Int J Clin Exp Pathol 2015;8(6):6255-6261
www.ijcep.com /ISSN:1936-2625/IJCEP0008394

Original Article

CXCR4 silencing inhibits invasion and migration of human laryngeal cancer Hep-2 cells

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Received March 24, 2015; Accepted May 22, 2015; Epub June 1, 2015; Published June 15, 2015

Abstract: CXCR4 has been reported in various types of human cancer, which is associated with cancer progression and metastasis. However, the investigation of CXCR4 in laryngeal cancer is extremely rare. In the present study, we used lentivirus-mediated shRNA targeting CXCR4 to silenced CXCR4 expression in Hep-2 cells and evaluated the effect of long-term suppression of CXCR4 on Hep-2 growth and metastasis. The Cell proliferation was analyzed by MTS assay, and the invasion and metastasis potentials were analyzed using wound healing and transwell assays, respectively. Our results showed that lentivirus-mediated shRNA effectively infected Hep-2 cells and suppressed CXCR4 expression, and inhibited cell growth of Hep-2 cells. Cell invasion and apoptosis were decreased concomitantly with the reduction in CXCR4 protein expression. Further analysis revealed that CXCR4 silencing caused the reducion of CXCR4, CXCL12, TIMP2, VEGF and MMP9, and the phosphorylation levels of IκB, AKT and MAPK, and also decreased the activity of NF-κB. These results suggested that knockdown of CXCR4 inhibits the invasion and metastasis of Hep-2 through PI3K/AKT and MAPK signaling pathways, by decreasing NF-κB activities to down-regulate VEGF, TIMP-2 and MMP-9 expression. These data demonstrate that the inhibition of CXCR4 may be an effective interventional therapeutic strategy in laryngeal cancer.

Keywords: CXCR4, invasion, laryngeal cancer

Introduction

Laryngeal cancer is one of the most common types of head and neck malignant tumors, and laryngeal cancer represents approximately 85-90% of all the malignant tumors of the larynx, which has a high mortality rate and a poor prognosis [1, 2]. Despite advances in diagnosis and treatment, laryngeal cancer remains an important cause of morbidity and mortality worldwide [3]. Tumor metastasis is the most cause of death for laryngeal squamous-cell carcinoma patients, but its mechanism is still unclear [4]. Therefore, studying the molecular mechanism of laryngeal cancer metastasis has important significance for the treatment and prevention of postoperative recurrence of metastatic laryngeal squamous-cell carcinoma and. prediction of recurrence to improve the clinical management of laryngeal cancer patients.

CXCR4 is an α-chemokine receptor; its ligand is stromal-derived-factor-1 (SDF-1, also known as CXCL12) that plays an important role in lymphocyte chemotaxis [5]. Although the intracellular expression level of CXCR4 was very low in a variety of normal tissues, CXCR4 is significantly expressed in more than 20 types of cancer cells, including laryngeal squamous-cell carcinoma, leukemia, breast cancer, ovarian cancer, prostate cancer [6, 7]. The binding of CXCL12 with CXCR4 can induce activation of relevant intracellular signaling pathways to regulate the expression of genes related to chemotaxis, growth and survival of the cells. Therefore, CXCL12/CXCR4 is closely related to tumor progression, angiogenesis and metastasis [8]. Clinical studies showed that CXCR4 expression predicted poor prognosis in patients with laryngeal squamous-cell carcinoma and other malignant tumors [9]. Inhibition of CXCR4 was able to suppress cancer cells growth and metastasis

