The CXCL7/CXCR1/2 Axis Is a Key Driver in the Growth of Clear Cell Renal Cell Carcinoma

Renaud Grépin, Melanie Guyot, Sandy Giuliano, Marina Boncompagni, Damien Ambrosetti, Emmanuel Chamorey, Jean-Yves Scoazec, Sylvie Negrier, Hélène Simonnet, and Gilles Pagès

Abstract

Mutations in the von Hippel–Lindau gene upregulate expression of the central angiogenic factor VEGF, which drives abnormal angiogenesis in clear cell renal cell carcinomas (ccRCC). However, the overexpression of VEGF in these tumors was not found to correlate with overall survival. Here, we show that the proangiogenic, proinflammatory cytokine CXCL7 is an independent prognostic factor for overall survival in this setting. CXCL7 antibodies strongly reduced the growth of ccRCC tumors in nude mice. Conversely, conditional overexpression of CXCL7 accelerated ccRCC development. CXCL7 promoted cell proliferation in vivo and in vitro, in which expression of CXCL7 was induced by the central proinflammatory cytokine interleukin (IL)-1β. ccRCC cells normally secrete low amounts of CXCL7; it was more highly expressed in tumors due to high levels of IL-1β there. We found that a pharmacological inhibitor of the CXCL7 receptors CXCR1 and CXCR2 (SB225002) was sufficient to inhibit endothelial cell proliferation and ccRCC growth. Because CXCR1 and CXCR2 are present on both endothelial and ccRCC cells, their inhibition affected both the tumor vasculature and the proliferation of tumor cells. Our results highlight the CXCL7/CXCR1/CXCR2 axis as a pertinent target for the treatment of ccRCC.

Introduction

Mutations in the von Hippel–Lindau gene cause overexpression of VEGF, resulting in clear cell renal cell carcinomas (ccRCC) to be one of the most vascularized tumors. Theoretically, ccRCC should be highly responsive to anti-VEGF therapy. Bevacizumab, a humanized monoclonal antibody targeting VEGF, in association with IFN-α, has obtained approval from the U.S. Food and Drug Administration for treatment (1). Despite the increased time to progression, the pivotal AVOREN study that compared the efficacy of IFN-α with IFN-α plus bevacizumab (2) showed that bevacizumab did not improve overall survival. However, a more detailed analysis of the results showed that some patients were high responders to treatment with prolonged survival, whereas the treatment was inefficient in other patients in which metastatic dissemination was observed (3, 4). Moreover, a recent meta-analysis showed that bevacizumab in combination with chemotherapy induced fatal adverse events (5). Our recent study also highlighted unexpected ccRCC-enhanced growth in mice treated with bevacizumab (6). We and others have also demonstrated a very important role for CXCL cytokines in the development of ccRCC progression, in particular CXCL8 (7). This suggested that ccRCC expresses a high amount of VEGF and others proangiogenic cytokines that play a key role when the VEGF/VEGFR axis is inhibited by either antibodies targeting VEGF or inhibitors targeting their tyrosine kinase receptors.

Cytokines of the CXCL family have angiogenic or antiangiogenic potency depending on the presence or absence of the amino acid triplet ELR in their protein sequence; ELR⁺CXCL (1–3, 5–8) have proangiogenic properties whereas ELR⁻CXCL (4, 9, 10) have antiangiogenic properties (8, 9). ELR⁺CXCL mediate their effect through their G-protein–coupled receptors CXCR-1 and CXCR-2, which leads to activation of the extracellular signal–regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways (8). The proangiogenic chemokine, CXCL8 also called interleukin (IL)-8, promotes angiogenesis, tumorigenesis, and metastasis, and it is overexpressed in many tumors, including ccRCC (10). Moreover, Ras-dependent secretion of CXCL8 enhanced tumor progression by promoting neovascularization (11). The CXCR2/CXCL8 axis was also described as a survival pathway for prostate (12), ovarian (13), brain (14), and skin cancers (15). Moreover, CXCR1 blockade inhibited the growth of human breast cancer stem cells (16). CXCL8 is not the only ELR⁺CXCL cytokine implicated in cancer progression as CXCL1 was shown to be important for the proliferation of esophageal (17) and...
Cancer Research
Cancer Res; 74(3) February 1, 2014

