RESEARCH COMMUNICATION

CXCR4 Expression in Patients with High-risk Locally Advanced Renal Cell Carcinoma Can Independently Predict Increased Risk of Disease Progression and Poor Overall Survival

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Abstract

Aims and background: CXC receptor 4 (CXCR4), one of chemokine receptor family, plays important roles in metastasis of solid malignancies. In the present study, we aimed to investigate the potential predictive value of CXCR4 in the metastasis of patients with high-risk locally advanced renal cell carcinoma (LARCC). Materials and Methods: From 2001 to 2005, the expression of CXCR4 in 117 high-risk LARCCs was evaluated with immunohistochemical staining and assessed for correlations with clinical characteristics, progression-free survival (PFS) and overall survival (OS) of the patients. Results: Mean duration of follow-up was 51 months. 4-year PFS and OS of patients was 55.6% and 69.2%, respectively. High expression of CXCR4 was associated with not only increased risk for disease progression (p=0.001), but also worse OS of high-risk LARCC patients (p=0.001). Further analysis also suggested that CXCR4 expression had a significant negative predictive value for the effect of interferon alpha (IFN-α) on PFS (p=0.003). Conclusions: CXCR4 is a novel biomarker for prognosis in high-risk LARCC, which might furthermore have promise to predict clinical response to adjuvant therapy.

Keywords: CXCR4 - locally advanced RCC - progression-free survival - overall survival - prognosis

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Introduction

Renal cell carcinoma (RCC) accounts for 2-3% of all malignancies in adults. The worldwide incidence of RCC is about 209,000 new cases per year and 102,000 deaths per year, therefore, it has been considered the most lethal among all the genitourinary tumors (Gupta K et al., 2008). Generally speaking, about 70% of patients were present with localized or locally advanced renal cell carcinoma (LARCC). Radical or partial nephrectomy could provide curative treatment of RCC, unfortunately, 20 to 40% of patients underwent surgical resection would experience recurrence and metastasis (Lam et al., 2005), suggesting that there are some individuals in whom surgical excision is necessary but insufficient. Once metastasis occurred, the patients with metastatic RCC (mRCC) would face a poor prognosis with a median survival of 6-10 months and a 10–20% 2-year survival rate (Janzen et al., 2003). Although, to date, there have been few effective adjuvant strategies for prevention recurrence of RCC, it is of necessity to evaluate progression risk for each individual with LARCC accurately. In order to improve disease surveillance, predict potential treatment response or clinical outcome, and make a decision on the need for probable adjuvant therapy, several prognostic nomograms and molecular biomarkers have been constructed and investigated. UISS, SSIGN and Leibovich scoring systems are the most well-known prognostic models for identification of patients at risk for disease progression after nephrectomy (Kattan et al., 2001; Frank et al., 2002; Leibovich et al., 2003). Several molecular biomarkers, including carbon anhydrase 9 (CAIX), Ki67 and CXC receptor 3 (CXCR3) (Bui et al., 2003; 2004; Klatte et al., 2008), have been sequentially discovered to be associated with clinical outcome in patients with high-risk LARCC.

The human chemokine system is currently known to include more than 50 chemokines and 20 chemokine receptors (Sun et al., 2010). Chemokines and chemokine receptors are now known to play important roles in inflammation, infection, tissue injury, allergy, cardiovascular diseases and malignant tumors (Lazennec and Richmond, 2010). One of the most intriguing and perhaps important roles that chemokines and the chemokine receptors have is to regulate metastasis of solid tumors. CXCR4 is one of the best studied chemokine receptors, which selectively binds to the CXC chemokine stromal cell-derived factor 1 (SDF-1), also known as CXCL12 (Fredriksson et al., 2003). To date, CXCR4 have been demonstrated to be overexpressed in over 20 human malignancies, including breast cancer, prostate cancer,

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kidney cancer, colon cancer, thyroid cancer and pancreatic cancer (Müller et al., 2001; Akashi et al., 2008; Maréchal et al., 2009; Wang et al., 2009; He X et al., 2010). Notably, overwhelming evidences have implied that CXCL12/CXCR4 axis plays a pivotal role in directing metastasis of CXCR4 positive tumor cells to organs expressed CXCL12 (Sun et al., 2010). Further analysis even suggested that overexpression of CXCR4 in resected primary tumor tissues could predict distal metastasis and poor prognosis of diseases (Kim et al., 2005; 2006; Maréchal et al., 2009).