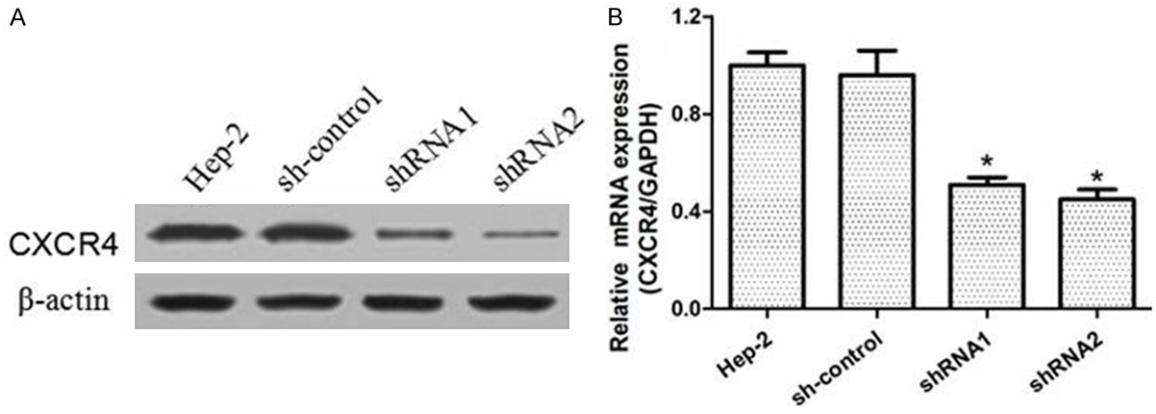

Figure 1. Knockdown effect of CXCR4 mRNA and protein expression by real-time PCR and western blot analysis. After transfected with CXCR4 shRNA or negative control for 48 h. A. Antibody specific for CXCR4 was used for the protein level change, while β-actin was used as an internal control. B. Relative level of CXCR4 mRNA expression was analyzed using the $2^{-\Delta\Delta C}$ method. *P<0.05 vs. the Hep-2 parental group. Hep-2, the Hep-2 parental group; sh-control, transfection of negative control of shRNA; shRNA1, transfection of shRNA1; shRNA2, transfection of shRNA2.

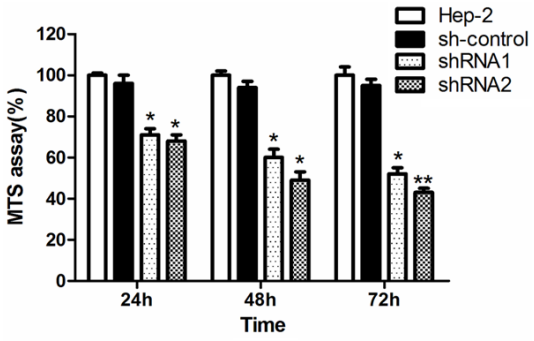

Figure 2. CXCR4 silencing inhibited cell growth of Hep-2 cells. The shRNA of CXCR4 was transfected into Hep-2 for 24 h, 48 h, 72 h, which were subjected to MTS assays. *P<0.05, **P<0.01 vs. the Hep-2 parental group.

[10, 11], but studies on its mechanism mainly focused on AKT and MAPK signaling pathways [11, 12]. It was showed that CXCR4, VEGF and MMP-9 together could predict lymph node metastasis in breast cancer [13]; CXCR4 could also regulate VEGF, MMP-9 and TIMP-2 to promote metastasis in prostate cancer [14]. The available studies showed that VEGF promoted laryngeal squamous-cell carcinoma lymphatic metastasis [15], while the clinical studies showed that MMP-9 expression was closely related to laryngeal squamous-cell carcinoma progression [16, 17]. Therefore, this study investigated whether the regulation of CXCR4 on laryngeal squamous-cell carcinoma cell metastasis was correlated with VEGF and MMP-9.

Materials and methods

Cell culture

Human laryngeal cancer Hep-2 cell line was obtained from the Cell Bank (Shanghai) of Type Culture Collection Committee of the Chinese Academy of Sciences. The Hep-2 cells were cultured in an incubator under 37°C, 5% CO_2 and saturated humidity condition. The culture medium was DMEM supplemented with 10% FBS. The cells were digested with 0.25% trypsin-EDTA for passaging. Cells in logarithmic growth phase were used in all experiments.

shRNA silences CXCR4

The shRNA vector expressing targeted human CXCR4 (GenBank®, NM_001008540) or the control (shRNA control) lentivirus transfection vector was constructed by Invitrogen (Shanghai); this vector simultaneously carried green fluorescent protein (GFP) used to screen the successfully transfected cells. The shRNA sequence was 5'-CGCCTGTTCTGCCTTACTA-3'. The cells were cultured to logarithmic growth phase and transfected, followed by screening for successfully transfected cells in accordance with the reagent operating instructions.

