Smoking-Induced CXCL14 Expression in the Human Airway Epithelium Links Chronic Obstructive Pulmonary Disease to Lung Cancer

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CXCL14, a recently described epithelial cytokine, plays putative multiple roles in inflammation and carcinogenesis. In the context that chronic obstructive pulmonary disease (COPD) and lung cancer are both smoking-related disorders associated with airway epithelial disorder and inflammation, we hypothesized that the airway epithelium responds to cigarette smoking with altered CXCL14 gene expression, contributing to the disease-relevant phenotype. Using genome-wide microarrays with subsequent immunohistochemical analysis, the data demonstrate that the expression of CXCL14 is up-regulated in the airway epithelium of healthy smokers and further increased in COPD smokers, especially within hyperplastic/metaplastic lesions, in association with multiple genes relevant to epithelial structural integrity and cancer. *In vitro* experiments revealed that the expression of CXCL14 is induced in the differentiated airway epithelium by cigarette smoke extract, and that epidermal growth factor mediates CXCL14 up-regulation in the airway epithelium through its effects on the basal stem/progenitor cell population. Analyses of two independent lung cancer cohorts revealed a dramatic up-regulation of CXCL14 expression in adenocarcinoma and squamous-cell carcinoma. High expression of the COPD-associated CXCL14-correlating cluster of genes was linked in lung adenocarcinoma with poor survival. These data suggest that the smoking-induced expression of CXCL14 in the airway epithelium represents a novel potential molecular link between smoking-associated airway epithelial injury, COPD, and lung cancer.

Keywords: CXCL14; airway epithelium; smoking; COPD; lung cancer

The airway epithelium, a pseudostratified layer of epithelial cells lining the tracheobronchial tree, is the initial cell population that interacts with inhaled environmental contaminants (1, 2). Among these, cigarette smoke, with its 4,000 compounds and greater than 10¹⁴ free radicals per puff, is a major stressor for airway epithelial cells, and is causally associated with the development

CLINICAL RELEVANCE

Chronic obstructive pulmonary disease (COPD) and lung cancer are the two commonest causes of mortality in the United States and worldwide. These two diseases share common features: most cases are caused by cigarette smoking, both begin in the airway epithelium and are associated with inflammation, and the presence of COPD increases the risk for lung cancer 4.5-fold. We found that CXCL14 expression is induced in the COPD airway epithelium in a smoking-dependent manner, and is correlated with genome-wide changes relevant to cancer. Further, a dramatic overexpression of CXCL14 occurred in lung adenocarcinoma and squamous-cell carcinoma. The high expression of genes coexpressed with CXCL14 in the COPD airway epithelium was associated with a significantly shorter survival of patients with lung adenocarcinoma. These observations suggest that CXCL14 is a potential link between smoking-associated COPD and lung cancer, and that CXCL14 represents a potential target of therapeutic approaches to prevent the development of lung cancer in susceptible individuals.

of chronic obstructive pulmonary disease (COPD) and lung cancer (1, 3–8).

COPD is a smoking-related disorder defined by irreversible airflow obstruction, associated with structural alterations in the small airways (9, 10), including the small airway epithelium (SAE), which becomes hyperplastic, secretes excessive amounts of mucus, and undergoes metaplastic changes (10–12). In addition to the epidemiological data establishing COPD as a risk factor for lung cancer, several lines of evidence indicate that the molecular mechanisms of COPD pathogenesis are relevant to smoking-induced lung cancer (5, 7, 8). Importantly, the airway epithelial pathology associated with COPD exhibits remarkable similarities to the pre-neoplastic phenotypes of smoking-induced lung cancer, with a continuum of hyperplastic, metaplastic, and dysplastic changes gradually evolving into an invasive phenotype (13).

Studies using mouse models suggest that COPD and lung cancer are both driven, in part, by chronic inflammation (14, 15). In response to cigarette smoke, cytokines orchestrate an abnormal inflammatory response of the airways, persisting even after smoking cessation, and resulting in reprogramming of the epithelial cell phenotype and function, airway remodeling, and tissue destruction (4, 6). Although the roles of classic inflammatory cytokines such as IL-6, IL-8, and TNF- α have been addressed in both COPD and lung cancer pathogenesis (7, 8, 16), little is known regarding the contributions of epithelial homeostatic cytokines to these smoking-related disorders.

