# SDF1β Expression in Renal Cell Carcinoma Correlates with Grading and Infiltration by CD8+ T-Cells

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**Abstract.** *For several tumor entities, a significant correlation between the chemokine stromal cell-derived factor 1 (SDF1) and its receptor C-X-C chemokine receptor type 4 (CXCR4), metastasis and tumor proliferation, as well as prognosis, has been described. In this study, a series of 105 renal cell carcinoma patients were analyzed in terms of expression of SDF1α and SDF1β and infiltration by CD4+ and CD8+ T-cells and the data correlated with TNM category, grading and survival. While the splice variant SDF1α had no impact on tumor grading, T-cell invasion or overall survival, expression of SDF1β showed a significant correlation with tumor grading and also suggested a correlation with metastasis, as well as CD8+ T-cell invasion. These results indicate a potential T-cell-mediated antitumor response induced by SDF1β up-regulation. Therefore targeting the SDF1β–CXCR4 signaling pathway may be a promising means for new therapeutic strategies in advanced tumor stages.*

Renal cell carcinoma (RCC) is the sixth leading cause of cancer-related deaths in the Western world. RCC makes up 2-3% of all newly diagnosed malignancies in adults and 85% of all kidney tumors (1). The age-adjusted incidence of RCC in Western nations is 5 and 12/100,000 in women and men, respectively (2), with a peak incidence in the 6th decade. Early diagnosed stages can be cured by nephrectomy. However, approximately one-third of the patients experience relapse and progression with metastatic disease. About 30-50% of patients already have metastatic disease at presentation. The preferential sites of metastasis are the regional lymph nodes, the lung, the liver and the bones. Survival strongly depends on the tumor stage at presentation. The 5-year survival rate is approximately 50%, whereas the median survival in cases of metastasis is less than one year (3-5). The current standard treatment for metastasized RCC consists of interferon-α (IFN-α) and interleukin-2 (IL-2) (6). Recently, phase II clinical trials using receptor tyrosine kinase (RTK) inhibitors have shown more promising results (2).

*In vivo* and *in vitro* results from different tumor entities suggest that organ-specific metastasis is partially governed by interactions between chemokine receptors on cancer cells and their corresponding chemokines expressed in target organs, therefore directing lymphatic and hematogenous spread and furthermore influencing sites of metastatic growth (7). Chemokines and their respective G-protein-coupled receptors were initially described as mediating different pro- and anti-inflammatory responses (8). In particular, a high expression of stromal-cell derived factor 1 (SDF1), also known as CXCL12, by endothelial cells, biliary epithelial cells, bone marrow stromal cells and lymph nodes results in a chemotactic gradient attracting C-X-C chemokine receptor type 4 (CXCR4)-expressing lymphocytes into those organs (9-13). SDF1 is a chemokine of the CXC subfamily originally characterized as a pre B-cell stimulatory factor and cloned from bone marrow supernatants. SDF1 exists in three alternative splicing variants (α, β, and γ) (14). The human *SDF1* gene is located on chromosome 10q (15).

Most recently, CXCR4 has shifted into focus as it is the most common chemokine receptor expressed on cancer cells (16). It was suggested to play an important role in tumor spread of colorectal, breast and oral squamous cell carcinoma as all of them commonly metastasize to SDF1-expressing organs (17-19). Data obtained from *in vitro* as well as from murine *in vivo* models, analyzing the metastatic

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ability of CXCR4-expressing cancer cells, underlined the key role of CXCR4 for tumor cell malignancy, as activation of CXCR4 by SDF1α induced migration, invasion and angiogenesis of cancer cells (20-22).

Therefore, we evaluated the expression of SDF1α and SDF1β in 105 RCC specimens and correlated these results with the patients' clinicopathological parameters and survival.

## Materials and Methods

*Tissue samples*. RCC samples were obtained intraoperatively, according to local Ethics Committee regulations, from 105 patients with RCC who underwent surgery at the Department of Urology of the University of Mainz. The morphological classification of the carcinomas was conducted according to World Health Organization (WHO) specifications (23). Patients were followed up on a regular basis depending on the procedure performed.

