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 VHL 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 pro-angiogenic, pro-inflammatory 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, where expression of CXCL7 was induced by the central pro-inflammatory cytokine IL-1β. ccRCC cells normally secrete low amounts of CXCL7, it was more highly expressed in tumors due 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 over-expression of VEGF, resulting in ccRCC to be one of the most vascularized tumors. Theoretically, ccRCC should be highly responsive to anti-VEGF therapy. Bevacizumab (BVZ), a humanized monoclonal antibody targeting VEGF, in association with interferon-alpha (interferon-α), has obtained approval from the American Food and Drug Agency for treatment (1). Despite the increased time to progression, the pivotal AVOREN study that compared the efficacy of interferon-α versus interferon-α plus BVZ (2) showed that BVZ 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 while the treatment was inefficient in others patients, in which metastatic dissemination was observed (3, 4). Moreover, a recent meta-analysis showed that BVZ in combination with chemotherapy induced fatal adverse events (5). Our recent study also highlighted unexpected ccRCC enhanced growth in mice treated with BVZ (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 pro-angiogenic 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 anti-angiogenic potency depending on the presence or absence of the amino acid triplet ELR in their protein sequence (ELR+CXCL (1-3, 5-8) have pro-angiogenic properties whereas ELR-CXCL (4, 9, 10) have anti-angiogenic 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 ERK and PI3 kinase pathways (8). The pro-inflammatory chemokine CXCL8 also named interleukin-8 promotes angiogenesis, tumorigenesis and metastasis and it is over-expressed in many tumors including ccRCC (10).
Moreover, Ras-dependent secretion of CXCL8 enhanced tumor progression by promoting neo-vascularisation (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 oesophageal (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 since CXCL4 and CXCL7 are markers of advanced disease for myelodysplastic syndromes (20). Hence, the ELR+CXCL/CXCR1, 2 axis is a key component implicated in tumor development. However, the meaning of co-expression in the same cell of CXCR and CXCL is controversial since 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 since 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. RCC10 were a kind gift from WH Kaelin (Dana Farber Cancer Institute). 786-O$^{\text{LUC}^+}$, RCC-10$^{\text{LUC}^+}$ and ACHN$^{\text{LUC}^+}$ cells were obtained by lentiviral transduction (pLenti6/V5-D-TOPO, Invitrogen, France) and blasticidin selection (10 μg/ml) (6). Tumor 1 was a non-metastatic pT3b, Fuhrman grade 2 tumor, tumor 2 was a non metastatic 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, Heidelberg Germany). 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’-ATGGAAGATTTTAACATGGAG-3’; reverse primer 5’-GAGAGTAGTGGAAGTGTGCC-3’.

Antibodies

The following antibodies were used for immunoblotting or immunohistochemistry respectively: anti-phospho ERK 1, 2 antibody (pERK, Sigma St Louis, MO), anti-phospho AKT (pAKT), anti-AKT, anti-ERKs, (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PARP (Cell Signaling, Cambridge, UK) and anti-CAIX (Bayer Health Care, Lyon France). 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, France) were diluted in PBS and injected intra-peritoneally (5mg/kg), as described (11). PBS was injected into the control group. SB225002 was synthesized by the Chemistry Department of the University of Nice as previously described (24) (Dr Rachid Benhida).

Tumor xenograft formation and size evaluation

786-O\(^{LUC+}\), RCC-10\(^{LUC+}\) or ACHN\(^{LUC+}\) cells (3.10\(^6\) to 10.10\(^6\) cells) were injected subcutaneously into the flanks of 5-week-old nude (nu/nu) female mice (Janvier, France). Bioluminescence was quantified using the In Vivo Imaging System (IVIS, Perkin Elmer, France) according to the manufacturer's instructions. Tumor volume (\(v = L \times l^2 \times 0.52\) (25)) was determined in parallel using a caliper. There was a linear relationship between values for bioluminescence and the tumor volume.

Immuno-histochemistry and immuno-fluorescence experiments

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

Measurement of hemoglobin and cytokines

Frozen tumor tissues were lysed in cell extraction buffer (Biosource, Belgium). The intra-tumor hemoglobin content was measured using a Drabkin reagent kit 525 (Sigma, France). CXCL cytokines, FGF, human and mouse VEGF were measured using PeproTech ELISA kits.
according to the manufacturer’s recommendations (PeproTech, Neuilly-sur-Seine, France). VEGF-C was measured using the Human DuoSet ELISA kits, VEGF-D using the Quantikine ELISA Kit (R&D Systems, Minneapolis, USA).

