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CHEMOKINE AND CHEMOKINE RECEPTOR EXPRESSION IN KIDNEY TUMORS: MOLECULAR PROFILING OF HISTOLOGIC SUBTYPES AND ASSOCIATION WITH METASTASIS

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Abstract

Purpose—Molecular characterization of renal cell carcinoma (RCC) may help in differentiating benign oncocytomas from malignant RCC subtypes and predict metastasis. Chemokines (e.g., interleukin-8 (IL-8)) and chemokine receptors (e.g., CXCR4, CXCR7) promote inflammation and metastasis. Stroma derived factor (SDF-1) is a ligand for CXCR4 and CXCR7, with six known isoforms. We evaluated the expression of these chemokines and chemokine receptors in kidney specimens.

Materials and Methods—Using quantitative PCR, mRNA levels of IL-8, CXCR4, CXCR7 and SDF1 isoforms α , β and γ were measured in 166 specimens from 86 patients (tumor: 86; matched normal kidney: 80); mean and median follow-up: 18.9 ± 12 ; Median: 19.5 months. RCC specimens included: clear cell RCC (ccRCC): 65; papillary: 10; chromophobe: 5; oncocytoma: 6; metastasis (+): 17.

Results—Median levels of CXCR4, CXCR7 and SDF1- γ were 2–10-fold elevated and SDF1- α and SDF1- β levels were either unchanged or lower in ccRCC and papillary tumors when compared to normal tissues. Median SDF1- γ , IL-8, CXCR4 and CXCR7 levels were 3–40-fold elevated in chromophobe tumors when compared to oncocytoma. Both CXCR4 and CXCR7 levels were elevated in tumors < 4 -cm (3057 ± 2230 ; 806 ± 691) when compared to oncocytoma (336 ± 325 ; 201 ± 281 ; $P = 0.016$). In multivariate analyses, CXCR4 ($P = 0.01$), CXCR7 (0.02) and SDF1- β

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($P=0.005$) were independently associated with metastasis. Combined CXCR7+SDF1- α and CXCR7+IL-8 markers showed the highest sensitivity (71–81%) and specificity (75–80%) among all individual or combined markers.

Conclusions—Chemokines and chemokine receptors differentiate between RCC and oncocytoma. Combined SDF1- α +CXCR7 and IL-8+CXCR7 markers have ~ 80% accuracy in predicting RCC metastasis.

Keywords

Prognostic markers; SDF-1, IL-8, CXCR4, CXCR7; metastasis, oncocytoma

INTRODUCTION

Renal cell carcinoma (RCC) comprises of a spectrum of tumors, ranging from tumors with indolent clinical course to highly metastatic¹. Histologically, clear cell RCC (ccRCC) comprises of > 70–80% of all RCC cases. The papillary subtype accounts for 10–15% cases followed by chromophobe (5–10%), oncocytoma and tumors of the collecting duct, medullary and undifferentiated histologies². The use of CT and MRI allows the detection of smaller tumors (< 4-cm), and therefore, limits the availability of tissues for sophisticated microscopic, ultrastructural and immunohistochemical techniques that can distinguish between benign oncocytomas and malignant RCC subtypes³. The five-year survival of patients with organ-confined disease varies between 75–90% but then drops to 60–70% among patients with locally advanced disease. For patients with lymph node positive and metastatic disease, the survival is a dismal 15–30%⁴. Thirty percent of all RCC patients present with disease that is too advanced for curative surgical resection. Furthermore, 30% of patients with local disease relapse after surgery^{1–4}. Molecular characterization can lead to identification of markers that distinguish between indolent versus malignant RCC tumor types, accurately predict metastatic potential and aid in designing targeted therapies^{5–7}.

Several chemokines and chemokine receptors promote RCC growth and metastasis. Interleukin-8 (IL-8) is a multifunctional ELR⁺-CXC chemokine that is known to promote tumor growth, motility, invasion and angiogenesis⁸. IL-8 levels were found to be significantly higher in primary renal tumors from patients with metastasis than those without and may contribute to sunitinib resistance^{9,10}. IL-8 also regulates the expression of chemokine receptors, such as CXCR7¹¹. CXCR7/RDC-1 and another chemokine receptor, CXCR4, bind to stromal cell derived growth factor-1 (SDF-1)^{12–14}. Six variant isoforms of SDF-1 have been identified. Among these SDF1- α and SDF1- β isoforms have been well characterized and shown to promote tumor metastasis and angiogenesis^{13–16}. In three studies, CXCR4 as determined by immunohistochemistry, has been shown to correlate with metastasis and/or disease-free survival in RCC^{17–19}. One study reported that CXCR7 expression also correlates with diseasefree survival in RCC patients¹⁷.