*Immunohistochemical staining of SDF1α and SDF1β*. The avidin-biotin complex method was used to detect the proteins SDF1α, SDF1β and the surface markers CD8 and CD4 respectively (anti-SDF1α: MBL, Code No. JM-5387-100, dilution 1:100; anti-SDF1β: MBL, Code-No. JM-5390-100, dilution 1:50; DakoCytomation Monoclonal Mouse Anti-Human CD8+, Dako Deutschland GmbH, Hamburg, Germany, Clone C8/144B: Code No. M 7103, dilution 1:50; Novocastra™ Lyophilized Monoclonal Mouse Antibody CD4+, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany, Product Code: NCL-CD4-1F6, dilution 1:50). Formalin-fixed and paraffin-embedded tissue were deparaffinized and subsequently microwaved in EDTA buffer. After pre-incubation with hydrogen peroxide, avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA) and human AB-serum the primary antibodies were applied for 2 h (SDF1α), 4 h (SDF1β) or 1 h (CD8 and CD4), respectively, at room temperature. After incubation with the secondary antibody (Dako LSAB+ System-HRP, Code K0679; Dako Deutschland GmbH), the avidin-biotin complex was added and the enzyme activity visualized with diaminobenzidine (DakoLiquid DAB+ Substrate Chromogen System Code K3468; Dako Deutschland GmbH). Counterstaining was performed with hematoxylin. For negative controls, only the secondary antibody was used. A negative control was performed for every RCC sample (N=105). For positive controls, formalin-fixed and paraffin-embedded tissue samples of human spleen were applied.

*Evaluation of immunostaining*. Immunostaining was evaluated by three authors independently (T.C.W., K.A., S.B.), blinded to patient outcome and all clinicopathological findings. The immunohistochemical staining was analyzed according to a scoring method as previously validated and described, elsewhere (17): the tumors were classified into four groups based on the homogenous staining intensity: 0: absent; 1: weak; 2: intermediate; 3: strong staining. In the case of heterogeneous staining within the same sample, the respective 0.5 points higher score was chosen if more than 50% of cells revealed the higher staining intensity. If evaluations did not agree, specimens were re-evaluated and re-classified according to the assessment given most frequently by the observers. $CD4^+$ and $CD8^+$ cells were counted per visual field using a forty-fold magnification in triplicate per slide; thereafter the average was calculated.

*Statistics*. The correlation of SDF1α and SDF1β staining intensity with clinicopathological patterns was assessed with the $\chi^2$ test, with the unpaired Student *t*-test and the SPSS-generated Spearman's rank correlation coefficient when appropriate. Survival rates were visualized applying Kaplan-Meier curves, and *P*-values were estimated by log-rank test. $p<0.05$ was considered significant and $p<0.001$ highly significant in all statistical analyses.

## Results

*SDF1α and SDF1β expression in RCC tissue and the corresponding healthy renal cells*. SDF1α and SDF1β expression of RCC tissue revealed varying expression intensities as depicted by Figure 1A and 1B.

*Tumor characteristics and patient profiles*. The selected group of patients presented the typical characteristics of RCC in industrialized countries, except for a lower percentage of cases with distant metastases as depicted in Table I.

*TNM-classification and grading*. In this study, 105 patients (66 males and 39 females) suffering from RCC were analyzed for TNM staging and grading, as well as for age. The age of the patients ranged from 33 to 95 years (mean 64 years). According to the TNM classification, most tumors were classified as T1 and only few as T4 (Table I). In about 53%, no metastases were found in the regional lymph nodes (Table I). However, it was not possible to analyze local lymph nodes (Nx) in 41%. Distant metastases were detected in 3% of the patients. Again, in 85% of patients, the existence of distant metastases was not evaluable. Most tumors were graded as G2 or higher (Table I). No significant difference was observed between the genders with regard to clinicopathological parameters.

*Immunohistochemical staining of SDF1α and SDF1β in RCC*. The staining of normal human kidney tissue for SDF1α and SDF1β revealed a predominantly cytoplasmatic location, and only in tubular regions was there additional membranous location (Figure 1A and 1B: co); no staining appeared in glomeruli. Nuclear staining of SDF1α and SDF1β was not observed.

