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

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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.

CXCL cytokines of the ccXCL 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, tumorogenesis, 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
melanoma cancer cells (18). CXCL7 is also implicated in the development of the lymphatic network through the regulation of VEGF-C and VEGF-D, two major growth factors for lymphatic endothelial cells (19). The role of CXCL cytokines is not restricted to solid tumors because CXCL4 and CXCL7 are markers of advanced disease for myelodysplastic syndromes (20). Hence, the ELR+ CXCL/CXCR1/CXCR2 axis is a key component implicated in tumor development. However, the meaning of coexpression in the same cell of CXCR and CXCL is controversial because this autocrine pathway can drive both senescence (21) and tumor development (16). This discrepancy can be explained by differential expression during tumor progression. CXCR expression may be beneficial during tumorigenesis because it drives senescence. However, its 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 previously (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-GLUC+, RCC-10LUC+, and ACHNLUC+ cells were obtained by lentiviral transduction (pLenti6/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 oligonucleotides: forward primer 5’-ATGGAA-GATTCTAACATGGAG-3’; reverse primer 5’-GAGAGTAGTG- GAAGTGCC-3’.

Antibodies

The following antibodies were used for immunoblotting or immunohistochemistry respectively: antiphospho ERK1/2 antibody (pERK; Sigma), antiphospho AKT (pAKT), anti-AKT, anti-ERKs (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 synthesised 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-GLUC+, RCC-10LUC+, or ACHNLUC+ cells (3 × 10^6 to 10 × 10^6) were injected subcutaneously into the flanks of 5-week-old nude (nu/nu) female mice (Janvier). Bioluminescence was quantified using the In Vivo Imaging System (Perkin Elmer) according to the manufacturer’s instructions. Tumor volume [(v = L × l × 0.52 (25)] was determined in parallel using a caliper. 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 \( \chi^2 \) 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 immunoassay 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 outcome, 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 ($P < 10^{-3}$ 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 Führman 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 independent ccRCC cell lines (RCC-10 and ACHN) with equivalent 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 onto 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).

Anti-CXCL7 antibodies do not alter the angiogenic profile of ccRCC tumors in nude mice but modify their growth

CXCL7 stimulates endothelial cell proliferation and angiogenesis via activation of two G-protein–coupled receptors, CXCR1 and CXCR2 (8). However, as tumor cells may express abnormal levels of CXCR1 and CXCR2, we hypothesized that anti-CXCL7 antibodies may have antiangiogenic and antiproliferative properties. First, we evaluated the production of angiogenic cytokines in tumors of placebo or anti-CXCL7–treated mice. As expected, anti-CXCL7 antibodies decreased CXCL7 levels within the tumors. However, they did not affect the production of other major angiogenic cytokines, including VEGF and CXCL8 (Fig. 2A). Tumor vascularization, assessed by measuring intratumor hemoglobin levels, was not affected by the anti-CXCL7 treatment (Fig. 2B). To gain a more precise evaluation of vascularization, we also analyzed the network of blood and lymphatic vessels. Again, no clear differences were noted for the anti-CXCL7 treatment on the number of CD31-labeled cells (Supplementary Fig. S2) and no positive cells for Lyve-1 were observed in the center of tumors in contrast to what we previously observed following treatment with bevacizumab (6). Hence, we hypothesized that the anti-CXCL7 treatment might affect the proliferation of tumor cells. To test this hypothesis, we first evaluated two major signaling pathways implicated in cell proliferation, the mitogen-activated protein kinase/ERK and PI3K pathways. ERK and AKT activities were significantly downregulated in mice treated with anti-CXCL7 antibodies (Fig. 2C). The tumors also showed a decreased number of Ki67-labeled cells (Fig. 2D) and an increased number of necrotic zones (Fig. 2E and Supplementary Fig. S3). 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 onto 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 maturates 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...
dies alone inhibit cell proliferation in the absence of IL-1 probably participate in PBP maturation. Anti-CXCL7 antibo-
PBP-maturating proteins (32, 33), are active in 786-O cells and matrix metalloproteases (MMP), the proteases described as exerts an autocrine proliferation loop via its receptors CXCR1