**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 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 ccRCC patients**

Previously, we utilized 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 intra-tumoral 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 (1152 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 pro-angiogenic forms of VEGF (Levels of total VEGF (V) minus the levels of the anti-angiogenic form of VEGF (Vb) (27)) did not significantly correlated with survival ($p = 0.26$ and $p = 0.49$) (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 $\alpha$ or 2 $\alpha$ (HIF-1$\alpha$ or 2$\alpha$) (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 to what was detected in human samples (mean 2000 pg/mg protein) (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 et al 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$^{\text{LUC+}}$ 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 results (Supplementary Fig. S1).

**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 anti-angiogenic and anti-proliferative 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 intra-tumor haemoglobin levels, was not affected by 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-labelled 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 BVZ (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 MAP Kinase/ERK and PI3 Kinase pathways. ERK and AKT activities were significantly down-regulated 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 poly-ADP ribose polymerase (PARP) cleavage) were detected in tumor samples. Carbonic anhydrase 9 (CAIX) is a major target of the hypoxia-inducible factor 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 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).

**Over-expression of CXCL7 accelerates the growth of ccRCC xenograft tumors in nude mice**

To definitively demonstrate the pro-tumor growth effect of CXCL7, we generated 786-O cells over-expressing CXCL7. Control 786-O cells produced levels of CXCL7 at the limit of the detection threshold of ELISA tests (50 pg/ml/24 hours/10^6 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/10^6 cells. Over-expression resulted in accelerated cell proliferation, reinforcing the concept of an autocrine proliferation loop driven by CXCL7 (Fig. 3B and 3C). Over-expression of CXCL7 resulted in accelerated tumor growth in xenografts onto the flanks of nude mice (Fig. 3D). Since tumors obtained with CXCL7 over-expressing cells were too big, mice were sacrificed for ethical purposes. Therefore extensive comparison of control and “CXCL7” tumors by immuno-histochemistry 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, CXCL8- were detected. The presence of equal haemoglobin 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 \textit{in vivo} (Fig. 3G).

**Regulation of expression of CXCL7 by tumor cells \textit{in vitro} and \textit{in vivo}**

We analysed 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 interleukin 1 β (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 \textit{in vitro} and \textit{in vivo} experiments were explained by the fact that tumors generated in nude mice produced IL-1β (mean 550 pg/ml/mg protein range 50-2000, 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 correlates with IL-1β levels. Moreover, cathepsin G and metallo-proteases (MMPs), the proteases described as PTB maturating 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 shRNA had no effect, CXCL7-directed shRNA inhibit 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 qRT-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 to human micro-vascular 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 pro-angiogenic 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 anti-tumor growth effects in ccRCC since they concomitantly inhibit cell proliferation, induce apoptosis of tumor and inhibit endothelial cells and expression of pro-angiogenic 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 labelling 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 intra-tumor 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 non-statistically drop in CXCL8 ($p = 0.06$). Intra-tumoral CXCL7 was equivalent in control and SB225002-treated mice. The statistically significant increase in CXCL4 ($p = 0.007$), an anti-angiogenic member of the CXCL cytokine family also favored the anti-angiogenic effect of SB225002 (Fig. 6E). CD31 labeling (Fig. 6F) confirmed the decrease in blood vessel density. HES staining highlighted the presence of numerous blood vessels in the core of control tumors while 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: 1) Are the levels of ELR+CXCL cytokines easily assayed on samples obtained from patients in a minimally invasive way? 2) If yes, can they represent new therapeutic targets? 3) 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 it is an independent prognostic marker may orientate clinicians’ treatment strategies for patients with \textit{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 since 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 while anti-CXCL8 antibodies only slow-down tumor growth highlighting CXCL7 as a more relevant target. Moreover, CXCL8 does not appear 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 appears as a marker of poor prognosis in univariate analysis but fails to reach statistical significance in multivariate analysis (p = 0.017 and p = 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-1β suggests the contribution of cytokines produced by inflammatory cells in the tumor context to favor CXCL7 production by cancer cells. Maturation towards the active NAP-2/CXCL7 is then mediated by metalloproteinases (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 chemo-attractants for neutrophils or macrophages.
to maintain the inflammatory context within the tumors. Moreover IL-1β 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-1β promotes tumor cell proliferation via CXCL7 production and enhances tumor aggressiveness.

