

# The post-transcriptional inhibition of CXCR4 expression by miR-139 regulates the proliferation of human kidney cancer cells

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The C-X-C chemokine receptor 4 (CXCR4) has been reported to be involved in several cancer related processes. The current study was designed to investigate the role of CXCR4 in human kidney cancer and to unveil the underlying molecular mechanisms. The results showed the expression of CXCR4 to be significantly ( $P<0.05$ ) up-regulated in human renal cancer tissues and cell lines. Silencing of CXCR4 lead to a significant ( $P<0.05$ ) decline of cell proliferation and colony formation of the Caki-1 and A498 kidney cancer cells. Moreover, the migration and invasion of the Caki-1 and A498 cells was also significantly ( $P<0.05$ ) inhibited upon CXCR4 silencing. TargetScan analysis and dual luciferase assay revealed that CXCR4 interacts with microRNA-139 (miR-139). The expression of miR-139 was found to be significantly ( $P<0.05$ ) downregulated in human kidney cancer cells lines. Overexpression of miR-139 caused post-transcriptional suppression of CXCR4 expression and significant ( $P<0.05$ ) inhibition of the Caki-1 and A498 cell proliferation. Nonetheless, CXCR4 overexpression could nullify the inhibitory effects of miR-139 on the proliferation of Caki-1 and A498 cells. Taken together, the results revealed that CXCR/miR-139 axis regulates the proliferation, migration, and invasion of human kidney cancer cells and may act as a therapeutic target

**Keywords:** chemokine receptor, microRNA-139, renal carcinoma, proliferation, migration, invasion.

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**Abbreviations:** CXCR4, C-X-C chemokine receptor 4; HIV, Human immunodeficiency virus; miRs, micro-RNAs; SDF1, stroma derived factor 1

## INTRODUCTION

There is growing evidence about the regulatory role of C-X-C chemokine receptor type 4 (CXCR4) and its binding-ligand, CXCL12 or SDF1 (stroma derived factor 1), in controlling the growth and metastatic behavior of various cancer cell types (Zlotnik, 2008; Guo *et al.*, 2016). CXCR4 acts as a receptor for specific chemokine peptides and falls in the broad category of serpentine heteromeric receptor class of G-protein coupled recep-

tor family (Evans *et al.*, 2019). CXCR4 became the focus of scientific research for its involvement in Human immunodeficiency virus (HIV) infection of human T-cells (Feng *et al.*, 2019). However, with the finding that CXCR4 participates in trafficking and tissue localization of B-cells in chronic leukemia together with its role in metastasis of breast cancer, research investigations were centered at characterization of its cancer-specific molecular role (Burger & Bürkle, 2007; Liang *et al.*, 2005). Up-regulation of CXCR4 is linked with many types of human cancers like those of liver and breast cancers and its overexpression has been reported to act as a poor disease prognostic factor of cancer. Moreover, CXCR4 controls the tumor growth and is essentially involved in the biological processes like angiogenesis and metastasis (Gravina *et al.*, 2015; Ping *et al.*, 2011). CXCR4 has been shown to be upregulated in renal cancer (Ierano *et al.*, 2016). Human renal carcinoma is one of the common epithelial kidney malignancies wherein near about one-third of the patients are diagnosed with metastatic condition (Hagenkord *et al.*, 2017). The patients with distant disease metastasis are seen with overall five-year survival rate of less than 10% (Capitani & Montorsi, 2016). Therefore, it becomes very crucial to evaluate the progression and regulatory aspects of renal cancer for identifying the molecular factors responsible for its growth and aggressiveness. This will further prove helpful in formulating better treatment strategies and improving the survival rate of cancerous malignancy of kidneys. Various human cancers have been shown to exhibit marked dysregulation of regulatory RNAs named micro-RNAs (miRs) and as such, miRs have become preferential exploratory targets of current scientific research (Jansson & Lund, 2012; Acunzo *et al.*, 2015; Di Leva, 2012). The miRs, by operating as post-transcriptional/translational level, regulate expression of more than 30% of protein coding genes and affect crucial biological and physiological pathways of human cells (Sato *et al.*, 2011). Studies have shown that miRs play key regulatory roles in tumorigenesis of human cancers and control the proliferation and metastasis of cancer cells by targeting specific down-stream genes (Hayes *et al.*, 2014). In the present study molecular characterization of CXCR4 was performed in renal cancer and its regulatory mechanism was explored. CXCR4 was highly upregulated in renal cancer tissues and cell lines. Silencing of CXCR4 in Caki-1 cell line remarkably reduced their growth and viability and restricted the migration and invasion, significantly. The miR-139 was shown to target CXCR4 at post-transcriptional level and miR-139/CXCR4 molecular axis was proven to be acting as the growth regulatory signal in renal carci-

noma. This study points towards the therapeutic potential of miR-139/CXCR4 molecular axis in the management of kidney cancer.

