Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation

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Summary Interleukin-8 (IL-8; CXCL8) is a cytokine of the CXC chemokine family that is involved in neutrophil recruitment and activation. In addition, IL-8 has been implicated in a wide variety of other processes, including angiogenesis and metastasis in lung cancer. Lung adenocarcinoma and muco-epidermoid carcinoma cells produce substantial amounts of IL-8, and express both CXCR1 and CXCR2 IL-8 receptors. We hypothesized that IL-8 stimulates proliferation of non-small cell lung cancer cells, involving transactivation of the epidermal growth factor receptor (EGFR). The EGFR plays a central role in regulating cell proliferation and it has been therefore implicated in lung cancer. Both EGFR ligands and transactivation of the receptor may lead to downstream signalling events, including mitogen-activated protein kinase (MAPK) activation. Transactivation of the EGFR has been shown to occur in response to ligands of various G-protein coupled receptors (GPCRs) and involves metalloproteinase-mediated release of membrane bound EGFR ligands. The aim of the present study was to investigate the effect of IL-8 on proliferation of lung adenocarcinoma and muco-epidermoid carcinoma cells, and to explore the mechanisms leading to this proliferation in two different non-small cell lung cancer cell lines (A549 and NCI-H292). In both NSCLC cell lines, we observed that IL-8 stimulates epithelial cell proliferation in a dose-dependent manner. The ability of IL-8 to increase cell proliferation was blocked both by an inhibitor of EGFR tyrosine kinase, by a specific anti-EGFR blocking antibody and by a panmetalloproteinase inhibitor. Similar results were obtained using the GPCR inhibitor pertussis toxin. Inhibition of the MAPK p42/44 (ERK1/2) also blocked the mitogenic effect of IL-8, while a p38 MAPK inhibitor did not affect IL-8-induced cell proliferation. These results suggest that IL-8 increases cell proliferation in NSCLC cell lines via transactivation of the EGFR and that this mechanism involves metalloproteinase activity.

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1. Introduction

Interleukin-8 (IL-8) is a cytokine of the CXC chemokine family that acts as a ligand for two G-protein coupled receptors (GPCRs): the chemokine receptors CXCR1 and CXCR2. CXCR1 also binds a second CXC chemokine, granulocyte chemotactic protein 2 (GCP-2), whereas CXCR2 mediates cell activation by several CXC chemokines, including IL-8 and growth-related oncogene (GRO) α and γ [1]. In addition to its role in neutrophil recruitment and activation, IL-8 is thought to be involved in a wide variety of other processes, including angiogenesis and metastasis formation in lung cancer. Whereas primary bronchial epithelial cells [2] express only CXCR2, human lung cancer cells express both CXCR1 and CXCR2 [3]. In non-small cell lung cancer (NSCLC), expression of IL-8 in the tumor was associated with angiogenesis [4] suggesting a role of IL-8 in tumor growth through increased angiogenesis [5]. Furthermore, IL-8 concentrations correlate with tumor progression, patient survival and timing and relapse in the same type of lung cancer [6]. In vitro studies showed that IL-8 stimulates the proliferation of various human melanoma cell lines in a dose-dependent manner, suggesting that IL-8 can be an autocrine growth factor for human epithelial cells and that IL-8 is involved in metastasis formation [7]. These findings indicate that epithelial cell proliferation is a key feature of the epithelial changes observed in human lung cancer cells, including lung adenocarcinoma and muco-epidermoid carcinoma.

The epidermal growth factor (EGF) receptor (EGFR) is a central regulator of epithelial cell proliferation, as well as various other cellular processes in epithelial cells. Analysis of EGFR expression in human lung disease has provided evidence for its role in lung cancer. EGFR is overexpressed in 40–80% of NSCLC [8], and at least a subgroup of patients with a specific mutation in the EGFR gene have a marked clinical response to EGFR tyrosine kinase inhibitors gefitinib [9] and erlotinib [10]. These studies emphasize a role for EGFR in lung cancer.