Recently, the role of CXCR4 in mRCC has been thoroughly elucidated. Results demonstrated that high expression of CXCR4 was strongly associated with poor survival of patients with mRCC (Wang et al., 2009; Zhao et al., 2011). However, there is a paucity of data related to the potential prognostic role of CXCR4 in LARCC. We are interested whether CXCR4 expression is of important value in prediction progression for high-risk LARCC after surgical resection.

In the present study, the expression of CXCR4 among patients with high-risk LARCC was evaluated by immunohistochemical staining. We hypothesized that CXCR4 expression in high-risk LARCC at the time of initial diagnosis could be associated with a high likelihood of subsequent distant metastases or shorter overall survival.

Materials and Methods

Patients and follow-up

A total of 117 patients with RCC (from 2001 to 2005) participated in the present study. The age distribution ranged from 16 to 85 years (mean: 57.7 years, median: 59 years). All the patients were histologically documented, previously surgically resected locally advanced clear cell renal cell carcinoma, and they were thoroughly classified into high-risk of progression according to Leibovich integrated stratification system (Leibovich score ≥ 5) (Leibovich et al., 2003).

Detailed follow-up data were available in all of patients, including 36 patients' death and 52 patients with disease progression. At the final cutoff day (June 30, 2010) for analysis, the median duration of follow-up was 51 months (ranging from 2 to 100 months). The clinicopathological data, including age, gender, diameter of tumor, TNM staging, Fuhrman grading, tumor necrosis, ECOG performance status at diagnosis and adjuvant therapy after surgery, had been recorded in detail. 55/117 patients were treated with interferon alpha (IFN-α) after surgery (600 million IU, subcutaneous injection, every other day for 9 to 12 months). The overall survival (OS) and progression-free survival (PFS) were two objective endpoints. OS duration was defined as the time between the dates of initial diagnosis and the last follow-up or death. PFS duration was defined as the time between the date of initial diagnosis and the date of the last follow-up or the first event defined as locoregional relapse, contralateral renal cancer, distal metastasis, or death.

Immunohistochemical analysis

Immunohistochemical staining was performed using the monoclonal antibody against CXCR4. Formalin-fixed and paraffin-embedded clear cell renal cell carcinoma sections (4μm) were dewaxed in xylene and rehydrated using graded ethanol. Endogenous peroxydase activity was blocked by 3% hydrogen peroxide in 50% methanol for 45 minutes. For antigen retrieval, sections were treated with citrate buffer saline (pH 6.0) for 3 minutes. After blocking with 10% normal goat serum for 45 minutes at room temperature, sections were incubated with primary antibody (mouse monoclonal anti-human CXCR4: R&D system, Minneapolis, MN, USA; dilution 1:100) for another 60 minutes at room temperature. The sections were subsequently incubated for 16-18 hours at 4°C. Using the EnVision System-labeled HRP anti-mouse (Dako, Denmark), the primary antibody was visualized with diaminobenzidine –H_2O_2 and counterstained with Mayer's hematoxylin. PBS was used instead of the primary antibodies for the negative controls. Lung adenocarcinoma tissue was used as positive control.

CXCR4 expression was viewed and scored independently by two pathologists who were blind to clinical characteristics. The expression of CXCR4 was scored by conventional four-tiered semi-quantitative scoring system (scores 0–3 for negative, weak, moderate, and strong staining, respectively) (Kunkle et al., 2007), based on staining intensity. Moderate to strong expression of CXCR4 was defined as positive expression, while negative or weak expression was defined as negative.

Statistics

Analyses were performed using the SPSS 17.0 software package (Chicago, IL, USA). The relationship between CXCR4 and clinical pathological characteristics was rested via χ^2 analysis. Kaplan–Meier survival curves were established and subsequently analyzed by log-rank tests to assess the prognostic significance of CXCR4 expression in tumor tissues. Multivariate analysis was performed with Cox regression model to assess additional prognostic values of CXCR4 and related clinical pathological variables. P values of 0.05 or less were deemed statistically significant.

Results

Tumor characteristics and immunohistochemical staining of CXCR4 in resected high-risk LARCC

The details of the clinical and pathological characteristics are shown in Table1. CXCR4 was exclusively expressed in the cytoplasm, but not expressed in the nuclear and membrane of renal tumor cells. In RCC tissues, the percentage of CXCR4 expression was 77.7% (91/117) and varied from negative (22.2%), weak (27.4%), moderate (23%), to strong (27.4%) (Figure1A, 1B, 1C, 1E). CXCR4 was not expressed in glomeruli of normal renal tissue(Figure 1D) but slightly expressed in a few of renal tubule cells.

