**ABSTRACT**

CXCR chemokine receptor 1 (CXCR1) signaling has been shown as an essential molecular nexus regarding cancer cell proliferation, tumor inflammation, and angiogenesis in clear cell renal cell carcinoma (ccRCC). The aim of this study was to investigate the prognostic significance of CXCR1 in patients with non-metastatic ccRCC. Data from 446 consecutive non-metastatic ccRCC patients, operated between 2003 and 2008 at a single institution, were evaluated retrospectively. The cohort was split into a training set (n = 223) and a validation set (n = 223). CXCR1 expression was assessed by immunohistochemistry staining and its association with clinicopathologic features and prognosis were evaluated. High CXCR1 epithelial expression presented prognostic value, and indicated poor overall survival (OS) (P = 0.010 and P = 0.015, respectively) and recurrence-free survival (P = 0.011 and P = 0.019, respectively) in the training and validation sets. The incorporation of CXCR1 into the T stage and SSIGN score would help to refine individual risk stratification. Multivariate analysis identified increased epithelial CXCR1 was statistically significantly associated with a poor outcome for OS (HR [95% CI] 1.570 [1.076–2.290]; P = 0.006) and RFS (HR [95% CI] 1.570 [1.076–2.290]; P = 0.019) in all non-metastatic ccRCC patients. Predictive nomograms were generated with identified independent prognosticators to assess patient overall survival and recurrence-free survival at 3, 5 and 10 y. Furthermore, high CXCR1 expression were correlated with elevated infiltrated neutrophils and enriched MMP family gene expression. To conclude, high CXCR1 level within epithelial area represented a potential independent negative prognostic factor regarding OS and RFS in non-metastatic ccRCC patients after nephrectomy.

**Introduction**

In recent decades, the incidence rates of renal cell carcinoma (RCC) have been steadily rising by 2–4% and RCC is now the 8th leading cancer type in the United States.1 The widespread use of abdominal imaging leads to a tumor size and stage migration toward small and asymptomatic organ-confined tumors,2 accounting for 48–66% of newly diagnosed RCC.3 However, tumor size alone is not sufficient to distinguish small RCC with benign behavior from aggressive small RCC.4 Moreover, despite organ-confined RCC can be early diagnosed and cured with existing surgical approach, 20–30% of patients eventually experience a relapse or develop metastases during follow-up.5 At present, there is no adjuvant therapy for small and organ-confined RCC, only observation.6 To reduce the risk of recurrence and progression of non-metastatic disease (N0M0) after surgery, prognostic biomarkers and models that can accurately predict the clinical outcome of these RCC patients are of paramount important for both individualized risk assessment of patient and comparison of clinical trials.7 Several prognostic models have been established to predict clinical outcome, such as UISS (UCLA), SSIGN (Mayo Clinic), Leibovich (Mayo Clinic), Kattan (MSKCC) and Karakiewicz (UM).8 The incorporation of efficient biomarkers can further improve the accuracy of above mentioned prognostic models.9

CXCR chemokine receptor 1 (CXCR1) is a class A, rhodopsin-like G-protein–coupled receptor (GPCR) responsible for cellular signal transduction.10 As one of 2 high-affinity receptors for the chemokine CXC ligand 8 (CXCL8)/Interleukin-8 (IL-8), CXCR1 is selectively activated by IL-8 only.11 This ligand-activated intracellular signaling pathway leads neutrophil migration to inflammation area.12 IL-8 signaling also promotes angiogenesis, tumorigenicity, and metastasis in different types of tumor.13 Researches also showed that CXCR1 could correlate with cell proliferation or migration in prostate,14 ovarian,15 colon16 and lung17 cancer. Grepin R and colleagues showed that pharmacological inhibition of CXCR1 and CXCR2 affected both tumor vasculature and tumor proliferation in vitro and xenograft.18 The only laboratory evidence supporting an oncogenic role for
CXCR1 in ccRCC is encouraging. However, the detailed function of CXCR1 might differ according to different location CXCR1 expressed. Therefore, validation of CXCR1 expression as a relevant prognostic marker in different compartments of ccRCC with a larger, independent cohort of patients must be urgently performed to extend above-mentioned results.