Figure 2. Anti-CXCL7 antibodies decrease the proliferation index and promote tumor necrosis. A, specificity of the anti-CXCL7 (A7) antibodies. Intratumor content of CXCL7, CXCL8, and VEGF in tumor extracts of mice treated with A7, B, the intratumoral amount of hemoglobin (Hg), which gives a global read out of the blood supply, is not affected by anti-CXCL7 antibodies. C, tumor extracts from control (C) or A7-treated mice 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 tumor extracts of PBS-treated mice were considered as the reference values (100%). D, the percentage of Ki67-labeled cells in control and A7-treated tumors is shown. Statistical differences are indicated. E, delimitation of necrotic zones was performed after hematoxylin-eosin-saffron staining of tumor slices. The graph gives the ratio of the areas of necrotic zones/total area of three independent tumors sections. F, CAIX was detected in tumor extracts from control (C) or A7-treated mice by immunoblotting and normalized to the tubulin content. Statistical significance: *, P < 0.05; **, P < 0.01.

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 antibo-
dies 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 deter-
mined 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 CXCRs 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
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.
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 univariate 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-1ß 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-1ß 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-1ß 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
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). Con-
sequently, there is an urgent need to identify predictive mar-
kers 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

Figure 6. SB225002 inhibits tumor growth by inhibiting cell proliferation, increasing necrosis, and decreasing angiogenesis. A, left, 3 × 10^6 786-O1UC1 cells were subcutaneously injected into nude mice (n = 10 per group). Fifteen days after injection, all mice developed tumors and were treated three times a week with PBS or with 200 μg of SB225002. Bioluminescence was measured weekly as described (6). Statistical differences between the luminescence of tumors of control and treated mice are presented. Middle, representative images of the tumor-bearing mice. Right, average volume ± SD and statistical analysis. B, control and (C) SB225002 (SB)-treated mice tumors were tested for ERK and AKT activity by immunoblotting using antibodies directed against the phosphorylated and nonphosphorylated forms of the proteins. The histograms show the ratio of phosphorylated ERKs or AKT to nonphosphorylated ERKs or AKT. The ERK and AKT activities in control tumors were considered as the reference values (100%). C, Ki67 labeling was performed on control and SB225002-treated tumor sections. The percentage of Ki67-labeled cells in control and SB225002-treated tumors was shown. D, delimitation of necrotic zones was performed after hematoxylin-eosin-saffron staining of tumor slices. The histograms give the ratio of the area of necrotic zones/total area of three independent tumors. E, the presence of proangiogenic CXCL1 (C1), CXCL2 (C2), CXCL8 (C8), VEGF and antiangiogenic CXCL4 (C4) cytokines, and fibroblast growth factor (FGF), for stromal cells, was tested by ELISA in tumor extracts of control and SB225002-treated mice tumors. F, the tumor vasculature in the two experimental groups (control and SB225002-treated) was evaluated by immunostaining for CD31. Quantification of the vascular density (number of vessels per field ± SD) resulted from analysis of four independent tumors and at least ten fields for each tumor were examined. Data (A and E), mean ± SD. Statistically significant difference: *, P < 0.05; **, P < 0.01.
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 IL-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.

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Acknowledgments
The authors thank Dr. Marc Colombel (patients' informed consent), Dr. Jean Claude Chambard ( lentivirus expressing the luciferase gene), Dr. Eodie Delaplanche (biopsies management), Dr. Florence Mege-Lechevallier (pathologic analyses), Jacky Papat (animal studies), and Dr. Scott Parks (editorial assistance). The authors greatly acknowledge the IRCAN 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 (ARRub)." 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.

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