GAAGTGTGCC-3
overnight at 37
fragments following surgery were treated with collagenase
3 was a metastatic pT3a, Fuhrman grade 4 tumor. Tumor
was a nonmetastatic pT3a, Fuhrman grade 4 tumor, and tumor
Invitrogen) and blasticidin selection (10
were obtained by lentiviral transduction (pLenti6/V5-D-TOPO;
progression. CXCR expression may be bene
can be explained by differential expression during tumor
senescence (21) and tumor development (16). This discrepancy
controversial because this autocrine pathway can drive both
component implicated in tumor development. However, the mean-
ing of coexpression in the same cell of CXCR and CXCL is
ponent implicated in tumor development. However, the mean-
expression during the late stages accelerates tumor growth because
stimulation of CXCR induces activation of ERKs, a major
signaling pathway implicated in cell proliferation (22). The
goal of our study was to identify within the family of CXCL
cytokines the one that is the most pertinent as a prognostic
marker for survival of patients with ccRCC and to determine if
targeting this cytokine or its receptors inhibits growth of an
experimental model of RCC.

Materials and Methods
Human kidney samples
The clinical characteristics of the patients and angiogenic
profile of the normal and tumor tissues were described pre-
viously (6).

Cell lines and molecular biology
786-O (CRL 1932), Caki-2 (HTB-47), and ACHN (CRL 1611)
cells were from American Type Culture Collection. RCC10 were
a kind gift from W.H. Kaelin (Dana-Farber Cancer Institute,
Boston, MA). 786-O (O), RCC-10 (L), and ACHN (L) cells
were obtained by lentiviral transduction (pLent6/V5-D-TOPO;
Invitrogen) and blasticidin selection (10 μg/mL; ref. 6). Tumor
1 was a nonmetastatic pT3b, Fuhrman grade 2 tumor, tumor 2
was a nonmetastatic pT3a, Fuhrman grade 4 tumor, and tumor
3 was a metastatic pT3a, Fuhrman grade 4 tumor. Tumor
fragments following surgery were treated with collagenase
overnight at 37°C and/or mechanically disaggregated with
scalpels. Tumor cells were suspended in cell culture medium
specific for renal cells (PromoCell). Further experiments
were performed after passage ten when the cell line was established.
Reverse transcription reactions were performed as described
(23). PCR analysis of CXCR2 expression was performed with the
following oligonucleotide pair: forward primer 5'-ATGGAA-
GATTTTACATGGGAG-3'; reverse primer 5'-GAGAGATTAGTG-
GAATGGTGGCC-3'.

Antibodies
The following antibodies were used for immunoblotting or
immunohistochemistry respectively: antiphospho ERK1/2
antibody (pERK; Sigma), antiphospho AKT (pAKT), anti-AKT,
antiphospho ERK1/2 (Santa Cruz Biotechnology), anti-PARP (Cell
signaling), and anti-CAIX (Bayer Health Care). The CD31 antibodies
were a rat monoclonal anti-mouse CD31 (clone MEC 13.3,
1:1000, BD Pharmingen) or a rabbit polyclonal anti-mouse
CD31 (ab124432; Abcam).

Drugs
The anti-human CXCL7 antibodies (Peprotech) were diluted
in PBS and injected intraperitoneally (5 mg/kg), as described
previously (11). PBS was injected into the control group.

SB225002 was synthesized by Dr. Rachid Benhida, the Chemistry
Department of the University of Nice, Nice, France, as
previously described (24).

Tumor xenograft formation and size evaluation
786-O (L), RCC-10 (L), or ACHNLUC (3 x 10⁶ to
10 x 10⁶) were injected subcutaneously into the flanks
of 5-week-old nude (nu/nu) female mice (Janvier). Biolumines-
cence was quantified using the In Vivo Imaging System (Perkin
Elmer) according to the manufacturer's instructions. Tumor
volume [v = L x W x 0.52 (25)] was determined in parallel
using a calliper. There was a linear relationship between values
for bioluminescence and the tumor volume.