The aim of our study was to further clarify the prognostic significance of CXCR1 in non-metastatic ccRCC patients and to investigate whether this parameter provides refinement to predictive accuracy to well-established prognostic models. We analyzed the both epithelial and stromal expression of CXCR1 by immunohistochemistry in non-metastatic ccRCC patients’ clinical specimens and its associations with clinicopathologic characteristics and clinical outcomes including survival and recurrence. Moreover, we explored the hypothesis that incorporation of CXCR1 expression into T stage, Fuhrman grade and ECOG-PS would refine individual risk stratification. Further differential immune cell infiltration and gene expression were also investigated in TCGA KIRC cohort, suggesting neutrophils recruitment as a possible mechanism.

Results

Immunohistochemistry findings

In an effort to investigate whether the expression of CXCR1 is associated with development and progression of non-metastatic ccRCC, we first evaluated the expression levels of CXCR1 by IHC staining in 446 matched non-metastatic ccRCC tissues from training and validation sets. We analyzed CXCR1 expression within epithelial and stromal area separately. CXCR1 positive staining was mainly located in the cell cytoplasm and/or membrane in the tumor area. Representative pictures of different staining intensity and corresponding isotype IgG were illustrated in Supplementary Figure 1. At the optimal cutoff value derived from H-score, we showed that 43.9% (training set, 98 of 223) and 50.7% (validation set, 113 of 223) were scored as high CXCR1 epithelial expression, and 49.8% (training set, 111 of 223) and 48.0% (validation set, 107 of 223) were scored as high CXCR1 expression within stromal area. Detailed clinical significance of CXCR1 epithelial expression in non-metastatic ccRCC patients was presented in Table 1 and Supplementary Table 1. Patients with higher CXCR1 epithelial expression tended to have higher grades in both 2 sets, with a statistical significance in validation set (P = 0.002), and a nearly significance in training set (P = 0.126) (Table 1). In validation set, gender (P = 0.034), tumor size (P = 0.024), ECOG-PS (P = 0.026) and UISS score (P = 0.043) presented a significant correlation with CXCR1 stromal expression, however, these correlations were not shown in training set (Supplementary Figure 1). Other clinical factors did not show any significant correlation either with CXCR1 epithelial expression or with CXCR1 stromal expression.

Figure 1. Kaplan–Meier analysis of overall survival (OS) and recurrence-free survival (RFS) according to the expression of epithelial CXC chemokine receptor 1 (CXCR1) in patients with non-metastatic clear-cell renal cell carcinoma (ccRCC). Kaplan–Meier analysis of OS in the training set (A) and validation set (B), Kaplan–Meier analysis of RFS in the training set (C) and validation set (D). P value was calculated by log-rank test.
CXCR1 expression within epithelial area presented prognostic value

To explore the clinical value of CXCR1 expression within epithelial and stromal area, we performed Kaplan-Meier survival analyses. As illustrated in Fig. 1, both in training set and validation set, patients with higher CXCR1 epithelial expression were inclined to have worse OS (P = 0.010 and P = 0.015, respectively; Fig. 1A and 1B) and RFS (P = 0.011 and P = 0.019, respectively; Fig. 1C and 1D). While within stromal area, CXCR1 expression could not stratify patients for OS and RFS in both study set (Supplementary Figure 2). We then focused on CXCR1 expression within epithelial area, and further explored its prognostic value. To increase the robustness of this study, we combined the training sets and validation sets in further analyses.

