Prevention of Bronchial Hyperplasia by EGFR Pathway Inhibitors in an Organotypic Culture Model

Jangsoon Lee$^{1,4}$, Seung-Hee Ryu$^{1,5}$, Shin Myung Kang$^{1,6}$, Wen-Cheng Chung$^1$, Kathryn Ann Gold$^2$, Edward S. Kim$^1$, Walter N. Hittelman$^3$, Waun Ki Hong$^1$, Ja Seok Koo$^1$

$^1$Department of Thoracic/Head and Neck Medical Oncology, $^2$Hematology and Oncology, $^3$Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, Texas.

Grant support: Department of Defense VITAL grant W81XW-04-1-0142 (J.S. Koo, E.S. Kim, W.K. Hong), National Heart, Lung and Blood Institute grant R01-HL-077556 (J.S. Koo), and NCI Cancer Center Support grant CA-16672 (The University of Texas MD Anderson Cancer Center).

Corresponding Author: Ja Seok Koo, Department of Thoracic/Head and Neck Medical Oncology, Unit 432, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030; phone: 713-792-8454; fax: 713-794-5997; e-mail: jskoo@mdanderson.org.

Note: Jangsoon Lee and Seung-Hee Ryu contributed equally to this work.
Prevention of Bronchial Hyperplasia

Present address: 4Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas; 5Department National Investment Project, Asan Institute for Life Sciences, Asan Medical Center, Seoul, Korea; 6Center for Lung Cancer, National Cancer Center, Goyang-si, Korea.

Running title: Prevention of Bronchial Hyperplasia

Keywords: NHBE, bronchial hyperplasia, dysplasia, erlotinib, MEK inhibitor

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest exist.
Prevention of Bronchial Hyperplasia

Abstract

Lung cancer is the leading cause of cancer-related mortality worldwide. Early detection or prevention strategies are urgently needed to increase survival. Hyperplasia is the first morphologic change that occurs in the bronchial epithelium during lung cancer development, followed by squamous metaplasia, dysplasia, carcinoma in situ, and invasive tumor. The current study was designed to determine the molecular mechanisms that control bronchial epithelium hyperplasia. Using primary normal human tracheobronchial epithelial (NHTBE) cells cultured using the 3-dimensional organotypic method, we found that the epidermal growth factor receptor (EGFR) ligands EGF, transforming growth factor-alpha, and amphiregulin induced hyperplasia, as determined by cell proliferation and multilayered epithelium formation. We also found that EGF induced increased cyclin D1 expression, which plays a critical role in bronchial hyperplasia; this overexpression was mediated by activating the mitogen-activated protein kinase pathway but not the phosphoinositide 3-kinase/Akt signaling pathway. Erlotinib, an EGFR tyrosine kinase inhibitor, and U0126, a MEK inhibitor, completely inhibited EGF-induced hyperplasia. Furthermore, a promoter analysis revealed that the activator protein-1 transcription factor regulates EGF-induced cyclin D1 overexpression. Activator protein-1 depletion using siRNA targeting its c-Jun component completely abrogated EGF-induced cyclin D1 expression. In conclusion, we demonstrated that bronchial hyperplasia can be modeled in vitro using primary NHTBE cells maintained in a 3-dimensional (3-D) organotypic culture. EGFR and MEK inhibitors completely blocked EGF-induced bronchial hyperplasia, suggesting that they have a chemopreventive role.
Prevention of Bronchial Hyperplasia

Introduction

Hyperplasia in the bronchial epithelium, as evidenced by increased cell proliferation, is associated with conditions such as trauma, smoking, chronic cough, chronic inflammatory airway disease, and cancer. It is the first of several progressive, cumulative, genetic and morphologic changes associated with lung squamous cell carcinoma, followed by squamous metaplasia, dysplasia, and carcinoma in situ (1-3). These extensive, multifocal changes occur throughout the respiratory tree when the lungs are chronically exposed to common carcinogens, a phenomenon known as field cancerization (4).

