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CXCR2 EXPRESSION IN TUMOR CELLS IS A POOR PROGNOSTIC FACTOR AND PROMOTES INVASION AND METASTASIS IN LUNG ADENOCARCINOMA

Erminia Massarelli

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CXCR2 EXPRESSION IN TUMOR CELLS IS A POOR PROGNOSTIC FACTOR AND PROMOTES INVASION AND METASTASIS IN LUNG ADENOCARCINOMA

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CXCR2 EXPRESSION IN TUMOR CELLS IS A POOR PROGNOSTIC FACTOR AND
PROMOTES INVASION AND METASTASIS IN LUNG ADENOCARCINOMA

A

THESIS

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Health Science Center at Houston
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of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Erminia Massarelli, MD, PhD

Houston, Texas

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CXCR2 EXPRESSION IN TUMOR CELLS IS A POOR PROGNOSTIC FACTOR AND PROMOTES INVASION AND METASTASIS IN LUNG ADENOCARCINOMA

Erminia Massarelli, MD, PhD
Supervisory Professor: Jonathan M. Kurie, MD

CXC chemokine receptor 2 (CXCR2) is a G-protein coupled receptor which mediates signaling by binding to CXC chemokines CXCL1-3 and 5-8. In non-small cell lung cancer CXCR2 has been studied mainly in stromal cells and is known to increase tumor inflammation and angiogenesis. However, there is controversial data in the literature about CXCR2 expression in tumor cells and its role in the tumor microenvironment. We hypothesized that tumoral expression of CXCR2 and its ligands promote tumor invasion and metastasis in non-small cell lung cancer.

The effect of CXCR2 expression on tumor cells was studied using stable knockdown clones derived from a murine KRAS/p53–mutant lung adenocarcinoma cell line (344SQ) with high metastatic potential and an orthotopic syngeneic mouse model and in vitro using a CXCR2 small molecule antagonist (SB225002). We showed that in vivo CXCR2 inhibition by knock-down reduces its invasive ability. In a murine model of orthotopic syngeneic lung adenocarcinoma CXCR2 knock-down 344SQ cell line was found to be associated with decreased tumor burden, local and distant metastases.

In order to translate our preclinical discoveries to human NSCLC, we explored CXCR2 tumoral immunohistochemical expression in 262 tissue microarrays created from tumor specimens of patients with surgically resected stage I-II lung adenocarcinoma and correlated it with patient clinic-pathological characteristics including smoking status, histological differentiation and survival outcomes. We considered also localization of CXCR2 expression in the cytoplasm, membrane and nucleus. High cytoplasmic CXCR2
was associated with smoking history, aggressive histological differentiation and worse survival. When we screened a publicly available large database of human NSCLC cell lines (N=52) and human lung adenocarcinomas (N=442), we found that at the gene expression level, CXCL5, a CXCR2-ligand, was the main driver of a cluster of cell lines and lung adenocarcinomas with high-risk features, including RAS and MET pathway activation, epithelial-to-mesenchimal transition and resistance to epidermal growth factor inhibition (i.e., gefitinib). We studied promoter methylation in 70 human non-small cell lung cancer cell lines and discovered that CXCL5 was regulated by promoter methylation. We concluded that the CXCR2 axis may be an important target in smoking-related lung adenocarcinoma.
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ABBREVIATIONS
CAF=cancer associated fibroblasts
COPD= chronic obstructive pulmonary disease
DSP=desmoplakin
DUSP4=dual specific phosphatase 4
EGFR=epidermal growth factor receptor
FBS=fetal bovine serum
FOLR1=folic acid receptor 1
GEO=gene expression Omnibus
GSEA=gene set enrichment analysis
HGF=hepatocyte growth factor
HBEC=human bronchial epithelial cells
HR=hazard ratio
IHC=immunohistochemistry
IPA=ingenuity pathway analysis
MAP=mitogen-activated protein
NKX2-1=thyroid transcription factor 1
NSCLC=non small cell lung cancer
PC1=principal component
PLC=phospholipase C
RRM2=ribonucleotide reductase M2
SPP1=osteopontin
SPINT1=hepatocyte growth factor activator inhibitor 1
SFTP=surfactant protein
TGFB1=tumor growth factor B1
TMA=tissue microarray
TTF1=thyroid transcription factor-1
VEGF=vascular endothelial growth factor
VIM=vimentin
INTRODUCTION

Lung cancer is responsible for over a million of deaths worldwide per year \(^1\). Diagnosis is often made at an advanced stage and the 5-years survival remains poor (about 15%) with only little progress in the last two decades. Lung cancer histology drives the therapeutic decisions and chemotherapy choice. Small-cell lung cancers and non-small-cell lung cancers (NSCLC) are the two major histological subtypes\(^2\). Lung adenocarcinoma, a subtype of NSCLC, represents about 50% of the total number of lung cancers. First-line chemotherapy for advanced/metastatic NSCLC consists of combination chemotherapy with platinum compounds\(^3\). In particular, in non-squamous histology, pemetrexed has shown to produce better outcomes than gemcitabine\(^4\).

The discovery of driver molecular alterations, such as \textit{EGFR} mutations\(^5\):\(^6\) and most recently ALK-fusion\(^7\) predominantly in never-smokers\(^8\), has unraveled a new horizon in the treatment of lung adenocarcinomas. The concept of “oncogene addiction”\(^9\) has created the basis for the successful use of targeted therapy of genetic alterations (i.e., erlotinib and crizotinib) changing therefore the therapeutic approach to this particular subtype of NSCLC\(^10\), as cancers harboring these genetic alterations are dependent on the expression of these single mutant oncogenes for survival. In the last decade, extensive literature has been published on genetic alterations that arise in lung adenocarcinoma from never smokers, however little is known about potential targets of smoking-related lung adenocarcinoma. Known driving mutations found in lung adenocarcinomas from smokers are \textit{KRAS} and \textit{BRAF}, however more efforts need to be implied to discover novel targets in this setting\(^11\).

The major role of chemokines is to act as a chemoattractant to guide the migration of cells. Cells that are attracted by chemokines follow a signal of increasing chemokine content.
concentration towards the source of the chemokine. Chemokines are functionally divided in two groups: homeostatic which are constitutively produced in certain tissues and are responsible for basal leukocyte migration (CCL14, CCL19, CCL20, CCL21, CCL25, CCL27 and CXCL12, CXCL13) and inflammatory which are produced under pathological conditions (i.e., on pro-inflammatory stimuli such as IL-1, TNF-alpha, viruses) and actively participate in the inflammatory response attracting immune cells to the site of inflammation (i.e., CXCL8, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL10)\textsuperscript{12}. Members of the chemokine family are divided into four groups depending on the spacing of their first two cysteine residues: CC chemokines with 2 adjacent cysteines, CXC chemokines in which the two N-terminal cysteines are separated by one amino acid, C chemokines with only two cysteines and CXC3C chemokines with 3 amino acids between the two cysteines. CXC chemokines have multiple roles in the tumor microenvironment\textsuperscript{13; 14; 15} and they are expressed on multiple cells including neutrophils, monocytes, eosinophils, mast cells, basophils, lymphocytes, epithelial cells, and endothelial cells\textsuperscript{16; 17}. One of the most important functions is to mediate communication between different cells in the tumor microenvironment and immune system\textsuperscript{16}. CXC chemokines containing the sequence Glu-Leu-Arg (ELR motif) as compared with members that lack these 3 amino acids are potent inducers of angiogenic activity as these 3 amino acids appear to be important in ligand/receptor interactions on neutrophils\textsuperscript{18}.