Table 1. Correlations between CXCR1 epithelial expression and clinical characteristics in non-metastatic ccRCC patients.

| Characteristic | Training set (n = 223) | Validation set (n = 223) |
|---------------|------------------------|--------------------------|
| Patients      | CXC chemokine receptor 1; SD, standard deviation; ECOG-PS, Eastern Cooperative Oncology Group performance status; UISS, University of California Los Angeles Integrated Staging System; SSIGN, Mayo Clinic stage, size, grade and necrosis. | |
| Mean age, years | 55.6 ± 12.3 | 55.7 ± 11.7 | 56.2 ± 11.3 |
| Gender | Male 161 72.2 87 74 | 158 70.9 73 85 |
| Tumor size, cm | 4.8 ± 2.7 | 4.4 ± 2.4 | 4.7 ± 2.6 |
| T stage | T1 152 68.2 87 65 | 146 65.4 72 74 |
| Fuhrman grade | 1 49 22.0 31 18 | 44 19.7 27 17 |
| Tumor necrosis | Absent 177 79.4 103 74 | 182 81.6 94 88 |
| ECOG-PS | 0 192 86.1 107 85 | 185 83.0 88 97 |
| UISS score | LR 102 45.7 60 42 | 89 39.9 51 38 |
| HR | 10 4.5 6 4 | 16 7.2 8 8 |
| SSIGN score | LR 138 61.9 82 56 | 134 60.1 69 65 |
| IR | 76 34.1 38 38 | 81 36.3 39 42 |
| HR | 9 4.0 5 4 | 8 3.6 2 6 |

Prognostic value of CXCR1 epithelial expression for clinical outcome of patients

To identify specific patients who mostly could benefit from CXCR1 expression prediction, we first applied Kaplan-Meier survival analyses and proportional hazard regressions in patients with different T stages. As shown in Fig. 2, CXCR1 expression could significantly stratify pT1 patients for the risk of death and recurrence (P = 0.007 and P = 0.011, respectively; Fig. 2A and 2D). However, this prognostic value was not observed in pT3+pT4 patients for death (P = 0.147, Fig. 2C). While for RFS, higher CXCR1 epithelial expression could significantly predict higher recurrence risk (P = 0.040, Fig. 2F). In pT2 patients, high CXCR1 expression could statistically represent worse OS (P = 0.022; Fig. 2B). While for the recurrence risk of pT2 patients, high CXCR1 expression only showed a tendency of shorten RFS which failed to reach statistic significance (P = 0.152; Fig. 2E). Further proportional hazard regression confirmed these phenomena (OS: Fig. 2G; RFS: Fig. 2H).

In accordance with these findings in T stage subgroups, patients in low and intermediate risk group based on SSIGN system also could be stratified by CXCR1 expression for both OS and RFS (OS: LR: P = 0.006, Fig. 3A; IR: P = 0.025, Fig. 3B and RFS: LR: P = 0.014, Fig. 3D; IR: P = 0.022, Fig. 3E). SSIGN high-risk patients could not be stratified by CXCR1 expression (OS: P = 0.535, Fig. 3C; RFS: P = 0.510, Fig. 3F). Further proportional hazard regression confirmed these findings as well (OS: Fig. 3G; RFS: Fig. 3H). Thus, CXCR1 epithelial expression had a better prognostic value in early disease patients for both OS and RFS.

We then conducted Cox regression to verify the independence of epithelial CXCR1 prognostic value. As listed in Supplementary Table 2, along with other well-known prognosticators, high CXCR1 epithelial expression presented poor
outcome of patients (OS: HR: 2.032, 95%CI: 1.360–3.037, P = 0.001; RFS: HR: 1.862, 95%CI: 1.300–2.666, P = 0.001). Further multivariate Cox regression showed that CXCR1 epithelial expression was an independent prognosticator for both OS and RFS (OS: HR: 1.808, 95%CI: 1.184–2.761, P = 0.006; RFS: HR: 1.570, 95%CI: 1.076–2.290, P = 0.019) (Fig. 4A).

