoid treatment was not associated with altered EGFr expression in bronchial epithelium. In contrast, t-RA increases EGFr protein expression in many epithelial and nonepithelial cells (32), but t-RA decreases EGFr mRNA and protein expression in epidermoid carcinoma cells and human trophoblast cells (33, 34). These studies demonstrate that the effects of retinoids on EGFr expression are cell type-specific. In addition to altering EGFr expression, retinoids affect EGFr function. For example, in normal HBE cells, retinol reduces EGFr binding through decreased secretion of EGFr ligands, and the addition of EGFr to the media abrogates retinol-induced growth arrest (28). These studies in normal HBE cells suggest that retinol interrupts EGFr autocrine signaling by inhibiting the secretion of EGFr ligands, contributing to the growth inhibitory effects of retinol. EGFr ligands include EGF, heparin-binding EGF-like growth factor, β-cellulin, amphiregulin, and transforming growth factor α (35–37). Future studies should examine 13cRA-induced changes in the expression of EGFr ligands in these bronchial biopsies.

Findings from this clinical trial revealed that bronchial metaplasia is prevalent in active smokers. Reversion of metaplasia occurred with smoking cessation (10), demonstrating that squamous differentiation is a reversible phenotype in these participants. The reversibility of bronchial metaplasia in this trial demonstrates that only a subpopulation of metaplastic lesions persist after smoking cessation. This stable subpopulation may represent premalignant foci of bronchial epithelium. Future chemoprevention trials should focus on these lesions. To identify this subpopulation, genetic and biochemical markers that are specific for premalignant HBE cells must be identified. This study revealed that EGFr is a marker of metaplastic lesions that revert after smoking cessation, demonstrating that it is not specific to premalignant foci. Other markers of premalignant lesions could include genetic abnormalities such as deletions on chromosomes 3 or 9 (6, 7) or point mutations of genes that undergo mutations during lung carcinogenesis including k-raz and p53. Once biomarkers that are specific for premalignant lesions are identified, the effects of retinoids as lung cancer chemopreventive agents can be better assessed.

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