In the developed lung, growth factors and their signaling receptors support cellular activities in equilibrium, preserving normal lung structure and function (5). However, this homeostatic control can be compromised during the accumulation of genetic and molecular alterations that lead to lung cancer. Several decades of research have revealed that the ErbB system is a critical growth factor system in normal and abnormal epithelial cell proliferation (6, 7). The ErbB family, ErbB1-4, plays an important role in lung cancer development. In addition, several ErbB ligands are aberrantly regulated in cancer cells. Therefore, we hypothesized that bronchial hyperplasia results from ErbB hyperactivation in bronchial epithelial cells. To test this hypothesis, we evaluated ErbB ligands for their ability to induce bronchial hyperplasia using a 3-dimensional (3-D) organotypic air-liquid interface primary bronchial epithelial cell culture system (8-10). We then determined which downstream signaling pathways and genes were involved in bronchial hyperplasia development. EGFR ligands induce bronchial hyperplasia via the MEK/ERK signaling pathway. EGF-induced cyclin D1 overexpression plays a critical role in the development of bronchial hyperplasia. EGFR and MEK inhibitors completely blocked EGF-induced bronchial hyperplasia.
Prevention of Bronchial Hyperplasia

As monotherapy, erlotinib, a small molecular inhibitor targeting the intracellular tyrosine kinase domain of EGFR, significantly prolonged survival in previously treated advanced non-small cell lung cancer (NSCLC) patients compared with placebo (13) and was recently approved by the FDA. Erlotinib has anti-proliferative effects arising from G1 arrest and pro-apoptotic effects on cancer cells (14). However, its effects on normal and hyperplastic bronchial epithelial cells are unknown. Our results showed that erlotinib blocks EGF-induced bronchial hyperplasia and can reverse hyperplasia, restoring normal bronchial epithelial morphologic characteristics. We identified several mechanisms involved in the onset of changes leading to lung cancer, such as abnormal cell proliferation, which may be targets for preventing malignant progression.

Materials and Methods

Chemicals

Erlotinib (LKT Laboratories, Inc.), U0126, LY294002, and Akt inhibitor VIII (Calbiochem) were dissolved in dimethylsulfoxide.

Cells

Normal human tracheobronchial epithelial (NHTBE) cells were obtained from the Lonza Walkersville, Inc. A549 lung cancer cells were obtained from the American Type Culture Collection. We authenticated A549 cell by genotyping through MD Anderson cancer center DNA analysis core facility.

3-D organotypic air-liquid interface cell culture and treatment

We cultured NHTBE, or A549 cells using the 3-D organotypic air-liquid interface method described previously (9, 11). The medium in the bottom chamber was changed every 24 h. We treated 7-day-old confluent NHTBE cells grown on a porous membrane of a Transwell
Prevention of Bronchial Hyperplasia

plate with various ligands (Sigma-Aldrich) for ErbB receptors EGF (10 ng/ml), transforming growth factor-alpha (TGF-α, 10 ng/ml), amphiregulin (AR, 50 ng/ml), or heregulin (HR, 100 ng/ml) for 4 days. The ligands were included only in basal media, and the apical side of the cultures was exposed to air by removing the media overlaying the cells in the upper side of the well. For dose-dependent experiments, we used 0.5, 2.0, 5.0, 10, and 25 μg/ml of EGF. For time-dependent experiments, we cultured cells with 5 ng/ml of EGF for 1 to 4 days.

Western blot analysis

We prepared total protein extracts using cold radioimmunoprecipitation assay lysis buffer (50 mM HEPES, pH 7.4; 1% NP-40, 150 mM NaCl, 1 mM EDTA, phosphates inhibitors, and protease inhibitors). Protein (15 μg) was resolved by 10% SDS-PAGE gel. Membranes were incubated with rabbit polyclonal antibodies against CREB, phospho-CREB-133 (Upstate Biotechnology), ERK, phospho-ERK-202/204, cyclin A1, cyclin B1, cyclin D1, cyclin E2, Akt, phospho-Akt-473, c-Jun, phospho-c-Jun-73, p-EGFR-1068, and EGFR (Cell Signaling Technology) overnight. β-actin (clone AC-15; Sigma-Aldrich) was used as a loading control. The proteins’ reaction with primary antibodies was visualized with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (GE Healthcare).