Before the development of anti-angiogenic 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 multi-region 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 anti-angiogenic 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 anti-angiogenic 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 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 observed 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” anti-angiogenic 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.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1: Kaplan-Meier analysis of overall survival of patients with renal cell carcinoma and role of CXCL7 blockade for 786-O xenograft tumor growth

(A) Correlation of overall survival with baseline levels of CXCL7 (first quartile value 1152 pg/mg protein). Overall survival was calculated from patient subgroups with baseline levels of CXCL7 that were less or greater than the first quartile value.

(B) $3 \times 10^6$ 786-O$^{LUC^+}$ cells were subcutaneously injected into nude mice (n = 7 per group). When the tumor volume was approximately 100 mm$^3$, mice were treated intra-peritoneally once per week with irrelevant IgG (control) or with 15 mg/kg of the anti-CXCL7 antibodies (anti-CXCL7). Bioluminescence was measured weekly as described previously (6). Results are presented as the mean ± SD. Statistical differences between the size of tumors of control and treated mice are presented: *$p<0.05$.

(C) Representative images of tumor-bearing mice.

(D) Mean tumor volume ± SD at the end of experiment; ***$p<0.001$

Fig. 2: Anti-CXCL7 antibodies decrease the proliferation index and promote tumor necrosis.

(A) Specificity of the anti-CXCL7 (A7) antibodies. Intra-tumor content of CXCL7, CXCL8 and VEGF in tumor extracts of mice treated with A7. Statistical differences are presented: *$p<0.05$.

(B) The intra-tumoral 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 non phosphorylated forms of the proteins. The graphs show the ratio of phosphorylated...
ERKs or AKT to non phosphorylated ERKs or AKT, respectively. The ERK and AKT activities from tumor extracts of PBS-treated mice were considered as the reference values (100 %). Statistical differences: **p<0.01.

(D) The percentage of Ki67 labeled cells in control and A7-treated tumors is shown. Statistical differences are indicated: **p<0.01.

(E) Delimitation of necrotic zones was performed after HES staining of tumor slices. The graph gives the ratio of the areas of necrotic zones/total area of three independent tumors sections. Statistical significance is shown; *p<0.05.

(F) CAIX was detected in tumor extracts from control (C) or A7-treated mice by immunoblotting and normalized to the tubulin content. Statistical significance is shown; *p<0.05.

**Fig. 3: Expression of CXCL7 accelerates tumor growth.**

(A) 3.10^5 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. Statistical significance of the results is shown; ***p<0.001.

(B) 3.10^5 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. Statistical significance of the results is shown; **p<0.01.

(C) Clonogenic assay with control 786-O cells (CT) or 786-O cells expressing CXCL7 (C7) following ten days of culture.

(D) 3.10^6 control 786-O^{LUC+} cells (CT) or 3.10^6 786-O^{LUC+} cells expressing CXCL7 (C7) were subcutaneously injected into nude mice (n = 7 per group). Bioluminescence was
measured weekly. Data are presented as the mean ± SD. Statistical differences between
the size of tumors of control and treated mice are presented: *$p<0.05$; **$p<0.01$.

(E) Intra-tumoral and plasmatic CXCL7 was measured by ELISA test

(F) The intra-tumoral 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 non phosphorylated forms of the proteins. The graphs show the ratio
of phosphorylated ERKs or AKT to non phosphorylated ERKs or AKT, respectively. The
ERK and AKT activities from control tumor extracts were considered as the reference
values (100 %). Statistical differences: **$p<0.01$.

**Fig. 4: Interleukin 1β (IL-1β) induces CXCL7 expression in 786-O cells**

(A) Exponentially growing 786-O cells were treated with 10 ng/ml IL-1β for 24 hours.
CXCL7 expression was tested by qPCR.

(B) Exponentially growing 786-O cells were treated with 10 ng/ml IL-1β for 24 hours.
Secreted CXCL7 during 24 hours was determined by ELISA in the conditioned medium.
Results are the mean ± SD of three independent experiments. Statistical significance of the
results is shown; ***$p<0.001$.

(C) The proliferative capacity of exponentially growing 786-O cells in the presence of 10
ng/ml IL-1β (IL1β), anti-CXCL7 (A7), and IL-1β + anti-CXCL7 antibodies (IL1β+A7)
was tested using the MTT assay following 48 hours of treatment. The values obtained for
untreated cells were taken as the reference values (100 %). Statistical significances of the
results compared to untreated cells are indicated; *$p<0.05$; ***$p<0.001$. 
(D) The proliferative capacity of exponentially growing 786-O cells stably expressing control (shC) or two independent CXCL7-directed (sh1, sh2) shRNA in the presence or absence of IL-1β was tested using the MTT assay three days after cell seeding. The values obtained for cells expressing control shRNA were taken as the reference values (100%). Statistical significances of the results compared to control cells are indicated; **p<0.01; ***p < 0.001.

