The Chronic and Short-Term Effects of Gefinitib on Airway Remodeling and Inflammation in a Mouse Model of Asthma

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Key Words
Asthma • Gefinitib • Airway remodeling • Inflammation • Epidermal growth factor receptor

Abstract
Background: Asthma is a complex and heterogeneous chronic inflammatory disorder which is characterized by airway remodeling and airway inflammation, including goblet cell and airway smooth muscle cell hyperplasia, mucus hypersecretion and eosinophil infiltration. Epidermal growth factor receptor (EGFR) plays an important role in goblet cell hyperplasia and mucus hypersecretion. We aimed to investigate the effects of gefitinib, an EGFR inhibitor, on ovalbumin (OVA)-induced airway remodeling and inflammation of a mouse model of asthma.

Methods: Pathological changes of OVA sensitization of BALB/c mice were measured by H&E and PAS staining; pEGFR, Bcl-2 and Bax expression was measured by western blot; ELISA was used to measure the level of muc5ac, IL-13 and IFN-γ; TUNEL staining was used to detect goblet cell apoptosis.

Results: At the present study, H&E and PAS staining showed that mice pretreated with gefitinib developed fewer pathological changes compared with asthmatic mice and gefitinib treatment asthmatic mice, such as a remarkable reduction in airway inflammation, goblet cell and airway smooth muscle cell hyperplasia. Chronic gefitinib treatment or short-term gefitinib treatment significant down-regulate the expression of pEGFR compared with asthma group. Also, chronic gefitinib treatment markedly decreased the levels of muc5ac and IL-13 in BALF, whereas the level of IFN-γ did not change obviously. TUNEL staining showed that the goblet cell apoptosis rate was much higher in the short-term gefitinib treatment group compared with the asthma and chronic gefitinib treatment group which was accompanied by a decrease in Bcl-2 levels and an increase in Bax expression in goblet cells.

Conclusion: In summary, our results suggested that gefitinib may have a potential role in airway remodeling and inflammation, and may be an effective pharmacotherapy for asthma.

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Introduction

Allergic asthma is increasingly regarded as a complex and multifactorial [1] chronic inflammatory disease. The symptoms of this disorder include wheezing, shortness of breath and coughing [2]. It is estimated that as many as 300 million people of all ages and all ethnic backgrounds suffer from asthma and the number of patients is increasing worldwide [3]. A large number of studies have suggested that increased mucus accumulation leads to obstruction in the airways of humans with asthma or COPD [4]. In addition to mucus hypersecretion, the pathological features of asthma also include pulmonary infiltration by mast cells, lymphocytes and eosinophils, and structural changes to persistent airways, such as subepithelial fibrosis, bronchial wall thickening, and goblet cell and airway smooth muscle (ASM) hyperplasia [5]. Allergic asthma is a complex chronic inflammatory disorder characterized by airway inflammation, mucus hypersecretion and airway remodeling [6]. Airway inflammation in chronic asthma may be the result of Th2 lymphocytes secreting cytokines (such as IL-13) that promote cellular inflammation. Airway remodeling in asthma refers to structural changes and is characterized by the hyperplasia of goblet cells and smooth muscle [7, 8].