Two G-protein-coupled receptors, CXCR1 and CXCR2, and the Duffy antigen receptor, are the three known receptors for these chemokines. Endothelial cells and neutrophils express CXCR2 and migrate toward sites of CXCR2 ligand production such as inflammatory foci or nascent tumors, thereby promoting angiogenesis and inflammation in tumors that express ELR-positive CXC chemokines\textsuperscript{19; 20; 21; 22}. CXCR2 binds CXCL8 (IL-8), CXCL1, 2, and 3 (GROα, β, and γ), CXCL5 (ENA-78), CXCL6 (GCP2), and
CXCL7 (NAP2), whereas CXCR1 binds only to GCP2, NAP2, and IL-8. Chemokine receptors associate with G-proteins to transmit cell signals following ligand binding. Activation of G proteins, by chemokine receptors, causes the subsequent activation of the phospholipase C (PLC) with subsequent activation of the mitogen-activated protein (MAP) Kinase pathway activation with induction of chemotaxis, degranulation, release of superoxide anions and changes in the avidity of integrins within the cell harboring the chemokine receptor23.

In particular CXCR2 couples to the pertussis toxin-sensitive G, proteins to stimulate PLC-β which ultimately results in hydrolysis of the lipid phosphatidylinositol 4,5-bisphosphate, generating diacylglycerol, which activates PKC isoforms, and inositol 1,3,4-triphosphate, which releases calcium from intracellular stores. In addition, recently CXCR2 has been found to contain specific modular protein interaction domains called PDZ domains that may be responsible to nucleate the formation of compartmentalized multiprotein complexes that are critical for efficient and specific cellular signaling with scaffold proteins24;25.

Expression of the ELR+ CXC chemokines in human NSCLC samples correlates with worse survival26;27. Wislez M. et al. showed that CXCR2 ligands are abundant in alveolar epithelial cells of lung tumors arising in mice expressing KRASG12D alone and in combination with a Tp53 mutation (p53R172H) or Pten deletion28;29;30;31;32;33. CXCR2 neutralization decreased tumor angiogenesis and neutrophilic inflammation and blocked the expansion of early alveolar neoplastic lesions in KRASLA1 mice without having direct effects on tumor cells, implicating the tumor microenvironment in the anti-tumor effect of CXCR2 inhibition33.
However, there is controversial data in the literature about CXCR2 expression in tumor cells and its role on tumor growth and angiogenesis\textsuperscript{20, 34, 35, 36, 37}. Preclinical models have shown that CXCR2 expression can impact cell proliferation, migration, invasion and stress-induced apoptosis evasion\textsuperscript{14, 38, 39, 40, 41}. There are missing data, however, about if these effects are mediated by a direct effect of CXCR2 expression on cancer cells or by an anti-angiogenesis effect\textsuperscript{20, 39, 42, 43}. Pharmaceutical inhibition of CXCR2 is currently undergoing clinical development in chronic obstructive pulmonary disease (COPD) to counter-act the damaging effects of cigarette smoking that produces inflammation with increase in alveolar destruction by neutrophils, goblet cell hyperplasia and pro-angiogenetic effects\textsuperscript{17, 44}. These compounds therefore may be eventually available to be used to target the CXCR2 expression on cancer cells in the future.

Among the CXCR2 inhibitors, SB225002 (N-(2-hydroxy-4-nitrophenyl)-N’-(2-bromophenyl)urea) was the first reported potent and selective non-peptide inhibitor of CXCR2. It is an antagonist of IL-8 binding to CXCR2 with an IC\textsubscript{50} = 22 nM. SB 225002 showed >150-fold selectivity over CXCR1 and four other chemokine receptors tested. This compound inhibited the binding of both CXCL8 and CXCL1 on recombinant and native CXCR2 and also blocked CXCL8 and CXCL1-induced chemotaxis and margination of human and rabbit neutrophils without having any affinity or activity on CXCR1\textsuperscript{45}.

To clarify the effect of CXCR2 inhibition in NSCLC, I hypothesized that CXCR2 ligands promote tumor invasion and metastasis in NSCLC. I proposed to test this hypothesis by investigating the CXCR2 role in a KRAS/p53\textsuperscript{−}\textsuperscript{−} mutant lung adenocarcinoma murine model \textit{in vitro} and \textit{in vivo}\textsuperscript{46, 47, 48}. To understand the potential translational significance of my work in human NSCLC, CXCR2 tumoral expression was assessed in tissue microarrays of human NSCLC from stage I-II patients in correlation with patients
clinicopathological characteristics and a systematic analysis of gene expression profiles of \textit{CXCR2} and its ligands (subsequently called the \textit{CXCR2} axis) was conducted in human NSCLC cell lines and lung adenocarcinomas.
MATERIALS AND METHODS


**Human lung tissues and tissue microarray**
A detailed description of the tissue microarray (TMA) construction is provided elsewhere. In summary, after histological examination of NSCLC specimens, the NSCLC TMAs were constructed by obtaining three 1-mm in diameter cores from each tumor at three different sites (periphery, intermediate and central tumor sites).

**Immunohistochemical analysis**
Mouse monoclonal anti-human CXCR2 antibody (R&D Systems, Minneapolis, MN) was used at a dilution of 1:200, according to the manufacturer's instruction. CXCR2 staining was examined using light microscopy by a lung cancer pathologist (Dr. Yuan Ping). An independent observer (Dr. Ignacio I. Wistuba) reviewed one third of the cores chosen randomly. In case of discordance (~10%), both pathologists reviewed the slides jointly in a multiheaded microscope and reached consensus. Both pathologists were blinded with respect to the patients' outcome. Only cytoplasmic CXCR2 expression was quantified using a four-value intensity score (0, 1+, 2+, and 3+) and extent of reactivity (0-100%). Final score was then obtained by multiplying the intensity and reactivity extension values (range, 0-300).

**Animal husbandry**
All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at MD Anderson Cancer Center. For syngeneic tumor experiments, 10- to 16- week-old 129/Sv mice were injected with the indicated numbers of tumor cells into the left lung and euthanized at the first signs of morbidity.

**Establishment of murine lung adenocarcinoma cell lines**
The methods used to establish lung adenocarcinoma cell lines in culture from murine tumors have been described previously. Cell lines were named according to the mouse number and site of derivation (e.g., 344SQ for mouse 344, subcutaneous metastasis). These cells have alveolar type II cell properties and variable propensities to undergo the epithelial-to-mesenchymal transition and metastasize following injection into syngeneic mice.