## MATERIALS AND METHODS

### Tissue procurement

The renal tumor and normal adjacent tissue specimens (45 in total) were obtained from the patients at the time of surgical procedure of radical nephrectomy at Shanghai Traditional Chinese Medicine-Integrated Hospital, The Second Affiliated Hospital of Zhejiang University School of Medicine, Nanjing First Hospital. The study was performed at Shanghai Traditional Chinese Medicine-Integrated Hospital. Written consent was taken from the patients prior to tissue procurement and the experimentation of human tissue specimens was approved by our institutional Ethics Committee under approval number TCMH/651/2019.

### Cell lines and culture conditions

The procurement of renal cancer cell lines (769P, 786-O, A498 and Caki-1) as well as the normal renal epithelial cell line (RPTEC) was made from American Type Culture Collection (ATCC, USA). The cell lines were propagated using high glucose supplemented Dulbecco's Modified Eagle Medium (DMEM) (Gibco) added with fetal bovine serum (FBS) (10%) and penicillin-streptomycin (1%) at 37°C with 5% CO<sub>2</sub> in humidified CO<sub>2</sub> incubator.

### Transfection

The Caki-1 and A498 cells were transfected with the help of Lipofectamine 2000 (Thermo Fisher Scientific) as per the manufacturer's instructions. All the constructs used in the present study (miR-NC, miR-139 mimics, si-NC, si-CXCR) were procured from GenePharma Co. Shanghai, China.

### Gene expression analysis by qRT-PCR

TRIzol reagent (Thermo Fisher Scientific) was used to homogenize the cell lines and tissue samples (cancer and normal) to isolate total RNA as per manufacturer's guidelines. PrimeScript™ 1st strand cDNA Synthesis Kit (Takara) was used to reverse transcribe the extracted RNA after quantification. Using cDNA as template, gene expression was assessed using qRT-PCR. Relative expression of CXCR4 and miR-139 was quantified through 2<sup>-ddCt</sup> method. The cycling conditions were 95°C for 20 sec, followed by 40 cycles of 95°C for 20 sec, and 57°C for 1 min. The snRNA U6 gene was used as an internal

reference for miR-139 while human Actin and GAPDH served as an internal control for CXCR4 gene. The primers used in this study are listed in Table 1.

### Immuno-histochemical staining

The paraffin-embedded tissue samples were sectioned and mounted on glass slides followed by staining with hematoxylin and eosin. To analyze the expression of CXCR4 protein expression, rabbit anti-human CXCR4 antibody (Abcam) staining was performed. This was followed by staining with peroxidase conjugated-secondary goat anti-rabbit antibody (DAKO, Heverlee, Belgium) and then with rabbit peroxidase-conjugated anti-goat tertiary antibody (DAKO). The 3,3'-diaminobenzidine was used for visualizing the stain. The quantitative scoring of staining intensity of CXCR4 was performed from O (unstained), I (moderate staining intensity) and II (strong staining intensity).

### Western blotting

Western blot analysis was made for protein expression studies. Briefly, the RIPA buffer (Beyotime Inst. of Biotech.) with PMSF and protease inhibitor cocktail was used for total protein extraction. Precisely 45 µg of total proteins were resolved on SD-PAGE gel and blotted to nitrocellulose membrane (Roche). The membrane was blocked using skimmed milk and then incubated with the specific primary antibodies at 4°C overnight. Following its washing with cold PBS, the membrane was again exposed to secondary antibodies at 25°C for 2 h. All the antibodies used were provided by Cell Signaling Technology. The β-actin was used as an internal reference protein in protein expression studies.

### 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The cell viability was deduced through MTT assay. Here, the cells were treated with 0.25% trypsin after gene transfection for 24 h and then added to 96-well plate with 10<sup>5</sup> cells per well density. After incubation of 0, 12, 24, 48 and 96 h at 37°C, MTT solution with final concentration of 0.5% was added per well and 37°C incubation was prolonged for 4 h. The wells were given 150 µL dimethyl sulfoxide (DMSO) for solubilizing formazan. Finally, optical density (OD) at 570 nm was determined for each well and the results were plotted as a curve of mean ± standard deviation (S.D.).