Cell proliferation analysis

The Hep-2 cells transfected with, shRNA control, shRNA1, and shRNA2, and their parent cells in logarithmic growth phase, 5×10^4 cells/

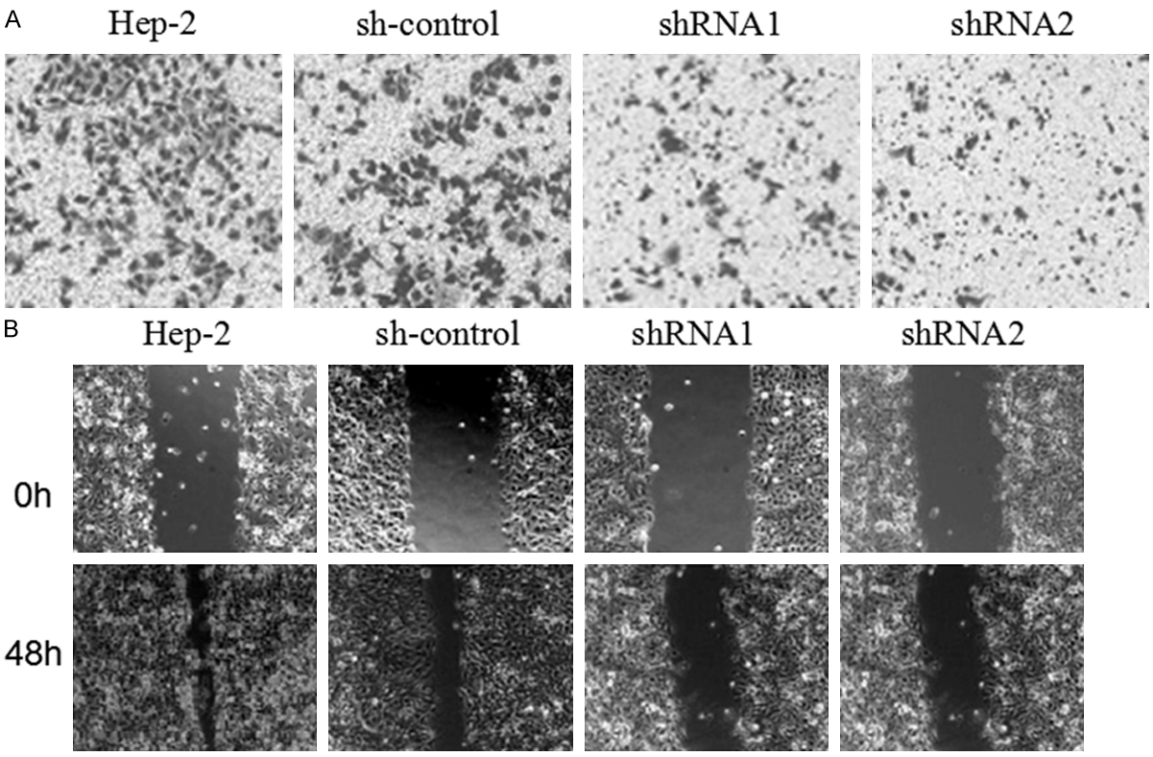

Figure 3. CXCR4 silencing suppresses migration and invasion of Hep-2 cells. A. After transfected with CXCR4 shRNA or negative control for 24 h. The cell viability of Hep-2 cells was measured by wound healing assay. Five random views were chosen along the scratch wound in each well at ×100. B. After transfected with CXCR4 shRNA or negative control for 24 h, cells which penetrated the membrane were fixed and stained after 48 h as described in the materials and methods.

ml, were seeded in 96-well microplates, 100 μl/well, and cultured overnight to allow cell adherence. The cell growth status was detected daily using MTS assay. The specific procedures were as follows: after removing the medium, MTS was added in accordance with reagent instructions to continue the culture for 4 h; the OD value was measured at 490 nm wavelength with a microplate reader to represent the cell counts; the measurement was carried out for 3 successive days.