**RNA extraction and quantitative reverse-transcription PCR**
RNAs were extracted using TRIzol (Invitrogen, Carlsbad, CA). mRNA was reverse transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). For quantitative PCR reactions, 1:10 dilutions of cDNA products were amplified by using SYBR Green PCR Master Mix (Applied Biosystems,
Carlsbad, CA) and analyzed by using ABI Prism 7500 Fast System (Applied Biosystems). mRNA expression values were normalized on the basis of L32 mRNA.

**Generation of shRNA transfectants**
The shRNA retroviral CXCR2 constructs were purchased (OriGene, Rockville, MD). The sequences of the CXCR2 and scrambled shRNA were as follow:
- CAAGGTGGATAAGTTCAACATTGAAGATT (CXCR2 clone 1),
- GTCTGCTATGAGGATGTAGGTAACAATAC (CXCR2 clone 3), and
- GCACTACCAGCTAACTCAGTACT (scrambled shRNA). Purified plasmids (1 μg of each) were transfected into 344SQ cells by using LipofectAmine and PLUS (Invitrogen). After 48 hours, transfectants were replated in RPMI 1640 medium containing 10% FBS and 15 μg/ml puromycin for selection and passed serially for 4 weeks to generate stable transfectants.

**Cell invasion assay**
As described previously\(^46\), cells (cancer-associated fibroblasts) were seeded first in the lower chambers (105 cells), and tumor cells (344SQ) were then seeded in the upper chambers (5x10^4) of 24-well Transwell invasion plates (BD Biosciences, Bedford, MA) in serum free medium containing mitomycin C to block proliferation. Cells in the upper chambers were allowed to invade for 14 to 16 hours. Cells on the inserts were fixed with 90% ethanol, stained with 0.1% crystal violet blue, and washed with ddH2O. Noninvaded cells on the upper side of the inserts were wiped off with a cotton swab. Invaded cells were counted in five microscopic fields at 4x magnification, and the counts were averaged. A small molecule antagonist of CXCR2 (SB225002) (Calbiochem) was used to inhibit CXCR2 invasive properties of 344P cells in Boyden chamber assays and included 344SQ cells as positive controls\(^45;50\).

**Gene expression analysis**
Publicly available gene expression profiles and clinical annotations of 442 lung adenocarcinomas were downloaded from the NCI Director’s Challenge Consortium for the Molecular Classification of adenocarcinoma (DCC)\(^51\). CEL files of 52 NSCLC cell lines (GSE4824)\(^52;53\), 130 lung squamous cell carcinomas with clinical annotations (GSE4573)\(^54\), and 7 NSCLC cell lines and 3 human bronchial epithelial cell lines (HBEC) before and after treatment with 5-aza-2'-deoxycytidine (decitabine) (GSE5816)\(^55\) were downloaded from Gene Expression Omnibus (GEO). The gene expression analysis was generated by using Array Studio software (Omicsoft Corporation, Research Triangle Park, NC). Raw microarray data were processed using quantile normalization and robust multi-array average algorithm. Probesets corresponding to CXCR2 axis were identified using the NetAffx Analysis Center from Affymetrix website. They were used to compute an unsupervised hierarchical clustering of the cell lines and of the lung adenocarcinomas using the Pearson’s correlation coefficient and Ward’s linkage method. To summarize the effect of CXCR2 axis, a principal component analysis was computed for the first two components. In the DCC, the first principal component (PC1) was used for correlative studies with tumor differentiation, smoking status, and overall survival. In the cell lines, PC1 was correlated with the whole genome. Gene Set Enrichment Analysis (GSEA) using the “pre-ranked” tool was done using either the genes ranked according to their correlation with PC1, or to fold-change between groups defined by the unsupervised hierarchical clustering. Probesets with an absolute Pearson correlation or a fold-change ≥0.5 were included in network analyses performed using Ingenuity Pathway Analysis.
(IPA) (Ingenuity® Systems, Redwood City, CA). Details of the GSEA and IPA are provided in Supplementary Material and Methods.

**CXCL5 promoter methylation study**

CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6 promoter methylation status was obtained from high-throughput promoter methylation profiles of 42 NSCLC cell lines overlapping with the panel of 52 NSCLC cell lines analyzed for gene expression. CXCL7 and CXCL8 results did not pass the quality control and were not included in the analysis. DNA methylation status of a set of 27,579 CpG sites around promoters of 14,475 consensus coding sequences was interrogated using the Illumina HumanMethylation27 Beadchip (Illumina, Inc., San Diego, CA). Genomic DNA (1 μg) was bisulfite-converted using the EZ DNA Methylation kit (Zymo Research Corp, Orange CA). Whole-genome amplification, fragmentation, hybridization, washing, counterstaining, and scanning were performed according to the manufacturer’s instructions. The scanner data and image output files were managed with the Illumina BeadStudio software Methylation module v.3.2. The normalized data, presented as beta values, represent the degree of methylation at each CpG site, 0 being unmethylated and 1 being methylated.

**Gene Set Enrichment Analysis**

GSEA is a robust computational method that determines whether an *a priori* defined set of genes shows statistically significant, concordant correlation with the CXCR2/CXCR2 ligands biological axis. GSEA aims to interpret large-scale expression data by identifying pathways and processes. The main advantage of this method is its flexibility in creating molecular signature databases of gene sets, including ones based on biologic pathways, or expression profiles in previously generated microarray data sets. The input data for the GSEA were the following: (1) a complete table of genes ranked according to their Pearson correlation with PC1, (2) a mapping file for identifying probesets in HG-U133A and B platforms, and (3) a catalog of functional gene sets from Molecular Signature Database (MSigDB, version 3.0, 30-September-2010 release, www.broad.mit.edu/gsea/msigdb/index.html). A total of 2,480 curated gene sets (canonical pathway gene sets, chemical and genetic perturbations gene sets, BioCarta gene sets, GenMAPP gene sets, and KEGG gene sets) were included in the analysis. Default parameters were used. Probesets (44,754 unique features in cell lines, 22,283 unique features in lung adenocarcinomas) were collapsed into gene symbols (18,770 unique genes in cell lines, 13,321 unique genes in lung adenocarcinomas). Inclusion gene set size was set between 15 and 500, and the phenotype was permuted 1,000 times. Gene sets that met the false discovery rate 0.25 criterion were considered.

**Ingenuity Pathway Analysis**

Probesets with an absolute Pearson correlation or a fold-change equal to or greater than 0.5 were included in network analyses performed using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). Network Generation: a data set containing gene identifiers and corresponding expression values was uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. Network/My Pathways Graphical Representation: a Network/My Pathways is a graphical representation of the molecular relationships between genes/gene products. Genes or gene products are
represented as nodes, and the biological relationship between two nodes is represented as an edge (line).
All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Pathways Knowledge Base, but are represented as a single node in the network. The intensity of the node color indicates the degree of positive (red) or negative (green) correlation. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with various labels that describe the nature of the relationship between the nodes.