In this study we investigated the expression of six chemokine and chemokine receptors (i.e., IL-8, CXCR4, CXCR7, SDF1- α , SDF1- β and SDF1- γ) in matched normal kidney and RCC tissues by quantitative reverse transcription PCR (Q-PCR) to distinguish oncocytoma from other histologic subtypes. We also investigated whether chemokine or chemokine receptor

expression either individually or as a combined marker correlates with metastasis. We hypothesized that since these chemokines and chemokine receptors function in similar biological pathways, the combination of these molecules might be a better predictor of prognosis than individual molecules.

MATERIALS AND METHODS

Patients and tissue specimens

Normal kidney (n=80) and tumor (n=86) specimens were collected from 86 patients undergoing nephrectomy for RCC. All specimens were obtained based on their availability for research purpose and under a protocol approved by University of Miami's Institutional Review Board; a written consent was obtained from the study individuals. Total RNA was isolated from tissues (~ 30 mg) using the miRNeasy Mini Kit (Qiagen, Valencia CA). Patient and tissue characteristics are presented in Table 1. Metastasis was present at the time of surgery in 7 patients, and 10 patients developed metastasis during the course of follow-up. Presence of metastasis was confirmed by biopsy and/or imaging; the frequency for follow-up imaging averaged at 6 months.

Quantitative RT-PCR (Q-PCR)

Total RNA isolated from tissues or exfoliated cells was subjected to Q-PCR using the iQ real time PCR system (BioRad, Hercules, CA) and primers and probes specific for each transcript (Table 2)²⁰. The transcript levels of each gene were normalized to Tata binding protein (TBP) mRNA levels as follows: $(1/2^{-Ct} \times 100)$; $Ct = Ct(\text{transcript}) - Ct(\text{TBP})$ ²¹. The variance of the PCR assay was examined by computing intra-class correlation, as described previously²⁰. The intra-class coefficient for all markers varied between 0.955 and 0.994 with $P < 0.001$.

Statistical analyses

Differences in biomarker levels among kidney tissues (e.g., normal versus CC, CC versus oncocytoma, etc) were compared by One-way ANOVA (Kruskal-Wallis test) followed by Dunn's multiple comparison test, because the data showed a non-normal distribution. All of the P-values reported in this study are two-tailed. Logistic regression single-parameter model (i.e., univariate analysis) was used to determine the association of clinical parameters, and transcript levels with metastasis. Cox-proportional hazards model (i.e., multivariate analysis) was used to determine which of the clinical and pathologic parameters (i.e., age, gender, tumor grade, stage, tumor size, renal vein involvement, lymphovascular invasion) and/or tissue biomarkers predict metastasis.

The levels of the combined biomarkers (e.g., SDF1- α +CXCR4 for each study subject were calculated as follows: $[\text{intercept} + (\alpha \times (\text{SDF1-}\alpha)_1) + (\beta \times (\text{CXCR4})_1)]$; α and β : SDF1- α and CXCR4 coefficients, respectively and $(\text{SDF1-}\alpha)_1$ and $(\text{CXCR4})_1$: SDF1- α and CXCR4 levels in subject # 1, respectively. The intercept and coefficients for each marker were computed by simultaneously analyzing the two variables (i.e, SDF1- α and CXCR4) in a regression model.

Receiver operating characteristic (ROC) curves were generated to determine the association between tissue biomarker levels and metastasis. Cut-off values were selected from the ROC curve data by a statistical program (JMP®6 Software from SAS) for calculating sensitivity and specificity of each biomarker. A biomarker level that yielded the highest efficacy (i.e., sensitivity – (1-specificity)) was selected by the program as the cut-off limit. Crossvalidation using boot-strap modeling (specific sampling rate = 0.5; re-sampling = 104) was performed to obtain the mean $\pm$ SD and 95% CI for the sensitivity, specificity and accuracy of each biomarker. Statistical analyses were carried out using the JMP® Software Program (version 6.0; SAS Institute, Cary, NC).

