Original Articles

Tobacco smoke induces production of chemokine CCL20 to promote lung cancer

Gui-Zhen Wang, Xin Cheng, Xin-Chun Li, Yong-Qiang Liu, Xian-Quan Wang, Xu Shi, Zai-Yong Wang, Yong-Qing Guo, Zhe-Sheng Wen, Yun-Chao Huang, Guang-Biao Zhou

Keywords: Tobacco smoke NNK Lung cancer CCL20

ABSTRACT

Tobacco kills nearly 6 million people each year, and 90% of the annual 1.59 million lung cancer deaths worldwide are caused by cigarette smoke. Clinically, a long latency is required for individuals to develop lung cancer since they were first exposed to smoking. In this study, we aimed to identify clinical relevant inflammatory factors that are critical for carcinogenesis by treating normal human lung epithelial cells with tobacco carcinogen nicotine-derived nitrosaminoketone (NNK) for a long period (60 days) and systematic screening in 84 cytokines/chemokines. We found that a chemokine CCL20 was significantly up-regulated by NNK, and in 78/173 (45.1%) patients the expression of CCL20 was higher in tumor samples than their adjacent normal lung tissues. Interestingly, CCL20 was up-regulated in 48/92 (52.2%) smoker and 29/78 (37.2%) nonsmoker patients (p = 0.05), and high CCL20 was associated with poor prognosis. NNK induced the production of CCL20, which promoted lung cancer cell proliferation and migration. In addition, an anti-inflammation drug, dexamethasone, inhibited NNK-induced CCL20 production and suppressed lung cancer in vitro and in vivo. These results indicate that CCL20 is crucial for tobacco smoke–caused lung cancer, and anti-CCL20 could be a rational approach to fight against this deadly disease.

Introduction

Lung cancer is the most common cause of death from cancer worldwide which accounts for 1.59 million deaths in 2012 [1] and represents one of the most fatal malignant neoplasms with a five year overall survival rate of only 15% for all stages combined [2]. It is estimated that 90% of the lung cancer deaths were caused by cigarette smoke [3]. Over 5000 compounds have been identified in cigarette smoke, including 73 compounds which are considered carcinogenic to either laboratory animals or humans by the International Agency for Research on Cancer. There are more than 20 compounds that are lung carcinogens, e.g., polycyclic aromatic hydrocarbons (PAHs) and nicotine-derived nitrosaminoketone (NNK), or tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [3]. Elucidation of molecular carcinogenesis of tobacco smoke and derived carcinogens is critical to develop successful prevention and treatment strategies for lung cancer.

Carcinogen NNK induces tumors of the lung in rats, mice, and hamsters, and plays a major role in lung carcinogenesis [4,5]. It is a genotoxic carcinogen, converted enzymatically in the body to highly electrophilic DNA-binding diazohydroxides and related intermediates which bind to DNA causing mutations in genes including KRAS [3]. Recent studies show that tobacco smoke promotes lung tumorigenesis by triggering IKKβ- and JNK1-dependent inflammation [6]. Cigarette smoke activates epithelial cells and macrophages and induces pulmonary inflammation and secretion of inflammatory cytokine and chemokine, such as tumor necrosis factor (TNF), transforming growth factor-β (TGF-β), interleukin-1 (IL-1), IL-6, IL-8, CXCL9 and CXCL10 [6–8]. NNK alone induced Akt-dependent proliferation and NFκB-dependent survival of non-small cell lung cancer cells, and NFκB is critical to NNK-induced inflammation [9]. NNK inhibits the production of IL-12, TNF and NO, but stimulates IL-10 and...
PGE2 release [10]. Lipopolysaccharide-elicited chronic lung inflammation significantly increases the risk of NNK-mediated lung tumorigenesis [11], and chronic extrinsic lung inflammation induced by NNK in combination with bacteria enhances lung tumorigenesis in mice [12]. The chronic inflammation may promote neoplasia by inducing preneoplastic mutation, cell proliferation, resistance to apoptosis, invasiveness, angiogenesis, and secretion of immune suppressive factors [8]. However, most of the above results were obtained from cellular and animal models, and studies in smoker patients are still warranted.