**Statistical analysis**
Wilcoxon rank sum test or Kruskal-Wallis test was used to test the differences of CXCR2 expression between/among categorical variable levels. Martingale residuals were performed from a Cox model that included only baseline hazard function but no covariate. By applying a nonparametric smoother, the plots allow one to examine visually the nature of the relationship between the residuals and CXCR2 H-scores and to define a reasonable cutoff point to dichotomize the population. The Kaplan-Meier method was used to construct overall and recurrence-free survival curves, and the log-rank test was used to test the difference by covariate levels. Univariate and multivariate Cox models were fitted to estimate the effect of prognostic factors, including patient age, sex, tumor histology, stage, and marker on time to event endpoint. For cell line experiments, comparisons between two groups were performed using the Wilcoxon rank sum test unless otherwise indicated. One-way ANOVA was performed to compare multiple experimental groups. All statistical tests were two-sided, and P-values of 0.05 or less were considered to be statistically significant.

**RESULTS**

*Creation of an orthotopic syngeneic lung adenocarcinoma metastasis model*
We recently described the creation of a panel of cell lines from *KrasLA1/+p53R172HΔG/+* mice, which develop aggressive and metastatic lung adenocarcinoma\(^48\). One of these cell lines, 344SQ, is highly metastatic when injected subcutaneously into syngeneic mice\(^46\). In order to refine our lung adenocarcinoma metastasis model, we used 344SQ to create a novel orthotopic syngeneic model. As previously described\(^57\), \(2 \times 10^4\) 344SQ cells were injected into the left lung of syngeneic mice. The mice were euthanized 21 days after injection and, at necropsy, had developed metastases to hilar and mediastinal lymph nodes (Fig. 1), chest wall, and contralateral lung as well as extrathoracic distant metastases in the paraaortic lymph nodes, liver, adrenal glands, kidneys, spleen and diaphragm.
CXCR2 knockdown decreased tumor cell invasion in vitro

To investigate the role of CXCR2 expression by tumor cells, we created CXCR2 shRNA clones from parental 344SQ lung adenocarcinoma cell lines isolated from KrasLA1/+p53R172HΔG/+ mice. CXCR2 shRNA clones exhibited significantly lower expression of CXCR2 mRNA than scrambled controls (Fig. 2A). To test the invasion potential of the shRNA clones, we used cocultures with cancer-associated fibroblasts, which produce high levels of CXCR2 ligands, as previously described. To block tumor cell proliferation, we added mitomycin C to the serumfree medium in which the tumor cells were cultured (344SQ). CXCR2 shRNA clones showed a significantly lower invasion potential than scrambled controls (Fig. 2B).

CXCR2 pharmacological inhibition decreased tumor cell invasion in vitro

To confirm our findings in the 344SQ cells, we carried out experiments by using 344P, a second highly invasive and metastatic lung adenocarcinoma cell line derived from KrasLA1/+p53R172HΔG/+ mice. To inhibit CXCR2 using a different approach, we used a small molecule antagonist of CXCR2, SB225002, that has demonstrated selectivity and potency in vitro and in vivo. We examined the effect of SB225002 on 344P cell invasive properties in Boyden chamber assay and included 344SQ cells as a positive control. Treatment with SB225002
inhibited tumor cell invasive properties with an IC50 of 3-4 μmol/L for both 344P cells and 344SQ cells (Fig. 2C-D).

**CXCR2 knockdown in tumor cells decreased 344SQ metastatic potential**

We used the orthotopic murine model already described to test whether the metastatic potential of 344SQ would be affected by CXCR2 knockdown. Two shRNA clones (clone-1 and clone-3) and scrambled controls were compared by injecting 2x10⁴ cells into the left lung of mice (N=10 in each group, for a total of 30 mice). Mice were euthanized at 21 days after injection because two of the 10 mice in the scrambled control group demonstrated poor physical conditions due to tumor burden. At necropsy, mice bearing CXCR2-shRNA lung adenocarcinoma had significantly fewer lung tumor nodules in the lung of primary injection (Fig.2E) and fewer distant metastases (Fig. 2F) than scrambled controls. Sites of distant metastases included liver, adrenal glands, ipsilateral and contralateral lung, diaphragm, spleen and paraaortic lymph nodes.

**Figure 2. Effect of CXCR2 downregulation and inhibition.** In vitro (A-B) properties of shRNA clones (clone-1 and -3) compared to scrambled control: (A) mRNA expression by reverse transcription PCR relative to standard, (B) invasion assay using co-cultures with cancer associated fibroblasts (CAF) and mitomycin C to block tumor cell proliferation. In vitro CXCR2 inhibition with CXCR2-antagonist (C, D): invasion assay using co-cultures of 344P (C) and 344SQ (D) cell lines treated with increasing concentrations of SB225002. In vivo properties of shRNA clones (clone-1 and -3) compared to scrambled control (E, F): number of left lung tumor nodules, (E) number of distant metastases (F). Median and inter-quartile range are shown in the dot plots (E, F). Wilcoxon rank sum test (A, B, E, and F) between scrambled control and shRNA single clones (clone-1 and clone-3) or between different concentrations of SB225002 and the control (C, D).
Figure 2

A

B

C

D

E

F
CXCR2 protein expression in human NSCLC tumor cells is associated with adverse outcome and tobacco smoking

To investigate the role of CXCR2 in human tumor cells, we stained a tissue microarray that included 370 resected NSCLC. For the purpose of this study, we considered only 262 patients with stage I and II disease who did not receive preoperative chemotherapy. Clinical and pathological characteristics of the patient population are described in Table 1. Median age was 67.4 years (range: 32.2-90.0). Adenocarcinoma was the most frequent histological subtype (N=173, 66%). With a median follow-up of 5.3 years, 133 patients had developed recurrence (50.8%) and 101 had died (38.5%).
Table 1. Clinical and pathological characteristics of patients included in the tissue microarray (N=262).

<table>
<thead>
<tr>
<th>Covariate</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>134 (51.1%)</td>
</tr>
<tr>
<td>M</td>
<td>128 (48.9%)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>22 (8.4%)</td>
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<tr>
<td>White</td>
<td>240 (91.6%)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>105 (40.1%)</td>
</tr>
<tr>
<td>Former</td>
<td>127 (48.5%)</td>
</tr>
<tr>
<td>Never</td>
<td>30 (11.5%)</td>
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<tr>
<td>Pathological stage</td>
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</tr>
<tr>
<td>I</td>
<td>201 (76.7%)</td>
</tr>
<tr>
<td>II</td>
<td>61 (23.3%)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>173 (66%)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>89 (34%)</td>
</tr>
<tr>
<td>Degree of differentiation</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>74 (28.2%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>155 (59.2%)</td>
</tr>
<tr>
<td>Well</td>
<td>33 (12.6%)</td>
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<tr>
<td>Mild</td>
<td>106 (41.1%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>106 (41.1%)</td>
</tr>
<tr>
<td>Severe</td>
<td>46 (17.5%)</td>
</tr>
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<tr>
<td>Adjuvant therapy</td>
<td></td>
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<tr>
<td>No</td>
<td>180 (71.4%)</td>
</tr>
<tr>
<td>Yes</td>
<td>72 (28.6%)</td>
</tr>
<tr>
<td>Unknown</td>
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</table>

CXCR2 was expressed mainly in the cytoplasm (mean 31.07±30.78, median 20, range 0-130). Distribution of cytoplasmic CXCR2 protein expression in the whole population is shown in Fig. 3.
Figure 3. Distribution of cytoplasmic CXCR2 protein expression. Distribution of cytoplasmic CXCR2 protein expression in 262 non small cell lung cancer cell lines by squamous cell carcinoma (SCC) and adenocarcinoma (Adeno) histology.