**Extension of CXCR1 epithelial expression with current prognostic models**

Since CXCR1 epithelial expression presented strong prognostic value, we sought to explore its potential of improving current prognostic models. After incorporating with T stage, UISS and SSIGN models, CXCR1 could increase their predictive accuracy and efficiency for both OS and RFS (Table 2). The combination of CXCR1 epithelial expression and other prognostic models had higher c-indices and lower AICs than other prognostic models alone. To visualize the prognostic application of CXCR1 epithelial expression, we then construct nomograms for OS and RFS based on CXCR1 epithelial expression and other well-known prognosticators (Fig. 4B and 4C, respectively). Fig. 4D–4G presented the calibration of nomograms for both OS and RFS (OS: Fig. 4D–4E; RFS: Fig. 4F–4G), showing a good prediction accuracy of CXCR1 nomograms.

**Immune cell infiltration and gene expression between CXCR1 high and low expression**

To further inquire the mechanism of prognostic value of CXCR1 expression, we applied the CIBERSORT methods to TCGA KIRC cohort and found significant differential immune cell infiltration between CXCR1 high and low group (Supplementary Figure 3A). Among these identified 22 immune cells, resting NK cells, monocytes and neutrophils were elevated significantly in high CXCR1 expression group. While in low CXCR1 group, Tfh cells, Tregs, and M0 macrophages were significantly increased. Further differential gene expression showed that CXCR2, IL8, CSF3 and MMP family
were highly enriched with high CXCR1 expression (Supplementary Figure 3B). These results suggested that high CXCR1 expression might correlate with recruitment of neutrophils and then facilitated tumor invasions (such as MMP family), thus presented an unfavorable prognostic value for both OS and RFS in patients with clear-cell renal cell carcinoma.

**Discussion**

IL-8 signaling pathway has been long considered to regulate various leukocytes functions and angiogenesis within tumor microenvironment, thus to promote tumor cell proliferation and metastasis. The activation of this significant signaling pathway requires its receptors, which are CXCR1 and CXCR2. While CXCR2 could bind to various ligands besides IL-8, such as GRO-α, GRO-β, and GRO-γ, neutrophil-activating peptide 2 (NAP-2), CXCR1 is only activated by IL-8, and represents a highly affinity to IL-8, thus the expression of CXCR1 seems more specific and unified toward IL-8 signaling. After activated by IL-8, CXCR1 then stimulates downstream signaling such as PI3K/Akt, PKC, MAPK and JAK/STAT to play its pluripotent roles in cell survival and proliferation, invasion and angiogenesis.

However, recent studies have focused more and more on tumor microenvironments (TME), rather than tumor cell itself. CXCR1 is not only expressed on tumor cells, but also on other leukocytes (such as neutrophils) and endothelial cells. CXCR1 could expressed on neutrophils and facilitated neutrophils chemotaxis to the TME (reviewed in ref.13). While in endothelial cell, CXCR1 expression could promote a rapid induction of Gho-GTPase activity, and lead to endothelial cells retraction and vascular permeability increase, which is associated with neutrophils recruitment and angiogenesis. Since CXCR1 function could differ depending on the location of CXCR1 expression, detailed biology mechanisms of CXCR1 expression in tumor remain blurred. Of our particular interest in RCC, which could be treated with anti-angiogenesis agents, CXCR1 was reported to induce the growth of ccRCC. Grepin reported that CXCL7/CXCR1/2
We have also reported previously that higher CXCR2 expression indicated worse clinical outcomes in non-metastatic ccRCC patients, suggesting activated IL-8/CXCR1/CXCR2 axis could promote tumor growth and lead to diminished prognosis of ccRCC patients. However, as CXCR1 and CXCR2 could present on tumor cells, endothelial cells and neutrophils, which could also direct worse prognosis via tumor cell proliferation, tumor angiogenesis and neutrophils recruitment, respectively, the detailed biology mechanism of CXCR1 expression in ccRCC remained to be explored.