RESULTS

Differential expression of chemokines and chemokine receptors in normal and RCC tissues

We measured the transcript levels of IL-8, SDF1- α , SDF1- β , SDF1- γ , CXCR4 and CXCR7 in 166 specimens collected from 86 patients. IL-8, SDF1- γ , CXCR4 and CXCR7 transcript levels were 2–4-fold higher in ccRCC tissues than in normal kidney specimens (Figure 1; $P < 0.001$). However, the median expression of SDF1- α and SDF1- β was lower in ccRCC (SDF1- α : 527.8; SDF1- β : 606.3; $P < 0.001$) when compared to normal kidney specimens (SDF1- α : 1056; SDF1- β : 1300). IL-8, CXCR4 and CXCR7 levels were also higher in papillary tumors (median levels: 76, 2786, 549, respectively) than in normal kidney tissues (median levels: 11, 528, 174; $P < 0.05$). Among all markers, SDF1- γ levels were significantly higher in chromophobe RCC when compared to normal kidney (median levels: 50 versus 2.0; Figure 1; $P < 0.01$).

Differential expression of chemokines and chemokine receptors in RCC subtypes

IL-8, CXCR4 and CXCR7 transcript levels were 10–20-fold higher in ccRCC and papillary subtypes when compared to oncocytoma (Figure 1; $P < 0.01$). Median levels of IL-8 and SDF1- γ transcripts were significantly higher in chromophobe subtype (16.5, 50) when compared to oncocytoma (median levels: 0.83, 1.4, respectively; Figure 1; $P < 0.05$). SDF1- γ levels were also significantly higher in chromophobe subtype when compared ccRCC ($P = 0.0167$) and papillary tumors ($P = 0.008$).

Stratification of RCC subtypes other than oncocytoma, with respect to tumor size, yielded 17 specimens from tumors that were < 4 cm (2.9 ± 0.55 ; median: 3.0). In these specimens only CXCR4 and CXCR7 transcript levels were significantly higher in RCC subtypes with tumor size < 4 cm when compared to oncocytoma (mean tumor size: 4.2 ± 0.98 ; median: 4.6; Figure 2).

Association of chemokine/chemokine receptors with metastasis

In the cohort of 86 patients, 17 patients were positive for metastasis during a median follow-up of 19.5 months. Among these 17 patients, 7 were positive for metastasis at surgery. TMN status of all patients is presented in Table 1. As shown in Figure 3, the mean $\pm$ SD levels of IL-8, SDF1- α , SDF1- β , CXCR4 and CXCR7 were higher in tumor specimens from patients who developed metastasis when compared to those who did not. Univariate analysis showed

that among clinical parameters, tumor grade, stage and tumor size were significantly associated with metastasis (Table 3). Except for SDF1- γ , all of the markers (i.e., IL-8, SDF1- α , SDF1- β , CXCR4 and CXCR7) significantly associated with metastasis (Table 3).

In the multivariate analysis, that included all of the clinical parameters and each marker, only SDF1- α and SDF1- β were independently associated with metastasis (Table 4). Although, the number of specimens was limited, analysis of the efficacy of each marker found to be significant in the univariate analysis showed that for individual markers either the sensitivity or the specificity was low for predicting metastasis. Only CXCR7 had reasonable sensitivity (70.6%) and specificity (69.5%) to predict metastasis.

Since CXCR4 and CXCR7 were independent prognostic markers, we determined whether their combination with SDF1 splice variants (which are ligands for CXCR4 and CXCR7) would improve the sensitivity and/or specificity to predict metastasis. As shown in Table 5, except for CXCR4+SDF1- β and CXCR4+IL-8 combinations all other combinations independently associated with metastasis. Furthermore, combination of CXCR7 with SDF1- β or IL-8 had the highest sensitivity (70.6%, 81.3%), specificity (79.7%, 75.4%) and accuracy (77.8%, 76.5%), respectively (Table 5). These results show that SDF1 family of molecules and possibly IL-8 together with chemokine receptors CXCR4 and CXCR7 interdependently associate with metastasis.