In this study, we used a cytokine/chemokine PCR array to uncover NNK-induced inflammatory factors in normal human epithelial 16HBE cells [13], and tested the expression of the most significant factor in 173 lung cancer patients. We also investigated how the inflammatory factor promoted lung cancer progression in vitro and in vivo. Our results showed that the expression of a chemokine CCL20 was elevated and inversely associated with prognosis in smoker lung cancer patients, providing a potential therapeutic target for this deadly disease.

Materials and methods

Patient samples
The study was approved by the research ethics committees of all participating sites. A total of 173 previously untreated non-small cell lung cancers (NSCLCs) were included (Table 1). Tumor and adjacent normal lung tissues were collected immediately after surgical resection and stored in liquid nitrogen until further use. Serum samples were obtained from 15 healthy donors and 30 NSCLC patients (15 smokers and 15 non-smokers).

Cell culture

The lung adenocarcinoma cell lines A549, NCI-H1795, NCI-H23 and HCC827, large cell lung cancer line NCI-H640 were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). Human bronchial epithelial cell line 16HBE [13] was obtained from Clonetics (Walkersville, MD). Human highly metastatic large-cell lung cancer line 95D, lung squamous cell carcinoma cell line L78, and lung adenocarcinoma cell line SPC-A-1 were obtained from the Cell bank of the Chinese Academy of Sciences (Shanghai). The cells were tested and authenticated by GoldeneyeTM20A STR method by Beijing Microreal Genetec Co., Ltd (Beijing, China) upon initiation of this study. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 (Gibco, California, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin [14]. Cell proliferation was evaluated using MIT assay [15].

Table 1
Baseline demographic characteristics of the 173 patients whose samples were analyzed for the expression of CCL20.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case, n</th>
<th>CCL20-high, n (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>173</td>
<td>78 (45.1)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>114</td>
<td>56 (49.1)</td>
<td>0.12</td>
</tr>
<tr>
<td>Female</td>
<td>55</td>
<td>20 (36.4)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>2 (50)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>92</td>
<td>48 (52.2)</td>
<td>0.05</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>78</td>
<td>29 (37.2)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>1 (33.3)</td>
<td>0.76</td>
</tr>
<tr>
<td>Age ≤65</td>
<td>115</td>
<td>51 (44.3)</td>
<td></td>
</tr>
<tr>
<td>≥65</td>
<td>49</td>
<td>23 (46.9)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>9</td>
<td>4 (44.4)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>102</td>
<td>42 (41.2)</td>
<td>0.34**</td>
</tr>
<tr>
<td>Squamous-cell carcinoma</td>
<td>55</td>
<td>27 (49.0)</td>
<td></td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>2</td>
<td>1 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>8</td>
<td>4 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>6</td>
<td>4 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td>0.001***</td>
</tr>
<tr>
<td>I</td>
<td>68</td>
<td>30 (44.1)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>24</td>
<td>12 (50.0)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>46</td>
<td>17 (40.0)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>15</td>
<td>10 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>20</td>
<td>9 (45.0)</td>
<td></td>
</tr>
</tbody>
</table>

* Chi-square test.

Adenocarcinoma versus squamous-cell carcinoma.