EGFR signaling is triggered by the binding of EGF and EGF-like growth factors, resulting in the homodimerization of the EGFR molecules or heterodimerization with other closely related receptors, such as c-erbB2. Autophosphorylation and transphosphorylation of the receptors through their tyrosine kinase domains lead to the recruitment of downstream effectors and the activation of proliferative and cell survival signals. Downstream signaling pathways that are activated via the EGFR include phosphorylation of mitogen activated protein kinases (MAPK). Their activation partly determines cell fate, since activation of the MAPK extracellular-regulated kinase p42/44 (or extracellular-regulated kinase (ERK) 1/2) has been associated with cell survival and proliferation, whereas c-jun N-terminal kinases (JNK) and p38 MAPK are linked to induction of apoptosis [11].

There are conflicting data regarding the effect of IL-8 on epithelial cell proliferation. In some studies IL-8 was found to induce epithelial cell proliferation [3,12–15], whereas in others it decreased the proliferation of neoplastic epithelial cells [16]. However, no data are available on the effect of IL-8 on human lung cancer cell proliferation and the mechanisms that regulate such a mitogenic effect. The receptors for IL-8, CXCR1 and CXCR2, are G-protein coupled receptors (GPCRs). Many cellular functions are regulated by GPCRs, but more recently they have been recognized also as important mediators of non-inflammatory cell proliferation, migration and differentiation [17]. They were found to activate growth factor signalling cascades via additional mechanisms involving receptor tyrosine kinases phosphorylation, which is illustrated by various studies that have shown a cross-talk between GPCRs and EGFR. This was found to involve metalloprotease-mediated release of membrane-bound EGFR ligands that subsequently activate the EGFR, in a process called transactivation [18–20]. This transactivation of the EGFR via GPCR results in cellular activity such as proliferation and migration. Therefore, the aim of the present study was to investigate the effect of IL-8 on the proliferation of two NSCLC cell lines (A549: adenocarcinoma and NCI-H292: muco-epidermoid carcinoma), and the involvement of metalloprotease-mediated transactivation of EGFR in the mitogenic effect of IL-8. Part of the data shown in this manuscript have been presented to the European Respiratory Society Congress in 2003 [21].

2. Materials and methods

2.1. Cell culture

The human NSCLC cell lines A549 (adenocarcinoma) and NCI-H292 (muco-epidermoid carcinoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were routinely cultured in RPMI 1640 (Gibco, Grand Island, NY) medium containing 2 mM L-glutamine, penicillin (20 U/ml), streptomycin (20 μg/ml) (all from Bio Whittaker, Walkersville, MD), and 10% heat-inactivated FCS (Gibco, Grand Island, NY) at 37 °C in a humidified 5% CO₂ atmosphere. Cells were passaged weekly using TrypStat Versene (Bio Whittaker, Walkersville, MD).

2.2. Cell proliferation

Cell proliferation was assessed using 5-bromo-2-deoxyuridine (BrdU) incorporation as previously described [22]. Briefly, subconfluent cultures of A549 and NCI-H292 cells (80–90%) were incubated for 20 h with IL-8 (Peprotech, Rocky Hill, NJ). Next, BrdU (Sigma–Aldrich, St. Louis, MO) was added and the cells were incubated for another 4 h. Cells were washed twice in PBS and fixed in ethanol 70% (v/v) for at least 1 h. Cells were then permeabilized with 1 M hydrochloric acid followed by subsequent washes with 0.1 M sodium tetraborate and PBS. BrdU incorporation was demonstrated by incubation with a mouse anti-BrdU mAb (Dako, Golstrup, Denmark) followed by incubation with a peroxidase-labeled rabbit anti-mouse polyclonal antibody (Dako, Glostrup, Denmark). BrdU incorporation was visualized using Nova RED (Vector Laboratories, Burlingame, CA) and the percentage BrdU-positive nuclei was calculated.