Goblet cell metaplasia is involved in mucus hypersecretion; both T helper 2 (Th2) cytokines and epidermal growth factor receptor (EGFR) signaling contribute to the process [9]. Substantial evidence has suggested that Th2 cells may play an important role in the pathology of allergic asthma, whereas Th1 cells have a protective effect [10]. Th2 cells are recognized by their secretion of Th2 cytokines, such as IL-4 and IL-13. These cytokines are implicated in the pathogenesis of asthma and show that this is an upstream cytokine that regulates allergic inflammation [11]. Current evidence indicates that EGFR is involved in IL-13-induced mucin expression in goblet cells. Mucus hypersecretion is the results of airway narrowing because of goblet cell hyperplasia [12]. Recently, some studies showed that the activation of EGFR could induce goblet cell hyperplasia and increase the secretion of MUC5AC in asthmatic patients [13]. Epidermal growth factor receptor (EGFR) signaling also has been involved in goblet cell metaplasia [14]. EGFR is a member of the receptor tyrosine kinase family. There is some evidence that increases in EGFR levels are involved in the severity of airway thickening in chronic asthmatic subjects, and the EGFR signal pathway is induced by asthma-related inflammation and cytokines [15]. There is also evidence that EGFR is upregulated by the proinflammatory cytokine tumor necrosis factor-α (TNF-α), which is increased in the lungs in asthma [16, 17]. This 170 kDa receptor tyrosine kinase plays a central role in the regulation of epithelial cell behavior by virtue of their capacity to stimulate migration or proliferation to differentiation and enhanced survival; EGFR could also regulate the expression of metalloproteinases, various inflammatory mediators, matrix proteins, adhesion molecules, and mucins [18]. There is some evidence to suggest that EGFR expression reflects damage and activation to the airway epithelia, and increases in proportion to asthma severity [19, 20]. There is growing evidence that EGF regulates MUC5AC secretion in goblet cells of the respiratory tract [21]. EGFR was originally reported to induce goblet cell hyperplasia and increase the expression of mucin genes and proteins (such as MUC5AC) in asthmatic patients [13]. Moreover, several studies suggested that EGF ligands induce MUC5AC production through the EGFR signaling pathway in the airway [14, 22].

M. Tamaoka et al. showed that short-term treatment by EGFR receptor inhibitor, AG1478 mediates allergic airway remodeling in the asthmatic rats model [23]. Also, Timothy D. Le Cras et al. suggested that EGFR receptor inhibitor erlotinib or transgenic mice which expressed mutant dominant negative EGFR could mediate airway hyperreactivity and remodeling in a mouse model of chronic asthma [15]. Yun M et al. showed that melatonin could active and somatic EGFR mutations, resulted in the sensitivity to gefitinib of H1975 cells increased [24]. Thus, we believed that inhibiting the expression of EGFR may alleviate the symptoms of asthma. Gefitinib, the EGFR tyrosine kinase inhibitor, is a reversible competitive inhibitor of the tyrosine kinase domain of EGFR that binds to its adenosine-5’ triphosphate-binding site [25]. Elevated levels of EGFR and/or its cognate ligands have been identified as a common
component of multiple cancer types and appear to promote solid tumor growth [26]. Evidence has suggested that gefitinib can block the signal transduction pathways implicated in the proliferation and survival of cancer cells via inhibiting the EGFR tyrosine kinase [27, 28]. Here, we investigated the prevention and treatment effects of gefitinib, the EGFR tyrosine kinase inhibitor, on OVA-induced allergic asthma in mice in vivo. OVA-sensitized mice were pretreated with gefitinib which inhibited the symptoms of asthma. However, the treatment of asthma mice with gefitinib did not alleviate the asthma symptoms.

**Materials and Methods**

**Antibodies**

Rabbit anti-mouse EGFR polyclonal antibody, rabbit anti-mouse pEGFR polyclonal antibody, rabbit anti-mouse Bax polyclonal antibody, rabbit anti-mouse Bcl2 polyclonal antibody and rabbit anti-mouse β-actin polyclonal antibody were all purchased from Abcam (Cambridge, MA, USA).

**Ethical standard**

All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Fourth Military University.

**Mice and sensitization**

Male BALB/c mice aged 6–8 weeks (15-20 g) were obtained from the Experimental Animal Centre of the Fourth Military University, Shaanxi Province, China. OVA sensitization of BALB/c mice was performed according to the methods described previously with minor modifications [29]. Briefly, mice were sensitized by i.p. injections of OVA (1 μg, Sigma, Saint Louis, MO) and Al(OH)₃ suspended (100 μg) in 0.5 ml saline on days 0 and 7. The mice were assigned randomly to a control group, an OVA-induced group, a chronic gefitinib treatment group and a short-term gefitinib treatment group. Ten animals were studied in each group. On days 14–20, the mice were challenged with 1% OVA aerosol (2.3 mg/m³) for 5 h each day to construct an asthmatic mouse model. On days 14–20, BALB/c mice were feeding with 50 mg/kg gefitinib (10mg/ml, Soluble in 10% glucose solution) for 12 h each day to construct the chronic gefitinib treatment mouse model. On days 18–20, BALB/c mice were treated with 50 mg/kg gefitinib (10mg/ml, Soluble in 10% glucose solution) for 12 h each day to construct short-term gefitinib treatment mouse model. Mice were sensitized to phosphate-buffered saline (PBS) and challenged with PBS aerosols (PBS-mice) to construct the control group.