Fig. 5: Expression of CXCR1 and CRCX2 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 micro-vascular 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, T3).

(B) The proliferative capacity of 786-O cells in the presence of increasing concentrations of SB225002 was tested using the MTT assay. Data are the mean fold increase ± SD. The fold increase of untreated cells was taken as the reference value for statistics. Statistical significances of the results compared to untreated cells are indicated; *p<0.05; **p <0.01; ***p < 0.001.

(C) The proliferative capacity of cells established from fresh tumors was measured as described above. The percent of inhibition of proliferation using 5 μM SB225002 compared to untreated cells is plotted. **p <0.01; ***p < 0.001.
(D) 786-O cells incubated with the indicated amounts of SB225002 for two hours were tested for ERK and AKT activities as indicated above. Statistical differences: * \( p< 0.05 \); ** \( p<0.01 \).

(E) 786-O cells incubated with varying amounts of SB225002 for twenty four hours were tested for the presence of cleaved forms of PARP indicated by an asterisk. Tubulin is shown as a loading control.

**Fig. 6: SB225002 inhibits tumor growth by inhibiting cell proliferation, increasing necrosis and decreasing angiogenesis**

(A) *Left:* \( 3.10^6 \) 786-O\(^{LUC}\) 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 \( \mu \)g of SB225002. Bioluminescence was measured weekly as described (6). Data are the mean \( \pm \) SD. Statistical differences between the luminescence of tumors of control and treated mice are presented. Statistical significance of the results is shown: *\( p<0.05 \); **\( p<0.01 \).

*Middle:* Representative images of the tumor-bearing mice.

*Right:* Average volume \( \pm \) SD and statistical analysis: **\( p<0.01 \).

(B) Control (C) and SB225002 (SB)-treated mice tumors were tested for ERK and AKT activity by immunoblotting using antibodies directed against the phosphorylated and non phosphorylated forms of the proteins. The histograms show the ratio of phosphorylated ERKs or AKT to non phosphorylated ERKs or AKT. The ERK and AKT activities in control tumors were considered as the reference values (100 %). Statistical differences are: **\( p<0.01 \).
(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. Statistical differences are indicated: **p< 0.01.

(D) Delimitation of necrotic zones was performed after HES staining of tumor slices. The histograms give the ratio of the area of necrotic zones/total area of three independent tumors: *p<0.05.

(E) The presence of pro-angiogenic CXCL1 (C1), CXCL2 (C2), CXCL8 (C8), VEGF and anti-angiogenic 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. Data are the mean ± SD. Statistical significant differences: *p<0.05; **p <0.01.

(F) The tumor vasculature in the two experimental groups (control, 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. Statistically significant difference: **p <0.01.
Intra-tumor CXCL7 > First quartile

\[ p = 0.0023 \]

B

Bioluminescence (photons/s x 10^7)

- Control
- Anti-CXCL7

Start of treatment

Time (days post injection)

C

Control
Anti-CXCL7

D

Tumor Volume (mm^3)

C
A7

Fig. 1: Grépin, R et al
A  

Cytokine amount (ng/mg prot.)

CXCL7  CXCL8  VEGF

B  

Hg

C

ERK/AKT activity (% of control)

C  A7  C  A7

pERK/ERK  pAKT/AKT

C  A7  C  A7

D  

Ki67

% positive cells

C  A7

**

E  

% of necrotic zone

C  A7

*

F  

CAIX amounts (arbitrary units)

C  A7

*

Fig. 2: Grépin, R et al
Fig. 3: Grépin, R et al
Fig. 4: Grépin, R et al
Fig. 5: Grépin, R et al
Fig. 6: Grépin, R et al.

A) Bioluminescence (photons/s x 10^7) over time (days post injection) for Control and SB225002 treatments. The start of treatment is indicated with an arrow.

B) ERK/AKT activity (% of control) for C and SB treatments, showing significant differences.

C) Ki67 cells (% of Ki67 positive cells) for C and SB treatments, indicating a decrease in cell proliferation.

D) Necrosis (% of necrotic areas) for C and SB treatments, with a decrease in necrotic areas in the SB treatment group.

E) Cytokine amount (ng/mg prot) for C and SB treatments, with significant differences for C1, C2, C4, C8, VEGF, and FGF.

F) Blood vessels with average blood vessel number/field for C and SB treatments, showing a decrease in blood vessel number in the SB treatment group.

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