Small interfering RNA

Human c-Jun (accession no. NM_002228) siRNAs were used to knock down c-Jun expression (Sigma-Aldrich), according to the manufacturer’s instructions. A mixture of several siRNAs ensured that the targeted gene product was effectively deleted. Cells at 60%-70% confluency were transfected for 48 h with a final concentration of 100 nM c-Jun siRNA or nonspecific control pooled siRNAs using the Dharmafect 1 transfection reagent (Dharmacon),
Prevention of Bronchial Hyperplasia

according to the manufacturer’s instructions. The cells were treated with EGF (5 ng/ml) for 24 h, when target protein levels had been reduced more than 70%, as assessed by Western blot analysis.

Immunohistochemistry and immunofluorescence

The NHTBE cells were fixed in neutral-buffered formalin and embedded in paraffin. Sections (5 μm each) were prepared using a microtome, mounted on slides, deparaffinized in xylene, rehydrated in graded alcohols, and washed in distilled water. Endogenous peroxidases were quenched by incubation in 3% H₂O₂. Antigens were retrieved by microwaving the sections in 10 mM citric acid (pH 6.0) for 5 min. The slides were washed three times with phosphate-buffered saline (PBS) and blocked for 30 min with 10% normal goat serum in 1% bovine serum albumin (BSA)/PBS. Immunohistochemical staining was visualized using the Histostain-Bulk-SP and the AEC red substrate kits (Zymed Laboratories). Immunohistochemical staining without a primary antibody was performed as a negative control. Immunofluorescence staining was visualized using Cy-3 (Jackson ImmunoResearch) and DAPI (Invitrogen). Stained slides were visualized with an Axioskop 40 fluorescence microscope (Carl Zeiss, NY); the images were captured at a magnification of 200× and stored using Axiovision LE software v4.5 (Carl Zeiss, NY) according to the manufacturer’s instructions.

Luciferase reporter assay

NHTBE cells (5×10⁴) were cultured in 12-well tissue culture plates (Corning, MA) overnight and co-transfected with cyclin D1 promoter-luciferase constructs (wild type, AP-1 site mutant, or CRE site mutant; kindly provided by Dr. Richard Pestell at Thomas Jefferson
Prevention of Bronchial Hyperplasia

University) and Renilla luciferase control vector using Lipofectamin 2000 (Invitrogen). After 24 h, the culture medium was changed to 0.1% BSA in BEBM (Lonza Walkersville, Inc.) and cells were treated with or without EGF (5 ng/ml) for 24 h. Luciferase activity was detected using the dual-luciferase reporter assay (Promega) and measured using a Lumat LB 9507 tube luminometer (Berthold, TN). All assays were performed in triplicate and repeated at least three times. Figures show representative results.

Hyperplasia evaluation via cell layer thickness and cell number

To determine ErbB receptors ligands’ effects on the histomorphologic characteristics of NHTBE cells, we incubated NHTBE cells with EGF (10 ng/mL), TGF-α (10 ng/mL), AR (50 ng/mL), or HR (100 ng/mL) for 4 days. After making paraffin-embedded blocks, we captured three images from each block within a 10-mm area from the center of the Transwell membrane with an Axioskop 40 microscope (Carl Zeiss, NY) under light microscopy (200×). To evaluate hyperplasia, we measured thickness using Axiovision LE software v4.5 (Carl Zeiss, NY) according to the manufacturer’s instructions. Total cell numbers in the captured area were counted manually under light microscopy (200X). Bars, standard error (SE); ** P<0.01 and *** P<0.001.

Statistical analysis

For each experimental outcome, descriptive statistics (mean, standard deviation, median, and range) were summarized for each group. An analysis of variance (ANOVA) model was used to detect any differences between treatment and control groups.

Results
EGFR ligands induce hyperplasia in bronchial epithelial cells grown in 3-D organotypic culture

To determine whether ErbB receptors are involved in bronchial hyperplasia morphologic changes, we cultured NHTBE cells using the organotypic air-liquid interface method with various ErbB receptor ligands, including EGF (10 ng/ml), TGF-α (10 ng/ml), AR (50 ng/ml), and HR (100 ng/ml), for 4 days. We histochemically evaluated ErbB ligands’ effects on morphologic changes in NHTBE cells and found that the NHTBE cell layer was statistically significantly thicker after treatment with EGFR ligands, EGF, TGF-α, and AR but not with HR (Fig. 1A). Quantitative changes are shown as mean thicknesses: control=13 (± 2) μm, EGF treated=42 (±6) μm, TGF-α treated=31 (±4) μm, AR treated=28 (±3) μm, and HR-treated=15 (±2) μm cells. These data clearly demonstrate that high EGFR ligand concentrations induce bronchial epithelial cell hyperplasia. The most prominent hyperplastic morphologic changes in NHTBE cell culture histologic patterns were induced by EGF; therefore, we selected EGF for subsequent study.