*** (1+II+III) versus IV.

RNA was extracted and transcribed to cDNA by using RT2 First Strand kit (Qiagen, Valencia, CA). The expression of inflammatory factors in 16HBE cells treated with or without NNK for up to 60 days was determined by Human Inflammatory Cytokines & Receptors RT2 Profiler PCR Array containing 84 cytokines/chemokines and their receptors (Qiagen, Valencia, CA). Real-time PCR was performed using the SYBR premixExTaP™ (Takara, Dalian, China) in CFX96 Real Time System (Bio-Rad, Hercules, CA) according to the manufacturer’s guidelines and the 2−ΔΔCT method [16]. Each sample was analyzed in triplicate and repeated 3 times, and the primer sequences were: GAPDH, forward, 5′-GAAAGTGAAGGTGCAGCT-3′, reverse, 5′-GGTTAGTATGGAATCGCCG-3′; β-actin, forward, 5′-GTGATGCTGTACCAAGG-3′, reverse, 5′-GCCATAGGTTGATCTGAACG-3′; Vimentin, forward, 5′-GACAACTTGGCGCTGAAACG-3′, reverse, 5′-GTCCTCGTGGTCTGGCAACT-3′; Snail, forward, 5′-CTCCCTCAGCATACAGGGAC-3′, reverse, 5′-CCAGGCGCTAAGTCTTGTCG-3′; CCL20, forward, 5′-GCCAGCAACTTTGAGCTGCT-3′, reverse, 5′-ATTTCGCCACACAAGACT-3′. For RT-PCR, the primer sequences were: CCL20, forward, 5′-ATGGTGTCAGAACAGATG-3′, reverse, 5′-TTTTCAGTTCTTGCACAT-3′; CCR6, forward, 5′-ATGACGCGGAATCTGATGA-3′, reverse, 5′-GCCAGCAGAAAGTTGAC-3′.

Clonogenic and migration assays

For foci formation, A549 cells were treated with or without CCL20 and seeded in triplicate onto 35 mm plates (300 cells per plate). After 14 days of culturing, cells were stained with Giemsa and clones containing more than 50 cells were counted. Quantitative transmigration assays were performed using an 8.0 μm polycarbonate membrane (Corning, NY, USA) [17]. A549 cells (1 × 10⁵) in DMEM were seeded in the upper chamber, and different concentrations of CCL20 in DMEM with 0.5% FCS were added to the lower chamber. After incubation for 24 hours at 37°C and 5% CO₂, the cells on the upper side were removed, and cells on the bottom side were fixed with methanol for 30 minutes, stained with 0.0005% Gentian Violet Solution and counted.

Immunohistochemical analysis

Immunohistochemical assay was performed using an anti-CCL20 antibody [18]. Briefly, formalin-fixed, paraffin-embedded human or mouse lung tissue specimen (5 μm) were deparaffinized by xylene and graded alcohol, and subjected to a heat-induced epitope retrieval step in citrate buffersolution. The sections were blocked with 5% bovine serum albumin (BSA) for 30 min and incubated with the antibody at 4°C overnight, followed by incubation with secondary antibodies for 90 min at 37°C. Cell smears were fixed by 4% paraformaldehyde, blocked with 5% BSA for 30 min and incubated with indicated antibodies at 4°C overnight, followed by H₂O₂ treatment and incubation with secondary antibodies for 90 min at 37°C. Detection was performed with 3,3′-diaminobenzidine (DAB, Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China) and counterstained with hematoxylin. The scoring of immunoreactivity was performed as described [19].

Enzyme-linked immunosorbent assay (ELISA)

Serum concentration of soluble CCL20 was determined using a commercially available ELISA kit (Shanghai GenePharma, Shanghai, China). The absorbance of the plates was read at 450 nm using an automated microplate reader (Bio-Tek, Winooski, VT, USA).