(Received in original form October 3, 2012 and in final form April 8, 2013)

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This study was supported by National Institutes of Health grants P50 HL084936 and 1R01HL107882 (R.G.C.), UL1-RR024996 (Julianne Imperato-McGinley), and R25 CA10112 (Susanna Cunningham-Rundles). Rachel Sackrowitz was also supported by National Institutes of Health grant R25 CA10112. Renat Shaykhiyev was supported by the Parker B. Francis Foundation.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 49, Iss. 3, pp 418–425, Sep 2013

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Originally Published in Press at DOI: 10.1165/rcmb.2012-0396OC on April 18, 2013

Internet address: www.atsjournals.org

CXCL14, a recently described epithelium-derived chemokine, has a variety of functions relevant to inflammation and carcinogenesis (17–19). Several lines of evidence suggest that CXCL14 functions as a chemoattractant for mononuclear cells, including monocyte-derived dendritic cells (20–23). Based on the knowledge that cigarette smoking is associated with the recruitment of mononuclear cells to the lung (4, 6) and that the development of both COPD and lung cancer is linked to inflammation (4, 5, 7, 8, 24), we hypothesized that the airway epithelium may respond to cigarette smoke with an altered expression of CXCL14 as a part of the disease-relevant phenotype.

The present study demonstrates that CXCL14 expression is up-regulated in the airway epithelium of healthy smokers, further up-regulated in COPD, and dramatically more up-regulated in both major subtypes of non-small cell lung cancer, namely, lung adenocarcinoma (AdCa) and squamous-cell carcinoma. *In vitro* experiments revealed that CXCL14 is induced in the differentiated airway epithelium by cigarette smoke extract (CSE), and epidermal growth factor (EGF) mediates CXCL14 up-regulation in the airway epithelium through its effect on basal cells (BCs), which comprise stem/progenitor cells of the airway epithelium. Further, at the genome-wide level, the induction of CXCL14 in the SAE of COPD smokers was associated with changes in molecular pathways relevant to the regulation of tissue integrity and carcinogenesis. In lung AdCa, the high expression of CXCL14-correlated genes was associated with shorter survival. Thus, the present study identifies CXCL14 as a novel potential molecular link between smoking, COPD, and lung cancer.

MATERIALS AND METHODS

Datasets and Gene Expression Analyses

The SAE dataset, including 53 healthy nonsmokers, 59 healthy smokers, and 23 COPD smokers, was processed using HG-U133A and HG-U133A version 2.0 microarrays (Affymetrix, Santa Clara, CA), as previously described (25). The primary lung AdCa dataset originally described by Chitale and colleagues (26) included 91 samples processed using Affymetrix HG-U133A microarrays and 102 samples processed using HG-U133A version 2.0 microarrays. Independent lung-cancer datasets analyzed included those described by Bild and colleagues (27), which included 58 AdCa and 53 squamous-cell carcinoma (SqCa) samples processed using Affymetrix HG-U133A, and the dataset described by Kuner and colleagues (28), which included 40 AdCa and 18 SqCa samples processed by Affymetrix HG-U133A version 2.0 microarrays. Validation of the microarray CXCL14 gene expression data was performed using TaqMan gene expression analyses of the SAE samples of randomly selected healthy nonsmokers ($n = 12$), healthy smokers ($n = 11$), and COPD smokers ($n = 10$), as previously described (25).

Based on CXCL14 expression in the SAE, healthy smokers were divided into “CXCL14-high” (normalized gene expression level > 5 , $n = 8$) and “CXCL14-low” (normalized gene expression level < 0.25 , $n = 8$) expressors. The SAE transcriptomes of “CXCL14-high” and “CXCL14-low” healthy smokers were then compared with COPD smokers (criterion, $P < 0.05$ with Benjamini-Hochberg correction). Multiplatform gene expression data were normalized according to housekeeping gene ribosomal protein S18 (29). Genome-wide Spearman correlation analysis was performed to identify the genes whose expression in the SAE positively ($\rho \geq 0.4$, $P < 0.05$) correlated with the expression of CXCL14 in COPD smokers. The set of CXCL14 coexpressed genes was characterized using Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood City, CA) and data from the literature.

Airway BCs and Air-Liquid Interface

Airway BCs were isolated from the airway epithelium, obtained by bronchoscopic brushings using a cell-culture method, as described previously (30). The BC phenotype of isolated cells was confirmed by

positive staining for the BC marker keratin 5 ($> 98\%$ positive cells) and negative staining for markers of other cell types (30). The progenitor cell function of obtained BCs was confirmed by their ability to generate differentiated airway epithelia in air-liquid interface (ALI) culture, as described elsewhere (30). Briefly, BCs were seeded at a density of 2.0×10^5 cells/cm² onto a 0.4- μ m-pore-sized Costar Transwells inserts (Corning Incorporated, Corning, NY) precoated with Type IV collagen (Sigma-Aldrich, St. Louis, MO). The apical surface of the cell population was exposed to air as soon as the cells reached confluence (ALI Day 0). Epithelial differentiation was assessed by monitoring transepithelial resistance using a Millicell-ERS epithelial ohmmeter (Millipore, Bedford, MA), and morphologically by determining airway cilia formation (30).