DISCUSSION

Molecular subtyping of kidney tumors is of clinical significance from the stand point of differentiating benign oncocytomas from other histologic RCC subtypes, and identifying the existence of metastasis or predicting its occurrence at the time of diagnosis. Although, the expression of IL-8, CXCR4 and CXCR7 has been correlated with RCC metastasis, no study has examined different chemokines and chemokine receptors simultaneously in the same set of tissues and evaluated their potential to predict metastasis.

SDF-1 isoforms have both overlapping and distinct biological functions^{15,22,23}. Although the function of SDF-1 isoforms has not been studied in RCC cell lines, isoforms α , β and γ are differentially expressed in RCC tissues. For example, the median levels of SDF1- α and SDF1- β in ccRCC tissues were ~ 50% less than in normal kidney tissues. Contrarily, SDF1- γ levels were significantly elevated in ccRCC tissues. While SDF1- α and SDF1- β levels were not significantly higher in chromophobe tissues when compared to oncocytomas, SDF1- γ levels were 35.7-fold higher in chromophobe tissues.

Although, there were only six oncocytoma patients in the study, the mean and median levels of all markers included in this study were lower in oncocytoma when compared to normal kidney tissues. CXCR4 and CXCR7 levels were significantly higher, not only in different RCC subtypes but the levels were also 4 and 10-fold higher in tumors < 4-cm when compared to oncocytoma, respectively.

Among the six transcripts evaluated in this study, the role of IL-8 has been extensively studied in inflammation and cancer^{24–27}. For example, IL-8 overexpression supports androgen independent growth in prostate cancer and associates with biochemical

recurrence^{26,27}. In this study IL-8 expression significantly associated with metastasis in univariate analysis. However, due to poor specificity, as a single marker IL-8 may not be a clinically useful predictor of metastasis. In this study, CXCR4, CXCR7 and SDF1- β were found to be independent predictors of metastasis, however only CXCR7 had both reasonable sensitivity and specificity to predict metastasis. The co-expression of CXCR4 and CXCR7 has been shown to associate with metastasis¹⁷. However, the combined CXCR4+CXCR7 marker was not better than the individual markers in this patient cohort. Interestingly, the combination of the ligand SDF1- β with its receptor CXCR7 had significantly higher sensitivity and specificity to predict metastasis. This suggests that the interaction of SDF1 with CXCR7 plausibly promotes metastasis.

IL-8 induces CXCR7 expression¹¹, suggesting a regulatory association between an ELR +CXC cytokine and a chemokine receptor, which functionally does not associate with IL-8. Consistent with this regulatory association, the combined CXCR7+IL-8 marker had 10% sensitivity without compromising the specificity when compared to individual markers. This suggests that a biomarker profile involving IL-8 and CXCR7 levels may be clinically useful in predicting RCC metastasis.

As mentioned above the limitations of this investigation include shorter follow-up, single institutional study and some technical limitations related to RNA quality preservation. Furthermore, since only one piece of tissue was analyzed, there may be some variation in biomarker levels as they relate to tumor heterogeneity. Another limitation would be the confirmation of chemokine and chemokine receptor expression at the protein level, especially for SDF-1 isoforms since this has not been done. However, the currently available SDF-1 antibodies do not distinguish between various isoforms and therefore, immunohistochemistry would yield spurious results. The major limitation of the study is the 18 months median follow-up, which is substantially less than the 2–2.5 year median time to recurrence. As a result, at least some of the patients who later might have developed metastasis were misclassified as false positive, resulting in lower specificity for several markers.

In a study of 880 patients, Smaldone et al showed that the majority of the small renal masses remain radiographically static after an initial period of active surveillance, with only a very percentage (~ 2%) of the patients developing metastasis (28). Although in this study nephrectomy specimens were utilized to measure the levels of different markers, one key clinical application of our study would be if a biopsy of a small renal mass could be molecularly characterized based on these markers.

Taken together in this prospective study, IL-8, CXCR4 and SDF1- γ mRNA levels were found to differentiate between different RCC subtypes and oncocytoma. The combined biomarker profiles of CXCR7+SDF1- β and CXCR7+IL-8 were found to be potentially accurate in predicting RCC metastasis.