Immunohistochemistry and immunofluorescence
experiments
Tumor sections were handled as described previously (26)
for immunofluorescence experiments. Sections were incubated
with rat monoclonal anti-mouse CD31 (clone MEC 13.3; BD
Pharmingen). For immunohistochemistry, a rabbit polyclonal
anti-mouse CD31 (ab124432; Abcam) was used. Vessel density
was evaluated using the ImageJ program. Three double-blind
counts were performed.

Measurement of hemoglobin and cytokines
Frozen tumor tissues were lysed in cell extraction buffer
(Biosource). The intratumor hemoglobin content was measured
using a Drabkin reagent kit 525 (Sigma), CXCL cytokines,
fibroblast growth factor (FGF), human and mouse VEGF were measured using Peprotech ELISA kits according to the
manufacturer's recommendations (Peprotech). VEGF-C was measured
using the Human DuoSet ELISA kits, and VEGF-D using
the Quantikine ELISA Kit (R&D Systems).

Statistical analysis
Statistical analyses were two-sided and were performed
using R-2.12.2 for Windows. Statistical comparisons were
performed using the 2 test or the Fisher exact test for
qualitative data, the Student t test or Wilcoxon test for
quantitative data, and the log-rank test for censored data.

Results
CXCL7 is an independent prognostic factor for overall
survival of patients with ccRCC
Previously, we used a minoassay to determine cytokines
that regulate the angiogenic balance in 51 ccRCC patient
samples and their normal tissue counterpart (6). To investigate
whether these cytokines were associated with patient out-
come, we measured the correlation between the overall patient
survival and the intratumoral levels of the different cytokines.
In total, 22 patients (43%) died during the follow-up period. It is noteworthy that patients who exhibited CXCL7 levels superior to the first quartile (1,152 pg/mg) had a significantly higher death rate (Fig. 1A). Univariate analysis showed that CXCL7 expression was a poor prognostic factor for overall survival ($P = 0.0015$; Supplementary Table S1). Furthermore, metastasis at diagnosis and the Fuhrman grade, which are both known to be poor prognostic factors for patient’s outcome, also correlated significantly with overall survival ($P \leq 10^{-3}$ and 0.001). Unexpectedly, the levels of the proangiogenic forms of VEGF [levels of total VEGF (V) minus the levels of the antiangiogenic form of VEGF (Vb; ref. 27)] did not significantly correlate with survival ($P = 0.26$ and 0.49; Supplementary Table S1). These prognostic factors (level of CXCL7, metastasis at diagnosis, and the Fuhrman grade) were then analyzed in a multivariate Cox regression model on overall survival (Supplementary Table S2). CXCL7 expression was identified as an independent prognostic parameter for overall survival ($P = 0.014$). Similar results were obtained for metastasis at diagnosis and the Fuhrman grade with respect to overall survival ($P = 0.0005$ and 0.007; Supplementary Table S2).