To study the effects of cigarette smoke on CXCL14 expression in airway epithelium *in vitro*, airway epithelium differentiated after 28 days of culture in an ALI was exposed to CSE from the apical surface by adding 2% CSE, prepared as previously described (25). Control cells were treated with culture medium only. The apical medium containing CSE remained in the upper chamber until the next day, when the resistance was measured and the medium was replaced with fresh CSE-containing medium. The CSE stimulations were performed for 24 hours every other day during a 2-week period. In additional experiments, 2% CSE was applied directly to airway BCs for 1, 3, or 5 days in a submerged culture. After CSE exposure was completed, RNA was extracted and processed for TaqMan RT-PCR analysis.

In separate experiments, EGF (10 ng/ml; Sigma-Aldrich) was applied to airway epithelium that had been fully differentiated in ALI culture from either apical (to expose differentiated cells) or basolateral (to expose BCs) sides every other day for 2 weeks. In control groups, ALI medium without added EGF was applied. After stimulation, RNA was isolated as already described for TaqMan RT-PCR.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded biopsies of airway epithelium obtained by flexible bronchoscopy, normal lung tissue samples (US Biomax, Rockville, MD), and biopsy samples from patients with lung AdCa undergoing lung resection at the Memorial Sloan-Kettering Cancer Center (MSKCC), collected according to the protocol approved by the Institutional Review Board at MSKCC, using primary monoclonal rabbit anti-human CXCL14 antibody (1.5 μ g/ml; Epitomics, Burlingame, CA) according to previously described protocols (25, 31). Rabbit IgG (Invitrogen, Carlsbad, CA) was used as control.

Survival Analysis

For the survival analysis, 182 of 199 individuals of the original lung AdCa cohort described by Chitale and colleagues (26) with available microarray data and detailed clinical information, and 53 lung SqCa samples from the dataset of Bild and colleagues (27), were used. To determine the effects of CXCL14-associated gene expression changes in the SAE of COPD smokers on lung cancer survival, the initial set of CXCL14-coexpressed genes, as already described, was restricted to genes with a high correlation ($\rho \geq 0.6$, $P < 0.05$), designated the “CXCL14 cluster.” Of the 125 CXCL14 cluster genes, 91 were present on HG-U133A and HG-U133A version 2.0 arrays. A “CXCL14-cluster index” was calculated as the number of CXCL14 cluster genes having, in a given subject, an expression level above the normal expression level defined as a mean ± 2 standard deviations of the expression of a given gene in the SAE of healthy nonsmokers. Subjects were then categorized into “CXCL14 cluster-high” (index $>$ median for the cohort) and “CXCL14 cluster-low” (index $<$ median for the cohort) subgroups. The survival analysis was performed according to the Kaplan-Meier method. Multivariate analysis was performed using the Cox proportional hazard model.

Statistical Analysis

GeneSpring software (Agilent Technologies, Palo Alto, CA) was used to analyze microarray data. Differences with $P < 0.05$ adjusted by Benjamini-Hochberg correction were considered statistically significant. The differential expression of CXCL14 and the CXCL14 index

in the independent lung cancer cohorts (26) versus SAE samples and survival were analyzed by Mann-Whitney test (SPSS Statistical Package; SPSS, Inc., Chicago, IL).

RESULTS

Smoking and COPD Are Associated with Increased CXCL14 Expression in the Airway Epithelium

Microarray analysis revealed that the CXCL14 gene is expressed in the normal healthy airway epithelium at relatively low concentrations, and that CXCL14 is significantly up-regulated in healthy smokers compared with healthy nonsmokers ($P < 10^{-3}$; Figure 1A). Further increase in CXCL14 gene expression

was evident in COPD smokers compared with healthy smokers ($P < 5 \times 10^{-3}$; Figure 1A). No correlation was evident between the CXCL14 expression in the SAE of smokers and pack-years (Figure E1 in the online supplement), suggesting that the progressive up-regulation of CXCL14 in the COPD SAE is attributable to the disease itself rather than the longer smoking history of COPD smokers.