In RCC, the respective expression rate for SDF1α was 92.4% (97/105), with the majority of tumors having at least intermediate staining (Table II). The expression rate for SDF1β was 98.1% (103/105), again with most tumors having at least intermediate staining (Table II). Negative controls of human RCC remained negative for all tissue samples (N=105, not shown). As positive control, human lymph node tissue revealed strong CD8 and CD4 expression and human renal tissue revealed strong expression of SDF1α and SDF1β exclusively in the tubuli.

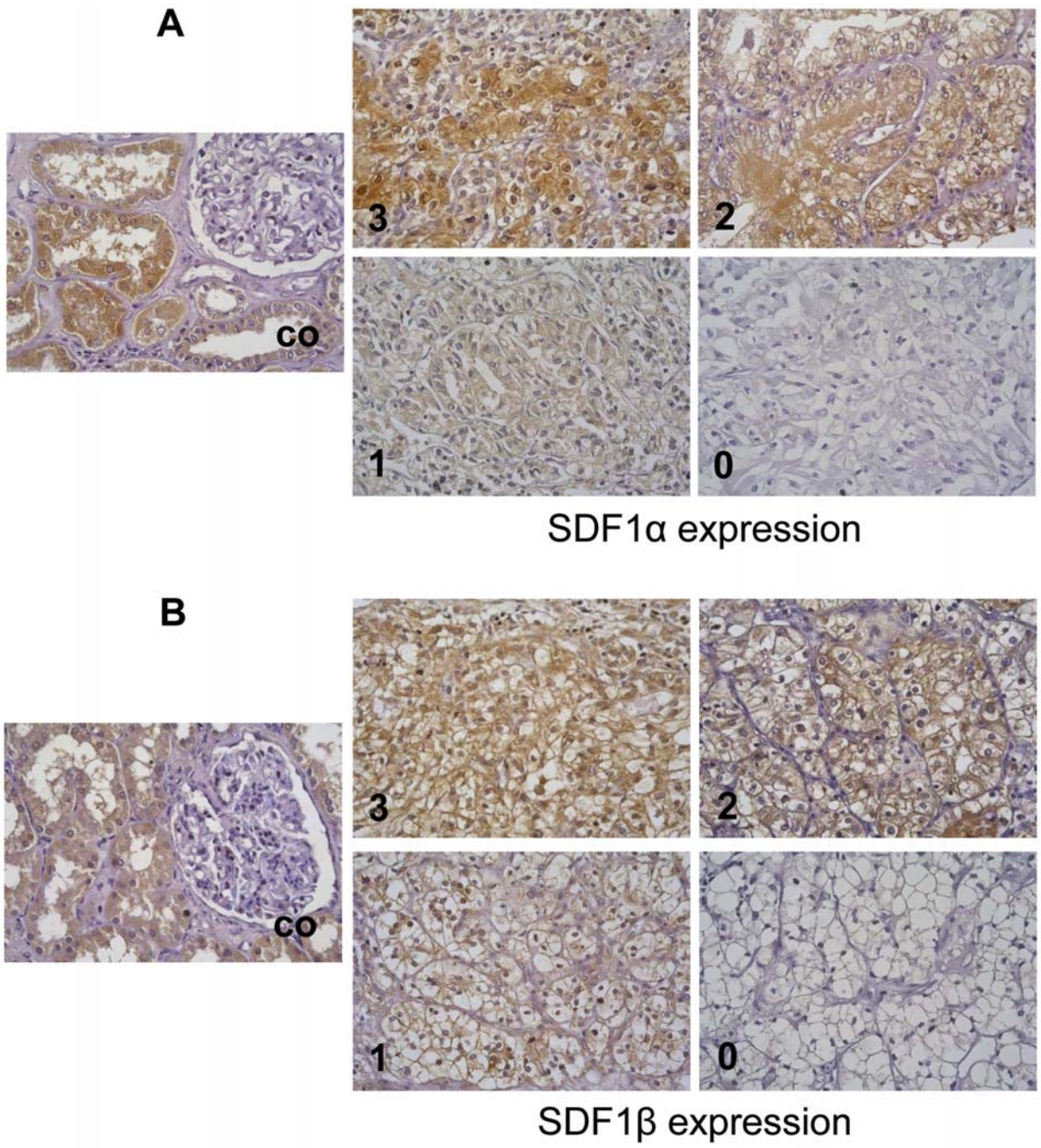


Figure 1. *SDF1α and SDF1β expression score in renal cell carcinoma (RCC). A total of 105 samples of RCC tissue were analyzed by staining with antibody to SDF1α (A) and SDF1β (B). Kidney tissue controls showed positive staining only in tubuli regions (co). Staining was evaluated according to the intensity of expression 0: no expression, 1: weak expression, 2: medium expression, 3: strong expression (fourty-fold magnification).*

*Relevance of SDF1α and SDF1β expression in renal cell carcinoma*. No significant correlation between SDF1α expression and TNM classification was detected. The tumors were also analyzed for a correlation of SDF1α expression and T-cell infiltration. No correlation was seen (data not shown).

A tendency for stronger SDF1β expression in older patients was detected (r=0.203, $p$=0.038). No significant correlation between SDF1β expression and TNM classification was detected. However, a higher grading correlated with a stronger SDF1β expression (r=0.194; $p$=0.048; Table III).

Table I. *Patient and tumor characteristics.*

| | n (%) |
|---|---|
| Total number | 105 |
| Median age (years) | 64 |
| Gender | |
| Female | 39 (37%) |
| Male | 66 (63%) |
| T – category | |
| 1 | 57 (54%) |
| 2 | 15 (14%) |
| 3 | 31 (30%) |