CXCL7 blockade decreases the growth of ccRCC xenograft tumors

The results described above prompted us to test the potential of CXCL7 as a new therapeutic target for the development of ccRCC. For this purpose, we analyzed expression of CXCL7 in the conditioned medium of different model cell lines of ccRCC, including 786-O, RCC-10, ACHN, and Caki-2. These cell lines have been extensively for their mutations in the von
Hippel–Lindau protein, which lead to constitutive expression of the hypoxia-inducible factor 1 α or 2 α (HIF-1α or 2α; ref. 28). These cell lines only produced levels of CXCL7, which are at the lower limit of the detection threshold. However, human CXCL7 (ELISA tests specific of human CXCL7 were used) was present in tumors generated from 786-O cells in nude mice in a range comparable with what was detected in human samples (mean, 2,000 pg/mg protein; ref. 6), suggesting that "factors" produced in the tumor’s microenvironment stimulate the production of CXCL7 by tumor cells. This result was in agreement with the data of Pillai and colleagues, who described that stromal-derived "activities" were required for optimal expression of CXCL7 by monocytes (29). These results provided the rationale for testing the impact of blocking human CXCL7 on the development of ccRCC xenografted tumors. Thus, 786-O-Luc+ cells were monitored by luminescence to evaluate the growth of tumors in vivo following treatment. Mice developed tumors seven days after cell injections and tumors remained latent for 40 days before developing exponentially. In contrast, when anti-CXCL7 antibodies were administered 40 days after the injection of 786-O cells, tumors remained latent for as long as 70 days (Fig. 1C and D). The effect of anti-CXCL7 antibodies was also tested on ccRCC generated with two others independent ccRCC cell lines (RCC-10 and ACHN) with equivalent proliferative properties. First, we evaluated the production of angiogenic cytokines in tumors of placebo or anti-CXCL7-treated mice. No major signs of apoptosis (assessed by PARP cleavage) were detected in tumor samples. Carbonic anhydrase 9 (CAIX) is a major target of the HIF and has been extensively studied as a marker of hypoxia (30). A significant decrease in CAIX protein levels was observed in tumors of anti-CXCL7–treated mice (Fig. 2F), suggesting that the anti-CXCL7 treatment decreases cell proliferation and therefore limits the development of hypoxic zones that have been correlated with the selection of more aggressive cells (31).

**Overexpression of CXCL7 accelerates the growth of ccRCC xenograft tumors in nude mice**

To definitively demonstrate the protumor growth effect of CXCL7, we generated 786-O cells overexpressing CXCL7. Control 786-O cells produced levels of CXCL7 at the limit of the detection threshold of ELISA tests (50 pg/mL/24 hours/10⁶ cells; Fig. 3A). The level of CXCL7 in the media of 786-O cells transduced with a CXCL7-expressing lentivirus was superior to 1 ng/mL/24 hours/10⁶ cells. Overexpression resulted in accelerated cell proliferation, reinforcing the concept of an autocrine proliferation loop driven by CXCL7 (Fig. 3B and C). Overexpression of CXCL7 resulted in accelerated tumor growth in xenografts on the flanks of nude mice (Fig. 3D). Because tumors obtained with CXCL7 overexpressing cells were too big, mice were sacrificed for ethical purposes. Therefore, extensive comparison of control and "CXCL7" tumors by immunohistochemistry was impossible because of the small size of control tumors. Only ELISA tests and Western blotting were possible. High levels of CXCL7 were detected in the plasma and the tumors (Fig. 3E). No modifications of other potent angiogenic factors—VEGF and CXCL8—were detected. The presence of equal hemoglobin content also suggests that the difference in tumor growth was not dependent on angiogenesis (Fig. 3F). Increased proliferating capabilities were noted by testing ERK and AKT activity (significant increase for ERK and a tendency to increase AKT activity), which almost mirrored the results obtained by blocking CXCL7, strongly suggesting that CXCL7 accelerates tumor cell proliferation in vivo (Fig. 3G).

**Regulation of expression of CXCL7 by tumor cells in vitro and in vivo**

We analyzed the discrepancy between CXCL7 production in cell lines and in tumor xenografts. CXCL7 is produced as a precursor protein platelet basic protein (PBP), which is then cleaved to give the connective tissue-activating peptide III (CTAP-III) and the β-thromboglobulin-antigen (βTG-Ag). βTG-Ag matures to generate active neutrophil-activated peptide 2 (NAP-2)/CXCL7 (32). PBP expression could be stimulated by IL-1β in colon carcinoma cell lines (32). IL-1β induces PBP/CXCL7 mRNA expression in 786-O cells (Fig. 4A). ELISA tests specific for the mature CXCL7 form show that IL-1β also stimulates the production of the mature protein (Fig. 4B). The discrepant results between in vitro and in vivo experiments were explained by the fact that tumors generated in nude mice produced IL-1β (mean, 550 pg/mL/mg protein range, 50–2,000; n = 15). Equivalent amounts of IL-1β were detected in the samples of the cohort of patients (mean, 500 pg/mL/mg protein). In both experimental and human tumors, CXCL7
levels correlate with IL-1β levels. Moreover, cathepsin G and matrix metalloproteases (MMP), the proteases described as PBP-maturing proteins (32, 33), are active in 786-O cells and probably participate in PBP maturation. Anti-CXCL7 antibodies alone inhibit cell proliferation in the absence of IL-1β, suggesting that even if CXCL7 is produced at low level it participates in an autocrine proliferation loop in 786-O cells. Even in conditions of exponential proliferation in the presence of serum, IL-1β stimulates 786-O cell proliferation (Fig. 4C). Whereas control short hairpin RNA (shRNA) had no effect, CXCL7-directed shRNA inhibits cell proliferation of 786-O cells stimulated or not with IL-1β. These experiments demonstrate by a different approach the involvement of CXCL7 produced by the 786-O cells in an autocrine proliferation loop (Fig. 4D).