To determine EGF’s dose-dependent effects on NHTBE cell hyperplasia, we incubated NHTBE cells with EGF for 4 days. Immunohistochemical analysis and cell quantitation clearly indicated that EGF induced hyperplasia in a dose-dependent manner. NHTBE cell layers were significantly thicker after treatment with 5 ng/ml of EGF, and cell quantitation showed a similar pattern (Fig. 1B). To determine EGF’s time-dependent effects, we incubated NHTBE cells with 5 ng/ml of EGF for 1 to 4 days. The NHTBE cell layer expanded in a time-dependent manner. Four days of treatment resulted in an approximately 2.5-fold increase in cell number and cell layer thickness (Fig. 1C).

EGF induces cell proliferation at only the basal layer of NHTBE cell cultures
Multilayered hyperplasia is believed to result from uncontrolled bronchial epithelial cell proliferation. To identify the specific NHTBE cell population after EGFR ligand exposure, we performed Ki-67 immunostaining in NHTBE cells grown in a 3-D organotypic culture system. We used A549 cells as a positive control. As shown in Fig. 2, immunohistochemical (Fig. 2A) and immunofluorescence (Fig. 2B) analyses of Ki-67 as a cell proliferation indicator revealed positive staining in cells in the basal layer of NHTBE cell cultures. In contrast, Ki-67-positive cells were detected randomly in both basal and parabasal layers in A549 cells. These results indicate that only NHTBE cells in the basal layer divide and grow in response to EGF; cancer cells are not limited to the basal layer.

**MEK/ERK pathway is a critical signaling pathway for EGF-induced NHTBE cell hyperplasia**

The MEK/ERK and PI3-K/Akt pathways are well-established downstream signaling pathways of the EGF-EGFR pathway (12). To determine the relative importance of these pathways for transmitting EGF-induced hyperplasia signals in NHTBE cells, we treated fully confluent 7d-old NHTBE cells with 5 ng/ml of EGF for 2 hr. EGF induced Akt, ERK, and CREB phosphorylation in NHTBE cells (Fig. 3A), demonstrating that the Akt and ERK pathways may participate in EGF’s induction of cell proliferation and layer thickening. To further understand the relative role of these pathways, we treated 7d-old NHTBE cells with EGF (5 ng/ml) alone or in combination with pharmacological inhibitors targeting select molecules in the PI3-K/Akt and ERK signaling pathways: erlotinib (EGFR-TKI, 1 μM), U0126 (MEK inhibitor, 5 μM), LY294002 (PI3-K inhibitor, 10 μM), and Akt inhibitor VIII (10 μM) for 15 min. Inhibitors were pretreated for 30 min before EGF treatment. Erlotinib completely inhibited EGFR, Akt, and ERK phosphorylation (Fig. 3B). U0126 inhibited ERK and CREB phosphorylation. However,
Prevention of Bronchial Hyperplasia

LY294002 and Akt inhibitor VIII had no effect on EGF-induced ERK and CREB phosphorylation (Fig. 3B).

To further determine the morphologic consequences of inhibiting critical molecules in signaling pathways, we cultured NHTBE cells for 7 days until they become confluent and then treated with EGF (5 ng/ml) alone or in the presence of various inhibitors for 4 days. NHTBE cells treated with EGF alone experienced hyperplastic changes and increased thickness, whereas those treated in the presence of erlotinib or U0126 underwent no such changes. However, LY294002- and Akt inhibitor VIII-treated NHTBE cells still showed hyperplasia and increased thickness (Fig. 3C). These results suggest that the MEK/ERK pathway is the main pathway by which EGF induces hyperplasia in NHTBE cells. We next determined whether inhibitors reverse EGF-induced hyperplasia in NHTBE cells. After they became hyperplastic, the cultures were maintained in media containing 0.5 ng/ml EGF in the absence or presence of erlotinib (1 μM), LY294002 (10 μM), U0126 (5 μM), or Akt inhibitor VII (10 μM) for 4 additional days. Only erlotinib successfully reverted EGF-induced hyperplasia such that normal morphologic characteristics were restored (Fig. 3D).