TaqMan RT-PCR was performed on RNA samples from the SAE of randomly selected healthy nonsmokers ($n = 12$), healthy smokers ($n = 11$), and COPD smokers ($n = 10$) (Table 1). The correlation between CXCL14 mRNA expression quantified by these two methods confirmed the microarray results ($\rho = 0.68$, $P < 0.002$; Figure 1B).

Figure 1. Expression of CXCL14 in the airway epithelium. (A) Normalized CXCL14 gene expression levels were based on microarray analyses in the small airway epithelium (SAE) of healthy nonsmokers ($n = 53$), healthy smokers ($n = 59$), and smokers with chronic obstructive pulmonary disease (COPD) ($n = 23$). (B) Comparison of normalized CXCL14 expression according to microarray (y axis) and TaqMan PCR (x axis). In A and B, each circle represents an individual subject. Green denotes healthy nonsmokers, orange denotes healthy smokers, and blue denotes COPD smokers. Normalized CXCL14 expression was determined according to microarray versus CXCL14 expression, as ascertained by TaqMan PCR. The correlation coefficient (ρ) is derived from Spearman correlation analysis. P values indicate the significance of correlations. (C) Immunohistochemical analysis of the CXCL14 protein expression in the airway epithelium of healthy nonsmokers (upper), healthy smokers (middle), and COPD smokers (lower). Scale bars = 20 μm . (D) Volcano plots compare genome-wide expression in the SAE of COPD smokers ($n = 23$) versus "CXCL14-low" healthy smokers (upper; $n = 8$; normalized CXCL14 expression level, < 0.25) and "CXCL14-high" healthy smokers (lower; $n = 8$; normalized CXCL14 expression level, > 5). (E) Normalized expression of the CXCL14 gene after stimulation of the air-liquid interface (ALI)-differentiated airway epithelium with cigarette smoke extract (CSE; 2%) from the apical side every other day for 14 days, compared with unstimulated cells. (F) Normalized expression of the CXCL14 gene in ALI-differentiated airway epithelium after 2-week stimulation with epidermal growth factor (EGF) (10 ng/ml) or medium alone (control) from the basolateral or apical sides. P values are shown for significant differences between groups. N.S., no significance.

TABLE 1. DEMOGRAPHICS OF THE STUDY POPULATION AND BIOLOGIC SAMPLES

Parameter	Healthy Nonsmokers	Healthy Smokers	Smokers with COPD
<i>n</i>	53	59	23
Sex, male/female	38/15	39/20	18/5
Age, years	41.0 ± 11.3	42.9 ± 7.2	51.7 ± 8.4 [§]
Race, B/W/O	24/20/9	35/14/10	8/10/5
Smoking history, pack-years		27.6 ± 16.9	40.8 ± 27.6 [‡]
Urine nicotine, ng/ml		1,216 ± 1,604	1,069 ± 1,126
Urine cotinine, ng/ml		1,273 ± 1,022	1,337 ± 592
Blood carboxyhemoglobin, %	0.4 ± 0.7	1.8 ± 1.8 [‡]	2.9 ± 1.9 [§]
Pulmonary function parameters			
FVC	107 ± 13	109 ± 13	97 ± 20 [§]
FEV ₁	105 ± 14	107 ± 14	74 ± 20 [§]
FEV ₁ /FVC	81 ± 6	80 ± 5	61 ± 8 [§]
TLC	101 ± 13	101 ± 12	103 ± 21
DLCO	99 ± 15	94 ± 11 [‡]	75 ± 18 [§]
GOLD stage, I/II/III			9/12/2
Medication use			
β-agonist			7
Anticholinergic			2
Inhaled corticosteroid			3
Epithelial cells*			
Number recovered × 10 ⁶	6.6 ± 2.9	7.2 ± 3.0	6.7 ± 3.1
Percent epithelial cells [†]	99.1 ± 1.4	99.0 ± 1.5	98.7 ± 1.6
Percent inflammatory cells	0.9 ± 1.4	0.9 ± 1.5	1.3 ± 1.6
Differential cell count			
Ciliated, %	73.0 ± 8.3	65.8 ± 12.4 [‡]	62.9 ± 10.9 [‡]
Secretory, %	6.7 ± 3.5	9.0 ± 4.5 [‡]	12.1 ± 5.6 [§]
Basal, %	12.0 ± 5.9	12.6 ± 6.6	12.1 ± 6.2
Undifferentiated, %	7.3 ± 3.2	11.9 ± 6.6 [‡]	11.5 ± 3.6 [‡]

Definition of abbreviations: B, black; DLCO, diffusing capacity; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; O, other; TLC, total lung capacity; W, white.