**Aberrant expression of CXCR1 and CXCR2 by ccRCC cells**

The experiments described above suggested that CXCL7 exerts an autocrine proliferation loop via its receptors CXCR1 and CXCR2 (8). To verify our hypothesis, we determined by quantitative real-time PCR the expression of CXCR1 and CXCR2 in different ccRCC cell lines. CXCR1 and CXCR2 were expressed in cell lines and in cells derived from independent fresh human ccRCC tumor samples in comparison with human microvascular endothelial cells (HMVEC) as a positive control (34; Fig. 5A). The functionality and the role of CXCR1 and CXCR2 expressed by ccRCC cells on proliferation was determined through the use of SB225002, a competitive inhibitor of CXCR1 and CXCR2, which has been described in the context of inflammatory pathologies and tumor development (16, 35). SB225002 reduced 786-O cells accumulation in a dose- and time-dependent manner (Fig. 5B). SB225002 also significantly inhibited accumulation of ccRCC cells derived from fresh human ccRCC tumors (Fig. 5C) and accumulation of HMVEC (Supplementary Fig. S4A). SB225002 inhibited ERK and AKT activities in a dose-dependent manner (Fig. 5D) and induced PARP cleavage (Fig. 5E) in 786-O cells and HMVEC (Supplementary Fig. S4B and S4C), suggesting a combined inhibition of proliferation and induction of apoptosis. Moreover, SB225002 inhibited the production of proangiogenic cytokines, including VEGF, CXCL1, and CXCL8, in a dose-dependent manner (Supplementary Fig. S5). These results strongly suggest that CXCR1/CXCR2 inhibitors could have potent antitumor growth effects in ccRCC because they concomitantly inhibit cell proliferation, induce apoptosis of tumor, and inhibit endothelial cells and expression of proangiogenic factors by tumor cells.

**Inhibition of CXCR1 and CXCR2 prevents ccRCC tumor growth in nude mice**

The results presented above suggest that CXCR1 and CXCR2 play a central role in ccRCC development. Hence, we predicted that inhibition of abnormally expressed CXCR1s in tumor cells
should efficiently inhibit tumor growth. Indeed, SB225002 slowed down tumor growth in nude mice (Fig. 6A), suggesting that CXCR1 and CXCR2 may represent therapeutic targets for ccRCC. SB225002 effect inhibited ERK and AKT activity, indicating that at least part of the SB225002 on tumor growth was the result of decreased cell proliferation (Fig. 6B). Ki67 labeling confirmed the inhibitory effect of SB225002 on tumor cell proliferation (Fig. 6C). An increase in the size of the necrotic zones, which is likely due to the SB225002-dependent induction of apoptosis observed in vitro, was also detected (Fig. 6D). We then evaluated the angiogenic profile of the tumors of control or SB225002-treated mice. Although SB225002 had no significant effect on intratumor hemoglobin levels, it significantly decreased the levels of CXCL1 (P < 0.05) and CXCL2 (P = 0.006), two ELR + CXCL involved in CXCR2 activation, and those of FGF involved in stromal cell growth (P = 0.03). We also observed a decrease in VEGF production (P = 0.04) and a nonstatistically drop in CXCL8 (P = 0.06). Intratumoral CXCL7 was equivalent in control and SB225002-treated mice. The statistically significant increase in CXCL4 (P = 0.007), an antiangiogenic member of the CXCL cytokine family also favored the antiangiogenic effect of SB225002 (Fig. 6E). CD31 labeling (Fig. 6F) confirmed the decrease in blood vessel density. Hematoxylin-eosin-saffron staining highlighted the presence of numerous blood vessels in the core of control tumors whereas only a few big vessels were observed at the