**BALF collection and tissue preparation**

BALF was collected 24 h after the last OVA challenge, as described in the literature, with minor modifications [30]. Briefly, mice were sacrificed after anesthesia using 10 mg sodium pentobarbital. Tracheotomy was performed, and a cannula was inserted into the trachea. Half a milliliter of cold saline was instilled into the trachea; the process was repeated three times and 1.2 ml BALF was collected. In order to obtain the supernatant, BALF was then centrifuged at 1500 rpm for 10 min at 4°C in a Beckman model TJ6 table-top centrifuge. The supernatant was stored at -20°C before examination.

The left lungs were cut into 0.3 cm-thick sections and placed in 4% para-formaldehyde-PBS for 24 h. Then, conventional dehydration was performed, and samples were embedded in paraffin blocks, and serially sectioned at 3-μm for histological analysis. The right lungs of mice were stored in liquid nitrogen before western blot analysis.

**Cell counts in BALF**

BALF was collected and centrifuged, and then resuspended in 0.5 ml PBS. BALF cells were stained by Wright-Giemsa and classified counting, the total cell counts and the eosinophil counts were measured [31].

**Histological examination**

Histological examination was performed by using H&E staining according to the method described previously [32]. Briefly, 3-μm sections of fixed embedded tissues were deparaffinized, and then stained...
with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Inflammation of the lungs was assessed by histological analysis of H&E-stained lung sections. Goblet cell hyperplasia of the airway epithelium was examined by histological analysis of PAS-stained sections. Lung inflammation and goblet cell hyperplasia were observed under light microscope. Five randomly selected lung tissue sections were analyzed per mice. Five round cross-sections of bronchus (Shortest diameter of lumen/longest diameter >0.6) were randomly selected in a blinded manner, and then observed and pictures were taken. According to the previous study, the bronchial smooth muscle area (Wam) [33], goblet cell area [34] and perimeter of the bronchial basement membrane (Pbm) of mice were assessed with Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD), and Wam/Pbm, goblet cell area /Pbm were calculated [33, 35]. Wam/Pbm and goblet cell area /Pbm were used for indicating the thickness of bronchial smooth muscle cell layer and goblet cell, respectively.

**ELISA**
The levels of MUC5AC, IFN-γ and IL-13 in BALF supernatant were detected using commercial ELISA kits according to the manufacturer’s instructions. MUC5AC ELISA was obtained from KeYingMei Technology Co. Ltd (Beijing, China). IL-13 and IFN-γ ELISA kits were obtained from R&D Systems (Minneapolis, MN).

**Western blot**
The expression of p-EGFR, EGFR, Bcl-2 and Bax in lung tissue was determined by western blot. The total protein of right lung tissue (100 mg) was extracted by using RIPA buffer (Beyotime, Nantong, China). Protein concentration was determined by the BCA protein assay kit (Beyotime, Nantong, China). Each sample (30 μg/ lane) was mixed with 12% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. Rabbit anti-mouse EGFR polyclonal antibody, rabbit anti-mouse p-EGFR polyclonal antibody, rabbit anti-mouse Bcl-2 polyclonal antibody and rabbit anti-mouse Bax polyclonal antibody were used as the primary antibodies and HRP-conjugated goat anti-rabbit IgG was used as the secondary antibody. All experiments included 12 mice per group and were repeated at least three times.

**TUNEL**
Goblet cell apoptosis was determined using a commercially available kit according to the manufacturer’s instructions (Roche, Nutley, NJ) and the method described previously [36]. After deparaffinization and rehydration, lung tissue sections (3-μm) were incubated with TdT enzyme and 11-digoxigenin dUTP at 37°C for 4 h. Sections were then incubated with anti-digoxigenin FITC-conjugated antibody for 60 min at room temperature. The cell nucleus was stained with hematoxylin. Yellowish-brown and brown color was obtained with diaminobenzidine and hematoxylin (DAB, 1:50). The image data were acquired using a light microscope. TUNEL-positive cells in the epithelia goblet cells were counted in the sections. The apoptotic goblet cells (Acm) and the bronchial basement membrane (Pbm) of mice were quantified; Acm/Pbpm cells/mm was calculated.