### 5-Ethynyl-2'-deoxyuridine (EdU) assay

For EdU (5-ethynyl-2'-deoxyuridine) proliferation assay, the transfected cells were cultured in 12-well plates for 24 h at 37°C with 5000 cells/well. EdU solution with final concentration of 10 µM was added per well and 37°C incubation was again used for 2 h more. The cells were harvested and fixed with 4% paraformaldehyde. The fixed cells were washed and subsequently the detection of EdU was performed using Click-iT® EdU 22 Kit at room temperature for 30 min. The cells were further stained with 4',6-diamidino-2-phenylindole (DAPI) and visualized using a fluorescent microscope (Olympus, Tokyo, 2 Japan). The quantitative analysis of EdU positive cells was made with the help of Image-Pro Plus (IPP) 3 6.0 software (Media Cybernetics, Bethesda, MD, USA).

### Cell migration and invasion assays

Transwell assays were used for determining the migration and invasion of transfected cancer cells. Precisely,

**Table 1. List of primers used in the study.**

Primer	Direction	Sequence
CXCR4	Forward	5'-CTCCTCTTTGTCATCACGCTTCC-3'
	Reverse	5'-GGATGAGGACACTGCTGTAGAG-3'
miR-139	Forward	5-GCGGCCCTGTTGGAGAAAAA-3'
	Reverse	5'-GCGACA GAGCCGAAAAA-3'
snRNA U6	Forward	5'-ACG AATTGC GTG TCATCCT-3'
	Reverse	5'-ACGAATTG CGT GTCATC CT-3'
GADPH	Forward	5'-AAG CCTGCCGGTGACTAA C-3'
	Reverse	5'-GCATCACCCGGAGAGAA AT-3'

after 24 h transfection was trypsinised and then cell suspension was diluted to  $10^5$  cells/ml. 200  $\mu$ l of cell suspension was added to the upper chamber of transwell while only DMEM with 10% FBS was added into the lower chamber. Cell culturing was carried out for 48 h at 37°C followed by ethanol fixing and staining of cells that migrated to the lower chamber with 0.1% crystal violet. Subsequently, the photographs were taken using an inverted microscope and relative percent cell migration was estimated after manual counting. The invasion of transfected cells was determined by using the same method except that 50  $\mu$ l Matrigel (BD biosciences) was used for transwell coating.

### Bioinformatics and dual luciferase reporter assay

TargetScan web server ([http://targetscan.org/vert\\_71/](http://targetscan.org/vert_71/)) was used for specifically predicting the miR targeting CXCR4. The prediction of miR binding site was also performed. Dual luciferase reporter assay was used to validate the interaction. Here, the 3'-UTR segment of CXCR4 with predicted miR-139 binding site was PCR amplified and cloned into pmirGLO luciferase vector (Promega) to generate the reporter construct (WT-CXCR4). Also, the binding site sequence was mutated from 5'-GUCUCC-3' to 5'-ACAGUA-3' for constructing mutated reporter construct (MUT-CXCR4). The reporter construct, either wild type or mutated, was co-transfected with miR-139 or miR-NC into the Caki-1 cancer cells. The cells were cultured for 48 h at 37°C and the Dual-Luciferase Reporter Assay System (Promega) was used for performing the interaction study. The *Renilla* luciferase activity was used for normalizing the luciferase activity.

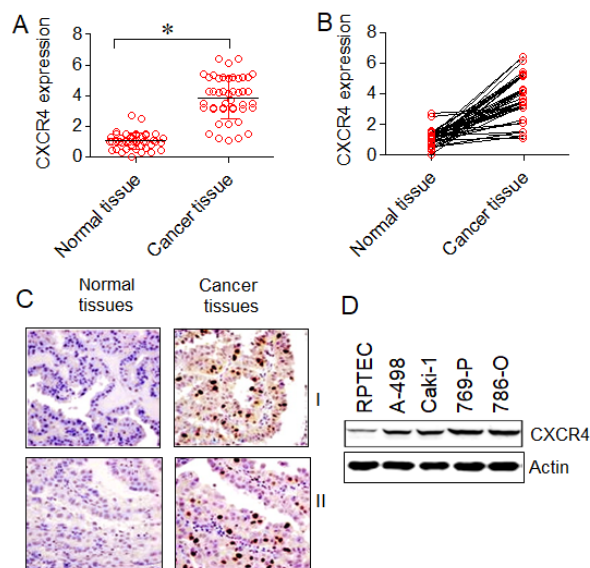
### Statistical analysis

Three replicates were used for each experimental set up and the results were expressed as mean  $\pm$  S.D. The SPSS13.0 offline software was used for all the statistical analyses. Student's *t*-test and one-way ANOVA were used for respectively comparing two groups or multi-groups. Statistical difference with *P*-value < 0.05 was taken to be significant.