Table 2. Comparison of the predictive accuracy of the prognostic models.

| Model                | Overall survival | Recurrence-free survival |
|----------------------|------------------|--------------------------|
|                      | C-index          | AIC                       | C-index          | AIC                        |
| CXCR1, epithelial    | 0.600            | 1156.16                   | 0.583            | 1419.78                    |
| T stage              | 0.633            | 1140.07                   | 0.650            | 1387.21                    |
| T stage + CXCR1, epithelial | 0.685      | 1129.40                   | 0.686            | 1377.47                    |
| UISS                 | 0.683            | 1108.08                   | 0.696            | 1349.41                    |
| UISS + CXCR1, epithelial | 0.718        | 1096.12                   | 0.723            | 1338.01                    |
| SSIGN                | 0.673            | 1121.94                   | 0.709            | 1359.60                    |
| SSIGN + CXCR1, epithelial | 0.704      | 1116.21                   | 0.725            | 1355.50                    |

C-index, Harrell’s concordance index; AIC, Akaike information criterion; CXCR1, CXC chemokine receptor 1; UISS, University of California Los Angeles Integrated Staging System; SSIGN, Mayo Clinic stage, size, grade and necrosis.

Figure 4. Multivariate analysis, nomogram, and calibration plots for the prediction of outcome in patients with non-metastatic clear-cell renal cell carcinoma (ccRCC). Multivariate analysis identified independent prognostic factors of overall survival (OS) and recurrence-free survival (RFS) (A), nomogram to predict OS (B) and RFS (C) at 3 years, 5 y and 10 y after nephrectomy, the calibration plots for predicting OS at 5 y (D) and 10 y (E), RFS at 5 y (F) and 10 y (G).
In this study, we analyzed CXCR1 expression within epithelial and stromal area separately, and tried to bring insight into the CXCR1 possible function in ccRCC. Our immunohistochemistry findings showed CXCR1 was mainly expressed within epithelial area, and epithelial expressed CXCR1 presented prognostic value while CXCR1 stromal expression failed. Further analyses suggested these risk-stratifying abilities were specifically existed in early disease patients. Multivariate analysis also confirmed the independence of epithelial CXCR1 prognostic value. We also presented that incorporation of CXCR1 epithelial expression into several mainstream prognostic models could promote their predictive accuracy by calculating c-indices and Akaike information criterion. Then, we constructed nomograms based on CXCR1 epithelial expression and other well-known prognostic factors to predict OS and RFS of non-metastatic ccRCC patients after nephrectomy. Furthermore, we tried to explore the differential immune cell infiltration and gene expression between high and low CXCR1 expression groups. Results showed that neutrophils and relevant neutrophils chemokines (such as CSF3) were elevated significantly in high CXCR1 group. We also observed several MMP family genes were enriched in high CXCR1 group. These results suggesting that in non-metastasis ccRCC, CXCR1 epithelial expression could affect patients’ prognosis, and might be correlated with neutrophils recruitment, which was already reported to be an independent unfavorable prognosticator in ccRCC. Recruited intratumoral neutrophils could then express MMPs and facilitate tumor invasion.

Other immune cells, such as resting NK cells and monocytes, were also elevated with increased CXCR1 expression, which implying that CXCR1 expression might not only lead to a certain biology pathway. As CXCR1 expression might present biology pathways other than directly induce angiogenesis, the combination of CXCR1 blockade and anti-VEGF treatment might benefit ccRCC patients. In fact, in pancreatic cancer, a study already showed that combination of IL-1, CXCR1/2, and TGF-β inhibition could reverse anti-VEGF therapy resistance in murine models. Besides, although the stromal expression of CXCR1 failed to present direct prognostic value, the possibility of indirect involvement of CXCR1 in tumor angiogenesis cannot be denied easily and further experimental researches were needed.

Several limitations of our study should be noted. First, due to the nature of retrospective study design, although we recruited relatively considerable study population size, a multicenter validation is needed. Secondly, although we reported the differential CXCR1 expression on epithelial and stromal area, the evaluation of CXCR1 expression on certain cell type could help to further interpret detailed mechanism. Moreover, biologic mechanisms of this IL-8/CXCR1/2 axis in ccRCC tumor microenvironment also require further explorations.

To conclude, our study indicated that higher epithelial expression of CXCR1 was an independent prognosticator for worse clinical outcomes in patients with non-metastatic ccRCC after nephrectomy. Patients with early stage disease could be further stratified according to CXCR1 epithelial expression. Higher CXCR1 expression might be correlated with elevated neutrophil infiltration. Thus, CXCR1 expression could refine current pathological based prognostic models.