Cyclin D1 is increased during EGF-induced NHTBE cell hyperplasia

Increased cell proliferation is partly responsible for hyperplasia induction. Because elevated cyclin levels play a role in enhancing cell proliferation, we determined cyclin levels in NHTBE cells after EGF treatment. EGF robustly increased cyclin D1 and slightly increased cyclin E2 expression (Fig. 4A). However, A1 and B1 were not significantly increased. The EGFR and MEK inhibitors erlotinib and U0126, respectively, markedly blocked EGF-induced expression of cyclin D1, but LY294002 and Akt inhibitor VIII did not (Fig. 4B).
Prevention of Bronchial Hyperplasia

To determine which transcription factors are involved in EGF-induced cyclin D1 gene expression, we performed a cyclin D1 promoter-luciferase activity assay (Fig. 4C). We transfected NHTBE cells with various cyclin D1 promoter-luciferase reporters (wild type, AP-1 site mutant, and CRE sites mutant in the cyclin D1 promoter region) and treated the transfected cells with or without EGF. EGF increased luciferase activity over 20 times when wild-type or mutated CRE cyclin D1 promoter reporters were introduced (Fig. 4C). However, when the AP-1 recognition sequence was mutated or removed, the EGF response was dramatically lower than that in the wild-type promoter. This result demonstrates the importance of the AP-1 transcription factor in EGF-induced cyclin D1 overexpression.

Next, we investigated the activation status of AP-1 components c-Jun and c-Fos. EGF induced c-Jun expression and phosphorylation (Fig. 4D) but not c-Fos (data not shown). In addition, erlotinib and U0126 markedly blocked EGF-induced c-Jun phosphorylation (Fig. 4E). c-Jun knockdown with c-Jun siRNA prevented EGF-induced cyclin D1 expression, suggesting that EGF-induced cyclin D1 expression is mediated by c-Jun (Fig. 4F). Thus, we concluded that EGF induces cyclin D1 overexpression and that this overexpression is mediated by AP-1 (c-Jun) transcription factor. In addition, EGF-induced cyclin D1 overexpression is blocked by EGFR and MEK inhibitors.

Discussion

We demonstrated that bronchial hyperplasia can be modeled and manipulated in vitro using primary NHTBE cells maintained in a 3-D organotypic air-liquid interface culture. The EGFR ligands EGF, TGF-α, and AR induce hyperplasia in NHTBE cells. This histomorphologic change is regulated by the MEK/ERK signaling pathway but not the PI3-K/Akt signaling
pathway. The MEK/ERK signaling pathway induces cyclin D1 expression by activating AP-1 transcription factor. The EGFR and MEK inhibitors erlotinib and U0126 completely blocked EGF-induced hyperplasia.

In view of multistep lung carcinogenesis and field cancerization, our results suggest that erlotinib may be useful as a chemopreventive agent as such agents inhibit, delay, or reverse carcinogenesis. First, erlotinib may be beneficial for high-risk patients, such as those with a strong smoking history. Erlotinib is currently being studied in the adjuvant setting after surgery and chemotherapy in NSCLC. Lung cancer develops in a field with extensive and multifocal hot spots throughout the respiratory trees, which are consistently exposed to common carcinogens. Even after tumor resection, these hot spots can develop into lung cancer. After resection, the risk of a second primary lung cancer is approximately 1% to 2% per patient per year, with a cumulative risk of up to 20% at 6 to 8 years after resection (15). Erlotinib would target two cell populations at once: micrometastatic NSCLC cells and evolving bronchial epithelial cells. Erlotinib’s inhibition and reversal of the first step in lung carcinogenesis in NHTBE cells warrant further investigation. We are conducting a clinical study and have enrolled 50 patients with early-stage lung cancer who have undergone neoadjuvant chemotherapy with cisplatin and docetaxel, followed by surgical resection and 1 year of adjuvant erlotinib. Patients will undergo bronchoscopy at 6 months and 1 year to assess possible changes in the bronchial epithelium after erlotinib treatment (16).