## RESULTS

### Significant upregulation of CXCR4 in kidney cancer tissues and cell lines

Whether the renal carcinoma associated with the dysregulation of CXCR4, transcript levels of CXCR4 were determined from normal and cancerous tissues of the kidney. It was found that CXCR4 is significantly (*P* < 0.05) upregulated in renal cancer in comparison to normal renal tissues (Fig. 1A). The expression analysis from paired (tumor and normal adjacent) renal tissues also interpreted the same relation (Fig. 1B). The immune-histochemical investigation of CXCR4 from tumor and normal renal tissues showed that tumor tissues exhibited considerably higher fluorescence intensity of CXCR4, depicting its higher expression in malignant tissues (Fig. 1C). The western blotting of CXCR4 was carried out from renal cancer cell lines (769P, 786-O, A498 and Caki-1) and normal renal epithelial cell line (RPTEC). It was found that CXCR4 has significantly higher expression in all the cancer cell lines with least expression in Caki-1 cells (Fig. 1D). Together, the results show that CXCR4 has significant upregulation in renal



**Figure 1. CXCR4 is overexpressed in kidney cancer.**

(A) Relative expression levels of CXCR4 in kidney cancer and normal renal tissues (B) relative expression of CXCR4 from paired kidney cancer and normal adjacent tissues (C) immune-histochemical staining of CXCR4 from kidney cancer and normal renal tissues (D) relative protein levels of CXCR4 in renal cancer cell lines (769P, 786-O, A498 and Caki-1) with reference to normal epithelial kidney cells, RPTEC. The experiments were performed in triplicate and values represent mean  $\pm$  S.D. (\**P* < 0.05 for normal vs Cancer tissues).

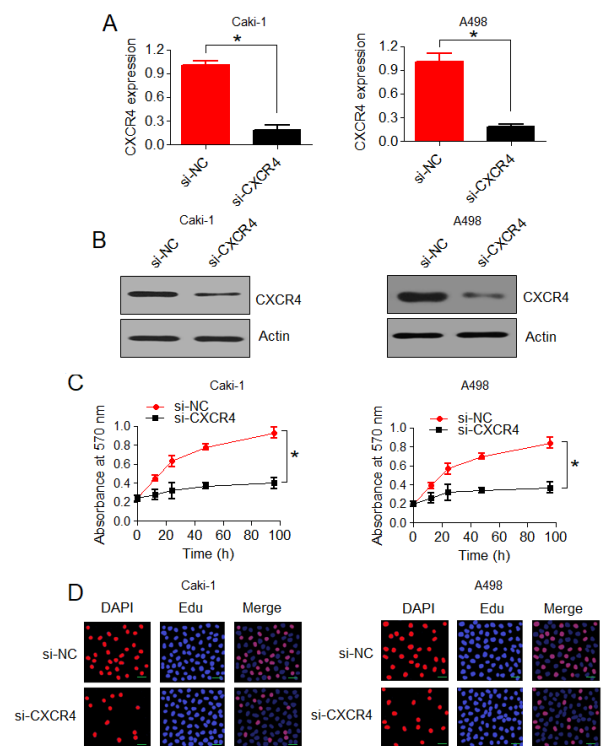
cancer suggesting its possible role in renal cancer development.

### Inhibition of cancer cell growth by transcriptional knockdown of CXCR4

To assess whether CXCR4 controls the growth and viability of renal cancer cells, the expression of CXCR4 was silenced in Caki-1 and A498 cancer cells through transfection of RNAi construct si-CXCR4 (RiboBio) and the same was confirmed by RT-PCR and western blotting (Fig. 2A and 2B). The si-NC transfected cells served as negative control. The MTT assay showed that silencing of CXCR4 in Caki-1 cancer cells significantly inhibited their viability (Fig. 2C). Again, the EdU absorption assay inferred that relative proportion of EdU positive cells was significantly lower confirming decline in Caki-1 and A498 cell proliferation under CXCR4 silencing (Fig. 2D). Thus, upregulation of CXCR4 positively correlates with the higher proliferation of kidney cancer cells and its silencing effectively reduced the cell growth and viability.