Correlations between CXCR4 expression and clinical pathological characteristics

Spearman's correlation analysis showed that there was significant statistically difference in CXCR4 expression

Table 1. Patient Characteristics According to CXCR4 Expression

Characteristic	CXCR4+ tumors (n=59)(%)	CXCR4- tumors (n=58)(%)	P value
Age			
≥60y	28	26	
<60y	31	32	0.854
Gender			
Male	38	40	
Female	21	18	0.696
Origination			
Left kidney	36	33	
Right kidney	23	25	0.709
Tumor size			
≥10cm	14	21	
<10cm	45	37	0.161
Tumor necrosis			
Yes	32	36	
No	27	22	0.455
T staging			
T2	12	8	
T3	42	46	
T4	5	4	0.462
N staging			
Nx or N0	41	44	
N1	18	14	0.535
Fuhrman grading			
2	10	13	
3	34	39	
4	15	6	0.825
Leibovich score			
<7(5/6)	6/12	9/21	
≥7(7/8/9)	16/17/8		0.025
IFNa-adjuvant therapy			
Yes	36	19	
No	23	39	0.003

according to tumor Fuhrman grading (p=0.02) (Figure 1A, 1B, 1C), and high expression of CXCR4 was also positively associated with high Leibovich score (score≥7). There was no correlation between CXCR4 expression and age, gender, diameter of tumor, T staging, N staging, tumor necrosis, and ECOG performance status (data not shown).

CXCR4 expression, clinical and pathological characteristics and survival of patients with high-risk LARCC

In the present study, lack of staining or weak expression were classified as CXCR4-negative expression (CXCR4-), whereas, moderate to strong expression were classified as CXCR4-positive expression (CXCR4+). The relationships between CXCR4 expression, clinical variables and survival of patients were analyzed via univariate and multivariate analyses.

Overall, 2-year and 4-year rates of disease progression was 21.4% (25/117) and 44.4% (52/117), respectively. The 2-year OS rate of the 117 patients was 84.6% and the 4-year rate decreased to 69.2%. The distribution of recurrences according to specific organ site was also identified. 33 (63.4%), 17 (32.7%), 15 (28.8%), 10 (19.2%), and 8 (15.4%) patients developed metastases in the lung, bone, lymph node, liver and brain, respectively.

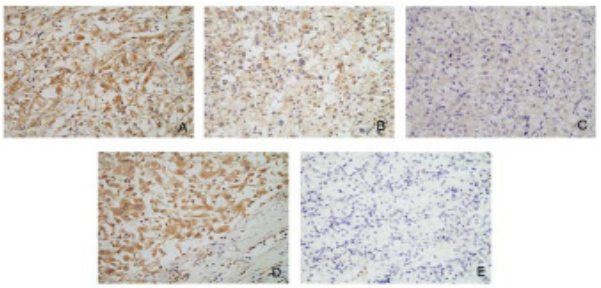

Figure 1. Illustrative Immunostaining for CXCR4 in High-risk Locally Advanced Renal Cell Carcinoma. (A) Strong expression of CXCR4 in tumor tissues with Fuhrman grading 4; (B) Moderate expression of CXCR4 in tissues with Fuhrman grading 3; (C) Weak expression of CXCR4 in tumor tissues with Fuhrman grading 2; (D) Strong expression of CXCR4 in tumor tissues vs. negative expression of CXCR4 in normal renal glomerular tissues; (E) negative expression of CXCR4 in tumor tissues. Original magnification: ×400

Remarkably, CXCR4 expression was not associated with any site of metastasis.

CXCR4 was highly expressed in patients with disease progression (42/52, 80.8%), whereas, high expression of CXCR4 in patients without progression was lowered to 26.5% (17/65). Univariate analysis demonstrated that, patients with CXCR4+ were predominately associated with shorter PFS and OS time than that of patients with CXCR4- (PFS time: 38.8±12.7 vs. 58.5±14.9 months, p=0.000; OS time: 58.3±16.7 vs. 74.7±17.1 months, p=0.000)(Figure 2A and 2B). The association of survival with other clinical variables of patients was also investigated. It was worthy of paying attention that higher Fuhrman grading (Fuhrman grading = 4) and higher Leibovich score (≥7) was related to poorer PFS time than lower grading and score (Figure 2C and 2D). Although, OS time of patients with lower Fuhrman grading (Fuhrman grading ≤3) was nearly 7 months longer than that of patients with higher grading (Fuhrman grading = 4), statistically significant difference was not found (p=0.052) (Figure 2D). And, Leibovich score was also not associated with overall survival of patients (Figure 2F). Association between PFS or OS time and age, gender, tumor location, diameter of tumor, tumor necrosis and TNM staging were not observed in our study (Table 2).