We found that EGF robustly increased cyclin D1 in primary NHTBE cells grown in organotypic culture. The malignant transformation of bronchial epithelial cells is driven by the dysregulation of oncogenes, growth factors, or tumor suppressor genes. Cyclin D1 is strongly implicated as an oncogene in lung cancer and several other human cancers, including B-cell
Cyclin D1 is part of the cyclin-dependent kinase (CDK)-cyclin complex that increases retinoblastoma (Rb) protein phosphorylation at the G1-S transition and may play a role in transcriptional regulation. Cyclin D deregulation, by amplification or transcriptional upregulation, has been found in many tumor types (18-22). The p53-Rb pathway that mediates G1 arrest is the most commonly affected pathway in lung cancer. Defects in G1-regulatory proteins, especially p53-p21WAF1, p16-Rb-cyclin D1, and cyclin E-p16 pathway deregulation, seem to be essential to lung cancer development (23, 24). An immunohistochemical analysis demonstrated cyclin D1 and E overexpression in bronchial preneoplasia that precedes the development of squamous cell carcinoma (25). These data imply that increased cyclin levels play a critical role in preneoplastic bronchial lesion progression. This conclusion was confirmed in bronchial epithelial cellular models (14) and carcinogen-induced lung tumors in animal models (26, 27).

Cyclin D1 overexpression may portend a worse prognosis in patients with resected lung cancer (28), although results have not been consistent (29). Cyclin D1 appears to be regulated by EGFR in gefitinib-resistant EGFR mutant cell lines, and these cell lines are sensitive to flavopiridol, a CDK inhibitor (30). Cyclin D1 repression is an indirect marker of erlotinib treatment response in aerodigestive tract cancers (31). Chemoprevention trials have found that rexinoid, a selective retinoid X receptor agonist, suppresses cyclin D1 expression in NSCLC (32), and low cyclin D1 expression predicts longer cancer-free survival in laryngeal premalignancy patients (33). Thus, cyclin D1 levels have been studied as markers for abnormal cell growth in chemoprevention trials (34). Cyclin D1 gene expression regulation has been reported to include the ras/raf/MAPK cascade in fibroblast cells (35, 36), p60Src pathways through CREB/activating transcription factor 2 activation in breast cancer cells (37), and PI3-K/Akt/NF-κB pathway-
involved pro-oncogenic effects in human bronchial epithelial cells (38). Our data showed that EGF activates the PI3-K/Akt and MEK/ERK pathways in a time-dependent manner. However, only the MEK/ERK pathway was involved in EGF-induced cyclin D1 expression, suggesting that it is involved in early-stage lung carcinogenesis.

As shown in Fig. 5, we found that bronchial hyperplasia can be modeled \textit{in vitro} using a 3-D organotypic culture method and prevented by blocking the EGFR/MEK signaling pathway. We further found that bronchial hyperplasia is dependent on cyclin D1, which is regulated by AP-1 activation through the MEK/ERK pathway rather than the PI3-K/Akt pathway. Our model system and results will help elucidate the molecular mechanisms of lung carcinogenesis at its early stages and may support the prophylactic use of EGFR-targeting agents in patients at high risk of tumor development.
Prevention of Bronchial Hyperplasia

References


Prevention of Bronchial Hyperplasia


Prevention of Bronchial Hyperplasia


Prevention of Bronchial Hyperplasia

35. Takuwa N, Takuwa Y. Ras activity late in G1 phase required for p27kip1 downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. Molecular and cellular biology 1997; 17:5348-58.


Acknowledgements

We thank Dr. Richard Pestell (Thomas Jefferson University, Philadelphia, PA) for the gift of cyclin D1 luciferase vectors.
Prevention of Bronchial Hyperplasia

Figure Legends

**Fig. 1.** EGFR ligands induce hyperplasia in NHTBE cells. NHTBE cells were cultured on a Transwell plate until they formed a monolayer. The culture condition was changed to an air-liquid interface, as described in Materials and Methods. Starting at day 7, cells were treated with various ErbB-ligands (EGF 10 ng/ml, TGF-α 10 ng/ml, AR 50 ng/ml, and HR 100 ng/ml) for 4 days (A). EGF induces hyperplasia in a dose- (B) and time-dependent (C) manner. Seven-day-old NHTBE cell cultures were treated with indicated concentrations (B) of EGF for the indicated time periods (C). Cells were then fixed with phosphate-buffered formalin and embedded in paraffin. The result was visualized using hematoxylin/eosin staining. Cells were counted as described in Materials and Methods. The results were summarized by descriptive statistics (mean, SE, and median), and box-plots were generated for each experiment, with different cell groups compared side by side. Data shown are representative of three experiments with similar results.