### Restriction of cancer cell migration and invasion under CXCR4 silencing

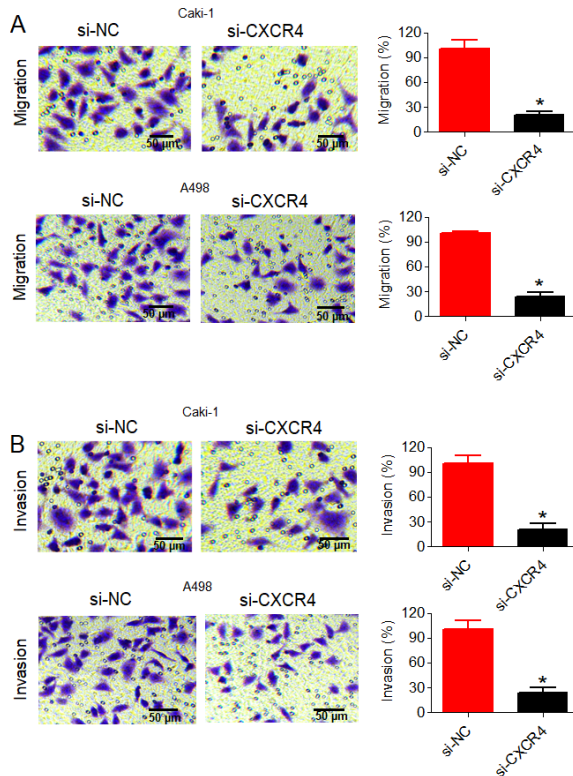
Transwell assays were used to determine the migration and invasion of Caki-1 and A498 transfected cancer cells and to analyze the effect of CXCR4 silencing on the aforementioned processes. The migration of Caski-1 and A498 cancer cells was reduced by 74% and 72% under CXCR4 repression, respectively (Fig. 3A). The cancer cells downregulating CXCR4 also exhibited significantly lower invasion which was only 22% and 26% with respect to that of the negative control Caki-1 and A498 cells, respectively (Fig. 3B). The results thus suggest that CXCR4 positively regulates the migration and invasion of renal cancer cells.



**Figure 2. CXCR4 upregulation inhibits kidney cancer cell proliferation** (A) qRT-PCR showing expression of CXCR4 silencing in si-NC and si-CXCR4 transfected Caki-1 and A498 cells (B) Western blots showing CXCR4 silencing in si-NC and si-CXCR4 transfected Caki-1 and A498 cells (C) determination of viability of Caki-1 and A498 cells transfected with si-CXCR4 or si-NC at varying culture durations by MTT assay (D) determination of proliferation by EdU assay of Caki-1 and A498 cells transfected with si-CXCR4 or si-NC. The experiments were performed in triplicate and values represent mean  $\pm$  S.D. (\* $P$ <0.05 for si-NC vs si-CXCR4).

#### Post-transcriptional regulation of CXCR4 by miR-139

The bio-informatics analysis predicted that miR-139 acts as the post-transcriptional repressor of CXCR4 and the former was predicted to be interacting with 3'-UTR of CXCR4 in sequence complementarity mode (Fig. 4A). From the luciferase assay, it was found that Caki-1 co-transfected with miR-139 mimics (overexpression construct, RiboBio) and wild type CXCR4 3'-UTR fragment (WT\_CXCR4) showed significantly lower luciferase activity in comparison to the ones where miR-139 binding site in CXCR4 3'-UTR was mutated (Fig. 4B). The reduction in luciferase activity confirmed the interaction between miR-139 and CXCR4 3'-UTR. Expectedly, the overexpression of miR-139 in Caki-1 cells led to significant decline in CXCR4 expression (Fig. 4C). Also, the cancer cell lines (769P, 786-O, A498 and Caki-1) were shown to possess significantly ( $P$ <0.05) lower transcript levels of miR-139 as compared to the normal epithelial cell line, RPTEC (Fig. 4D). The higher transcript levels of CXCR4 in cancer cell lines thus negatively correlate with miR-139 expression. The miR-139 was overexpressed in Caki-1 and A498 cancer cells through miR-139 mimics transfection (Fig. 4E). The overexpression of miR-139 in Caki-1 and A498 cancer cells significantly reduced the cell proliferation (Fig. 4F and 4G). Next, CXCR4 was overexpressed in miR-139 mimics transfected Caki-1 and A498 cells and confirmed by western blotting (Fig. 5A). It was found that the tumor-suppressive effect of miR-139 overexpression was attenuated by