Figure 3. Expression of CXCL7 accelerates tumor growth. A, 3 × 105 control 786-O cells (CT) or 786-O cells expressing CXCL7 (C7) were plated at time 0. Secreted CXCL7 during 48 hours was determined by ELISA in the conditioned medium. Results are the mean ± SD of three independent experiments. B, 3 × 105 control 786-O cells (CT) or 786-O cells expressing CXCL7 (C7) were plated at time 0. The mean cell counts ± SD after 48 hours of culture was given. C, clonogenic assay with control 786-O cells (CT) or 786-O cells expressing CXCL7 (C7) following ten days of culture. D, 3 × 105 control 786-O(ΔCCL7) cells (CT) or 3 × 105 786-O(CCL7) cells expressing CXCL7 (C7) were subcutaneously injected into nude mice (n = 7 per group). Bioluminescence was measured weekly. Data, mean ± SD. Statistical differences between the size of tumors of control and treated mice are shown with asterisks. E, intratumoral and plasmatic CXCL7 was measured by the ELISA test. F, the intratumoral amount of hemoglobin (Hg) that gives a global read out of the blood supply is not affected. G, tumor extracts from control (CT) or tumors overexpressing CXCL7 (C7) were tested for ERK and AKT activities by immunoblotting using antibodies directed against the phosphorylated and nonphosphorylated forms of the proteins. The graphs show the ratio of phosphorylated ERKs or AKT to nonphosphorylated ERKs or AKT, respectively. The ERK and AKT activities from control tumor extracts were considered as the reference values (100%). Statistical differences: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
periphery of tumors of SB225002-treated mice (Supplementary Fig. S6). These results further confirmed the decrease in vessel density, which is probably correlated with decreased proliferation and increased apoptosis of endothelial cells. Hence, our results strongly suggest that CXCR1/CXCR2 inhibitors simultaneously decrease angiogenesis and the proliferation of ccRCC and thus they might be considered as potent therapeutic agents for such cancers.

Discussion

Anti-inflammatory drugs like aspirin reduce the incidence of cancers (36–38). This feature reflects the fact that inflammation drives different mechanisms involved in tumor growth and dissemination, including proliferation of tumor cells, angiogenesis, and metastasis (39). These mechanisms are, in part, driven by secreted molecules such as ELR+CXCL cytokines, which play a key role in tumor development and invasion. Although they were first described to be produced by inflammatory cells notably neutrophils, they are also secreted by other cell types, including tumor cells of different origin and endothelial cells (8). Their receptors CXCR1 and CXCR2 are also not restricted to neutrophils with expression in both endothelial and tumor cells (16, 17, 21, 40).

Three questions arise from our finding: (i) Are the levels of ELR+CXCL cytokines easily assayed on samples obtained from patients in a minimally invasive way? (ii) If yes, can they represent new therapeutic targets? (iii) If this latter hypothesis is true, is it better to target specifically ELR+CXCL or their receptors? Our study begins to answer these questions although we have to be cautious because some of our experiments constitute a preclinical approach. Analysis of patient samples has clearly shown that CXCL7 represents a strong independent prognostic marker of tumor aggressiveness. The quantification of its intrinsic level allowed determination of a specific threshold for pejorative outcome. This quantitative parameter is of interest to clinicians who need reproducible methods that can be included in clinical practice. The fact that