Figure 3

Figure 4 shows one adenocarcinoma and one squamous cell carcinoma expressing CXCR2 with an intensity of 1 in 100% of tumor cells, and an H-score of 100. CXCR2 expression was low in the nucleus (mean 16.15±24.88, median 3.33, range 0-120). Cytoplasmic and nuclear CXCR2 expression levels were not correlated (rho=-0.02, \( P=0.76 \)). No association was observed between cytoplasmic CXCR2 and patient sex, race, tumor histology, stage or degree of inflammation. Cytoplasmic CXCR2 expression was higher in current smokers (32.57±28.28) and former smokers (31.19±33.15) than in never smokers (25.28±29.00), although it did not reach statistical significance. Similarly, poorly differentiated tumors had higher cytoplasmic CXCR2 levels (39.84±32.20) than moderately (29.99±30.18) or well-differentiated (16.46±23.98) tumors (\( P<0.0001 \)).
Figure 4. Expression of CXCR2 in tumor cells and tissues. Immunohistochemical expression of CXCR2 in two NSCLC tissue specimens, (A) squamous cell carcinoma and (B) adenocarcinoma.

The Martingale residual plots showed that median CXCR2 H-score was a reasonable cutoff point (Fig. 5).
**Figure 5. Median CXCR-2 immunohistochemistry score.** Martingale residuals analysis from a Cox model that includes only baseline hazard function but no covariate, are represented on the vertical axis and CXCR2 H-scores (CXCR2c) are represented on the horizontal axis. The median H-score for CXCR2 expression is shown by a vertical bar.

When cytoplasmic CXCR2 level was dichotomized using the median expression of 20, 141 (53.8%) tumors expressed low CXCR2 levels (<=20) and 121 (46.2%) tumors expressed high CXCR2 levels (>20). **EGFR** and **KRAS** mutational status was available for 157 of the lung adenocarcinomas; Figure 6 shows that cytoplasmic CXCR2 protein expression was lower, although not reaching statistical significance, in **EGFR**-mutant lung adenocarcinomas (N=17, mean 24.71±29.49, median 13.33, range 0-90), which are known to be more frequent among never smokers, than in **KRAS**-mutant (N=41, mean 31.02±31.65, median 20, range 0-110) or wild-type **EGFR** and wild-type **KRAS** lung adenocarcinomas (N=99, mean 33.05±33.61, median 20, range 0-130), which are more frequent among smokers.
Figure 6. CXCR2 expression according to **EGFR** and **KRAS** mutation status. CXCR2 expression in tumor cells according to **EGFR** and **KRAS** mutation status. **EGFR** and **KRAS** mutation status was available for 157 lung adenocarcinomas analyzed for cytoplasmic CXCR2 protein expression.
Using Kaplan-Meier curves, high CXCR2 expression was associated with poor overall survival and recurrence-free survival in patients with lung adenocarcinoma, although not reaching statistical significance (Fig. 7A-B).

**Figure 7. Overall survival and recurrence-free survival as a function of cytoplasmic CXCR2 expression in lung adenocarcinoma.** Kaplan-Meier curves for (A) overall survival and (B) recurrence-free survival as a function of cytoplasmic CXCR2 expression in 173 patients who underwent resection for lung adenocarcinoma.

No such association was observed in patients with squamous cell carcinoma (Fig. 8A-B).
Figure 8. Overall survival and recurrence-free survival as a function of cytoplasmic CXCR2 expression in lung adenocarcinoma. Kaplan-Meier curves for (A) overall survival and (B) recurrence-free survival as a function of cytoplasmic CXCR2 protein expression in 89 patients who underwent resection for lung squamous cell carcinoma.

Univariate Cox proportional hazards model assessed the effect of covariates on survival. High CXCR2 expression was associated with worse overall survival in both subtypes [hazard ratio (HR) = 1.488 95% confidence interval (95%CI): 0.905-2.448; HR=1.520 95%CI: 0.798-2.894] (Table 2A and 3A), although not reaching statistical significance. For recurrence-free survival, a similar trend was observed in adenocarcinoma (HR=1.284 95%CI: 0.830-1.985), but not in
squamous cell carcinoma (HR=1.028 95%CI 0.593-1.782) (Table 2B and Table 3B).

**Table 2.** Univariate Cox model assessing the effect of covariates on overall survival (A) and recurrence-free survival (B) (M: male; F: female; HR: hazard ratio; 95CI: 95% confidence interval), in adenocarcinoma patients (N=173).

### A

<table>
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<th>Covariate</th>
<th>HR</th>
<th>95%CI</th>
<th>P</th>
</tr>
</thead>
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<td>0.969</td>
<td>1.041</td>
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<td>Sex (M vs. F)</td>
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<td>0.356</td>
<td>1.923</td>
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<td>Smoking history</td>
<td></td>
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<tr>
<td>Former vs. Never</td>
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<td>Poor vs. Well</td>
<td>1.324</td>
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<td>Moderate vs. Mild</td>
<td>0.971</td>
<td>0.552</td>
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<td>Severe vs. Mild</td>
<td>0.986</td>
<td>0.504</td>
<td>1.928</td>
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<td>Adjuvant treatment (Yes vs. No)</td>
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<td>CXCR2 (&gt;20 vs. ≤20)</td>
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<td>0.905</td>
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### B

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<td>Sex (M vs. F)</td>
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<td>Race (White vs. Other)</td>
<td>1.129</td>
<td>0.491</td>
<td>2.596</td>
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<tr>
<td>Smoking history</td>
<td></td>
<td></td>
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<tr>
<td>Former vs. Never</td>
<td>1.134</td>
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<td>Current vs. Never</td>
<td>0.996</td>
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<td>2.471</td>
<td>1.520</td>
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<td></td>
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<td>Moderate vs. Mild</td>
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<td>Severe vs. Mild</td>
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<td>0.830</td>
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Table 3. Univariate Cox model assessing the effect of covariates on overall survival (A) and recurrence-free survival (B) (M: male; F: female; HR: hazard ratio; 95CI: 95% confidence interval), in squamous cell carcinoma patients (N=89).

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<td>Race (White vs. Other)</td>
<td>0.163</td>
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<td></td>
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<td>Former vs. Never</td>
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<td>0.034</td>
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</tr>
<tr>
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<td>Current vs. Never</td>
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<td>0.047</td>
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<td>Pathological stage (II vs. I)</td>
<td>1.042</td>
<td>0.523</td>
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<td>Degree of differentiation</td>
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<td>Poor vs. Well</td>
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<td>0.032</td>
<td>1.831</td>
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<td>Moderate vs. Mild</td>
<td>0.878</td>
<td>0.456</td>
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<td>Former vs. Never</td>
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<td>0.067</td>
<td>3.634</td>
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<td>0.035</td>
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Final multivariate Cox models are presented in Table 2. Combining all patients together, after adjusting for patients’ age, sex, and tumor stage, high cytoplasmic CXCR2 expression remained associated with poor overall survival (HR=1.559; 95%CI: 1.051-2.312, P=0.0273) (Table 4A).
Table 4. Final multicovariate Cox models assessing the effect of covariates on overall survival in the whole population (N=262), in patients with lung adenocarcinoma (N=173) (B), and in patients with lung squamous cell carcinoma (N=89) (C) (M: male; F: female; HR: hazard ration; 95CI: 95% confidence interval).