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Abbreviations used

CC	clear cell
IL-8	Interleukin-8
RCC	Renal cell carcinoma
SDF	Stroma-derived factor

REFERENCES

1. Basu B, Eisen T. Perspectives in drug development for metastatic renal cell. *Target Oncol.* 2010; 5:13.
2. Cheville JC, Lohse CM, Zincke H, et al. Comparisons of outcome and prognostic features among histologic subtypes of renal cell carcinoma. *Am J. Surg Pathol.* 2003; 27:612. [PubMed: 12717246]
3. Yusenko MV. Molecular pathology of renal oncocytoma: a review. *Int J Urol.* 2010; 17:602. [PubMed: 20590944]
4. Pantuck AJ, Zisman A, Belldegrun AS. The changing natural history of renal cell carcinoma. *J Urol.* 2001; 166:1611. [PubMed: 11586189]
5. Ficarra V, Brunelli M, Cheng L, et al. Prognostic and therapeutic impact of the histopathologic definition of parenchymal epithelial renal tumors. *Eur Urol.* 2010; 58:655. [PubMed: 20727666]
6. Paret C, Schön Z, Szponar A, et al. Inflammatory protein serum amyloid A1 marks a subset of conventional renal cell carcinomas with fatal outcome. *Eur Urol.* 2010; 57:859. [PubMed: 19747761]
7. Eichelberg C, Junker K, Ljungberg B, et al. Diagnostic and prognostic molecular markers for renal cell carcinoma: a critical appraisal of the current state of research and clinical applicability. *Eur Urol.* 2009; 55:851. [PubMed: 19155123]
8. Xie K. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev.* 2001; 12:375. [PubMed: 11544106]
9. König B, Steinbach F, Janocha B, et al. The differential expression of proinflammatory cytokines IL-6, IL-8 and TNF-alpha in renal cell carcinoma. *Anticancer Res.* 1999; 19:1519. [PubMed: 10365136]
10. Huang D, Ding Y, Zhou M, et al. Interleukin-8 mediates resistance to antiangiogenic agent sunitinib in renal cell carcinoma. *Cancer Res.* 2010; 70:1063. [PubMed: 20103651]
11. Singh RK, Lokeshwar BL. The IL-8-Regulated Chemokine Receptor CXCR7 Stimulates EGFR Signaling to Promote Prostate Cancer Growth. *Cancer Res.* 2011; 71:3268. [PubMed: 21398406]
12. Maksym RB, Tarnowski M, Grymula K, et al. The role of stromal-derived factor-1--CXCR7 axis in development and cancer. *Eur J Pharmacol.* 2009; 625:31. [PubMed: 19835865]
13. Sun X, Cheng G, Hao M, et al. CXCL12/CXCR4/CXCR7 chemokine axis and cancer progression. *Cancer Metastasis Rev.* 2010; 29:709–722. *Erratum:* *Cancer Metastasis Rev.* 2011;30:269. [PubMed: 20839032]
14. Vandercappellen J, Van Damme J, Struyf S. The role of CXC chemokines and their receptors in cancer. *Cancer Lett.* 2008; 267:226. [PubMed: 18579287]
15. Altenburg JD, Broxmeyer HE, Jin Q, et al. A naturally occurring splice variant of CXCL12/stromal cell-derived factor 1 is a potent human immunodeficiency virus type 1 inhibitor with weak chemotaxis and cell survival activities. *J Virol.* 2007; 81:8140. [PubMed: 17507482]
16. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res.* 2010; 16:2927. [PubMed: 20484021]
17. D'Alterio C, Consales C, Polimeno M, et al. Concomitant CXCR4 and CXCR7 expression predicts poor prognosis in renal cancer. *Curr Cancer Drug Targets.* 2010; 10:772. [PubMed: 20578990]
18. D'Alterio C, Cindolo L, Portella L, et al. Differential role of CD133 and CXCR4 in renal cell carcinoma. *Cell Cycle.* 2010; 9:4492. [PubMed: 21127401]