Western blot analysis

The cells were seeded in 6-well culture plates, treated with CCL20 and lysed in RIPA lysis buffer supplemented with the protease inhibitors of cocktail (Sigma, St Louis, MO), NA, sodium vanadate, and PMSF. Whole-cell lysates were collected by centrifugation and quantitated by Coomassie Blue Staining. Lysates containing equal amounts of protein were separated by SDS-PAGE (8–12%) and transferred onto nitrocellulose membrane (Millipore Corporation, Darmstadt, Germany). After blocking in 5% non-fat milk in Tris-buffered saline, the membranes were incubated with certain primary and then secondary antibodies, and detected by Luminescent Image Analyzer LSA 4000 (GE, Fairfield, CO, USA). Antibodies used in this study were: goat anti-human CCL20 polyclonal antibody, rabbit anti-human CCR5 polyclonal antibody, rabbit anti-human AKT1/2/3 polyclonal antibody, goat anti-human Lamin B polyclonal
antibody, mouse anti-human α-tubulin mAb, rabbit anti-human pAKT1/2/3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human ERK2 polyclonal antibody (Abcam, Cambridge, MA), human CCL20 ELISA Kit (Shanghai GenePharma, Shanghai, China), rabbit anti-E–Caderhin monoclonal antibody (CST), and Epithelial–Mesenchymal Transition (EMT) Antibody Sampler Kit (CST). Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) and Recombinant Human CCL20 protein (Peprotech, Rocky Hill, NJ) were purchased from the indicated vendors, respectively.

siRNA assays

Using HiPerFect Transfection Reagent (Qiagen, Crawley, UK) according to the manufacturer’s protocol, cells were transfected with 50 nM double-stranded siRNA oligonucleotides twice. The siRNA sequences were 5′-GAUCAAUGGCUACACAGGA-3′ (NF-κB siRNA1) and 5′-AAGGUGCAGAAAGAGGACA-3′ (NF-κB siRNA2).

Construction of stable knockout cell lines using the CRISPR/Cas-9 system

The experiments were conducted as described [20,21]. The two specifically targeting sequences for the CCL20 locus are 5′-GGCAGAAATCCAAACAGCT-3′ and 5′-AATATATTGTGCGTCTCCT-3′, and the identification and verification of the gene knockout events were based on sequencing analysis of the genomic PCR fragments of the target loci.

Animals

Female A/J mice weighing 18–22 g were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and fed in a specific pathogen-free environment and treated in accordance with the guidelines of the Institutional Animal Use and Care Committee of our institute. The mice were exposed to NNK (0, 50 mg/kg, twice per week for 5 weeks) in corn oil via oral gavage and/or dexamethasone (0.5 mg/kg per day for 12 weeks in diet). For in vivo scans, mice were anesthetized by a mixture

---

**Fig. 1.** NNK significantly up-regulates CCL20 in normal human lung epithelial cells. (A) A PCR array analysis of the expression of genes encoding cytokines/chemokines in 16HBE cells treated with NNK at 10 or 50 μM for 60 days. (B) Dots represent mean expression of single genes in NNK treated and un-treated cells after normalization to housekeeping genes (GAPDH, B2M, HPRT1, RPL13A, ACTB). Dotted lines indicate the arbitrary cutoff value of 1.2 fold over- or under-expression in treated versus un-treated cells. (C) The microarray analysis revealed the expression of 13 genes at mRNA level that showed at least 1.2 times higher or lower in treated than non-treated cells. Red columns represent genes up-regulated and blue columns down-regulated by NNK treatment.
of oxygen/isoflurane inhalation and positioned with legs fully extended. The entire lung of each mouse was scanned by a microCT (PerkinElmer, Waltham, MA). When the mice became moribund, they were sacrificed, lung tissues were isolated and cells were harvested for immunohistochemistry or flow cytometry assays.

Statistical analysis

Differences between data groups were evaluated for significance using two-sided Student’s t-test. p values < 0.05 were considered statistically significant. All

Fig. 2. The expression of CCL20 in lung cancer. (A–C) The expression of CCL20 in lung cancers was evaluated by real-time PCR (A), Western blot (B), and immunohistochemical (IHC) assays (C). N, normal; T, tumor. (D) Serum concentration of CCL20 in samples from healthy donors (HD), smoker (S) or non-smoker (NS) NSCLCs detected by ELISA.
experiments were repeated at least three times and the data are presented as mean ± SD, unless noted otherwise.