In vitro invasion and migration assay

CultreCoat® 96-Well BME-Coated Cell Invasion Optimization Assay Kit supplied by R&D Systems was used to assess the in vitro invasion ability of cancer cells. The cells were harvested after starvation in serum-free medium for 16 h and seeded, 25000 cells per well, in the upper Transwell chamber. After continuing the culture for 48 h, the invasion cell counts were analyzed in accordance with the kit instructions. The wound-healing assay was used to assess the in vitro migration ability of these cancer cells. The specific procedures included culturing the cells in 6-well plates until formation of single-layer confluence. After starving overnight in serum-free medium, the 200-μL pipette tips were used to make scorings in the cell layer; after continuing the culture for 48 h, the width of scorings was observed and measured under a microscope.

Western blotting assay

The cells were lysed to extract proteins from the cell lysate. The proteins were separated in 12% SDS-PAGE and then transferred onto a PVDF membrane; the target proteins were detected with different antibodies (4°C overnight). After washing off the primary antibodies, the membrane was incubated with HRP-conjugated secondary antibody for 1 h. After several washes, ECL kit was used to develop the immunoreactive bands. Then β-actin was used as an internal control to determine not only the expression levels of CXCR4, CXCL12,

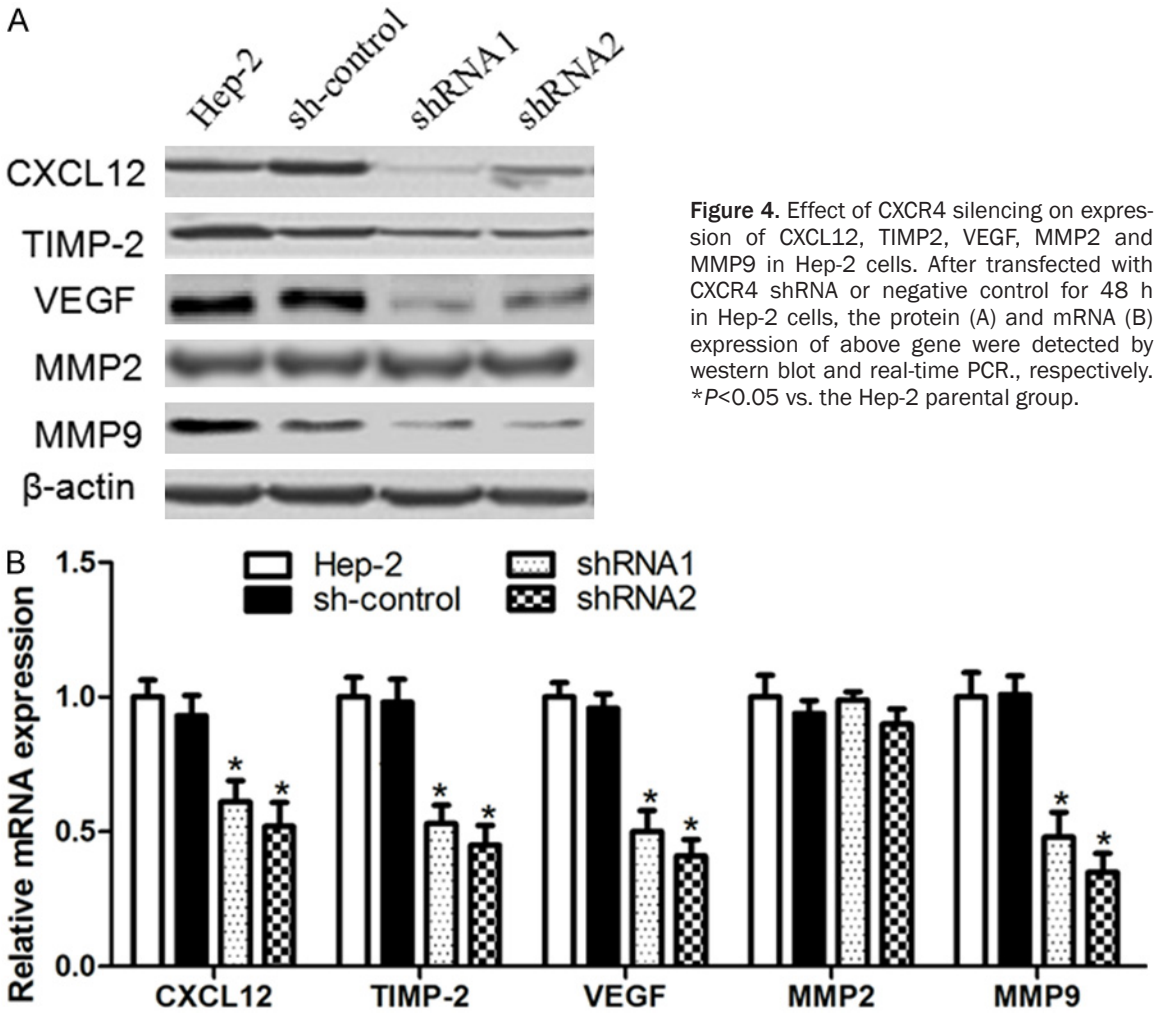

Figure 4. Effect of CXCR4 silencing on expression of CXCL12, TIMP2, VEGF, MMP2 and MMP9 in Hep-2 cells. After transfected with CXCR4 shRNA or negative control for 48 h in Hep-2 cells, the protein (A) and mRNA (B) expression of above gene were detected by western blot and real-time PCR., respectively. **P*<0.05 vs. the Hep-2 parental group.