Data are presented as means ± standard deviations. Smokers with “established COPD” were defined by the GOLD criteria (9). The COPD smoker group included GOLD I (*n* = 9), GOLD II (*n* = 12), and GOLD III (*n* = 2). Pulmonary function testing parameters are given as percentages of the predicted value, with the exception of FEV₁/FVC, which is reported as percent observed. For individuals with COPD, the FVC, FEV₁, and FEV₁/FVC comprise postbronchodilator values.

* Small airway epithelium.

[†] As a percentage of small airway epithelium recovered.

[‡] *P* < 0.05, versus healthy nonsmokers.

[§] *P* < 0.05, versus both healthy nonsmokers and healthy smokers.

Immunohistochemical analysis demonstrated that CXCL14 protein expression in the healthy airway epithelium was heterogeneous, with remarkable up-regulation in smokers.

Whereas in smokers with morphologically normal airway epithelium, CXCL14 was predominantly expressed in ciliated cells (Figure 1C, *middle upper*), in smokers with BC hyperplasia and squamous metaplasia, virtually all epithelial cells were strongly positive, with basal and intermediate cells demonstrating the highest expression levels of the CXCL14 protein (Figure 1C, *middle lower*). In the airway epithelia of COPD smokers, high CXCL14 expression was observed within the hyperplastic and metaplastic regions (Figure 1C, *right*).

We next determined whether high CXCL14 expression defines a subset of healthy smokers, who, despite being clinically healthy, at the global gene expression level exhibit a COPD-like molecular phenotype. Genome-wide analysis revealed that, whereas 610 gene probe sets were differentially expressed in the CXCL14-low healthy smokers versus COPD smokers (Figure 1D, *upper*; see MATERIALS AND METHODS for details), CXCL14-high healthy smokers were indistinguishable from COPD smokers (Figure 1D, *lower*). Thus, high expression of the CXCL14 gene

reflects the acquisition of the COPD-like molecular phenotype in the airway epithelium of smokers.

Cigarette Smoke–Dependent and EGF Receptor–Mediated CXCL14 Up-Regulation in the Airway Epithelium *In Vitro*

To determine whether CXCL14 up-regulation in the airway epithelium of smokers is attributable to the direct effect of smoking on airway epithelial cells, we used an *in vitro* model in which airway epithelium differentiated from airway BC stem/progenitor cells on ALI was exposed to a nontoxic concentration of CSE (2%) from the apical surface for 2 weeks. The analysis revealed that persistent CSE exposure resulted in a significant CXCL14 up-regulation in the differentiated airway epithelium (Figure 1E). By contrast, the direct stimulation of undifferentiated airway BCs with CSE in a submerged culture did not induce CXCL14 expression (not shown).

To provide insights into the mechanism regulating CXCL14 gene expression in the airway epithelium, we focused on the EGF/EGF receptor (EGFR) pathway, known to be activated by cigarette smoking in association with hyperplastic/metaplastic changes in the airway epithelium (32, 33). Based on our previous observations that smoking induces EGF in differentiated cells of the airway epithelium, and that EGFR expression is enriched in BC stem/progenitor cells (33), we stimulated differentiated airway epithelium with EGF in the ALI model for 2 weeks. The data demonstrated that, when EGF was added from the basolateral side, but not from the apical surface, significant CXCL14 up-regulation occurred (Figure 1F).

Up-Regulation of CXCL14 in the COPD SAE Is Associated with Cancer-Related, Transcriptome-Wide Changes

The data from the genome-wide analysis we have described suggest that CXCL14 up-regulation in the SAE is a feature of the COPD-like molecular phenotype. To better understand the biological phenotype associated with CXCL14 overexpression in the SAE of COPD smokers at the transcriptome-wide level, we performed a genome-wide correlation analysis, which identified 2,336 gene probe sets significantly correlating with CXCL14 expression ($\rho \geq 0.4$, *P* < 0.05) in the SAE of COPD smokers (Table E1). This gene set was enriched in molecular pathways related to the regulation of tissue integrity and cancer (Figure 2A), and in molecular features relevant to various aspects of epithelial differentiation, such as components of epithelial junctions, keratins, membrane-associated mucins, squamous metaplasia, and epithelial carcinogenesis (Figure 2B).