Multivariate analysis by Cox proportional hazard regression showed that, among the clinical and pathological characteristics, CXCR4 expression was not only an independent prognostic factor for disease progression in patients with high risk LARCC (RR=6.710, 95% CI=3.165–14.233, p=0.001), but also for overall survival (RR=4.120, 95%CI=1.792–9.473, p=0.001). Otherwise, Leibovich score was not an independent factor for PFS or OS in high-risk LARCC.

Relationship between CXCR4 expression and response to IFN alpha

Among 117 patients, forty-seven percentage (55/117) of them had been treated with IFN-α as adjuvant therapy after surgery. Totally, the mean PFS time of patients with IFN-α treatment was 46.6 months, surprisingly, the mean PFS time of patients without adjuvant therapy increased to

Table 2. Univariate Analysis of Survival in Patients with High-risk Locally Advanced RCC

Grouping	N	OS	p (Log rank test)	PFS	p (Log rank test)
Age					
≥60y	54	18/54	0.65	26/54	0.494
<60y	63	18/63		26/63	
Gender					
M	78	23/39	0.495	35/78	0.621
F	39	13/39		17/39	
Tumor Location					
Left	69	19/69	0.301	30/69	0.569
Right	48	17/48		22/48	
Tumor Size					
<10cm	82	24/82	0.605	35/82	0.625
≥10cm	35	12/35		17/35	
Tumor Necrosis					
N	49	20/49	0.068	25/49	0.316
Y	68	16/68		27/68	
T Staging					
≤T2	20	12-7-20	0.809	13/20	0.104
>T2	97	29/97		39/97	
N Staging					
Nx or N0	85	29/85	0.275	40/85	0.457
N1	32	7/32		12/32	
Fuhrman Grading					
≤3	96	25/96	0.052	35/96	0.001
4	21	11/21		17/21	
IFNa-adjuvant therapy					
Y	55	22/55	0.099	34/55	0.027
N	62	14/62		18/62	
CXCR4 expression					
+	59	28/59	0.001	42/59	0
-	58	8/58		10/58	
Leibovich score					
<7	48	10/48	0.053	15/48	0.033
≥7	69	26/69		37/69	

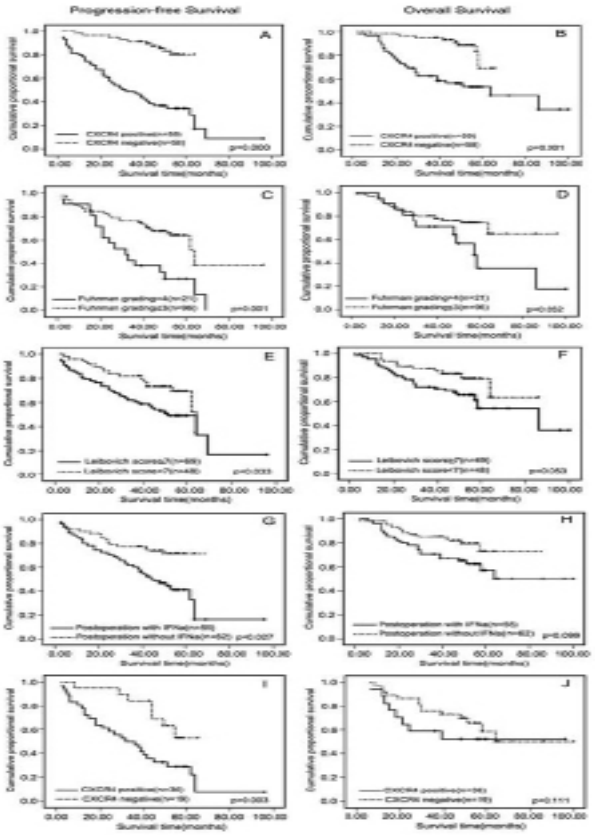

Figure 2. Association of CXCR4 Expression, Fuhrman Grading, IFN Therapy and Leibovich Score with Survival in Patients with High-risk Locally Advanced Renal Cell Carcinoma. Kaplan–Meier survival analysis of progression-free survival(left array) and overall survival (right array) for CXCR4 (Figure 2A and 2B), Fuhrman grading (Figure 2C and 2D), Leibovich score (Figure 2E and 2F), IFN as adjuvant therapy (Figure 2G and 2H) and CXCR4 expression in patients with IFN (Figure 2I and 2J). Log rank test P-values are listed for each parameter