In selected experiments using both cell lines, cells were preincubated for 1 h with various inhibitors: epidermal growth factor receptor (EGFR) inhibitor AG1478 (Calbiochem, La Jolla, CA); anti-EGFR monoclonal antibody (BD Transduction Laboratories); the metalloprotease inhibitor GM6001 (Chemicon, Temecula, CA); the GPCR
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2.3. Immunoblotting for MAPK p42/44

MAPK p42/44 phosphorylation was assessed using Western blotting technique as previously described [23]. Cells were cultured to near confluence, starved overnight for growth factors and subsequently stimulated for 5 min with IL-8 or transforming growth factor (TGF)-α (as a positive control). After washing with washing buffer (5 mM Tris, pH 6.4, 100 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂), cells were lysed in ice-cold lysis buffer (0.5% (v/v) Triton X-100, 0.1 M Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM Na₃VO₄, mini complete protease inhibitor cocktail [Roche, Basel, Switzerland]). Following incubation for 10 min on ice, cell lysates were centrifuged at 13,000 rpm for 5 min at 4°C to remove insoluble debris. Aliquots of the samples containing equal amounts of protein were suspended in reducing SDS-PAGE sample buffer and boiled for 5 min. Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes using the Mini-transblot System (both Biorad, Hercules, CA). These membranes were incubated in blocking buffer (0.05% Tween-20 in PBS containing 0.5% (w/v) casein) for 1 h, followed by overnight incubation with rabbit antibodies directed against phosphorylated (p) MAPK p42/44 at 4°C (New England Biolabs, Beverly, MA). After incubation with a secondary horseradish peroxidase (HRP) conjugated goat anti-rabbit polyclonal antibody (BD Transduction Laboratories, Franklin Lake, NJ), immunoreactivity was detected by electrochemiluminescent (ECL) detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.4. Statistical analysis

The data are expressed as mean ± S.E.M. Statistical analysis was performed with Student’s t-test for paired samples. Differences were considered statistically significant when P < 0.05.

3. Results

3.1. IL-8 induces proliferation of NSCLC cells

A549 and NCI-H292 NSCLC cells were treated with 0–500 ng/ml of IL-8 for 24 h. IL-8-induced cell proliferation in a concentration-dependent manner, an effect that reached statistical significance at the two highest concentrations in both A549 and NCI-H292 cells (500 and 200 ng/ml; Fig. 1A and B, respectively).

3.2. IL-8 stimulates cell proliferation via an EGFR- and metalloprotease-dependent pathway in NSCLC cells

To investigate the potential role of metalloproteases in GPCR-induced cell proliferation, we pretreated A549 adenocarcinoma cells with the metalloprotease inhibitor GM6001 (also known as Ilomastat or Galardin), the EGFR-specific inhibitor AG1478 (tyrphostin) or a blocking antibody against the EGFR, prior to IL-8 stimulation. The results show that IL-8-induced cell proliferation was blocked by AG1478 (1 μM), a blocking antibody against EGFR (2 μg/ml) and GM6001 (25 μM) (Fig. 2A–C). Similarly, inhibition of IL-8-induced cell proliferation by AG1478 and GM6001 was obtained using NCI-H292 cells (Table 1). In addition, pertussis toxin (PT) caused a dose-dependent inhibition of IL-8-induced cell proliferation of A549 cells (Fig. 2D).

3.3. Phosphorylation of MAPK p42/44, but not of p38 MAPK pathway, mediates IL-8-induced NSCLC cell proliferation

Since the EGFR plays a central role in the regulation of the MAPK signal, the MAPK pathways involved in IL-8-induced cell proliferation were explored. To study the role of MAPK p42/44 in IL-8-induced cell proliferation, MEK activity was inhibited using U0126, which is known to inhibit MEK phosphorylation by Raf. Preincubation of both A549 and NCI-H292 cells for 1 h with U0126 (25 μM) caused a signifi-
Fig. 2 Effect of signalling inhibitors on EGFR transactivation in A549 cells. The EGFR inhibitor, AG 1478 (1 μM) (A), the anti-EGFR monoclonal antibody (2 μg/ml) (B), the metalloprotease inhibitor GM6001 (25 μM) (C) and various concentrations of the GPCR inhibitor pertussis toxin (20–160 ng/ml) (D) have been used in these experiments. After preincubation for 1 h with the above described inhibitors, subconfluent cultures of A549 cells were incubated for 20 h with various concentrations of IL-8. Subsequently,
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Fig. 3  Effect of inhibiting signaling downstream of EGFR phosphorylation. The MEK inhibitor U0126 (25 μM; A), and the p38 inhibitor SB 203580 (25 μM; B) were used in these experiments. Subconfluent cultures of A549 cells were incubated for 20 h with various concentrations of IL-8. Next, BrdU was added and the cells were incubated for another 4 h and subsequently washed and fixed. BrdU incorporation was detected by immunocytochemistry (for details, see Section 2). The results are mean ± S.E.M. of three independent experiments, each performed in duplicate. *P < 0.05 vs. medium alone, +P < 0.05 vs. interleukin-8.