CXCL14 Is Up-Regulated in Human Lung Cancer

Based on the data indicating that CXCL14 gene expression in the SAE of COPD smokers is associated with transcriptome-wide changes related to carcinogenesis, we hypothesized that lung cancer is characterized by a further up-regulation of CXCL14 gene expression. The analysis of three independent non-small-cell lung cancer (NSCLC) cohorts revealed dramatic CXCL14 gene overexpression in two major NSCLC subtypes, namely, AdCa and SqCa (Figure 3A). The up-regulation of CXCL14 expression in lung cancer was confirmed by an immunohistochemical analysis of lung AdCa samples (Figure 3B).

High Expression of the COPD–SAE CXCL14–Coexpressed Genes Correlates with Poor Survival in Patients with Lung AdCa

Next, we asked whether molecular features associated with CXCL14 expression in the SAE of COPD smokers correlated with tumor

Figure 2. Characterization of CXCL14-associated, transcriptome-wide changes in the SAE of COPD smokers. (A) Ingenuity molecular pathway analysis. Molecular pathways with strong contributions by genes correlating with CXCL14 expression in the SAE of COPD smokers (Spearman correlation $\rho \geq 0.4$, $P < 0.05$; Table E1) are shown on the *abscissa*. The “ratio” (the number of pathway genes that correlate with CXCL14 expression, compared with the total number of genes in the pathway) is plotted on the *ordinate*. The significance of enrichment (P value based on the Fisher exact test) is indicated for each pathway. (B) Functional category analysis. Genes correlating with CXCL14 in the SAE of COPD smokers, identified as already described, were grouped into dominant functional categories, based on a search of the available literature. Nodes indicate individual genes; gene symbols and coefficients of correlation with CXCL14 (Spearman ρ) are shown. Edges indicate the relationship between the genes and associated functional categories.

aggressiveness, when overexpressed in lung cancer. To accomplish this, we first increased the robustness of the CXCL14-associated COPD–SAE gene set we have already described by restricting it to genes with the highest level of correlation with CXCL14 gene expression ($\rho \geq 0.6$, $P < 0.05$). The generated gene set was designated a “CXCL14 cluster.” Consistent with the differential CXCL14 gene expression in lung cancer, the overall expression of the CXCL14-cluster genes was higher in lung AdCa and SqCa versus nonmalignant airway epithelium (Figure E2A).

Based on Kaplan-Meier analysis, the CXCL14-cluster-high lung AdCa patients (see MATERIALS AND METHODS) demonstrated shorter overall survival compared with the CXCL14-cluster-low AdCa patients (log rank, $P < 0.003$; Figure 3C). Multivariate analysis, in which other clinical covariates such as age, sex, smoking status, and pathological stage were included, demonstrated that the high expression of CXCL14-cluster genes was an independent prognostic factor associated with the shorter survival of individuals with lung AdCa (hazard ratio, 2.14; 95% confidence interval, 1.38–3.34; $P < 0.002$; Table 2). By contrast, in squamous-cell carcinoma, the high expression of CXCL14 cluster genes was not associated with shorter survival (Figure E2B).

DISCUSSION

Evidence is accumulating that the pathogenesis of COPD and lung cancer, the major smoking-associated lung diseases, is dependent, at least in part, on progressive changes in the airway epithelium evoked by chronic exposure to cigarette smoking (1, 4–7). The two diseases are further linked by the epidemiologic data demonstrating that COPD *per se* is a risk factor for lung cancer, independent of smoking (7, 8). The results of the present study indicate that CXCL14, a recently described

epithelium-derived chemokine implicated in the homeostatic regulation of local immune processes and tissue integrity (17–23), is expressed in the human airway epithelium, where it is induced by cigarette smoking, and further increased in COPD smokers and lung-cancer tissue in association with the genome-wide molecular changes related to cancer and to the aggressiveness of lung cancer. CXCL14, therefore, represents a novel molecular link between smoking, COPD, and lung cancer.

CXCL14 Regulation in the Airway Epithelium

The data in the present study show that CXCL14 is expressed in the human airway epithelium, similar to other epithelia (18–23, 34–37), and is up-regulated in response to chronic cigarette smoking. In smokers with preserved airway epithelial architecture, ciliated cells comprised the major population of CXCL14-expressing cells. However, in smokers with disordered airway epithelium, the highest CXCL14 protein expression levels were detected within the areas of BC hyperplasia and squamous metaplasia. The change in the histologic pattern of CXCL14 expression in smokers parallels the progressive rearrangement of airway epithelial architecture by smoking, in which ciliated cells, the major differentiated cell population in the normal airway epithelium (2), are gradually replaced by squamous cells, a phenomenon known as squamous metaplasia (11, 13). Both ciliated cells in normal airways and squamous cells in metaplastic lesions originate from BCs, the stem/progenitor cell population of the airway epithelium (12, 38, 39), suggesting that smoking can modify airway BCs to generate CXCL14-expressing progenies.