19. Wang L, Wang L, Yang B, et al. Strong expression of chemokine receptor CXCR4 by renal cell carcinoma cells correlates with metastasis. *Clin Exp Metastasis*. 2009; 26:1049. [PubMed: 19859817]
20. Kramer MW, Escudero DO, Lokeshwar SD, et al. Association of hyaluronic acid family members (HAS1, HAS2, and HYAL-1) with bladder cancer diagnosis and prognosis. *Cancer*. 2011; 117:1197. [PubMed: 20960509]
21. Chi A, Shirodkar SP, Escudero DO, et al. Molecular characterization of kidney cancer: association of hyaluronic acid family with histological subtypes and metastasis. *Cancer*. 2011 In Press.
22. Ho TK, Tsui J, Xu S, et al. Angiogenic effects of stromal cell-derived factor-1 (SDF-1/CXCL12) variants in vitro and the in vivo expressions of CXCL12 variants and CXCR4 in human critical leg ischemia. *J Vasc Surg*. 2010; 51:689. [PubMed: 20206813]
23. Niedermeier M, Hennessy BT, Knight ZA, et al. Isoform-selective phosphoinositide 3'-kinase inhibitors inhibit CXCR4 signaling and overcome stromal cell-mediated drug resistance in chronic lymphocytic leukemia: a novel therapeutic approach. *Blood*. 2009; 113:5549. [PubMed: 19318683]
24. Ning Y, Manegold PC, Hong YK, et al. Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models. *Int J Cancer*. 2011; 128:2038. [PubMed: 20648559]
25. Ginestier C, Liu S, Diebel ME, et al. CXCR1 Blockade selectively targets human breast cancer stem cells in vitro and in xenografts. *J Clin Invest*. 2010; 120:485. [PubMed: 20051626]
26. Singh RK, Lokeshwar BL. Depletion of intrinsic expression of Interleukin-8 in prostate cancer cells causes cell cycle arrest, spontaneous apoptosis and increases the efficacy of chemotherapeutic drugs. *Mol Cancer*. 2009; 8:57. [PubMed: 19646263]
27. Araki S, Omori Y, Lyn D, et al. Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer. *Cancer Res*. 2007; 67:6854. [PubMed: 17638896]
28. Smaldone MC, Kutikov A, Egleston BL, et al. Small renal masses progressing to metastases under active surveillance: A systematic review and pooled analysis. *Cancer*. 2011 Jul 15. [Epub ahead of print].

Take home message

This is the first study that simultaneously evaluated chemokine and chemokine receptor expression in RCC. SDF1- γ , CXCR4, CXCR7 and IL-8 transcript levels differentiated RCC subtypes from oncocytomas. Combined CXCR7+ SDF1- β and CXCR7+IL-8 markers independently predicted metastasis with ~ 80% accuracy.

Figure 1. Scatter diagrams of chemokine and chemokine receptors in kidney tissues

Each symbol represents mRNA levels of a marker in a study subject. The mean $\pm$ SD scores for each biomarker are indicated; horizontal line: median levels. CC: clear cell RCC; Chromo: chromophobe; Oncocyt: oncocytoma.

Figure 2. Comparison of the expression of CXCR4 and CXCR7 levels between small RCC tumors (< 4 cm) and oncocytoma

In a subgroup analysis with respect to tumor size, among the six chemokines and chemokine receptors, CXCR4 and CXCR7 levels were found to be significantly higher in tumors < 4-cm when compared to oncocytoma. Scatter diagram of mRNA levels of CXCR4 and CXCR7 are shown for 17 tumors that were < 4-cm and 6 oncocytomas. The mean ± SD scores for each biomarker are indicated; horizontal line: median levels.

Figure 3. Comparison of the expression of chemokine and chemokine receptors with respect to metastasis

mRNA levels of the mean ± SD scores of each biomarker are indicated; horizontal line: median levels.

Table 1
Description of tissues and clinical characteristics of 86 patients with RCC

A: papillary sub-types: Type I: 5 patients; papillary type I with focal clear cell: 1; undefined: 2; papillary oncocytoma: 1; Mixed: 1. Percentages in parentheses were calculated by dividing the number of patients in each subcategory by total number of patients, i.e., 86

