Cyclooxygenase-2-Dependent Expression of Angiogenic CXC Chemokines ENA-78/CXC Ligand (CXCL) 5 and Interleukin-8/CXCL8 in Human Non-Small Cell Lung Cancer

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ABSTRACT

Elevated tumor cyclooxygenase (COX)-2 activity plays a multifaceted role in non-small cell lung cancer (NSCLC). To elucidate the role of COX-2 in the in vitro and in vivo expression of two known NSCLC angiogenic peptides, CXC ligand (CXCL) 8 and CXCL5, we studied two COX-2 gene-modified NSCLC cell lines, A549 and H157. COX-2 overexpression enhanced the in vitro expression of both CXCL8 and CXCL5. In contrast, specific COX-2 inhibition decreased the production of both peptides as well as nuclear translocation of nuclear factor kBF. In a severe combined immunodeficient mouse model of human NSCLC, the enhanced tumor growth of COX-2-overexpressing tumors was inhibited by neutralizing anti-CXCL5 and anti-CXCL8 antisera. We conclude that COX-2 contributes to the progression of NSCLC tumorigenesis by enhancing the expression of angiogenic chemokines CXCL8 and CXCL5.

INTRODUCTION

Cyclooxygenase (COX) is the rate-limiting enzyme for the production of prostaglandins (PGs) and thromboxanes from free arachidonic acid (1). The enzyme is bifunctional, with fatty acid COX and PG hydroperoxidase activities. Two forms of COX have been described: (a) a constitutively expressed enzyme, COX-1, present in most cells and tissues; and (b) an inducible isoenzyme, COX-2, expressed in response to cytokines, growth factors, and other stimuli (2, 3). We and others have reported that COX-2 is frequently constitutively elevated in human non-small cell lung cancer (NSCLC (4–7)). High levels of COX-2 mRNA and protein expression correlate with a poor prognosis in this disease (8, 9). In agreement with these clinical data, lung tumors with high COX-2 expression metastasize at high frequency (10), promote angiogenesis (11–15), and are more resistant to apoptosis by various stimuli (16–19). Our previous studies documented that COX-2 expression in NSCLC promotes immune suppression and tumor invasion (4, 20–22).

Tumor progression is dependent on angiogenesis. Net tumor-induced angiogenesis is due to an imbalance in the overexpression of angiogenic factors as compared with angiostatic factors (23). Previous studies have documented the role of two CXC family chemokines, CXC ligand (CXCL) 8 and CXCL5, in human NSCLC (24–33). The CXC chemokine family consists of a number of structurally related peptides that are either angiogenic or angiostatic. All angiogenic members of this family, including CXCL8 and CXCL5, possess a 3-amino acid motif, the glutamic acid-leucine-arginine (ELR) motif, which immediately precedes the first cysteine in their CXC motif located in their NH2 terminus (34). The angiogenic activity of ELR+CXC chemokines is mediated via the CXC motif ligand receptor 2 [CXCR2 (35)]. CXCR2 has been shown to bind all ELR+CXC chemokines, including CXCL8 and CXCL5, with high affinity (36–39). The expression of CXCL8 and CXCL5 is up-regulated by nuclear factor (NF)-kBF (40–43). NF-kBF, a heteromeric transcription factor, is activated in response to many stress signals. Factors activating NF-kBF-related gene expression initiate degradation of the cytoplasmic NF-kBF/IkB complex and subsequent translocation of NF-kBF into the nucleus, where it activates gene expression (40, 44, 45).

The current study defines a role for COX-2 in the expression of NSCLC angiogenic peptides CXCL8 and CXCL5. We have found that COX-2 up-regulates both the in vitro and in vivo expression of CXCL8 and CXCL5 in NSCLC cells. Our data suggest that COX-2 up-regulates the expression of these chemokines by activating NF-kBF nuclear translocation. In vivo, the COX-2-enhanced expression of CXCL8 and CXCL5 was associated with enhanced NSCLC tumor growth and angiogenesis.