<table>
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<tr>
<th>Parameter</th>
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<th>P-value</th>
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<td>1.051</td>
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<th>P-value</th>
<th>HR</th>
<th>95% HR CI</th>
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<td>0.850</td>
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<td>1.592</td>
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Gene expression pattern of CXCR2 axis is associated with human smoking-related adenocarcinoma and adverse clinical features
High-throughput gene expression profiles offer the opportunity to study CXCR2 as well the genes that encode for its known ligands. We took advantage of publicly available profiles of 52 NSCLC cell lines and 442 early stage resected lung adenocarcinoma. Gene expression patterns of CXCR2 ligands in NSCLC cell lines and lung adenocarcinoma were comparable (Fig. 9). Unsupervised hierarchical clustering using the CXCR2 axis identified a cluster of cell lines with
high expression of CXCR2 ligand genes, which we called the CXCR2/CXCR2 ligands cluster, that included nine (17%) of the cell lines analyzed (Fig. 9A). This group was enriched in KRAS mutations (Fisher’s exact test, \( P = 0.0548 \)). HCC827, an EGFR-mutant cell line with an exon 19 deletion, was also part of this group. In lung adenocarcinomas, a similar cluster of 115 (26%) tumors was identified (Fig. 9B). EGFR mutation status was available for 170 adenocarcinomas. None of the 30 tumors included in the CXCR2/CXCR2 ligands cluster harbored EGFR mutations, whereas 24 of the remaining 140 adenocarcinomas did harbor an EGFR mutation (Fisher’s exact test, \( P = 0.0295 \)). A similar CXCR2/CXCR2 ligands cluster was observed in each of the four individual cohorts forming the DCC (data not shown). From Massarelli E. and Saintigny P., Cancer Res. 2013 Jan 15; 73(2):571-82. DOI: 10.1158/0008-5472.

Figure 9. Distribution of expression of CXCR2, its ligand genes and IL8 in non-small cell lung cancer cell lines and lung adenocarcinomas. Distribution of expression of CXCR2 and its ligand genes (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, PPBP [CXCL7], and IL8 in non-small cell lung cancer cell lines (panel A; \( N = 52 \)) and lung adenocarcinomas (panel B; \( N = 442 \)).

A trend toward a worse prognosis in the high CXCR2/CXCR2 ligands cluster was observed (Fig. 10A). Using a similar approach in 130 patients resected for squamous cell carcinoma and included in the GEO series GSE4573\textsuperscript{54}, we did not see any association between high CXCR2/CXCR2 ligands cluster and poor outcome (Fig. 10B,C).
Figure 10. *CXCR2/CXCR2* ligands association with overall survival in patients with lung adenocarcinoma. *CXCR2/CXCR2* ligands cluster is associated with a worse overall survival in patients with lung adenocarcinoma (N=442) (A). *CXCR2/CXCR2* ligands cluster is not associated with outcome in patients with lung squamous cell carcinoma (N=130) (B, C).
In both cell lines and lung adenocarcinoma, we compared the genes differentially expressed between the CXCR2/CXCR2 ligands cluster and the remaining samples across the whole genome. In both cases, CXCL5 gene was the most frequently upregulated in the CXCR2/CXCR2 ligands cluster. Members of the aldo/keto reductase superfamily (AKR1B10, AKR1C2, AKR1C3), associated with tobacco exposure, were also upregulated. In cell lines, TGFB1, vimentin (VIM), and osteopontin (SPP1) were upregulated in the CXCR2/CXCR2 ligands cluster, while desmoplakin (DSP) and hepatocyte growth factor activator inhibitor 1 (SPINT1) were downregulated. These changes may be associated with the epithelial-to-mesenchymal transition and promote invasion and metastasis. In lung adenocarcinomas, genes encoding the matrix metalloproteinases were upregulated in the CXCR2/CXCR2 ligands cluster, as was the dual specificity phosphatase 4 (DUSP4) gene, which is known to be downregulated in EGFR-mutant NSCLC. Consistent with the association between poor differentiation and CXCR2 protein expression observed in the tissue microarray, another striking change was the downregulation of differentiation-associated genes, including thyroid transcription factor 1 (NKX2-1) (Fig. 11A) and surfactant proteins B (SFTPB), C (SFTPC), and D (SFTPD), in the CXCR2/CXCR2 ligands cluster. Interestingly, ribonucleotide reductase M2 (RRM2) was upregulated in the CXCR2/CXCR2 ligands cluster, while folic acid receptor 1 (FOLR1) was downregulated. RRM2 and FOLR1 have been reported to modulate response to gemcitabine and pemetrexed, respectively, in NSCLC. Similar trends were observed in cell lines for FOLR1 and NKX2-1 downregulation (Fig. 11B).
Figure 11. TTF-1 gene expression in lung adenocarcinomas and cell lines. TTF-1 gene expression was more frequently lower in lung adenocarcinomas (A) and cell lines (B) included in the CXCR2/CXCR2 ligands cluster.
The most significant network associated with differential gene expression, with an absolute fold-change ≥0.5 between the CXCR2/CXCR2 ligands cluster and the remaining samples, was related to NFKB in both the cell lines and lung adenocarcinoma (data not shown). Using as input the fold-change in gene expression between the CXCR2/CXCR2 ligands cluster and the remaining lung adenocarcinomas, GSEA showed enrichment of genes associated with poor survival (Fig. 12C) (28). We also found enrichment in gene sets associated with poor differentiation and proliferation (data not shown), as well as MET transcriptionally co-regulated genes (Fig. 12D). Using as input the fold-change in gene expression between the CXCR2/CXCR2 ligands cluster and the remaining cell lines, GSEA showed a significant upregulation of gene sets related to the RAS pathway (Fig. 12E) and resistance to gefitinib (Fig. 12F), and enrichment of target genes of hsa-miR-let7, a known regulator of KRAS expression, and of genes associated with the epithelial-to-mesenchymal transition was observed (data not shown).