Tissue specimens: Normal: 80; Tumor 86	
Age:	63.4 ±14 ; Median: 64 Range 19 – 88
Gender	Male: 60 Female: 26
Tumor Type	Clear Cell: 65 (75.6%) Papillary ^A : 10 (11.6%) Chromophobe: 5 (5.8%) Oncocytoma: 6 (7%)
Tumor grade	Stage 0 (Oncocytoma): 6 (7%) Grade 1: 5 (5.8%) Grade 2: 32 (37.2%) Grade 3: 25 (29.1%) Grade 4: 18 (20.9%)
Tumor stage(n)	Stage 0 (Oncocytoma): 6 (7%) T1 (a+b): 40 (46.5%) T2: 13 (15.1%) T3: 21 (24.4%) T4: 6 (7%)
Metastasis at surgery (M)	(+) 6 (7.5%) (–) 80 (92.5%)
Lymph node (N)	(+) 3 (3.5%) (–) 4 (4.6%) Unknown 79 (91.9%)
Renal vein involvement	Positive: 15 (17.4%) Negative: 68 (79.1%) No data: 3 (3.5%)
Lymphovascular invasion	Positive: 9 (10.5%) Negative: 45 (52.3%) No data: 32 (37.2%)
Tumor size (excluding oncocytoma)	< 4 cm: 17 (19.8%) > 4 cm: 68 (79.1%) Unknown: 1 (1.1%)
Follow-up	18.9 ± 12; Median: 19.5; 1 – 38.5 months
Metastasis	Negative: 70; Age: 64.3±13.4 years; Median: 66 years Positive: 16; Age: 59.5±16.1 years; Median: 61 years

Table 2
Sequences of the primers and probes used in Q-PCR assays

Marker	Forward Primer	Reverse Primer
IL-8	5'TTCGTCATCGGCATGATTGCCAAC3'	5-ATGTAGCAGTGCGTGTGTCAGCCT-3
SDF1- α	5'CACAGAAGGTCCTGGTGGTA3'	5'CATTGAAAAGCTGCAATCACA3'
SDF1- β	5'CGCCTTTCCCAGGTGCTAAC3'	5'TGGTCTGCTTAGGGGATTGG3'
SDF1- γ	5'GTGCCCTTCAGATTGTAGCC3'	5'GGGCAGCCTTTCTCTTCTTC3'
CXCR4	5'TCATCAAGCAAGGGTGTGAG3'	5'GGCTCCAAGGAAAGCATAGA3'
CXCR7	5'TACCCCGAGCACAGCATCAA3'	5'TGGAGAAGGGAACGGCAAAG3'
TBP	5'TGCACAGGAGCCAAGAGTGAA3'	5'CACATCACAGCTCCCCACCA3'

Table 3
Determination of the association between metastasis, clinical parameters, chemokines and chemokine receptors

The pre- and post-operative parameters included age, gender, tumor grade, stage, tumor size, renal vein invasion and lymphovascular invasion. Logistic regression analysis was used to determine the association of pre- and post-operative parameters and biomarker levels with metastasis.

Marker	Chi-Square	P value	Odds Ratio	95% CI
Age	1.4	0.237	ND	ND
Gender	0.65	0.42	ND	ND
Grade	12.8	0.0004 *	4.4	2.1 – 11.1
Stage	11.3	0.0009 *	2.9	1.6 – 5.5
Tumor size	8.85	0.003 *	1.3	1.09 – 1.5
Renal vein invasion	1.87	0.17	ND	ND
Lymphovascular invasion	2.96	0.085	ND	ND
IL-8	5.2	0.0226 *	1.02	1.01 – 1.03
SDF1- α	9.23	0.0029 *	1.02	1.01 – 1.04
SDF1- β	5.7	0.017 *	1.01	1.00 – 1.01
SDF1- γ	1.4	0.24	ND	ND
CXCR4	7.7	0.008 *	1.02	1.01 – 1.04
CXCR7	8.4	0.004 *	1.02	1.01 – 1.04
CXCR4 + SDF1- α	11	0.0009 *	2.6	1.6 – 5
CXCR7 + SDF1- α	11.7	0.0006 *	3.03	1.75 – 6.3
CXCR4 + SDF1- β	6.1	0.013 *	1.5	1.1 – 2.2
CXCR7 + SDF1- β	8.72	0.003	1.64	1.2 – 2.3
CXCR4 + IL-8	6.6	0.012 *	1.11	1.04 – 1.23
CXCR7 + IL-8	7.4	0.007 *	2.7	1.4 – 5.9

* : Significant parameter

Table 4
Multivariate analyses of pre- and post-operative parameters and chemokines and chemokine receptors to predict metastasis

Cox proportional hazards analysis was performed by including all of the pre- and post-operative parameters and transcript levels of IL-8, SDF1- α , SDF1- β , SDF1- γ , CXCR4 and CXCR7. All biomarkers (including the combined biomarkers) were included as continuous variables. The pre- and post-operative parameters included age, gender, grade, stage, tumor size, lymphovascular invasion, renal vein involvement. Since lymph node dissection was performed only on 7 patients, lymph node did not reach statistical significance. For biomarkers, risk-ratios shown are per unit changes (i.e., change in risk per unit change in a biomarker level).