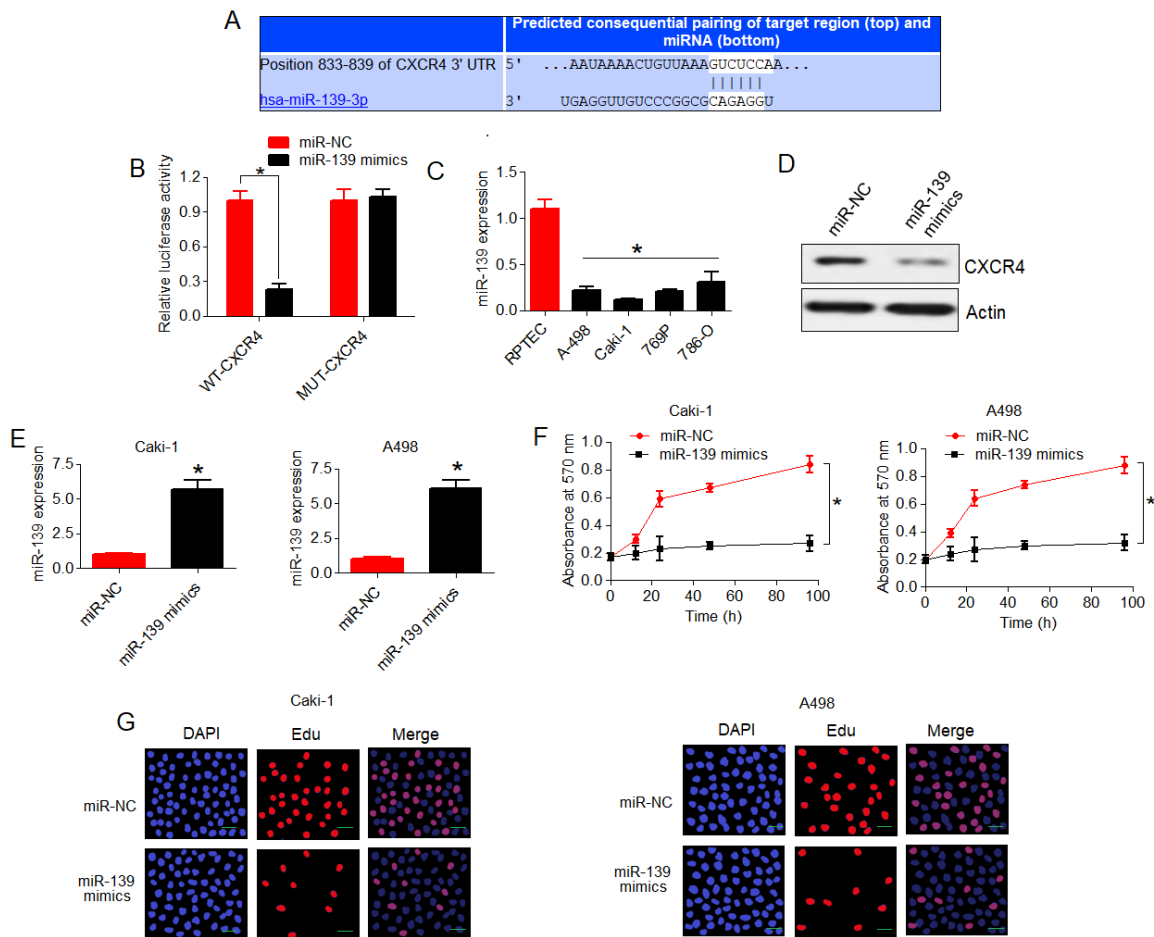
**Figure 3. Migration and invasion of kidney cancer cells is inhibited by CXCR4 upregulation.**

(A) Transwell assay for analysis of migration of Caki-1 and A498 cells transfected with si-CXCR4 or si-NC (B) transwell assay for analysis of invasion of Caki-1 and A498 cells transfected with si-CXCR4 or si-NC. The experiments were performed in triplicate and values represent mean  $\pm$  S.D. (\* $P$ <0.05 for si-NC vs si-CXCR4).