**Patients and methods**

**Patients**

One independent set comprising 446 non-metastatic ccRCC patients that received radical or partial nephrectomy between 2003 and 2008 from Shanghai Cancer Center, Fudan University (Shanghai, China) was enrolled in our study. The study was approved by the research medical ethics committee of Fudan University Shanghai Cancer Center and written informed consent was provided by each patient. The inclusion criteria for our study were as follows: (1) no history of anticancer therapy; (2) no history of other malignant tumors; (3) histopathologically proven ccRCC. All specimens were reassessed independently by 2 urology pathologists. Patients with N1 or M1 tumors were considered to have metastatic disease and were excluded from this study. Clinicopathological variables including age, gender, ECOG-PS, tumor size, TNM stage, Fuhrman grade and necrosis were collected for each patient. Patients were staged using radiographic reports and postoperative pathological data, and were reassigned according to 2010 AJCC TNM classification. The SSIGN scores were applied to each patient. Survival status of each patient was updated in October 2015. Overall survival (OS) was calculated from the day of surgery to death or to the date of the last follow-up. Recurrence-free survival (RFS) was calculated from the day of curative surgery to recurrence.

**Immunohistochemistry**

Primary anti-CXCR1 antibody (ab150548, diluted 1:200; Abcam) was applied for immunohistochemistry (IHC) staining according to the procedure as described previously. The specificity of the antibody was demonstrated by IHC with peptide competition. The staining intensity of each specimen was scored independently by 2 urology pathologists who blinded to the clinicopathological information. A semi-quantitative H-score ranged from 0 to 300 was calculated for each specimen by multiplying the distribution areas (0–100%) at each staining intensity level by the grade of intensities (0, negative; 1, weak; 2, moderate; 3, strong). To determinate the high or low expression, the “minimum P value” approach was applied to obtain the optimal cutoff value which provides the best separation between the groups of patients related to their overall survival. We selected the optimum cutoff score of 140 for the epithelial expression and score of 40 for the stromal expression of CXCR1 using the X-tile plots v3.6.1 (Yale University School of Medicine).

**Differential expression analysis**

The clear-cell renal cell carcinoma (KIRC) data from The Cancer Genomics Atlas (TCGA) was extracted to evaluate immune cells infiltrated fraction in tumor tissues. The CIBERSORT method was a technique to evaluate immune cell infiltration according to tumor RNA expression, was applied to analyze the differential immune cells fractions between high and low CXCR1 expression. The cutoff value of CXCR1 was determined as median. EdgeR method were also applied to explore significant differential gene expression (defined as
FDR-adjusted p-value ≤ 0.05 and fold change of at least 2x) between high and low CXCR1 expression.

Statistical analysis

Statistical analysis was performed with SPSS (version 21.0; SPSS Inc.) and Medcalc for windows (version 12.7.0.0; MedCalc Software). Pearson χ² test or Fisher’s exact test was applied for categorical variables and the t test was applied for continuous variables. Mann-Whitney U test were applied to compare different immune cell fractions between CXCR1 expression groups. Survival curves were built by the Kaplan-Meier method and compared by log-rank test. Numbers at risk were calculated for the beginning of each time period. Univariate and multivariate Cox proportional-hazard models were used to evaluate the hazard ratios of prognostic factors. We applied the R software version 3.0.2 and the ‘rms’ package (R Foundation for Statistical Computing) to perform the nomogram analysis and calibration plot. All P value was 2-tailed, and differences were considered significant at values of P < 0.05.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

Y. Zhu, Z. Liu and Y. Wang and for acquisition of data, analysis and interpretation of data, statistical analysis and drafting of the manuscript; H. Fu, Z. Wang, H. Xie, J. Zhang, G. Li and B. Dai for technical and material support; D. Ye and J. Xu for study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding and study supervision. All authors read and approved the final manuscript.

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