Table 1  Effect of inhibiting EGFR transactivation on IL-8-induced proliferation in NCI-H292 cells

<table>
<thead>
<tr>
<th>Stimulusa</th>
<th>Inhibitor</th>
<th>%BrdU positive nuclei</th>
<th>P-Value vs. control without inhibitor</th>
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<tbody>
<tr>
<td></td>
<td>Without inhibitor</td>
<td>With inhibitor</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>AG1478</td>
<td>29.7 ± 2.6</td>
<td>27.3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>GM6001</td>
<td>30.3 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>IL-8 (500 ng/ml)</td>
<td>AG1478</td>
<td>51.1 ± 1.3b</td>
<td>32.5 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>GM6001</td>
<td>29.8 ± 2.3</td>
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a For experimental details, see legend in Fig. 2. Both positive controls (TGF-α and FCS) caused a significant increase in the %BrdU positive nuclei— TGF-α: 48.7 ± 1.8 (P = 0.043 vs. medium); FCS: 53.8 ± 2.9 (P = 0.049).

b P = 0.029 vs. medium alone.

cant inhibition of IL-8-induced cell proliferation (Fig. 3A). In contrast, the p38 MAPK inhibitor SB203580 (25 μM) did not affect IL-8-induced cell proliferation (Fig. 3B) in A549 lung adenocarcinoma cells. Comparable results were obtained using NCI-H292 mucoepidermoid carcinoma cells (data not shown). Involvement of MAPK p42/44 in IL-8-mediated cellular activation was confirmed by Western blot analysis of phosphorylated ERK1/2 showing maximal activation of this MAPK after 5 min of exposure to IL-8 (Fig. 4).

BrdU was added and the cells were incubated for another 4 h and subsequently washed and fixed. BrdU incorporation was detected by immunocytochemistry (for details, see Section 2). The results are mean ± S.E.M. of three independent experiments, each performed in duplicate. *P < 0.05 vs. medium alone, +P < 0.05 vs. interleukin-8.
growth factors\[26\], cytokines and chemokines \[27,28\]. These mediators include essentially inflammatory cells. These mediators may be affected by growth factors produced by respiratory time (2 and 4 h in their study versus 24 h of exposure in proliferation of A549 lung carcinoma cells after IL-8 stimulation studies and our data, Wang et al. observed a decrease in proliferation, IL-8 increases the mitotic activity in explants of human epithelial cell proliferation\[3,12—15,24\]. In addition to producing IL-8 themselves, also tumor infiltrating inflammatory cells may increase production of IL-8 and ENA-78 \[30\]. This IL-8 may contribute to tumor cell proliferation and angiogenesis, but may also help to explain the marked association between neutrophilia and squamous cell carcinoma \[30,33,34\]. This is in line with our observation that the presence of neutrophil defensins and elastase is increased in areas with squamous cell metaplasia in smokers \[35\].