| 4 | 2 (2%) |
| N – category | |
| 0 | 56 (53%) |
| 1 | 1 (1%) |
| 2 | 5 (5%) |
| M – category | |
| 0 | 13 (12%) |
| 1 | 3 (3%) |
| Grading | |
| 1 | 11 (11%) |
| 2 | 56 (54%) |
| 3 | 35 (33%) |
| 4 | 3 (3%) |

Table II. *Expression of SDF1α and SDF1β in renal cell carcinoma.*

| Expression score | SDF-1α | | SDF-1β | |
|---|---|---|---|---|
| | n | % | n | % |
| 0 | 8 | 7.6 | 2 | 1.9 |
| 1 | 28 | 26.7 | 25 | 23.8 |
| 2 | 46 | 43.8 | 48 | 45.7 |
| 3 | 23 | 21.9 | 30 | 28.6 |
| Total | 105 | 100 | 105 | 100 |

Table III. *Correlation of SDF1β and grading of renal cell carcinoma.*

| | Grading | | | | |
|---|---|---|---|---|---|
| Expression | G1 | G2 | G3 | G4 | n |
| score | | | | | |
| 0 | 0 | 1 | 0 | 1 | 2 |
| 1 | 3 | 14 | 8 | 0 | 25 |
| 2 | 7 | 30 | 9 | 2 | 48 |
| 3 | 1 | 11 | 18 | 0 | 30 |
| Total | 11 | 56 | 35 | 3 | 105 |

The tumors were also analyzed for a correlation of SDF1β expression and T-cell infiltration. A significant correlation was seen for $CD8^+$ T-lymphocytes (r=0.244, $p$=0.012) but not for $CD4^+$ T-cells (r=0.029, $p$=0.772). Correlating with stronger SDF1β expression, the number of $CD8^+$ tumor-infiltrating lymphocytes rose significantly. This correlation is demonstrated in the box and whisker plot (Figure 2).

Furthermore, strong SDF1β expression revealed a trend towards being associated with hematogenous dissemination (M category) (r=0.284; $p$=0.286). The $\chi^2$ test suggests the existence of a significant correlation ($\chi^2$=6.373, $p$=0.041). Higher M category might correlate with SDF1β expression. Yet due to the very low sample number, this statistical result needs to be proven with a higher sample count.

No correlation was seen for lymphatic dissemination (N category) or local tumor progression (T category). SDF1α and SDF1β expression had no prognostic impact on overall survival (OS).