MATERIALS AND METHODS

Cell Lines. The NSCLC cell lines used in this study have been described previously by us in detail (21, 22). Briefly, A549 (human lung adenocarcinoma) and H157 (squamous cell carcinoma) were obtained from American Type Culture Collection (Manassas, VA) and National Cancer Institute, NIH, respectively. A 2.0-kb cDNA fragment of human COX-2 (generously provided by Dr. Harvey Hershman, University of California, Los Angeles) was expressed in these cell lines in sense and antisense orientation. The following cell line terminology is used in the text: (a) A549-S and H157-S are the cell lines transfected with COX-2 in sense orientation; (b) A549-AS and H157-AS are the cell lines transfected with COX-2 in antisense orientation; and (c) A549-V and H157-V are the cells transfected with the expression vector pLNCX only.

Reagents. SC58236 (provided by Pharmacia, Peapack, NJ) was dissolved in 100% ethanol at 100 mM; BAY-11-7082 [(E)-3-{[4-(methylphenyl)sulfonyl]-2-propenenitrile} (Calbiochem, La Jolla, CA) was dissolved in DMSO at 50 mM, and interleukin (IL)-1β (BD Biosciences, Palo Alto, CA) was dissolved in PBS at 2.000 units/ml. Anti-PGE2 [monoclonal antibody (mAB) 2B5] and isotype-matched control mouse IgG1 (MOPC21) were provided by Pharmacia and dissolved in PBS at 1 mg/ml. Polyclonal goat antimiurine CXCR2 (SCBBio, Peapack, NJ) was dissolved in PBS at 2,000 units/ml. Anti-PGE2 [monoclonal antibody (mAB) 2B5] and isotype-matched control mouse IgG1 (MOPC21) were provided by Pharmacia and dissolved in PBS at 1 mg/ml. Polyclonal goat antimurine CXCR2 was produced by the immunization of a goat with a peptide containing the ligand-binding sequence MEFEFKVSKFQNFDEGSG of CXCR2. This neutralizing polyclonal anti-CXCR2 antibody blocks mouse CXCR2 and detects CXCR2 by Western blot and fluorescence-activated cell-sorting analysis of neutrophils in vivo (46, 47). Neutralizing antihuman CXCL5 and anti-human CXCL8 sera were used for in vivo experiments are highly specific neutralizing antisera described in our previous studies (24, 25). They were produced by immunization of rabbits or goats with CXCL5 or CXCL8 (R&D Systems, Minneapolis, MN) in multiple intradermal sites with complete Freund’s adjuvant. Direct ELISA was used to evaluate antisera titers, and sera were used for Western blot, ELISA, and neutralization assays when titers had reached >1/1,000,000. Furthermore,
in a sandwich ELISA, these antibodies are specific for either CXCL5 or CXCL8 without cross-reactivity to a panel of 12 other recombinant human cytokines or the murine chemokines CXCL1, CXCL2, and CXCL3.

**Growth Conditions.** The A549 and H157 cell lines were plated in standard 6-well plates (Corning Inc., Corning, NY) at 20 × 10^4 and 40 × 10^4 cells/well, respectively. The cells were grown for 24 h, at which point SC58236 at 12.5 μM was added into the plating medium. Seventy-two-hour-later supernatants were harvested, and cells were counted by hemocytometer. Cells were grown in duplicates for all conditions. For purification of total RNA, the experiment was scaled up in 10-cm Petri dishes (Becton Dickinson, Franklin Lakes, NJ). In experiments with BAY-11-7082, the cells were plated in 6-well plates as described above and grown for 72–96 h, at which time-point the wasted medium was removed and replaced with fresh 10% FBS-RPMI 1640 containing BAY-11-7082. The cells were then grown for an additional 24 h, trypsinized, and counted, and the supernatants were analyzed for CXCL8 and CXCL5.

**Blocking PGE2 with Neutralizing Anti-PGE2 Monoclonal Antibody.** H157-S, H157-V, A549-S, and A549-V were plated as described above and grown for 96 h in 10% FBS-RPMI 1640, at which time-point fresh medium was added for the final 24 h of incubation. For the final 24 h, cells were divided into four treatment groups: (a) control mAB; (b) neutralizing anti-PGE2 mAB; (c) control mAB with IL-1β at 200 units/ml; and (d) anti-PGE2 mAB with IL-1β at 200 units/ml. All groups were grown in duplicates. Previous studies show that IL-1β is a potent inducer of both COX-2 and PGE2 in A549-S, A549-V, H157-S, and H157-V cells. In contrast, the A549-AS and H157-AS cells produce very low levels of PGE2, and they were therefore excluded from the anti-PGE2 mAB experiments (21, 22).