**Statistical analysis**
All experiments in this study were repeated triplicate. Data are presented as the mean ± SEM. Statistical analysis was performed using ANOVA followed by Student’s t-test. P<0.05 was considered statistically significant.

**Results**

**Effect of gefinitib on OVA-induced airway inflammation and airway smooth muscle hypertrophy**
The effects of gefinitib on OVA challenge induced mice airway inflammation were detected by H&E staining. The results showed that, compared with control saline challenge, there is a significant increase of airway epithelial cell and smooth muscle cell proliferation, and cell infiltration (such as monocytes, lymphocytes, and polymorphonuclear cells) in the OVA challenge group. Meanwhile airway wall thickening, luminal stenosis, trachea epithelial cell shedding, and mucus plug were observed in the OVA challenge mice. After treatment with gefinitib, the prevention group showed a marked improvement in epithelial cell shedding,
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Airway wall thickness, airway epithelial cell proliferation, smooth muscle hyperplasia, luminal stenosis, and mucus plug, whereas the improvement effects in the treatment group were not obvious (Fig. 1A-1D). The inflammation score in the asthma group was much higher than in the control group (P<0.05). After treatment with gefinitib, the prevention group showed markedly decreased inflammation scores compared with the asthma group. The treatment group showed no obvious difference compared with the asthma group, whereas a significant difference was found between the gefinitib prevention group and the treatment group (Fig. 1E).

Airway smooth muscle hypertrophy in different groups was also measured; the results showed that the airway smooth muscle area is markedly increased in the asthma group compared with the control group, and hypertrophy of the airway smooth muscle was ameliorated in prevention group (Fig. 1F). Wam/Pbm values were significantly increased in asthma, and decreased in the gefinitib prevention group. Differences between the short-term gefinitib treatment group and the asthma group in Wam/Pbm were not significant (P > 0.05). Also, a significant difference in Wam/Pbm was found between the gefinitib prevention group and the treatment group (P > 0.05) (Fig. 1F).
Effect of gefinitib on cell count, MUC5AC and cytokine levels in BALF

Compared with control saline challenge, there is a marked increase of the total cell count and the eosinophil count in the BALF of OVA challenged mice. OVA challenge induced eosinophil recruitment compared with the control group, and the recruitment effect was a dramatic decline in the chronic gefitinib treatment group. However, there was a slight change of eosinophil recruitment in BALF of the short-term gefitinib treatment group (Fig. 2A). MUC5AC, IL-13, IFN-γ levels in BALF were determined by ELISA. As shown in Fig. 2B and 2C, OVA challenged mice induced substantial MUC5AC and IL-13 expression in BALF compared with the control group. In contrast, IFN-γ level was not different between the OVA challenged group and the control group (Fig. 2D). Gefitinib pretreated significantly reduced the MUC5AC and IL-13 levels in BALF ($p < 0.05$), whereas gefitinib treated did not show any significant effects on MUC5AC and IL-13 expression. Moreover, IFN-γ level did not show any large changes between the control and asthma groups, and the gefitinib pretreated or treated groups did not show significantly altered IFN-γ expression. These data indicated that the prevention effects of gefitinib on asthma are very effective, whereas the treatment effects produce little effect.

Fig. 2. Effects of gefitinib on the cell count, MUC5AC and cytokine levels in BALF of OVA-induced mice. (A) Gefinitib inhibit the total cell count and eosinophils in the BALF; (B-D) muc5ac level (B), IL-13 (C) and IFN-γ (D) levels in BALF of all groups. BALF was collected 24 h after the last OVA challenge, and then the cell count and the muc5ac and cytokine levels were detected. Data are shown as mean ± SEM. *$p < 0.05$, compared with the control group; # $p < 0.05$, compared with the asthma group; & $p < 0.05$, compared with the short-term gefinitib treatment group.
Effect of gefinitib on pEGFR expression

The above results indicated that chronic gefitinib treatment can decrease the inflammatory cells and inflammatory cytokines. EGFR expression reflects the airway epithelial damage and activation, and increases in proportion to asthma severity [19, 20]. Gefinitib is a tyrosine kinase inhibitor that targets EGFR, thus, we first detected the EGFR expression in all groups of mice using western blotting. The results showed that p-EGFR is poorly expressed in lung tissue of normal mice, whereas there is a marked up-regulation of p-EGFR level in the asthmatic mice. In the chronic gefitinib treatment asthmatic mice model, the expression of p-EGFR was significantly decreased (P<0.05). However, p-EGFR level was slightly reduced in the short-term gefinitib treatment asthmatic mice model (Fig. 3).