## Discussion

Despite the knowledge about the pro-metastatic function of the CXCR4/SDF1 axis (24, 25), little attention has been devoted to the precise contribution of SDF1. SDF1 is the exclusive ligand of CXCR4 and is involved in tumor spread by promoting proliferation, inhibiting of apoptosis and inducing of angiogenesis (26), working synergistically with vascular endothelial growth factor (VEGF) (27). The expression of the chemokine receptor CXCR4 has been reported in various epithelial, mesenchymal and hematopoietic tumors. In several entities, its expression was linked to tumor dissemination and poor prognosis (24, 28, 29). Therefore, CXCR4-expressing cancer cells are certainly attracted to the typical 'homing organs', such as lungs, bone marrow, liver and lymph nodes with a high SDF1α expression (11, 19, 22).

CXCR4 expression can be increased as a result of intracellular second messengers, such as calcium (30) and cyclic AMP (31), by the inactivation of the tumor suppressor gene *p53* and overexpression of nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) (32), by cytokines such as IL-2, IL-10 and transforming growth factor-1beta (TGF-1β) (29, 33), and by growth factors such as VEGF and epidermal growth factor (EGF) (34, 35). Differences in the tumor biological function of the two splicing variants SDF1α and SDF1β are not yet known.

Due to this fact, our study investigated the correlations between the expression of SDF1α and SDF1β, respectively, with clinicopathological parameters, namely age, gender, TNM classification, grading and tumor size in RCC. To our knowledge, this is the first study investigating the expression of SDF1α and SDF1β separately.

We analyzed the expression profile of SDF1α and SDF1β in a large series of patients' samples of human RCC tissue

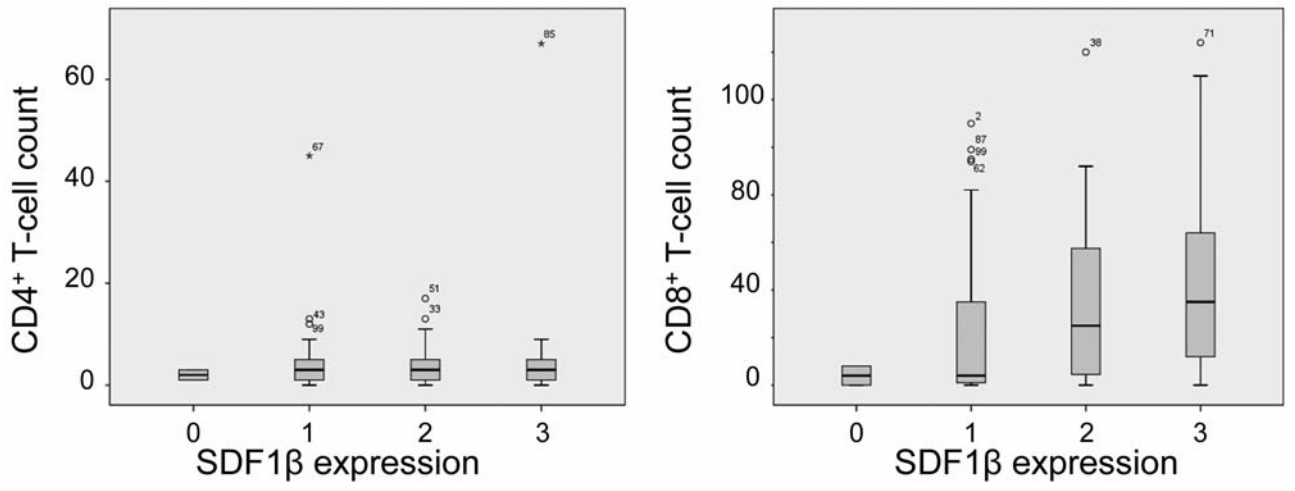


Figure 2. *Correlation of SDF1β expression and CD8+ or CD4+ T-cell infiltration in renal cell carcinoma (RCC). A total of 105 samples of RCC tissue were analyzed by staining with anti-SDF1β and anti-CD8 or anti-CD4 antibody. Staining of SDF1β was evaluated according to the intensity of expression 0: no expression, 1: weak expression, 2: medium expression, 3: strong expression. The T-cell number per visual field using a forty-fold magnification was determined. Box and whisker plots are shown.*

for which exact tumor staging and follow-up data were available and correlated. The human RCC samples revealed different intensities of SDF1α and SDF1β expression. SDF1α, as well as SDF1β, was seen to correlate positively with patient age.