Results

**NNK induces production of CCL20 in normal human lung epithelial cells**

To mimic the phenomenon that cigarette smoke usually causes lung cancer after a long latency, normal human lung epithelial 16HBE cells were treated with NNK at 10 or 50 μM for 60 days, and a real-time PCR-based microarray was used to explore the cytokines/chemokines induced by NNK. We found that the expressions of 3 cytokines (IL-5, IL-8, IL-9) and 4 chemokines (CCL20, CCL24, CXCL11, CXCL12) were significantly up-regulated and 2 cytokines (IL-1α, IL-1β), 3 chemokine/chemokine receptors (CXCL5, CCR3, CCR7) and IL-1 receptor antagonist (IL1RN) were down-regulated by NNK treatment (Fig. 1A–C). Among them, CCL20 was the most significantly elevated gene (Fig. 1A–C). We therefore chose it for further investigation.

**CCL20 is overexpressed in smoker lung cancers**

We tested the expression of CCL20 in 173 NSCLCs (Table 1), 119 patients determined by real-time PCR (Fig. 2A) and 54 patients assessed by Western blot (Fig. 2B). We found that in 78/173 (45.1%) patients, the expression of CCL20 was higher in tumor samples than their adjacent normal controls (Table 1). These results were confirmed by immunohistochemical assay (Fig. 2C). Moreover, CCL20 was up-regulated in 48/92 (52.2%) smoker and 29/78 (37.2%) nonsmoker patients, and the expression of CCL20 in smoker patients was higher than in non-smoker NSCLCs (p = 0.005; Table 1, Fig. 2A–C). By ELISA, we found that the serum concentration of CCL20 in NSCLCs was higher than in healthy donors, while smoker patients had higher CCL20 concentration than non-smoker NSCLCs (Fig. 2D). These results suggested a potential association between tobacco smoke and CCL20 expression.

We analyzed the relationship between CCL20 expression and tumor stages, and found that patients with relatively earlier disease (stages I, II, III) had lower CCL20, while those with advanced disease (stages IV) had higher CCL20 (p = 0.001, Table 1 and Fig. 3A). Furthermore, in 54 lung cancer patients whose survival information was available, the median survival time of CCL20-high patients (821 days) was much shorter than those with low expression of CCL20 (1017 days, p = 0.031; Fig. 3B). These results indicate that the expression of CCL20 is associated with advanced lung cancer and is inversely associated with clinical outcome.

**NNK induces CCL20 production by lung epithelial cells in vitro and in vivo**

We tested whether NNK could induce generation of CCL20 or not. To do this, normal human lung epithelial 16HBE cells and lung cancer A549 cells were treated with NNK at 10 and 50 μM for 48 h or 10 μM for 60 days, and the expression of CCL20 was determined by real-time PCR. We found that CCL20 was up-regulated by NNK in a dose- and time-dependent manner (Fig. 4A). In A/J mice treated by gavage with NNK at 50 mg/kg twice per week for 5 weeks, lung cancer was developed (Fig. 4B, C), and CCL20 was up-regulated (Fig. 4D). Immunohistochemical analysis showed that CCL20 was induced by NNK treatment in tumor samples of the mice (Fig. 4B, E), while serum concentration of CCL20 was also increased as detected by ELISA (Fig. 4F). An anti-inflammation drug dexamethasone (DEX) [22] could reduce NNK-induced tumor burden (Fig. 4B, C) and prolong the survival of the mice exposed to NNK (Fig. 4G). Interestingly, NNK-induced CCL20 was inhibited by DEX in the mice (Fig. 4B, D–F).

We investigated how NNK modulates CCL20 expression. By analyzing the promoter sequence of CCL20, we identified three potential NFκB binding sites (TGGGGAAAACCCC, GGGAAAACCCC, and AGGGAAATTCC), nine C/EBPα binding sites, and two Hlf binding sites. NNK is shown to be able to activate NFκB [23,24], and CCL20 is a target gene of NFκB [25], therefore NNK might up-regulate CCL20 via NFκB pathway. To test this possibility, 16HBE and A549 cells were treated with NNK in the absence or presence of NFκB inhibitor BAY11-7082 [26] or NFκB-specific siRNA, and the expression of CCL20 was assessed. We found that while NNK increased CCL20, BAY11-7082 and siNFκB significantly inhibited this effect (Fig. 4H–J), suggesting the role for NFκB to play in NNK-induced up-regulation of CCL20.