What is the mechanism involved in the proliferative response observed in NSCLC cells following IL-8 exposure? G-protein-coupled receptors (GPCR) are important mediators of cellular growth and differentiation \[17\]. It has been observed that GPCR activation induces a rapid increase in tyrosine phosphorylation of adaptors proteins \[36\]. This convergence of GPCR on receptor tyrosine kinases (RTK) is supported by observations that at least three RTKs (those for platelet-derived growth factor (PDGF) \[37,38\], EGF \[38,39\], and insulin-like growth factor-1 (IGF-1) \[39\]) become phosphorylated on tyrosine residues after GPCR activation). Furthermore, Prenzel et al. demonstrated that EGFR transactivation upon stimulation of GPCR involves proheparin binding (proHB)-EGF and a metalloproteinase-dependent cleavage of proHB-EGF that is rapidly induced upon GPCR–ligand interaction \[18\]. Our results are in line with this model, because we observed that IL-8-induced bronchial epithelial cell proliferation is blocked by inhibition of EGFR or of metalloproteinase activity. The EGFR effect was observed both when using the selective inhibitor of the phosphorylation of EGFR, tyrphostin AG1478, and by a blocking anti-EGFR antibody. Furthermore, we observed the same inhibition of NSCLC cell proliferation induced by IL-8 using the synthetic metalloproteinase inhibitor GM6001. Downstream signaling pathways that are (trans)activated via EGFR include phosphorylation of MAPK. Their activation partly determines cell fate, since activation of the MAPK p42/44 has been associated with cell survival and proliferation, whereas c-jun N-terminal kinases (JNK) and p38 MAPK are mainly linked to induction of apoptosis \[40\]. Using specific inhibitors, we demonstrated the involvement of the mitogenic MAPK pathway MAPK p42/44, but not of the pro-apoptotic MAPK pathway p38. Because the pro-apoptotic MAPK pathway p38 was not involved, we did not pursue to explore other MAPK pro-apoptotic pathways mediated by JNK.

Previous to our study, Venkatakrishnan et al. showed that stimulation of ovarian epithelial cells with recombinant human IL-8 resulted in the rapid activation of MAPK p42/44 and that EGFR participated in the transduction of GPCR-mediated IL-8 signals \[41\]. Using a human colon carcinoma cell line, Itoh et al. observed that IL-8-induced EGFR-mediated transactivation of the EGFR resulting in receptor phosphorylation. Finally, activity of MAPK p42/44 was found to be involved in the downstream signalling pathways from the EGFR.

This is not the first report to show that IL-8 increases epithelial proliferation, but it is the first to demonstrate the involvement of transactivation of the EGFR in this process. Various in vitro studies have demonstrated an effect of IL-8 on epithelial cell proliferation \[3,12—15,24\]. In addition, IL-8 increases the mitotic activity in explants of human duodenal mucosa in vivo \[25\]. However, in contrast to these studies and our data, Wang et al. observed a decrease in proliferation of A549 lung carcinoma cells after IL-8 stimulation \[16\], which may be explained by differences in the stimulation time (2 and 4 h in their study versus 24 h of exposure in the present study).

Cell proliferation is a key feature of tumor progression, which may be affected by growth factors produced by resident cells including epithelial cells and mediators produced by inflammatory cells. These mediators include essentially growth factors \[26\], cytokines and chemokines \[27,28\]. A role for IL-8 in lung cancer is supported by the observation of Arenberg et al. showing that treatment of mice inoculated with human NSCLC cells with neutralizing antibodies directed against IL-8 resulted in a marked reduction in tumor size \[5\].

Various studies have provided data supporting the concept that inflammation not only serves to suppress tumor growth, but also may contribute to tumor cell proliferation, survival and migration \[29\]. Our observations and studies showing the association between NSCLC and inflammation lend further support to this concept. It is highly likely that NSCLC are exposed to IL-8 in vivo. IL-8 and the related CXC-chemokine ENA-78 are markedly increased in the bronchoalveolar lavage fluid from patients with bronchoalveolar carcinoma, and their expression is observed in the majority of tumors \[30\]. IL-8 can be derived from inflammatory cells that infiltrate and surround the tumor and from the tumor itself. That the tumor itself may contribute to IL-8 production is suggested by various studies showing that epithelial IL-8 production is markedly increased by a wide range of stimuli, including cytokines \[30\], the EGFR ligands EGF and TGF-\(\alpha\) \[31\], but also by cigarette smoke, the main risk factor for lung cancer \[32\]. We confirmed that the EGFR ligand TGF-\(\alpha\) stimulates IL-8 release by both primary bronchial epithelial cells and epithelial cell lines (NCI-H292 and A549) (data not shown). Finally, in addition to producing IL-8 themselves, also tumor infiltrating inflammatory cells may increase production of IL-8 and ENA-78 \[30\]. This IL-8 may contribute to tumor cell proliferation and angiogenesis, but may also help to explain the marked association between neutrophilia and squamous cell carcinoma \[30,33,34\]. This is in line with our observation that the presence of neutrophil defensins and elastase is increased in areas with squamous cell metaplasia in smokers \[35\].
transactivation was mediated by an ADAM-dependent pathway, and that HB-EGF plays an important role as the major ligand for this pathway [12]. Our findings are in line with those observations, and extend these by showing that similar mechanisms are operative in NSCLC cells, and that a metalloprotease activity is likely involved in IL-8-induced transactivation of the EGFR.