55.4 months, there was a statistically significant difference between these two groups (p=0.027) (Figure 2G). At the same time, univariate analysis for OS suggested that, the patients without IFN therapy had a relative longer OS time than that of patients with IFN-α (estimated OS time: 71.8±15.1 vs. 66.7±15.9months, p=0.099) (Figure 2H). Subgroup analysis for association of CXCR4 with individuals' response to IFN-α showed that, patients with CXCR4+ was significantly associated with worse response to IFN-α. In patients with IFN-α therapy (n=55), The median PFS time associated with CXCR4- tumors (19/55) was 54 months, while, the median PFS time associated with CXCR4+ tumors (36/55) was only 36 months (p=0.003). Although the median OS time in patients with CXCR4- expression was over 10months longer than patients with CXCR4+, univariate analysis still suggested that CXCR4 expression was not linked with overall survival in this subgroup (p=0.111) (Figure 2I and 2J).

Discussion

CXCR4 is expressed in a broad range of tissues, including immune and the central nervous systems, but lowly or absently expressed in many normal tissues including breast and ovary. Previous studies have indicated that CXCR4 is involved in the development, hematopoiesis, organogenesis and vascularization of cancer (Nagasawa T et al., 1998; Zou YR et al., 1998; Nielsen TO et al., 2003). CXCL12 (SDF-1) is now known as an exclusive ligand for CXCR4. High expression of CXCL12 has been discovered in lymph node, bone marrow, liver, lung and brain, which are common sites of metastasis from various malignant cancers. A growing body of evidence now demonstrates that CXCR4/CXCL12 axis has an important role in directing metastasis of CXCR4 positive tumor cells to organs expressed CXCL12. In vitro, breast and prostate cancer cells have been confirmed to follow hypothetic pattern of metastasis, migrating primarily to the lymph nodes, lung, liver, and bone marrow tissue, all of which have high levels of SDF-1 expression (Sun X et al., 2010). In vivo, high expression of CXCR4 has been found in many types of cancers [Tachibana K, et al., 1998], furthermore, CXCR4 positive tumor cells were related to high possibility of distal metastasis, and CXCR4 expression in patients with various tumors were confirmed to be associated with prognosis.

In 2003, Staller and his colleagues firstly illustrated the close relationship between CXCR4/CXCL12 and

metastasis of RCC in vitro (Furusato B et al., 2010). Subsequently, expression profiles of CXCR4 in RCC tissues have been reported (Staller P et al., 2003; Pan J et al., 2006; Struckmann K et al., 2008; Wehler TC et al., 2008; Wang L et al., 2009). Based on results from researches mentioned above, it is credible and believable that CXCR4+ expression was inversely associated with survival of patients with mRCC. D'Alterio even reported CXCR4 could be considered as an independent prognostic factor for RCC (Struckmann K et al., 2008). The involvement of CXCR4 in cancer metastasis suggests that CXCR4 may be a promising prognostic biomarker, and its antagonists may be a potential option for prevention of metastasis. As one of novel biomarkers for RCC, the association of CXCR4 with mRCC was already definitive, however, the role of CXCR4 in patients with LARCC, especially high risk LARCC, has not yet been investigated.

In the current study, the expression profile and potential prognostic role of CXCR4 in patients with high-risk LARCC was firstly systemically analyzed. Positive expression rate of CXCR4 was similar to the other reports (50.4%). In 2009, Wang and his colleagues discovered the translocation of CXCR4 from membrane to cytoplasm in RCC (Wang L et al., 2009). CXCR4 expression was exclusively detected in cytoplasm of tumor cells in our study. Membrane or nucleus localization of CXCR4 was not detected. Till now, the significance of CXCR4 localization in tumor cells is still equivocal, one of reasonable explanation might be that, translocation of CXCR4 could be a sign and switch of metastasis. In order to identify this hypothesis, further investigation of CXCR4 expression both in primary and metastatic tumor cells should be designed. Here we also stratified CXCR4 expression values into CXCR4+ and CXCR4- groups. Our findings suggested that CXCR4+ in patients with high-risk LARCC was an independent negative predictive factor for PFS (RR=6.710, p=0.001) and OS (RR=4.120, p=0.001). To the best of our knowledge, this is the first report demonstrating the important prognostic role for CXCR4 in high-risk LARCC. The potential of LARCC from primary site to metastatic organ may be dependent on the differential expression of CXCR4.