**PGE2 Enzyme Immunoassay.** Cells were stimulated with IL-1β (200 units/ml; Genzyme, Cambridge, MA) for 24 h. PGE2 concentration in each group (with or without IL-1β stimulation) was measured by enzyme immunoassay using a PGE2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) as reported previously (21). All measurements were made in duplicates.

**CXCL8 and CXCL5 ELISA.** The concentrations of CXCL8 and CXCL5 were determined as described previously (24, 25). The antibody pairs and standards were purchased from R&D Systems. For CXCL8 ELISA, MAB208 was used as a coating antibody, and BAF254 was used as a biotinylated antibody. For CXCL5 ELISA, MAB254 was used as a coating antibody, and BAF254 was used as a biotinylated antibody. The 96-well MaxiSorp ELISA plates (Nunc, Rochester, NY) were coated with a capture antibody overnight, washed three to four times in PBS/0.5% Tween 20, and blocked with PBS/2% BSA for 1–2 h. After washing the plates as described above, the wells were incubated with supernatants (50 μl) for 1–3 h. The biotinylated antibody was added after washing the plates, and cells were incubated for 1 h. Horseradish peroxidase-conjugated streptavidin (Jackson Laboratory, Bar Harbor, ME) was added after washing the plates, and plates were incubated for 30–45 min, followed by three to four washes. Peroxidase and tetramethylbenzidine peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersburg, MD) was added for 5–10 min, and the reaction was stopped by 1 m phosphoric acid. The A450 nm was determined by Bio-Lab Benchmark Microplate reader (Bio-Rad, Hercules, CA). Each plate contained appropriate standards for the standard curve.

**Northern Blots.** Total RNA was purified using the RNeasy Midi kit from Qiagen (Valencia, CA). Ten μg of total RNA from each cell line were fractionated in formaldehyde gel, blotted onto nylon membrane, and hybridized under high-stringency conditions with CXCL8- and CXCL5-specific probes. The CXCL8- and CXCL5-specific probes were generated by PCR using [α-32P]dCTP (specific activity, 3000 Ci/mmol; Blue/Neg/513H; Perkin-Elmer, Franklin Lakes, NJ) for 12 weeks of age) mice were divided into two groups: (a) control mAB with IL-1β (21, 22) and (b) control mAB; (c) control mAB with IL-1β at 200 units/ml; and (d) anti-PGE2 mAB with IL-1β at 200 units/ml. All groups were grown in duplicates. Previous studies show that IL-1β is a potent inducer of both COX-2 and PGE2 in A549-S, A549-V, H157-S, and H157-V cells. In contrast, the A549-AS and H157-AS cells produce very low levels of PGE2, and they were therefore excluded from the anti-PGE2 mAB experiments (21, 22).

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**Rat Corneal Micropocket Assay of Angiogenesis.** Equal volumes of lyophilized supernatants normalized to total protein were combined with sterile Hydron (IFN Sciences Inc., New Brunswick, NJ) casting solution. The anti-CXCR2 (see above) or control antibody (goat polyclonal) was mixed at a 1:100 dilution in 1.0 ml of antiprotease buffer. These tumor lysates were cleared of insoluble debris by centrifugation and frozen at −80°C. In three separate series of experiments, the tumor growth of NSCLC lines was monitored. In the first series, the tumor growth of untreated NSCLC cell lines was monitored. In the second series, the tumor growth of both SC58236-treated parental NSCLC cell lines was compared with that of their respective diluent-treated control. SC58236 was injected i.p. at 3 mg/kg body weight, 3×/week. In the third series, the tumor growth of antihuman CXCL8 and antihuman CXCL5 (see above) antisera-treated NSCLC cell lines was compared with that of untreated control NSCLC cell lines. Both CXC antiserum and the control antisera were injected at 0.5 ml, i.p., 3×/week. The tumor volume was determined as described above.