**Fig. 2.** Proliferating cells are differentially localized in NHTBE or A549 cancer cells. Both cell lines were grown using the ALI method, as described in Materials and Methods. The cultures were starved without supplements in their respective culture media for 24 h and treated with 5 ng/ml of EGF (0.5 ng/ml as control). The cultures were fixed, and paraffin blocks were prepared. Slide sections were stained with anti-Ki-67. The result was visualized using immunohistochemical (A) and immunofluorescence (B) analysis. Data shown are representative of three experiments with similar results.
Prevention of Bronchial Hyperplasia

Fig. 3. MEK/ERK pathway is involved in EGF-induced hyperplasia in NHTBE cells. (A) EGF induced Akt, ERK, and CREB phosphorylation. Seven-day-old NHTBE cell cultures were maintained in BEBM without supplements overnight, and then treated with EGF (5 ng/ml) for the indicated time periods. (B) Effect of pharmacologic inhibitors on EGF-induced Akt, ERK, and CREB phosphorylation. The same 7d-old NHTBE cell cultures maintained in BEBM without supplements overnight were treated with EGF (5 ng/ml) alone or in combination with the indicated pharmacologic inhibitors for 15 min. Inhibitors were pretreated 30 min before EGF treatment. (C) Histologic evaluation of pharmacologic inhibitors’ effects. Seven-day-old NHTBE cell cultures were incubated with 5 ng/ml EGF alone or in combination with the various inhibitors for 4 days. Control cultures were maintained in 0.5 ng/ml EGF media. Cell numbers and thickness of cultures were determined as described in Materials and Methods. (D) Treatment effects of erlotinib on EGF-induced hyperplasia. Seven-day-old NHTBE cell cultures were treated with EGF (5 ng/ml) for 4 days to generate hyperplasia. The cultures were then maintained in culture media with 0.5 ng/ml EGF for 4 more days with or without inhibitors. The result was evaluated using hematoxylin/eosin staining. Cells were counted, as described in Materials and Methods. The results were summarized by descriptive statistics (mean, SE, and median).

Fig. 4. AP-1 is a critical transcription factor in EGF-induced cyclin D1 overexpression in NHTBE cells. (A, B, D and E) NHTBE cells were cultured on a Transwell plate until they formed a monolayer. The culture condition was changed to an air-liquid interface. Before EGF stimulation or pharmacologic inhibitor treatment, cells were incubated with 0.1% BSA/BEBM medium for 24 h before treatment. (A) EGF upregulates cyclin D1 expression in a time-dependent manner. (B) Erlotinib and U0126 inhibit EGF-induced cyclin D1 expression. (C) The
Prevention of Bronchial Hyperplasia

AP-1 binding site is important for cyclin D1 promoter activity. Cells were transfected with indicated plasmids using a 12-well tissue culture plate. After 48 hr of incubation, cells were maintained in 0.1% BSA/BEBM without supplements overnight and treated with EGF (5 ng/ml) for 24 hr. (D) EGF induces c-Jun phosphorylation in a time-dependent manner. (E) Erlotinib and U0126 inhibit EGF-induced c-Jun phosphorylation. (F) c-Jun siRNA abrogated EGF-induced cyclin D1 expression. Cells were transfected with siRNA using a 12-well tissue culture plate. After 48 hr incubation, cells were maintained in 0.1% BSA/BEBM without supplements overnight and treated with EGF (5 ng/ml) for 24 hr. Data shown are representative of three experiments with similar results.

Fig. 5. EGF induced increased cyclin D1 expression, which plays a critical role in bronchial hyperplasia; overexpression was mediated by activating the mitogen-activated protein kinase (MAPK) pathway.
# Cancer Prevention Research

## Prevention of Bronchial Hyperplasia by EGFR Pathway Inhibitors in an Organotypic Culture Model


*Cancer Prev Res* Published OnlineFirst April 19, 2011.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-10-0364</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

**E-mail alerts** Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions** To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.