As expected, no significant correlation was seen between the expression of SDF1α or SDF1β and gender. We observed a clear, although not significant, trend towards M1 category correlating with a stronger SDF1β expression. However, due to the very limited sample number in which distant metastasis was exactly known, no clear statement to the significancy of this result can be made. Nevertheless, this is more likely for SDF1-dependent tumor migration and metastasis as has been reported for RCC (21, 22) as well as for many other CXCR4-expressing tumor entities (25, 37).

In the work presented here, a clear significant correlation between the SDF1β expression and tumor grading was shown. A similar observation was made for brain tumors (38).

A pathophysiologically relevant fact worth mentioning is that endothelial cells coexpress SDF1α and vascular cell adhesion molecule 1 (VCAM-1), thus mediating tumor cell to endothelial cell attachment. CXCR4 activation by SDF1α induces β-integrin expression, binding VCAM-1 on endothelial cells (39, 40). Similar pathophysiological processes can be proposed for RCC dissemination. In addition to its chemotactic action on CXCR4-expressing cancer cells, SDF1 inhibits apoptosis through the induction of NFκB and stimulates proliferation *via* the extracellular-signal-regulated kinase and serine/threonine protein kinase pathways. SDF1 also induces the production of matrix metalloproteinases and integrins (26, 35, 41).

Together with the induction of angiogenesis, these factors contribute to metastasis. Based on the multiple functions of the chemokine SDF1 in tumor biology, the question whether SDF1α or SDF1β could serve as prognostic markers was addressed. In our study, we did not find any influence of SDF1 expression on the OS, whereas the TNM classification and grading showed a clear correlation with OS. The usefulness of SDF1 as a prognostic marker is discussed controversially in the literature (17, 42-46).

Using immunohistochemistry, we analyzed the SDF1α and SDF1β expression with respect to the presence of $CD4^+$ and $CD8^+$ T-cells. No correlation was seen for SDF1α, whereas a strong positive correlation was detected for SDF1β expression and the presence of $CD8^+$ T-lymphocytes. In a mouse model, Dunussi-Joannopoulus *et al*. showed that the peritumoral secretion of SDF1β attracts $CD8^+$ T-cells, inducing a T-cell-dependent antitumor response (47). In addition, there seems to be an association between tumor grading and T-cell infiltration. This dependency has already been reported for other tumor entities (48). The presence of tumor-infiltrating lymphocytes has always been reported to be more frequent in undifferentiated tumors. Thus, up-regulation of SDF1β expression by undifferentiated tumors might be a mechanism by which the organism induces immunoreactions to combat immortalized tumor cells. Recent SDF1α data support the view that it contributes to tumor evasion and that it has more likely an inhibitory effect on T-cell infiltration (49). Hence, our data suggest a relevant involvement of SDF1β in tumor progression of RCC and T-cell infiltration with regard to the *in vivo* situation. SDF has a rather a dual nature, correlating not only with de-differentiation but also with $CD8^+$ T-cell infiltration.

As the SDF1/CXCR4 interacts in various ways with the immune system and tumor progression, the targeted blockade of this signaling pathway seems to be a promising mechanism in the development of new therapeutic strategies. Several studies report the blunting of CXCR4 function by different means (50-52). Neutralization of SDF1 function by specific antibodies has been reported for non-small cell lung cancer (NSCLC) and RCC, where it reduces metastasis (11, 21). Recent data indicate that by the blockade of CXCR4, tumor dissemination was not only inhibited, but the efficacy of a subsequent immunotherapy was also potentiated. Thus, it seems reasonable to combine targeting therapies of the CXCR4/SDF1 axis with other therapeutic strategies such as the inhibition of angiogenesis by anti-VEGF antibodies.

## Conclusion

Chemokines have veritably been related to tumor growth, dissemination and local immunescape (36, 53). Our *in vivo* results expand these data for human RCC, as expression of SDF1β was significantly associated with de-differentiation of this tumor and $CD8^+$ T-cell infiltration. Thus, inhibition of RCC progression by CXCR4 antagonists might be a promising therapeutic option in the near future.

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