**The expression of CCL20 receptor CCR6 in lung cancer**

The function of CCL20 is mediated by binding to the G-protein coupled 7-transmembrane receptor CCR6, which is expressed by most B cells and subsets of T cells and dendritic cells (DCs) [27]. By RT-PCR and immunofluorescence analysis, we found that CCR6 was expressed in normal lung epithelial cells and lung cancer cell lines (Fig. 5A, B). By real-time PCR, we showed that the expression of CCR6 in lung cancer tumor samples was approximately equal to that in their adjacent normal lung tissues (Fig. 5C). By immunohistochemical assay, we confirmed that CCR6 was expressed in tumor samples of NSCLCs and NNK-treated A/J mice (Fig. 5D).

---

**Fig. 3.** The expression of CCL20 is related to advanced disease and poor prognosis. (A) Relationship between the expression of CCL20 and tumor stages of lung cancer patients. (B) Overall survival of 54 NSCLC patients.
Fig. 4. NNK induces secretion of CCL20 in vitro and in vivo. (A) NNK up-regulated CCL20 at mRNA level in a time- and dose-dependent manner in 16HBE and A549 cells. The cells were treated with NNK, and the expression of CCL20 was detected by real-time PCR. (B) The A/J mice treated with NNK and/or dexamethasone were detected by microCT, and when they became moribund the mice were sacrificed. The images of the lung, HE staining, and immunohistochemical analysis of CCL20 are shown. (C) Tumor volume of the mice treated with NNK and/or dexamethasone. (D) The expression of CCL20 in the lung tissue of mice treated with NNK and/or dexamethasone. (E) The immunoreactivity score of lung tissues analyzed by immunohistochemical assay in (B). (F) Serum concentration of CCL20 in mice treated with NNK and/or dexamethasone. (G) The survival curve of the mice treated with BaP and/or DEX (n = 8 for each group). (H) The expression of CCL20 in cells treated with NNK and/or NFκB inhibitor BAY11-7082. (I, J) The cells were transfected with NFκB-specific or negative control siRNA, treated with or without NNK, and the expression of CCL20 was tested by real-time PCR.
CCL20 secreted by lung epithelial cells is critical to cancer cell proliferation and migration

CCL20 is secreted by immune cells including macrophages, T-cells and B-cells [28]. To address whether these cells were other sources of CCL20 and were involved in NNK-induced lung cancer, lung tissue samples were harvested from mice treated with NNK or vehicle. By analyzing cell surface markers in the tumor microenvironment using flow cytometry and antibodies against CD45, CD4, CD19 and CD68, we tested which cell types were increased in NNK-induced lung cancer. The results showed that as compared to those in mice treated with vehicle control, CD68\(^+\) macrophages, CD4\(^+\) T cells or CD19\(^+\) B cells were not significantly increased in mice upon NNK (Fig. 6A). By immunohistochemistry assay of human NSCLC tumor samples, we showed that while the expression of CCL20 was high in cancer cells (Fig. 6B), CD68\(^+\) macrophages were few (mean, 2.7%). These results suggested that macrophages, T and B cells were not the main source of elevated CCL20 in tobacco smoke-induced lung cancer.

We then tested the role that CCL20 plays in lung cancer proliferation by knockdown of its expression using CRISPR/Cas-9 system in A549 cells (Fig. 6C), and found that the clonogenic activity of the cells was inhibited (Fig. 6D, E), and the proliferation of the cells (assessed by cell counting) was suppressed (Fig. 6F). In a transwell assay, we found that knockdown of CCL20 also inhibited migration of A549 cells (Fig. 6G).