**Figure 12. Identification of a CXCR2/CXCR2 ligands cluster.** Unsupervised clustering using gene expression of CXCR2 and its ligands (CXCL6, IL8, CXCL2, CXCL1, CXCL3, PPBP [CXCL7], and CXCL5) in (A) 52 NSCLC cell lines and (B) 442 lung adenocarcinomas. For Gene 23 Set Enrichment Analysis, genes were preranked according to the fold-change observed between samples with the CXCR2/CXCR2 ligands cluster and the remaining samples in both lung adenocarcinomas (C, D) and cell lines (E, F); representative gene sets enriched with a *P*-value and a false discovery rate <0.0001 are shown for lung adenocarcinomas (C, D) and NSCLC cell lines (E, F).
As an alternative approach to studying the effect of the CXCR2/CXCR2 ligands axis, we summarized the effect of this axis by computing a principal components analysis (Fig. 13A and 14A). The distribution of PC1 in cell lines was bimodal, a group of 12 cell lines having a high PC1 (≥1.5) and the remaining cell lines having a low PC1 (Fig. 13B). Interestingly, cell line H1395, which has been reported to harbor an inactivating CXCR2 G354W mutation, had a low PC1 (Fig. 13B). Cell lines included in the CXCR2/CXCR2 ligands cluster or with a high PC1 had low levels of NKX2-1 gene expression, except HCC827 (Fig. 13C).

Figure 13. Expression of CXCR2 and its ligand genes in NSCLC cell lines. The first two principal components were computed using expression of CXCR2 and its ligand genes (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, PPBP [CXCL7], and IL8) in 52 NSCLC cell lines. (A) The first principal component (PC1) accounted for 48.25% of the variation of the principal components analysis and allowed identification of two groups of cell lines (bimodal distribution). (B) Cell lines with a high PC1 had a significant overlap with cell lines included in the CXCR2/CXCR2 ligands cluster. (C) TTF-1 gene expression was low in almost all the cell lines with high PC1 outlined by the blue box. NCI-H1395 (shown by a star) has an inactivating CXCR2 G354W mutation.
Figure 13

A

Second Principal Component (16.11%)

First principal component (48.25%)

B

Cell lines included in the CXCR2/CXCR2 ligands cluster
Other cell lines

C

TTF-1 gene expression
In lung adenocarcinomas, a similar bimodal distribution of PC1 was observed (Fig. 14A). Consistently with our immunohistochemical results, PC1 was statistically significantly higher in poorly differentiated adenocarcinomas than in moderately ($P=0.0065$) and well-differentiated tumors ($P=0.0006$) (Fig. 14B), and higher in current smokers than in former ($P=0.0010$) and never smokers ($P=0.0085$) (Fig. 14C).
Figure 14. Expression of CXCR2 and its ligand genes in lung adenocarcinomas.
The first two principal components were computed using expression of CXCR2 and its ligand genes (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, PPBP [CXCL7], and IL8) in 444 lung adenocarcinomas. (A) The first principal component (PC1) accounted for 46.15% of the variation of the principal component analysis in lung adenocarcinomas. (B) PC1 was statistically higher in poorly differentiated versus moderately (P=0.0065) or well differentiated (P=0.0006) lung adenocarcinomas and (C) in current versus former (P=0.0010) or never smokers (P=0.0085).
When PC1 was dichotomized based on the median, tumors with low PC1 linked with longer OS (Fig. 15); low PC1 was associated with HR of 0.6907 (95Cl: 0.5302-0.9000, P-value= 0.0061). After adjusting for patient age, sex, tumor stage, and institution, low PC1 remained associated with longer overall survival (HR of 0.6827; 95Cl: 0.5046-0.8701, P=0.0031) (Fig 15).

**Figure 15. PC1 and overall survival.**

![Graph showing comparison of PC1 based on median](image)

**CXCL5 is the main driver of the CXCR2/CXCR2 ligands cluster in adenocarcinomas and is regulated through promoter methylation**

CXCL5 was the gene most upregulated in the CXCR2/CXCR2 ligands cluster in comparison to other samples across the whole genome, in both the cell lines and lung adenocarcinomas. CXCL5 upregulation was associated with poor overall survival (Fig. 16).
Its distribution was bimodal in both cell lines and lung adenocarcinomas (Fig. 17A and B). This led us to hypothesize that promoter methylation might regulate its expression. CXCL5 gene expression was inversely correlated with the average beta-value, which measures the degree of methylation of the promoter (Fig. 17C). Data generated in an independent study with publicly available raw data confirmed high levels of expression of CXCL5 in cell lines included in the CXCR2/CXCR2 ligands axis, and low levels in other cell lines, including immortalized HBEC lines (Fig. 17D).
Figure 17. **CXCL5** drives the **CXCR2/CXCR2** ligands cluster and is regulated through promoter methylation. **CXCL5** is the gene most frequently upregulated across the whole genome in samples with the **CXCR2/CXCR2** ligands cluster compared to other samples, both *in vitro* (A) and *in vivo* (B). (C) **CXCL5** gene expression was inversely correlated with the average beta-value, and (D) was increased after treatment with decitabine in most of the cell lines with low baseline **CXCL5** expression.

**Figure 17**
Moreover, CXCL5 expression was induced by decitabine in most of the cell lines with low CXCL5 expression that were shown to be methylated, as well as in HBEC cells. Expression of CXCL1 \((r = -0.46, P=0.0021)\), CXCL2 \((r = -0.49, P=0.0010)\), CXCL3 \((r = -0.32, P=0.0390)\), and CXCL6 \((r = -0.46, P=0.0022)\) genes was significantly inversely correlated with the average beta-value of their respective promoter, suggesting regulation through promoter methylation (Fig. 18A-D).
Figure 18. Promoter methylation of CXCR2 ligand genes. Expression of (A) CXCL1, (B) CXCL2, (C) CXCL3, and (D) CXCL6 genes was negatively correlated with the level of their promoter methylation as indicated by average beta-value.

However, only CXCL1 and CXCL3 had patterns similar to CXCL5 in terms of response to decitabine (Fig. 19A-D).
Figure 19. Effect of decitabine on CXCR2 ligand genes. The effect of two doses of decitabine on (A) CXCL1, (B) CXCL2, (C) CXCL3, and (D) CXCL6 gene expression levels in 10 lung cell lines is shown.
DISCUSSION

We show that *in vivo* CXCR2 inhibition by knock-down in a murine cell line (344SQ), which has a known high metastatic potential, reduces its invasive ability. In a murine model of orthotopic syngeneic lung adenocarcinoma CXCR2 knock-down 344SQ cell line was found to be associated with decreased tumor burden, local and distant metastases. In order to translate our preclinical discoveries to human NSCLC, we explored CXCR2 tumoral immunohistochemical expression in tissue microarrays from patients with surgically resected stage I-II lung adenocarcinoma and correlated it with patient clinic-pathological characteristics including smoking status, histological differentiation and survival outcomes. We considered also localization of CXCR2 expression in the cytoplasm, membrane and nucleus. High cytoplasmic CXCR2 was associated with smoking history, aggressive histological differentiation and worse survival. When we screened a publicly available large database of NSCLC cell lines and lung adenocarcinomas, we found that at the gene expression level, CXCL5, a CXCR2-ligand, was the main driver of a cluster of cell lines and lung adenocarcinomas with high-risk features, including RAS and MET pathway activation, epithelial-to-mesenchimal transition and resistance to epidermal growth factor inhibition (i.e., gefitinib). We have named this CXCR2/CXCR2 ligands cluster and we discovered that CXCL5 in this cluster was regulated by promoter methylation.