**Preparation of Nuclear Extracts.** Cells (1 × 10^5) were washed twice with cold PBS, and the cell pellet was suspended in 40 μl of cell lysis buffer for 10 min on ice. Nuclei were extracted by sedimentation (microcentrifugation at 6,500 rpm) for 10 min at 4°C. The resulting nuclear pellet was then suspended in 15 μl of extraction buffer C [20 mM HEpes (pH 7.9), 3% glycerol, 0.4 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] and incubated for 10 min at 4°C with brief intermittent mixing. The mixture was microcentrifuged (14,000 rpm for 10 min at 4°C), and the nuclear protein was resuspended in 60 μl of extraction buffer D [20]
Results

COX-2 Determines the Expression of CXCL8 and CXCL5 in NSCLC. To assess whether COX-2 regulated the expression of ELR+CXC chemokines CXCL5 and CXCL8, NSCLC cell lines transfected with COX-2-S, COX-2-AS, or pLNCX (vector control) were examined for the expression of CXCL5 and CXCL8 (Fig. 1). The overexpression of COX-2 in NSCLC cell lines significantly increased expression of both CXCL8 and CXCL5. Conversely, genetic ablation of COX-2 expression significantly reduced the expression of CXCL8 and CXCL5. In fact, the mRNA and protein expression of CXCL8 and CXCL5 in H157 cells was completely suppressed in H157-AS (Fig. 1). Furthermore, a COX-2 inhibitor drug, SC58236, inhibited the expression of CXCL8 in A549-S and H157-S cells (data not shown).

Specific Genetic Inhibition of COX-2 in NSCLC Cells Decreases NF-κB Nuclear Translocation. As shown in earlier studies, NF-κB activity regulates the expression of CXCL5 and CXCL8 and angiogenesis (43, 48–52). In addition, nonsteroidal anti-inflammatory drugs modulate NF-κB activity (53). Thus, we sought to elucidate whether the expression level of COX-2 modulates the nuclear translocation of NF-κB in NSCLC cells, and whether the expression of CXCL8 and CXCL5 in NSCLC cells is NF-κB dependent. Consistent with previous reports, pharmacological inhibition of NF-κB by BAY-11-7082 inhibited the expression of CXCL8 (41), as well as CXCL5 (Fig. 4A). Also, genetic inhibition of COX-2 clearly down-regulated the nuclear translocation of NF-κB in both sets of NSCLC cells (Fig. 4B). These findings suggest that NF-κB nuclear translocation plays an important role in the COX-2-regulated production of CXCL8 and CXCL5.

Endogenously Produced PGE₂ Modulates the in Vitro Expression of CXCL8. To assess whether PGE₂ modulates the expression of CXCL5 and CXCL8 in NSCLC cell lines, we cultured the A549 and H157 cell lines in presence of specific neutralizing anti-PGE₂ mAB or isotype-matched control mAB. Our previous studies show that IL-1β up-regulates both COX-2 and PGE₂ in H157 and A549.
Therefore, we tested whether neutralization of IL-1β/H9252-induced PGE₂ in H157 and A549 culture supernatants modulates the production of CXCL8 and CXCL5. We expected IL-1β/H9252 to increase the production of CXCL8 and CXCL5, as well as PGE₂, and the anti-PGE₂ mAB to decrease this IL-1β-stimulated CXCL8 and CXCL5 production. Consistent with our previous data, IL-1β up-regulated PGE₂ in H157 and A549 cells (21) and was accompanied by sharply elevated levels of CXCL5 and CXCL8, as compared to the control cells (Fig. 5). The anti-PGE₂ mAB, however, significantly down-regulated only the CXCL8 production of H157-S (Fig. 5), whereas the production of CXCL5 remained unchanged (data not shown). Thus, our results suggest a partial role for PGE₂ in ELR/CXC chemokine expression.

**DISCUSSION**

Lung cancer accounts for more than 28% of all cancer deaths each year and is the leading cause of cancer-related mortality in the United States (55). Despite focused research in conventional therapies, the 5-year survival rate remains 14% and has improved only minimally in the past 25 years. Newly discovered molecular mechanisms in the pathogenesis of lung cancer provide novel opportunities for targeted therapies of NSCLC (56). These investigations in the molecular pathogenesis of lung cancer have presented translational researchers with new targets that may specifically impact carcinogenesis (57). COX-2 is one of the novel targets under evaluation for lung cancer therapy and chemoprevention (58).