CCL20 promotes proliferation and metastasis of lung cancer

By clonogenic assay, we found that addition of CCL20 to A549 cells significantly increased the colony forming activity of the cells (Fig. 7A). MTT assay demonstrated that CCL20 promoted cell proliferation which could be inhibited by an anti-CCR6 antibody (Fig. 7B). We showed that in A549 and H460 cells, CCL20 up-regulated p-Erk and p-Akt in a dose-dependent manner (Fig. 7C); in tumor samples of NNK-treated A/J mice, the expression of p-Erk and p-Akt was up-regulated, while DEX attenuated this effect (Fig. 7D). CCL20 is a target gene of STAT3 [29], and NNK was shown to be able to activate this transcription factor [30]. We showed that in tumor samples of NNK-treated A/J mice, the expression of p-Stat3 but not total Stat3 was also up-regulated, while DEX attenuated this effect (Fig. 7D).

CCL20 can promote hepatocellular carcinoma cell proliferation and migration by inducing epithelial–mesenchymal transition (EMT)-like changes via PI3K/AKT and Wnt/β-catenin pathways [31]. We tested the effects of CCL20 on lung cancer cell migration by a transwell migration assay, and showed that CCL20 induced migration of A549 cells, while anti-CCR6 antibody and β-Catenin inhibitor ICG-001 [32] partially inhibited this effect (Fig. 7E). In A549 and H460 cells, CCL20 increased nuclear β-Catenin (Fig. 7F) and down-regulated the expression E-Cadherin and up-regulation of Vimentin and Snail (Fig. 7G). By real-time PCR experiments, we found that CCL20 induced down-regulation of E-Cadherin and up-regulation of N-Cadherin, Vimentin and Snail at mRNA level, while ICG-001 reversed these phenomena (Fig. 7H). Up-regulation of β-Catenin (in the nucleus) and Vimentin and down-regulation of E-Cadherin were also seen in A/J mice treated with NNK (Fig. 7I, J).

Discussion

Tobacco kills nearly 6 million people each year (up to half of its users), and the annual death toll could rise to more than eight million by 2030 unless urgent action is taken [33,34]. Comprehensive data have illustrated pathways of cancer induction, involving carcinogen exposure, metabolic activation, DNA adduct formation, and consequent mutation of critical genes along with the exacerbating influences of inflammation, cocarcinogenesis, and tumor promotion [3]. However, many of the studies were conducted in cellular and animal models, and more research using human samples
is needed to identify biomarkers to screen for those most prone to develop lung cancer, and to uncover targets for development of therapeutic and chemopreventive approaches.

The key carcinogens of tobacco smoke are NNK and PAHs [3,35]. These carcinogens induce chronic inflammation to facilitate carcinogenesis. For example, NNK stimulates IL-10 and PGE2 release [10], PAHs induce secretion of CCL1, CCL5, TNF-α, IL-1β, IL-6 and IL-8 [36–39]. On the other hand, a long latency (a hypothesized 10-year period at least) is required for the development of clinical recognition of lung cancer since the individuals have been exposed to cigarette smoke [40]. In this study, we screened for NNK-induced inflammatory factors by using normal human lung epithelial
Fig. 7. CCL20 promotes lung cancer cell proliferation and migration. (A) Soft agar assay of A549 cells treated with or without CCL20. (B) The growth curve of A549 cells treated with CCL20 and/or anti-CCR6 antibody. (C) Western blot assays using lysates of cells treated with CCL20 and indicated antibodies. (D) Western blot assays using lysates of lung samples harvested from mice treated with NNK/DEX and indicated antibodies. (E) Transwell assay using A549 cells co-incubated with CCL20, β-catenin inhibitor ICG-001, and anti-CCR6 antibody. (F) Western blot assays using cytoplasmic and nuclear fractions of cells treated with CCL20 and indicated antibodies. (G) Western blot assays using lysates of cells treated with CCL20/ICG-001 and indicated antibodies. (H) The expression of the indicated genes in cells treated with CCL20/ICG-001 detected by real-time PCR. (I) Western blot assays using cytoplasmic and nuclear fractions of lung samples harvested from mice treated with NNK and/or DEX and indicated antibodies. (J) Western blot assays using lysates of lung samples harvested from mice treated with NNK and/or DEX and indicated antibodies.
cells exposed to NNK for a long period (60 days) to reflect the long latency of smoker patients to develop lung cancer. We used a microarray containing 84 known cytokines/chemokines to systemically identify abnormalities in chronic inflammatory pathway critical to lung cancer pathogenesis. We found that in cellular and animal models, NNK induced the secretion of CCL20 ([Fig. 4]), in patients with lung cancer, CCL20 was elevated in tumor samples compared to their adjacent normal lung tissues, and higher expression of CCL20 was associated with advanced disease and poorer prognosis (Figs. 2 and 3). Knockdown of CCL20 in lung cancer cells led to inhibition of cell proliferation and migration ([Fig. 6]). These results demonstrate that CCL20 is a critical inflammation factor induced by NNK to facilitate cigarette smoke-caused lung cancer, and may represent a potential therapeutic target.