Consistent with these *in vivo* data, CSE induced CXCL14 expression in the differentiated airway epithelium *in vitro*, suggesting that CXCL14 up-regulation is a result of the direct

Figure 3. Analysis of the relationship between CXCL14 and lung cancer. (A) Box plot shows normalized expression of the CXCL14 gene (log 2, y axis) in the SAE of healthy nonsmokers, the SAE of healthy smokers, the SAE of COPD smokers, primary lung adenocarcinoma (AdCa) from the dataset of Chitale and colleagues (26), and lung AdCa and SqCa samples from the datasets of Bild and colleagues (27) and Kuner and colleagues (28). *P* values are based on the Mann-Whitney test. (B) Immunohistochemical analysis shows the up-regulation of the CXCL14 protein in lung AdCa (right), compared with normal lung tissue (left). Scale bar = 50 μ m. (C) Kaplan-Meier analysis-based estimates of overall survival of the CXCL14 cluster-high (red) versus CXCL14 cluster-low (blue) subjects with lung AdCa from the dataset of Chitale and colleagues (26). The number of individuals in each group, the median survival time (MST) for each group, and the *P* value determined by log-rank test are indicated.

effect of cigarette smoke components on the airway epithelium. Importantly, CXCL14 was not induced when CSE was applied to undifferentiated BCs in a submerged culture, suggesting that the presence of differentiated cells is important for the induction of CXCL14 via smoking. Previous studies showed that smoking induces EGF in differentiated cells of the airway epithelium, that EGFR expression is enriched in airway BCs (33), and that EGFR mediates smoking-related metaplastic changes in the airway epithelium (32, 33). Our *in vitro* experiments showed that CXCL14 is induced after the EGF stimulation of airway BCs, but not of differentiated cell populations. Thus, one mechanism of CXCL14 up-regulation in the airway epithelium may involve the smoking-induced, EGF-mediated reprogramming of the differentiation of BC stem/progenitors toward CXCL14-expressing progenies, in association with squamous metaplasia.

CXCL14 Is a COPD-Associated Gene in the Airway Epithelium

The persistent inflammation induced in response to cigarette smoke has been proposed to drive the structural changes in the lung leading to COPD (4–6, 8, 9). Multiple studies showed

that components of cigarette smoke are capable of inducing proinflammatory cytokines in human airway epithelial cells *in vitro* and in experimental animal models (4, 6, 14, 15). However, little is known about the contributions of homeostatic epithelial chemokines such as CXCL14 to the COPD-relevant molecular phenotype. The present study demonstrates that in the SAE of COPD smokers, further significant up-regulation of CXCL14 occurs, compared with both healthy nonsmokers

TABLE 2. MULTIVARIATE COX REGRESSION ANALYSIS OF THE EFFECT OF THE CXCL14 CLUSTER EXPRESSION ON LUNG ADENOCARCINOMA SURVIVAL*

Covariates	Hazard Ratio	95% Confidence Interval	<i>P</i> Value
Age, continuous	1.03	1.01–1.06	< 0.007
Sex, female	0.72	0.47–1.10	> 0.1
Smoking	1.95	1.05–3.65	< 0.04
Advanced stage [†]	3.89	2.52–6.02	< 0.001
CXCL14 cluster-high [‡]	2.14	1.38–3.34	< 0.002

*Based on the lung adenocarcinoma dataset of Chitale and colleagues (26).

[†]Based on TNM Classification of Malignant Tumours staging (26, 49).

[‡]Based on the chemokine (C-X-C motif) ligand 14-cluster index (see MATERIALS AND METHODS for details).

and healthy smokers, implying that progressive CXCL14 up-regulation in COPD versus healthy smokers occurs not simply because of longer smoking histories, but rather because of the disease itself.

In further support of the concept that CXCL14 up-regulation in the airway epithelium is a COPD-associated molecular feature, high variability of CXCL14 gene expression was observed among clinically healthy smokers, with a subset exhibiting a relatively high ("COPD-like") expression of this gene. At the genome-wide transcriptome level, these healthy smokers were indistinguishable from smokers with COPD. At the protein level, the highest amounts of CXCL14 expression were detected in the areas of squamous metaplasia in the airways of COPD smokers and in a subset of healthy smokers. Squamous metaplasia is a smoking-inducible lesion in the airway epithelium associated with the development of COPD (12). Together, these data indicate that increased CXCL14 gene expression in the SAE of smokers defines a COPD-like molecular phenotype that likely precedes clinical manifestations of the disease.