### Table 1 In vivo expression of CXCL5 and CXCL8 in a SCID mouse model of NSCLC

<table>
<thead>
<tr>
<th>A. In vivo expression of CXCL5 and CXCL8 (pg/ml/mg protein)</th>
<th>A549-S</th>
<th>A549-AS</th>
<th>A549-V</th>
<th>H157-S</th>
<th>H157-AS</th>
<th>H157-V</th>
</tr>
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<tbody>
<tr>
<td>CXCL5</td>
<td>22.1 ± 1.1ᵃ</td>
<td>.02 ± .001</td>
<td>16.9 ± 0.1</td>
<td>38.4 ± 0.4ᵇ</td>
<td>0</td>
<td>26.6 ± 1.1</td>
</tr>
<tr>
<td>CXCL8</td>
<td>550 ± 130ᵇ</td>
<td>220 ± 10</td>
<td>640 ± 20</td>
<td>540 ± 70ᵇ</td>
<td>300 ± 20</td>
<td>620 ± 90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. SC58236 inhibits in vivo expression of CXCL8 and CXCL5</th>
<th>A549-P</th>
<th>A549-P + SC58236</th>
<th>H157-P</th>
<th>H157-P + SC58236</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL5</td>
<td>15.5 ± 0.2ᶜ</td>
<td>7.9 ± 1.4</td>
<td>29.6 ± 0.8ᶜ</td>
<td>19.4 ± 1.5</td>
</tr>
<tr>
<td>CXCL8</td>
<td>650 ± 110</td>
<td>400 ± 100</td>
<td>630 ± 60</td>
<td>540 ± 60</td>
</tr>
</tbody>
</table>

ᵃ P < 0.00001, sense versus antisense.
ᵇ P < 0.05 sense versus antisense.
ᶜ P < 0.01, untreated parental versus SC58236-treated parental cells.
Expansion and maintenance of a functional vascular network serving the tumor are required for propagation, invasion, and subsequent metastasis. Thus, angiogenesis is requisite for tumor growth (23) and has been specifically implicated in the pathogenesis and prognosis of lung cancer (59–64). Several growth factors and cytokines have been implicated in tumor-related angiogenesis in lung cancer including vascular endothelial growth factor, macrophage migration inhibitory factor, and ELR + CXC family chemokines such as CXCL8 and CXCL5 (24–33, 65–68). As shown recently, a high level coexpression of vascular endothelial growth factor, macrophage migration inhibitory factor, and CXC family chemokines in NSCLC tumor samples is associated with high risk of NSCLC recurrence after resection (33). Thus, it could be concluded that multiple angiogenic factors are simultaneously up-regulated in NSCLC.

Here we demonstrate the importance of COX-2-dependent regulation of ELR + CXC family chemokines. Genetic and pharmacological inhibition of COX-2 significantly decreased these chemokines in lung cancer cells in vitro. Similarly, in vivo, COX-2 inhibition also reduced the production of CXCL5 and CXCL8. Most importantly, anti-CXCL5 and anti-CXCL8 antisera significantly inhibited the enhanced tumor growth of COX-2-overexpressing lung cancer cells. Thus, COX-2-dependent regulation of these ELR + CXC family chemokines is important in NSCLC tumor growth (Figs. 1 and 2; Table 1) but may not be the only activity that modulates NSCLC tumor growth. As shown by Hida et al. (69), inhibition of COX-2 may directly reduce the viability of tumor cells. Therefore, the COX-2-antisense-mediated inhibition of NSCLC tumor growth (Fig. 2, A and C) should be interpreted as resulting from the net effect of COX-2 inhibition, in which down-regulation of CXCL5/CXCL8 plays a prominent role (Figs. 1–3).