It has been postulated that organ specific metastasis might be governed, at least in part, by interactions between CXCR4 on cancer cells and SDF-1 produced by specific organs. In the previous studies, CXCR4 expression was associated with direction metastasis to specific SDF-1 expressed organ, such as bone, liver, lung and lymph node (Tachibana K et al., 1998; Sun X et al., 2010). In the present study, it is plausible that renal tumor cells with CXCR4+ expression have a higher potential to spread to distant tissues that produce CXCL12. Although CXCR4 expression was associated with high rate of distal metastasis, our data failed to detect an association between CXCR4 expression and site of metastases in any SDF-1 produced organs. However, it should be noteworthy that almost all of metastatic sites were abundant in SDF-1 production, which still suggested the potential role of CXCR4 in directing metastasis of renal tumor cells.

Based on current stratification systems, survival analyses suggested that, compared to low to intermediate risk LARCC, high-risk LARCC was associated with high 5-year recurrence rate of 38-69% (Frank I et al., 2002). Every prognostic model seems to performance well, but actually, almost all of them are based on TNM system and only have concordance rates between 60-85%. There are clearly a significant number of patients whose risk is inappropriately determined using existing models. For this and other reasons, investigators have attempted to identify molecular biomarkers associated with RCC outcomes to improve the risk stratification of patients after nephrectomy. In our study, the predictive values of CXCR4 and current prognostic model were firstly evaluated and compared at the same time. In the present study, although Leibovich score system could be associated with disease progression in univariate analysis, it was not an independent prognostic factor for patients with high-risk LARCC in multivariate analysis. It was worthy of paying attention that, in patients with high-risk LARCC, prognostic nomogram (Leibovich score) could not be considered as a criteria for outcome prediction, while, CXCR4 expression could be expected to be an independent predictive factor for high-risk LARCC.

In the past 30 years, drugs that have been studied as adjuvant treatment for high-risk LARCC have included radiotherapy, hormonal therapy, and immunotherapy. Unfortunately, a majority of clinical trials have unable to demonstrate a significant benefit in terms of PFS or OS (D'Alterio C et al., 2010). In our retrospective study, despite the rather small sample size, we were again disappointed with the effect of IFN-α as adjuvant therapy. Patients with IFN-α after surgery (n=55) were even related to shorter PFS (a decrease of 9 months) and OS (a decrease of 5 months) than those observation (n=62). Furthermore, CXCR4 expression was associated with a significant negative predictive value for the effect of IFN-α on PFS (p=0.003). CXCR4 could become not only a promising prognostic factor, but also a predictive biomarker of some adjuvant therapy for patients with high-risk LARCC. In recent years, significant improvements in survival have been subsequently reported when target small molecules, including sunitinib and sorafenib, were used for mRCC. To date, all of prospective clinical trials about adjuvant therapy were focused on the role of targeted molecules, and the favorable effect of these agents in the treatment of locally advanced RCC is worthy of expectation.

In summary, the clinical evidence of our study leads to a homing mechanism for 'direction' metastasis of renal cell carcinoma. In patients with high risk LARCC, CXCR4 could be an independent predictive factor for recurrence or distal metastasis of disease and overall survival. Since CXCR4 is not associated with other clinical and pathological prognostic factors, except for Fuhrman grading, the abnormality of CXCR4 expression could improve, supplement, and even replace the current evaluation criteria for LARCC, and could facilitate to make a decision of imperative adjuvant therapy to some individual.

Totally, in the present study, IFN-α, as one of adjuvant therapeutic regimen, was again validated to be no benefit for patients with high-risk LARCC. It is important to note that, high expression of CXCR4 was inversely related

to potential effect of IFN-αin adjuvant therapy, which implied the probable role of CXCR4 in prediction clinical response to adjuvant therapy. Ideally, clinicians will be able to identify high-risk patients and to offer treatment to those who would benefit most from adjuvant therapy. In the current area of targeted molecules, efficacy of small molecules in adjuvant therapy is worthy of expectation.

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