Several studies have reported a critical role of CXCR2 inhibition in melanoma\textsuperscript{43,59}, ovarian\textsuperscript{59}, prostate\textsuperscript{60}, and esophageal cancers\textsuperscript{61} mainly by regulating the cell cycle, apoptosis and angiogenesis via multiple signaling pathways including PI3K/AKT, NF-kB, MAPK and STAT3. However, there is not a clear understanding if CXCR2 inhibition plays a direct effect on tumoral cells. We have tested our hypothesis using a novel orthotopic syngeneic lung cancer metastasis model with preserved immunity via
injection of a highly metastatic cell line (344SQ) derived from
*KrasLA1/+p53R172HΔG/+* mice. This murine model reliably represents the
developmental process of human lung adenocarcinomas\(^{29}\). The effect of CXCR2
knock-down in shRNA stable clones on the substantial reduction of the thoracic
tumors, lymphatic and distant metastases, is unlikely due to regulation of angiogenesis.
In fact, data published showing the effect of CXCR2 knockdown in ovarian cancer cell
lines on decreasing tumor angiogenesis by VEGF activation, were obtained using
immunosuppressed murine xenografts tumor models which do not take into
consideration the interaction between the tumoral cells and the tumor
microenvironment\(^{62}\). We understand that our work lacks of a direct evaluation on tumor
angiogenesis, therefore, we cannot completely rule out a direct effect of CXCR2
knockdown in the 344SQ cells on tumor angiogenesis.

When we explored in human NSCLC samples the tumoral cell localization of CXCR2
by IHC, we observed that only cytoplasmic expression was associated with significant
difference in terms of smoking history, histologic differentiation and survival. Although
many chemokine receptors internalize through clathrin-coated pits, regulation of the
receptor trafficking is not fully understood. Fan GH et al. showed colocalization of
CXCR2 with transferrin and low-density lipoprotein (LDL) after agonist treatment for
different periods of time, suggesting 2 intracellular trafficking pathways for this
receptor. CXCR2 was colocalized with Rab5 and Rab11a, which are localized in early
and recycling endosomes, respectively, in response to agonist stimulation for a short
period of time, suggesting a recycling pathway for the receptor trafficking\(^{63}\). The altered
distribution of CXCR2 into the cytoplasm may start an autocrine loop that may
contribute as a transcriptional signaling to augment expression of receptor-ligand
production as previously suggested in prostate cancer progression\(^{60}\).
CXCR2 inhibition has been reported to also affect the epithelial to-mesenchymal transition via abrogation of the Snail-mediated increase in tumor burden in murine models of NSCLC\textsuperscript{39,64}. However, contrasting results were described on this topic in a small cohort of 20 NSCLC showing a correlation between CXCR2 expression and improved survival\textsuperscript{65}. In addition, a role of CXCR2 as tumor suppressor gene was also underscored in studies about tumor senescence and response to oncogenic signals\textsuperscript{66}. Our GSEA analysis results show that c-MET oncogenic pathway is expressed in the CXCR2/CXCR2 ligands cluster, which is in line with the observation of downregulation of \textit{SPINT1}, a potent inhibitor specific for HGF activator, and upregulation of \textit{TGFB1} and \textit{VIM} in the CXCR2/CXCR2 ligands cluster\textsuperscript{67}.

Another interesting finding of our tissue microarray and GSEA analysis was the association of CXCR2 protein expression, the CXCR2/CXCR2 ligands cluster and high histological grade. The thyroid transcription factor 1 (\textit{TTF1, NKX2-1}), which is a lineage survival gene abnormally expressed in about 70\% of lung adenocarcinoma, was one of the genes most frequently downregulated in the cluster compared to other lung adenocarcinomas. Interestingly, NKX2-1 protein expression in human NSCLC has been reported in the literature to be prevalent in lung adenocarcinoma, female gender, non-smoking history, presence of epidermal growth factor receptor (EGFR) mutation and better overall survival and it is one of the routinely tested immunohistochemical markers for the pathological diagnosis of NSCLC\textsuperscript{68}. Absence of NKX2-1 expression may identify lung adenocarcinomas with CXCR2 activation, with interesting repercussion on the identification of tumors which may be targeted by CXCR2 inhibition. In addition, the correlation between NKX2-1 protein expression and presence of epidermal growth factor receptor (\textit{EGFR}) mutation, is in line with the findings of absence of \textit{EGFR} mutations in the lung adenocarcinoma included in the
CXCR2/CXCR2 ligands cluster in the DCC dataset which doesn’t express NKX2-1 protein. Our findings in the DCC dataset also highlight the association of CXCR2 expression and KRAS mutation which is indication of EGFR tyrosine kinase inhibitor therapy resistance\textsuperscript{69}. These observations are in line with the strong inhibitory effect of CXCR2 knockdown in our orthotopic syngeneic lung adenocarcinoma metastasis model, which harbors a KRAS mutation. Therefore, CXCR2 may represent an important target of KRAS-driven lung adenocarcinomas.

The findings of CXCL5 being the main driver gene in the CXCR2/CXCR2 ligands cluster add to the current literature where there are controversial reports about its significance in different tumor types. In head and neck squamous cell carcinoma, CXCL5 has been associated with tumor cell proliferation, migration and invasion\textsuperscript{70}. On the other hand, knock down cell clones of colon carcinoma resulted in a rapid tumor growth and in a number of tumor metastases \textit{in vivo}\textsuperscript{71}. In the same study, low expression of CXCL5 by immunohistochemistry was significantly associated with poor outcome in human colon cancer patients\textsuperscript{71}. In human lung adenocarcinoma there is no data on the association of CXCL5 expression and prognosis. However, in NSCLC methylation appears the main mechanism of regulation\textsuperscript{72}. We found that about 75-80% of NSCLC harbor CXCL5 promoter methylation with sub sequential silencing of the gene. These observations may suggest that CXCL5 might promote tumorigenesis in 20% of lung adenocarcinomas through CXCR2 autocrine and paracrine loop.

In conclusion, our data show that tumoral CXCR2 inhibition has an important role on regulation of invasion, growth \textit{in vitro} and \textit{in vivo} in an orthotopic syngeneic lung adenocarcinoma metastasis model with KRAS and TP53 mutations. We identified a cluster of human NSCLC cell lines and lung adenocarcinomas, in which prognosis seems to be affected by the CXCR2-CXCL5 axis with an interesting association to aggressive histology and smoking history. Pharmaceutical inhibition of CXCR2 is
currently undergoing clinical development in COPD to counter-act the damaging effects of cigarette smoking that produces inflammation with increase in alveolar destruction by neutrophils, goblet cell hyperplasia and pro-angiogenetic effects\textsuperscript{17, 44}. In addition, the finding of a cluster of lung adenocarcinomas with CXCR-2 ligand overexpression may of great value in directing novel approaches in T cell adoptive therapy of lung cancer, as showed by Peng W. et al, who introduced the CXCR2 gene into tumor-specific T cells to enhance their localization to tumors and improve antitumor immune responses\textsuperscript{73}. Therefore, with the future availability of CXCR2 targeted therapy in clinical cancer research, CXCR2 expression may become an interesting target to validate in prospective clinical trials in NSCLC.
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VITA

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