![Figure 4](Image 455x452 to 470x518)
it is an independent prognostic marker may orientate clinicians’ treatment strategies for patients with a priori good prognosis according to clinical parameters. CXCL7 is probably the least investigated cytokine of the ELR\(^+\) CXCL family in the context of cancer development. Only a few articles describe its role as a marker of advanced disease in myelodysplastic syndromes (20), in malignant pancreatic diseases (41), and in breast cancers (42). In breast cancers, CXCL7 also promoted tumor cell dissemination because it induced expression of VEGF-C and VEGF-D, two mediators in the development of lymphatic vessels (19). We and others have recently published that targeting CXCL8, another member of the ELR\(^+\) CXCL family, inhibits the growth of 786-O xenografts (6, 7). However, anti-CXCL7 antibodies block tumor growth whereas anti-CXCL8 antibodies only slow down tumor growth highlighting CXCL7 as a more relevant target. Moreover, CXCL8 does not seem as a prognostic marker of survival in univari- or multivariate analysis in the cohort of patients that we analyzed \((P = 0.49)\). Among all the other ELR\(^+\) CXCL cytokines we analyzed in our cohort of patients, only CXCL1 seems as a marker of poor prognosis in univariate analysis but fails to reach statistical significance in multivariate analysis \((P = 0.017\) and 0.06, respectively). Like for anti-CXCL8 antibodies, anti-CXCL1 antibodies alone only slow down tumor growth. Anti-CXCL1 (not shown) or anti-CXCL8 (6) antibodies did not decrease the intratumoral levels of CXCL7. These results probably explain their weaker effects on tumor growth.

Discrepancies between basal production of CXCL7 by ccRCC cells \(in vitro\) and secretion \(in vivo\) were intriguing. Although active factors produced by stromal cells have already been suspected to stimulate CXCL7 production (29), the role of IL-1\(\beta\) suggests the contribution of cytokines produced by inflammatory cells in the tumor context to favor CXCL7 production by cancer cells. Maturation toward the active NAP-2/CXCL7 is then mediated by MMPs like in colon tumor cells or epithelial cells from patients with colitis (32) or by cathepsin G present at the surface of neutrophils (33). Hence, our results strongly suggest that in addition to hematopoietic cells, epithelial cells have the ability to produce mature cytokines, which serve as chemoattractants for neutrophils or macrophages to maintain the inflammatory context within the tumors. Moreover, IL-1\(\beta\) was shown to be produced by ccRCC cells with a high malignancy potential and to participate in the epithelial–mesenchymal transition (43). Our experiments suggest that IL-1\(\beta\) promotes tumor cell proliferation via CXCL7 production and enhances tumor aggressiveness.

Before the development of antiangiogenic therapies, the prognosis of metastatic ccRCC was poor. Although they have

---

Figure 5. Expression of CXCR1 and CXCR2 in ccRCC cells; effects of a pharmacological inhibitor of CXCR1 and CXCR2 (SB225002) on ccRCC cell proliferation. A, different ccRCC cell lines were tested for the presence of CXCR1 and CXCR2 mRNA by quantitative PCR in human microvascular endothelial (EC), 786-O (786), ACHN (AC), and RCC10 (R10) cells. The presence of the receptors was also tested in cell lines established from fresh tumors (T1, T2, and T3). B, the proliferative capacity of 786-O cells in the presence of increasing concentrations of SB225002 was tested using the MTT assay. Data, mean fold increase \(\pm\) SD. The fold increase of untreated cells was taken as the reference value for statistics. Statistical significances of the results compared with untreated cells are indicated. C, the proliferative capacity of cells established from fresh tumors was measured as described above. The percentage of inhibition of proliferation using 5 \(\mu\)mol/L of SB225002 compared with untreated cells is plotted. D, 786-O cells incubated with the indicated amounts of SB225002 for two hours were tested for ERK and AKT activities as indicated above. E, 786-O cells incubated with varying amounts of SB225002 for 24 hours were tested for the presence of cleaved forms of PARP indicated by an asterisk. Tubulin is shown as a loading control.
revolutionized the treatment of this pathology, the benefit in terms of overall survival is either null or measured in months (2, 44, 45). This has been putatively linked to the heterogeneity of ccRCC as evaluated by multiregion sequencing (46). Consequently, there is an urgent need to identify predictive markers of outcome for these expensive therapies or to develop new drug delivery systems (47) or alternative therapeutic targets must be found in case of evasion to antiangiogenic...
agents. These points are essential to reduce the health cost but more importantly to limit the number of patients developing adverse events (5, 6). Beyond the role as prognostic markers CXCL cytokines could also be considered as predictive markers of response to antiangiogenic therapies, not only in ccRCC but also in other cancer types, as we suggested recently (6, 23). A clinical assay aimed at correlating the plasma level of CXCL cytokines and the response to sunitinib in metastatic ccRCC patients is currently ongoing.