TIMP2, VEGF, MMP2 and MMP9, but also the phosphorylation levels of IκB, AKT and MAPK in these cells.

RT-PCR assay

Total RNA was extracted from each group using Trizol method. Real-Time PCR Kit was used to carry out reverse transcription to obtain the cDNA; then, CXCR4, VEGF, MMP-9 mRNA levels were detected; CXCR4 upstream primer sequence: 5'-GCCAACGTCAGTGAGGCAGA-3', downstream primer sequence: 5'-GCCAACCATGATGTGCTGAAAC-3'; CXCL12 upstream primer sequence: 5'-ATT CTCAACACTCCAAACTGTGC-3', downstream primer sequence: 5'-ACTTTA GCTTCGGGTCAATGC-3'; TIMP2 upstream primer sequence: 5'-GCTGCGAG TGCAAGATCAC-3', downstream primer sequence: 5'-TGGTGCCCGTTGAT GTTCTTC-3'; MMP2 upstream primer sequence: 5'-TGATCTTGACCAGAATAC CATCGA-3', downstream primer sequence: 5'-GGCTTGCGAGGGAAGAAGTT-3'; VEGF upstream primer sequence: 5'-CAACATCACCATGCAGATTATGC-3', downstream primer sequence: 5'-CCACAGGGACGGGATTTCTTG-3'; MMP9 upstream primer sequence: 5'-CATTTCG ACGATGACGAGTTGT-3', downstream primer sequence: 5'-CGGGTGTAGAG TCTCTCGC-3'; GAPDH upstream primer sequence: 5'-CTTAGATTTGGTCGTATTGG-3', downstream primer sequence 5'-GAAGATGGTGATGGGATT-3'.

Detection of intracellular NF-κB transcriptional activity using reporter gene technique

In 96-well plates, 1×10^4/well cells were seeded and cultured for 24 h to allow cell adherence. Then, 0.1 μg of pGL 4.32 [luc2P/NF-kB-RE/Hypro] plasmid was added to each well to transfect the cells; after continuing the culture for 24 h, the chromogenic reagent provided in the kit

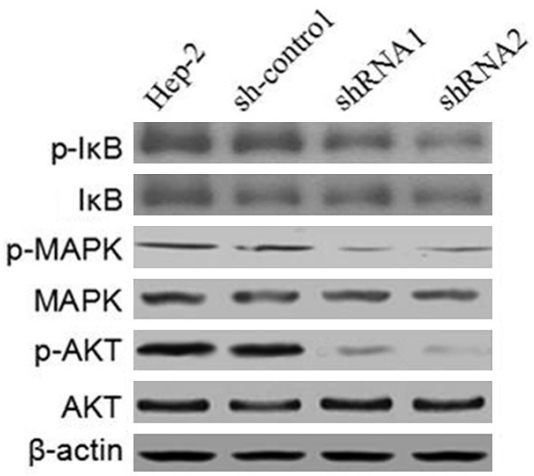

Figure 5. Effect of CXCR4 silencing on IκB, AKT and MAPK phosphorylation in Hep-2 cells. After transfected with CXCR4 shRNA or negative control for 48 h, the IκB, AKT and MAPK phosphorylation in Hep-2 cells were detected by western blot.