CXCL14 Links COPD to Lung Cancer

Several lines of evidence indicate that COPD constitutes a risk factor for lung cancer, and that the mechanisms involved in COPD pathogenesis are relevant to smoking-induced lung cancer (5, 7, 8). However, the molecular pathways linking these two smoking-associated diseases remain unclear. Considerable data support the hypothesis that the persistent inflammation characteristic of COPD creates a microenvironment conducive to carcinogenesis (5, 7, 8, 14). Chronic inflammation has been definitively linked to carcinogenesis in a number of organs, with cytokines and chemokines playing a critical role in these processes (7, 8, 14, 15, 24, 40). With this background, and based on our observation that CXCL14 (34, 35, 41) is up-regulated in the COPD airway epithelium in a smoking-dependent manner, we hypothesized that transcriptome-wide changes in the airway epithelium associated with CXCL14 up-regulation in COPD smokers will provide clues about the molecular pathways common to COPD and lung cancer (34, 35, 41).

Genome-wide analysis identified more than 2,000 gene probe sets significantly correlated with CXCL14 gene expression levels in the SAE of COPD smokers, suggesting that the up-regulation of CXCL14 represents a part of complex molecular processes. Consistent with this observation, the forced expression of CXCL14 in a lung cancer cell line altered the expression of more than 1,000 genes (42). Functional analysis of the CXCL14-associated gene set revealed a significant enrichment of molecular features related to epithelial differentiation and carcinogenesis, including membrane-tethered mucin genes (MUC1, MUC4, and MUC16), keratin family genes (KRT6 and KRT7), and a diverse set of genes encoding intercellular-junction proteins relevant to the biologic phenotype of AdCa and SqCa, the two major NSCLC subtypes (12, 13, 43–45). Consistent with the activation of cancer-related features within the CXCL14-coexpressed gene set, an analysis of three independent NSCLC datasets revealed a dramatic up-regulation of CXCL14 in AdCa and SqCa, compared with both healthy and COPD airway epithelium. Moreover, the present study provides evidence that the high expression of the CXCL14-coexpressed genes in the COPD–SAE represents an independent prognostic factor associated with poorer survival in lung AdCa. Interestingly, the expression of the CXCL14 gene cluster in squamous-cell carcinoma did not correlate with the survival of patients with this subtype of NSCLC, suggesting that CXCL14-associated genome-wide changes may differentially contribute to the biologic and clinical heterogeneity of different subtypes of lung cancer.

Based on the available data, the relationship between CXCL14 expression and tumorigenesis is complex, tissue-specific, and cancer type-specific. In head, neck, and cervical squamous-cell carcinomas (34, 46) and lung and breast carcinoma cell lines (34, 35), CXCL14 expression is down-regulated, suggesting a role in tumor inhibition. In contrast, data from AdCas of the pancreas and breast show a stimulatory role for CXCL14 in tumorigenesis (34, 35). In breast cancer, the expression of CXCL14 has been associated with an aggressive clinical phenotype (47). Although CXCL14 expression suppresses growth in prostate cancer cell xenografts, CXCL14 mRNA expression is increased in localized human prostate cancer (48), and CXCL14 enhanced prostate tumor cell proliferation and migration (17). One recent study revealed that the CXCL14 gene is methylated in a significant proportion of lung AdCas, and that the re-expression of this gene in a lung cancer cell line, in which CXCL14 is epigenetically silenced, contributes to increased tumor cell death (42), suggesting that in lung cancer, CXCL14 may serve as a tumor suppressor. One limitation, however, makes it difficult to compare these earlier observations with the results of the present study, namely, the expression levels of the CXCL14 gene in lung AdCa in that previous study (42) were not compared with those in the SAE and/or lung tissue of subjects without cancer. Although the biologic role of CXCL14 in human lung cancer in the complex *in vivo* environment remains unclear, the data from our study provide direct evidence that the expression of CXCL14 is up-regulated in lung cancer patients at both the gene and protein levels, and that transcriptome-wide, CXCL14-associated molecular changes in the SAE of COPD smokers are associated with shorter overall survival in lung AdCa. Importantly, CXCL14-associated molecular changes in the airway epithelium of COPD smokers are relevant to the biologic and clinical phenotypes of lung cancer, suggesting that CXCL14 may represent a novel molecular link between smoking, COPD, and lung cancer.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank N. Mohamed for help in preparing the manuscript, and B. Ferris for help with CXCL14 immunohistochemistry.

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