Our study questions the use of specific antibodies against CXCL7 or of CXCR1/CXCR2 inhibitors. When taking into consideration the development of inflammatory zones during the first phases of tumor development, we hypothesized that targeting specifically CXCL7 could be a good choice and would probably be a less aggressive treatment with limited side effects. Moreover, expression of CXCR1/CXCR2 might represent a "protective barrier" for cancer development through induction of senescence (21). However, the most aggressive forms of ccRCC apparently use the CXCR1/CXCR2 pathway to drive an autocrine proliferation loop. This more aggressive tumor is characterized by an active inflammatory component and angiogenesis dependent on cytokines secreted by tumor and inflammatory cells. CXCL7 is involved in this autocrine loop, but other members of the family of ELR + CXCL cytokines, including CXCL1 and CXCL8, which are expressed by ccRCC, as we have previously shown (6) and whose role in tumor development has been widely described (48–50). Then, an inhibitor of CXCR1/CXCR2 might target at the same time tumor, endothelial, and inflammatory cells thereby limiting tumor cell proliferation, angiogenesis, and inflammation. However, in tumor treated by SB225002, we did not observe a decrease of CXCL7 levels probably because SB225002 enhances II-1β-dependent CXCL7 expression by an as yet unknown mechanism (Supplementary Fig. S7). In conclusion, our study provides the framework for new therapeutic approaches targeting ccRCC subsequent to the failure of the "gold standard" antiangiogenic treatment sunitinib (51). Validation of CXCL7 as a relevant prognostic/predictive marker of response to standard therapy in a larger, independent cohort of patients must now be performed to extend our results.

Disclosure of Potential Conflicts of Interest
S. Negrier has honoraria from speakers' bureau of Pfizer, GSK, and Novartis.

Authors' Contributions
Conception and design: R. Grépin, M. Guyot, S. Negrier, G. Pagès
Development of methodology: R. Grépin, M. Guyot, S. Giuliano
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Guyot, S. Giuliano, M. Boncompagni, D. Ambrossetti, E. Chamorey, J.-Y. Scoazec, S. Negrier, G. Pagès
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Grépin, M. Guyot, M. Boncompagni, D. Ambrossetti, E. Chamorey, S. Negrier, G. Pagès
Writing, review, and/or revision of the manuscript: D. Ambrossetti, J.-Y. Scoazec, S. Negrier, H. Simonnet, G. Pagès
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Pagès
Study supervision: G. Pagès

Acknowledgments
The authors thank Dr. Marc Colombel (patients' informed consent), Dr. Jean Claude Chambard (latentivirus expressing the luciferase gene), Dr. Eloi Delaplanche (biopsies management), Dr. Florence Mège-Lechevalier (pathologic analyses), Jacky Paput (animal studies), and Dr. Scott Parks (editorial assistance). The authors greatly acknowledge the IBICN animal core facility.

Grant Support
This study was financially supported by the National Institute of Cancer (INCA/VEGFIL contract), the French Association for Cancer Research (ARC), the Fondation de France, the Conseil Général des Alpes Maritimes, Roche France, and The "Association pour la Recherche sur les Tumeurs du Rein (ARTuR)."

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 3, 2013; revised October 28, 2013; accepted November 21, 2013; published OnlineFirst December 12, 2013.

References
The CXCL7/CXCR1/2 Axis Is a Key Driver in the Growth of Clear Cell Renal Cell Carcinoma

Renaud Grépin, Mélanie Guyot, Sandy Giuliano, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-1267

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/12/12/0008-5472.CAN-13-1267.DC1

Cited articles
This article cites 51 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/3/873.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/74/3/873.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/74/3/873.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.