Our data agree with earlier studies demonstrating that COX-2 inhibitors limit the growth of tumors expressing COX-2. Previously, we have shown that both SC58236 and NS398 inhibit the growth of Lewis lung carcinoma (20). Likewise, Masferrer et al. (13) found that a COX-2 inhibitor drug, Celecoxib, reduced the growth of both Lewis lung carcinoma and HT-29 colon carcinoma cells, whereas Williams et al. (70) showed that Celecoxib inhibits the tumor growth of HCA-7 colon carcinoma and Lewis lung carcinoma cells (71). In keeping with these studies, Howe et al. (72) demonstrated that Celecoxib decreased the number of breast tumors in mouse mammary tumor virus/neu mice. A recent study also indicates that COX-2 inhibition reduces tumor growth in transgenic mice that spontaneously develop lung cancer (73). Although a variety of antitumor mechanisms may be operative during COX-2 inhibition, it has been suggested that inhibition of angiogenesis is one of the important pathways (11–15, 72). Our study indicates that the ELR + CXC chemokines are critically important in COX-2-dependent NSCLC angiogenesis as determined by a rat micropocket model for angiogenesis (Fig. 3). Specific antibody-mediated blockade of CXCR2 reduced the angiogenic capacity of the supernatants of H157-S and H157-V (Fig. 3). The effect of anti-CXCR2 was comparable with that of genetic inhibition of COX-2 (see Fig. 3, H157-AS). Thus, this finding supports our earlier data demonstrating that the majority, if not all, angiogenic activity of ELR + CXC chemokines is mediated through CXCR2 (35).

As shown in previous studies, the following regulatory events occur in NSCLC and the other types of tumors: (a) NF-κB nuclear translo-
cation up-regulates CXCL8, CXCL5, and COX-2; (b) pharmacological and genetic inhibition of NF-κB nuclear translocation decreases the expression of CXCL8 and CXCL5; (c) both COX-2 and ELR+CXC chemokines promote angiogenesis; and (d) nonsteroidal anti-inflammatory drugs inhibit the activity of NF-κB (24, 25, 27, 28, 33, 41–43, 48–53). Here, we explored the relationship between the expression of COX-2, NF-κB nuclear translocation, and ELR+CXC chemokine production in NSCLC. The current study implicates NF-κB as a downstream mediator of COX-2 in the COX-2-dependent enhancement of tumor growth and angiogenesis. The apparent COX-2-driven expression of NF-κB-dependent genes such as CXCL8 and CXCL5 in NSCLC cells gave rise to a more malignant NSCLC phenotype in vivo as demonstrated by a SCID mouse model for NSCLC, as well as a rat corneal pocket model for angiogenesis (Figs. 2 and 3). Our data suggesting that COX-2 drives the level of NF-κB-dependent CXCL8 and CXCL5 expression are in agreement with earlier studies showing that nonsteroidal anti-inflammatory drugs inhibit the activity of NF-κB (53). Also, our results for NSCLC tumor growth and angiogenesis are compatible with the recent clinical studies demonstrating that high levels of COX-2 mRNA and protein expression (8, 9) as well as angiogenic chemokines including CXCL8, CXCL5, and CXCL1 (27–31) in NSCLC tumor samples are associated with a poor prognosis.

The current study suggests a role for PGE2 in the expression of ELR+CXC chemokines in NSCLC cells (Fig. 5). We interpret the fact that only the CXCL8 expression of H157-S was modulated by anti-PGE2 as an indication that COX-2-enhanced NSCLC angiogenesis and tumor growth may have two distinct mechanistic pathways: (a) a PGE2-dependent pathway, as also described in previous studies (20, 74–76); and (b) a PGE2-independent pathway. Our findings implicate both of these pathways in COX-2-dependent ELR+CXC chemokine expression in H157 cells. It is possible that a unique repertoire of E-prostanoid cell surface receptors is required for PGE2 to modulate the expression of individual ELR+CXC chemokines. Additional studies, however, are needed to address this issue.

In conclusion, NSCLC COX-2 expression appears to be an important determinant for ELR+CXC chemokine expression. As the current study shows, a high NSCLC ELR+CXC chemokine content is associated with enhanced tumor growth, which can be reduced by inhibition of COX-2. Thus, our study confirms that COX-2 is a potential target for NSCLC chemoprevention or therapy, and it also implies that CXCL8 and CXCL5 could be used as markers of COX-2 inhibitor effects in clinical trials. Furthermore, our data suggest that CXCL8, CXCL5, and their receptors could be used as direct targets in future therapies of NSCLC.

REFERENCES


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