CCL20 belongs to the CC chemokine which is responsible for the chemotraction of immature dendritic cells, effector/memory T-cells and B-cells [27]. CCL20 is the only ligand that activates CCR6. CCL20 and CCR6 are overexpressed and associated with poor prognosis in colorectal cancer [41]. CCL20/CCR6 enhances proliferation of some types of cancerous cells and promotes metastasis and invasion [42–45]. Airway epithelial cells release CCL20 in response to cytokines and PAs-containing ambient particulate matter [46], and in patients with chronic obstructive pulmonary disease (COPD) the expressions of CCL20 mRNA in total lung and CCL20 protein in sputum are significantly higher compared to never-smoker COPD and smokers without COPD [47]. We showed that the expressions of CCL20 at both mRNA and protein levels were increased in lung cancer tumor samples compared to their adjacent normal lung tissues (Figs. 2 and 5), and smoker patients had higher CCL20 than non-smoker patients (Table 1, Fig. 2). Together, the above results indicated that CCL20 and CCR6 are critical to tumorigenesis of some subtypes of cancers, and cigarette smoke or second hand smoke may be responsible for up-regulation of the chemokine.

Previous study showed that transcription factor β-catenin is critical for EMT phenotype [48]. We found that CCL20 promoted lung cancer cell migration and induced EMT-like phenotype in vitro and in vivo (Fig. 7). We further showed that CCL20 activated β-catenin by inducing its translocation to the nucleus (Fig. 7). CCL20 is shown to be able to activate EGF [42,49], and the activated EGF induces β-catenin dissociation from E-cadherin, translocation to the nucleus, and increased transactivation by GSK-3β-independent mechanisms [50]. EGFR activation also results in the disruption of the β-catenin/α-catenin complex, thereby abrogating the inhibitory effect of α-catenin on β-catenin transactivation [51]. In addition, EGFR activation induces translocation of PKM2 into the nucleus, where K433 of PKM2 binds to c-Src-phosphorylated Y333 of β-catenin [52]. Therefore, CCL20 may activate β-catenin via the EGFR and its downstream signal molecules.

Chemokines have become rational drug targets [53]. We showed that a wide spectrum anti-inflammation agent dexamethasone [54] inhibited the production of CCL20 by epithelial cells ([Fig. 4]), inhibited EMT ([Fig. 7]), reduced tumor burden and prolonged the life span of the mice ([Fig. 4]), unveiling a new mechanism of this old drug. Since dexamethasone is a wide-spectrum anti-inflammatory drug which may cause severe side effects, more specific, CCL20-targeting antibodies or small molecules should be developed to combat lung cancer.

Authors’ contributions

The project was conceived by G.B.Z. The experiments were designed by G.B.Z. The experiments were conducted by G.Z.W., X.C., X.C.L., and Y.Q.L. Patient samples were provided by X.Q.W., X.S., Z.Y.W., Y.Q.G., Z.S.W., and Y.C.H. Data were analyzed by G.B.Z. The manuscript was written by G.B.Z.