CXCR4 overexpression (Fig. 5B and 5C). It suggested that decline in cell proliferation by miR-139 overexpression is exercised through CXCR4 targeting. Therefore, CXCR4 is post-transcriptionally repressed by CXCR4 in renal cancer by miR-139 and lowered expression of miR-139 is manifested as CXCR4 overexpression enhancing the cancer cell growth and metastasis.

#### DISCUSSION

Renal carcinoma is one of the highly prevalent cancers in developed countries like USA (Znaor *et al.*, 205). A significant proportion of renal cancer patients are diagnosed with advanced metastatic disease and thus possess exceptionally low survival rate (Hagenkord *et al.*, 2017; Capitanio & Montorsi, 2016). The risk factors of renal cancer include but are not limited to smoking, obesity, and hypertension (Scelo *et al.*, 2018). It is thus needed to investigate molecular mechanism of renal cancer growth and progression to identify therapeutic targets for effectively curbing this malignancy. The molecular characterization of mechanism of action of the chemokine receptor-4 (CXCR4) in renal cancer was performed in the current study. CXCR4 became the focus of intensive research for its role to act as the co-receptor for HIV infection (Feng *et al.*, 1996). However, it has also drawn much scientific attention for its regulatory role in growth and metastasis of human cancers. CXCR4 belongs to G-protein coupled receptor family and is specifically bound by CXCL12 to commence the active signaling cascade



**Figure 4. miR-139 post-transcriptional targets and inhibits CXCR4 in renal carcinoma.**

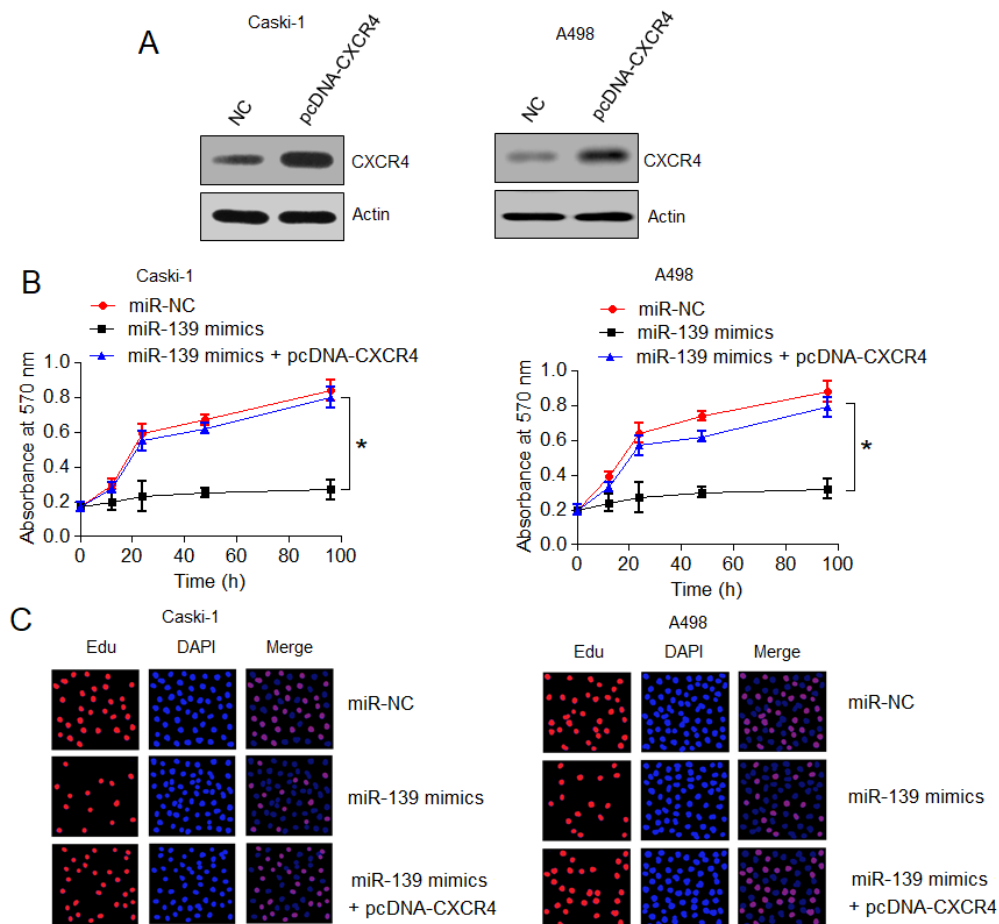
(A) TargetScan analysis of CXCR4 post-transcriptional miR-139 interaction (B) dual luciferase reporter assay for CXCR4 3'-UTR: miR-139 interaction (C) relative transcript levels of miR-139 in renal cancer cell lines (769P, 786-O, A498 and Caki-1) with reference to normal epithelial kidney cells, RPTEC (D) western blotting of CXCR4 from Caki cancer cells transfected with miR-139 mimics or miR-NC (E) RT-PCR based confirmation of miR-139 overexpression in miR-139 mimics transfected Caki-1 and A498 cancer cells (F) determination of viability of Caki-1 and A498 cells transfected with miR-139 mimics or miR-NC at varying culture durations by MTT assay (G) determination of proliferation of Caki-1 and A498 cells transfected with miR-139 mimics or miR-NC at varying culture durations by Edu assay The experiments were performed in triplicate and values represent mean  $\pm$  S.D. (\* $P$ <0.05 for miR-NC vs miR-139 mimics).