Effect of gefinitib on goblet cell hyperplasia

There is some evidence to suggest that EGFR activation leads to mucin muc5ac synthesis and goblet cell production in rats [13], and is an inhibitor of EGFR tyrosine kinase-inhibited goblet cell production in OVA-sensitized rats [14]. OVA challenge induced mice goblet cell hyperplasia and mucus production was determined by PAS staining. The results suggested that there was a significant increase in goblet cell hyperplasia and mucus hypersecretion in the lung tissue in OVA-challenged mice, but not in control mice. The OVA-induced goblet cell hyperplasia and mucus secretion was significantly abated by chronic gefitinib treatment compared with the control group. The effects of short-term gefinitib treatment on goblet cell hyperplasia and mucus hypersecretion were insignificant (Fig. 4A-4D).

Goblet cell area and perimeter of the bronchial basement membrane (Pbm) of mice were also measured. Goblet cell area /Pbm values were increased in asthma compared with the control group, and decreased in gefitinib prevention group compared with the asthma group (P<0.05). Differences between short-term gefitinib treatment group and asthma group
in goblet cell area /Pbm were not significant (P>0.05), whereas a significant difference was found between the gefinitib prevention group and the treatment group (P>0.05) (Fig. 4E).

**Effect of gefinitib on goblet cell apoptosis**

A large body of evidence suggested that gefinitib could induce cancer cell apoptosis through blocking the expression of EGFR, such as lung adenocarcinoma and gallbladder adenocarcinoma cells [37-39]. EGFR is highly expressed in the asthma mice model, and the above results suggested that gefinitib could inhibit the expression of EGFR. We therefore hypothesized that gefinitib may induce goblet cell apoptosis in the asthmatic mice model. The apoptosis of goblet cells in all groups was detected by the TUNEL staining method. The results showed that OVA challenged mice, chronic gefitinib treatment group mice and short-term gefinitib treatment group mice induced an increase in goblet cell apoptosis compared with the control group, and the short-term gefinitib treatment group induced apoptosis of a large number of goblet cells. Differences between the gefinitib prevention group and the asthma group in goblet cell apoptosis were not significant (P > 0.05) (Fig. 5).

**Effect of gefinitib on apoptosis-associated genes**

Above results indicated that short-term gefinitib treatment group induced an increase in goblet cell apoptosis. A large body of evidence suggested that cell apoptosis was controlled by a series of genes, such as Bcl-2 and Bax; the up-regulation of Bcl-2 inhibits multiple forms of cell apoptosis, whereas overexpression of Bax accelerates cell apoptosis [40, 41]. We next measured the effect of gefinitib on the expression of Bcl-2 and Bax by western blot. The results in Fig. 6 indicate that down-regulation of the anti-apoptosis gene Bcl-2 was observed in asthmatic mice, chronic gefitinib treatment mice and short-term gefinitib treatment mice, whereas the expression of Bax was up-regulated in mice of the three groups. Moreover, a
A drastic decrease in Bcl-2 level was observed in the short-term gefinitib treatment group, and there was a significant increase of Bax expression in the group compared with the asthma and chronic gefitinib treatment groups (Fig. 6).