A: In the multivariate model all patients with metastasis were included, i.e., those who had metastasis at the time of surgery (n=7), as well as, those who subsequently developed metastasis (n=10). Running the Cox Proportional Hazard model with only clinical parameters renal vein involvement and tumor stage reached statistical significance. **B:** In the multivariate model patients who had metastasis at the time of surgery were excluded. When only clinical parameters were included in the model, renal vein involvement and tumor stage significantly associated with metastasis. Logistic multivariate analysis was performed for patients positive for metastasis at the time of surgery. However, due to the small samples size (n=6), no parameter (i.e., clinical or biomarkers) reached statistical significance.

Parameter	Chi-square	P-value	Hazard ratio	95% CI
A: All metastasis				
Stage	14.75	0.0001	12.4	3.3 – 59.4
Renal vein	7.7	0.006	52.6	3.2 – 1429
CXCR4	6.4	0.011	1.02	1.0 – 1.03
CXCR7	5.5	0.019	1.01	1.0 – 1.04
SDF1- β	7.9	0.005	1.01	1.0 – 1.03
CXCR4 + SDF1- α	7.8	0.005	1.7	1.2 – 3.0
CXCR7 + SDF1- α	6.9	0.009	1.7	1.1 – 2.9
CXCR4 + SDF1- β	8.2	0.004	1.3	1.0 – 1.7
CXCR7 + SDF1- β	8.9	0.003	1.3	1.03 – 1.6
CXCR7 + IL8	4.2	0.04	2.3	1.0 – 6.0
B: Metastasis at follow-up only				
Stage	8.2	0.004	12.5	2.2 – 113
Renal vein	5.0	0.026	45.4	1.6 – 1667
CXCR4	8.3	0.004	1.01	1.0 – 1.04
SDF1- β	7.0	0.008	1.02	1.01 – 1.04
CXCR4 + SDF1- α	4.3	0.039	1.7	1.03 – 3.4
CXCR4 + SDF1- β	10.4	0.001	2.7	1.3 – 9.9
CXCR7 + SDF1- β	15.2	<0.0001	4.4	1.6 – 151

Please note that data are presented for only those parameters that reached statistical significance. *:

Table 5
Determination of Efficacy of chemokines and chemokine receptors for predicting metastasis

Sensitivity, specificity and accuracy of the biomarkers were determined using the cut-off values determined from the ROC curves.

Marker	Cut-off	AUC	Sensitivity	Specificity	Accuracy
IL-8	46.7	0.757	68.8% (11/16)	75.4% (49/65)	74% (60/81)
SDF1- α	566	0.746	82.3% (14/17)	61% (39/64)	65.4% (53/81)
SDF1- β	1056	0.654	64.7% (11/17)	67.7% (44/65)	67.1% (55/82)
CXCR4	1970	0.669	70.6% (12/17)	49.3% (32/65)	53.7% (44/82)
CXCR7	857	0.746	70.6% (12/17)	69.3% (45/65)	69.5% (57/82)
CXCR4+ SDF1- α	4.51	0.764	64.7% (11/17)	87.5% (56/64)	82.7% (67/81)
CXCR7+ SDF1- α	3.9	0.803	70.6% (12/17)	79.7% (51/64)	77.8% (63/81)
CXCR4+ SDF1- β	4.7	0.683	52.9% (1/17)	83.1% (54/65)	76.8% (63/82)
CXCR7+ SDF1- β	4.5	0.732	70.6% (12/17)	63.1% (41/65)	64.6% (53/82)
CXCR7+IL-8	2.7	0.8	81.3% (13/16)	75.4% (49/65)	76.5% (62/81)

AUC: area under the curve. The sensitivity, specificity and accuracy values for each marker were confirmed by performing bootstrap modeling.