(Pawig *et al.*, 2015). The signal is transmitted through multiple down-stream targets and regulates many biological processes and also the tumor proliferation, angiogenesis, and metastatic behavior of cancer cells (Gravina *et al.*, 2015; Ping *et al.*, 2011). It has been reported that CXCR4 is overexpressed in different types of human cancers like breast cancer, lung cancer, and colon cancer (Zhang *et al.*, 2014; Wald *et al.*, 2013; Romain *et al.*, 2014). The expression of CXCR4 was also shown to be consistently higher in renal carcinoma in the current study. Previously, it was shown that enrichment of CXCR4 expression in renal cancer cells significantly enhances their proliferation and viability together with its correlation with poor prognosis of renal cancer (Wang *et al.*, 2012). Here, we showed that silencing of CXCR4 in kidney cancer cells significantly inhibited growth, migration, and invasion of renal cancer cells. CXCR4 was shown to be targeted by miR-126 in colon cancer to regulate the growth, migration, and invasion of colon cancer cells (Li *et al.*, 2013). The factors controlling CXCR4 expression and thus signaling in renal cancer have not yet been identified. The results of present work indicated that miR-139 acts as a post-transcriptional regulator of CXCR4 in renal cancer. The miR-139 acts as tumor-

suppressor and its downregulation has been shown to be linked with many human cancers, including renal carcinoma (Shu *et al.*, 2017). The lowered expression of miR-139 was reported to be associated with higher recurrence of renal cancer. Herein, we found that miR-139 interacts with CXCR4 in renal cancer to post-transcriptionally inhibit its expression. The results suggested that downregulation of miR-139 might be responsible for enrichment of CXCR4 transcripts in renal cancer and it was found that miR-139/CXCR4 axis exercises considerable regulatory control over the growth and metastatic potential of human renal cancer cells. It might thus emerge as an important therapeutic target against the renal cancer. However, more studies directed to evaluate the role miR-139/CXCR4 *in vivo* are urgently required.

## CONCLUSION

Collectively, the results of the current study clearly revealed that human renal carcinoma is associated with CXCR4 overexpression which positively regulates the growth and metastatic potential of kidney cancer cells. CXCR4 is post-transcriptionally inhibited by miR-139. Renal cancer has miR-139 downregulation allowing the



**Figure 5. Overexpression of CXCR4 avoids tumor-suppressive effects of miR-139**

(A) Western blots showing the overexpression of CXCR4 in Caski-1 and A498 cells (B) determination of viability of Caski-1 and A498 cells transfected with miR-139 mimics, miR-139 mimics+ pcDNA-CXCR4 or miR-NC at varying culture durations by MTT assay (C) determination of proliferation of Caski-1 and A498 cells transfected with miR-139 mimics, miR-139 mimics+ pcDNA-CXCR4 or miR-NC at varying culture durations by Edu assay. The experiments were performed in triplicate and values represent mean  $\pm$  S.D. (\* $P$ <0.05 for miR-NC vs miR-139 mimics + pcDNA-CXCR4).

CXCR4 overexpression and thus enabling kidney cancer cells to proliferate at significantly elevated rates. The miR-139/CXCR4 axis might emerge as crucial therapeutic target against the human renal carcinoma.

### Ethics approval

The study was approved by the research ethics committee of Shanghai Traditional Chinese Medicine-Integrated Hospital under approval number TC-MIH/651/2019. Informed written consent was sought from the patients before participation in the study.

### Conflict of interest

Authors declare that there are no conflicts of interest.

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