**Discussion**

Takeshi Kitazaki and his colleague demonstrated that gefitinib could effectively improve mucin secretion within 24 h in patients with bronchioloalveolar carcinoma [42].
EGFR signaling also has been involved in goblet cell metaplasia [14]. There is some evidence to suggest that increases in EGFR have been involved in the severity of chronic asthmatic subjects. Moreover, B. Boris Vargaftig et al. showed that AG-1478, an EGFR tyrosine kinase inhibitor, could inhibit inflammation, BHR, and lung remodeling in Ova model and could regulate LT, IL-13, and monocyte chemoattractant protein-1 induced lung injury [43]. These reports indicated that EGFR pathway is involved in asthma-like syndrome. We therefore investigated whether the EGFR tyrosine kinase inhibitor gefitinib can suppress the symptoms in allergic asthma, such as airway inflammation and airway remodeling. A well-characterized murine model of OVA-induced allergic asthma was used, in which OVA exposure results in airway inflammation, goblet cell and smooth muscle hyperplasia, and mucus hypersecretion. Consistent with B. Boris Vargaftig reports[43], our results show that chronic gefitinib treatment of asthmatic mice effectively ameliorated the symptoms of asthma, such as the recruitment of eosinophils, the release of IL-13, MUC5AC secretion, and goblet cell and smooth muscle hyperplasia. However, the most symptoms of allergic asthma did not effectively improve in the short-term gefitinib treatment mice compared with the asthma group in addition to goblet cell hyperplasia. The results indicated that the development of airway inflammation, mucus hypersecretion and airway remodeling was negatively correlated with the chronic treatment of gefitinib.

Some evidence suggested that EGF and EGFR expression is up-regulation in asthmatic human airway epithelium compared with control population, suggesting that EGF may play an important role in the pathophysiology of bronchial asthma [44]. The present study indicated that chronic gefitinib treatment and the treatment of asthmatic mice markedly inhibited goblet cell hyperplasia. Moreover, we also observed that the apoptosis rates of goblet cell in the OVA challenged group, chronic gefitinib treatment group and short-term gefitinib treatment group were all higher than in the control group, and the highest apoptosis rate appeared in the short-term gefitinib treatment group. There is some evidence that Bcl-2 family has various pairs of antagonist and agonist proteins (such as Bax and Bcl-2) that regulate apoptosis [45, 46]. Our study showed that the expression of Bax was up-regulated after gefitinib short-term gefitinib treatment. In contrast, the expression of Bcl-2 was significantly down-regulated in gefitinib-treated mice. These results suggested that gefitinib promoted the apoptosis of goblet cells in asthmatic mice, potentially by regulating the expression of Bax and Bcl-2.

Emerging studies have revealed that short-term treatments by using EGFR inhibitors in the ovalbumin (OVA)-induced allergic asthma model could mediates allergic airway remodeling, allergic airway inflammation, mucin production in airways and BHR. Also, a recent study found that chronic treatment by using EGFR inhibitor in a chronic allergic asthma model could reduce AHR and ASM thickening. These results indicated that EGFR signaling play an important role in asthma. Consistent with these previously published studies, our study showed that chronic gefitinib treatment group resulted in airway epithelial shedding, smooth muscle hyperplasia improvement and goblet cell hyperplasia decrease, whereas there was no significant change in cell apoptosis. Moreover, our study showed that short-term gefitinib treatment significantly increased cell apoptosis, whereas chronic gefitinib treatment has no obvious effects on goblet cell apoptosis compared with the asthma group. These results suggested that gefitinib treatment or pretreatment may have a different mechanism on asthma airway remodeling. Thus, the main mechanism of the effects of chronic gefitinib treatment on asthma mice may inhibit cell proliferation. While short-term gefitinib treatment resulted in an increase in cell apoptosis, airway epithelial shedding and a decrease in goblet cell hyperplasia. These results indicated that gefitinib-induced apoptosis may be a pathway by which gefitinib reduces asthma symptoms. However, the specific mechanism of gefitinib on asthma needs further studies.
Conclusion

In conclusion, the findings from our study suggested that gefitinib effectively inhibits goblet cell and smooth muscle hyperplasia, airway inflammation and airway mucus hypersecretion. Moreover, we first observed that gefitinib induced goblet cell apoptosis in short-term gefitinib treatment asthmatic mice. However, there were no effects in the asthmatic mice after short-term gefitinib treatment, and the specific cause and mechanism are still unclear. Chronic gefitinib treatment through the allergen exposure period inhibits the asthma pathology significantly whereas late EGFR inhibition does not apart from some effects on apoptosis. This raises the possibility that EGFR inhibitor therapy would need to be chronic and start early to potentially be of benefit. Further studies will focus on providing some evidence for the mechanism of gefitinib. These findings indicate that chronic gefitinib treatment group may be an effective therapy against human asthma.

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Disclosure Statement

The authors have declared that no competing interests exist.

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