In most of the studies exploring IL-8 levels in biological fluids, lower IL-8 concentrations have been detected compared to those used in the present study [42,43]. However, our results are in line with data obtained by Itoh et al. [12], who observed a significant IL-8-induced cell proliferation at the same IL-8 concentrations utilized in our study. Furthermore, IL-8 concentrations detected in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome (ARDS) are in the similar range as those used in the present study [44]. We speculate that, in addition to IL-8 produced by inflammatory cells, also epithelial cell-derived IL-8 (that may reach high local concentrations) may act in a paracrine or autocrine way to increase epithelial cell proliferation. In our study, the involvement of the GPCR CXCR2 chemokine receptor was supported by the observed inhibitory effect of pertussis toxin (PTX) [45], which inhibits G proteins by causing ADP-ribosylation of the β-subunit of G proteins. Furthermore, we used specific concentrations of the p38 phosphorylation inhibitor SB203580 [46–48], the EGFR phosphorylation inhibitor AG1478 [49–51] and the MEK inhibitor U0126 [47,52,53] based on the large body of evidence present in literature. The concentration of the GM6001 inhibitor is also in line with concentrations used in other studies [52,54,55]. In contrast, we tested a dose-response curve of the GPCR inhibitor pertussis toxin, because of the great variability reported in other studies concerning epithelial cells and particularly A549 cells.

Our results may add new elements to our insight into the role of inflammation in the local and systemic progression of lung cancer [56]. It lends further support to a role of neutrophils, that may be attracted by tumor-derived chemokines, in the progression of lung cancer. Their presence in the vicinity of the tumor may increase tumor growth by the release of various mediators that have been shown to increase proliferation of lung epithelial cells, including not only IL-8, but also growth factors, reactive oxygen intermediates and the antimicrobial peptides neutrophil α-defensins [22] and LL-37 [57] (Fig. 5). Furthermore, our study might provide a mechanistic basis for the observation that COPD patients are at increased risk for the development of lung cancer [58,59]. In addition, IL-8-induced cell proliferation may in part explain the increased number of proliferating cells observed in bronchial biopsies obtained from smokers [60] and may be involved in the epithelial changes observed both in smokers with and without airflow limitation [61]. Furthermore, overexpression of EGFR is an early and consistent finding in bronchial epithelium of smokers and in most NSCLC. It is present in basal cell hyperplasia and squamous metaplasia and reversal of bronchial metaplasia is associated with decreased EGFR expression [62], suggesting a pivotal role of this receptor in both cell proliferation, inflammation and in metaplastic changes. Therefore, a better understanding of the interrelationship between growth factors, inflammatory response and epithelial hyperplasia/metaplasia may lead to improved therapeutic interventions for the prevention and treatment of lung cancer. Our finding that IL-8-induced proliferation is mediated via EGFR also provides us with a therapeutic target for inhibition of this effect of IL-8.

5. Conclusion

In conclusion, the results from this study show a mitogenic effect of IL-8 on NSCLC cells which is mediated via transactivation of the EGFR. These results support the idea that IL-8 is involved in tumor progression in airway mucosa. Thus, IL-8 may be a key player in a complex network involving inflammation and cancer progression.

Conflict of interest

The authors declare that they do not have any conflict of interest and do not have any financial and personal relationships with other people or organisations that could have inappropriately influenced or biased this work.

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