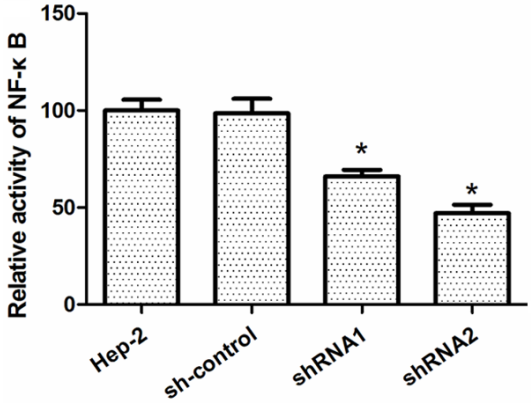

Figure 6. Effect of CXCR4 silencing on the activity of NF-κB. After transfected with CXCR4 shRNA or negative control for 24 h, then 0.1 μg pGL 4.32 [luc2P/NF-kB-RE/Hypro] plasmid was transfected for another 24 h, and the activity of NF-κB was detected with a fluorescence microplate reader. *P<0.05 vs. the Hep-2 parental group.

was used to determine the luciferase activity, which was detected with a fluorescence microplate reader.

Statistical analysis

Experimental data were expressed as mean ± standard deviation; SPSS13.0 software was used for analysis. One-way ANOVA was carried out for comparison; P<0.05 indicated statistically significant differences.

Results

shRNA silences CXCR4 expression

Cell clones transfected with lentivirus were obtained successfully. WB and RT-PCR results showed that, in Hep-2 cell clones selected for subsequent experiments, the intracellular CXCR4 expression level significantly decreased to approximately 23% and 19% of the shControl group, respectively, while no significant difference was observed between shControl group and non-transfected cells (**Figure 1**).

Effect of CXCR4 silencing on cell growth

MTS cell proliferation assay showed that CXCR4 silencing by shRNA affected cell growth, which became more apparent over time, as shown in **Figure 2**.

CXCR4 silencing inhibits the in vitro invasion and migration

It was observed after the cells have been cultured in Transwell for 24 h that, when compared with shControl group, invasion cell counts in shRNA group significantly decreased, as shown in **Figure 3**. The wound-healing assay also showed that, 48 h later, the width of scorings was significantly reduced in shControl group, while the tendency of width reduction was significantly inhibited in shRNA group, as shown in **Figure 3**. These assay results suggested that silencing CXCR4 was able to inhibit the in vitro invasion and migration of cancer cells.

CXCR4 silencing down-regulates the expression of VEGF and MMP-9, and inhibits the phosphorylation of IκB, AKT, MAPK

Western blot assay results showed that, when compared with the cells in shControl group, the intracellular expression levels of VEGF and MMP-9 proteins decreased significantly in shRNA group. The RT-PCR results showed that the regulation of these protein expression occurred at the transcriptional level, as shown in **Figure 4**. In the meantime, the IκB, AKT and MAPK phosphorylation level decreased in shRNA transfected cells, as shown in **Figure 5**.

CXCR4 inhibits NF-κB transcriptional activity

The reporter gene assay showed that the intracellular luciferase activity decreased signifi-

cantly after CXCR4 silencing, suggesting that NF-κB transcriptional activity was inhibited (As showed in **Figure 6**).

Discussion

In this study, we first studied if CXCR4 short hairpin RNA (shRNA) expression vector was able to efficiently inhibit the expression of CXCR4 in Hep-2 cells. Further study showed that the growth, invasion and migration capacity of tumor cells decreased significantly after CXCR4 was silenced by shRNA. Therefore, we further investigated and verified the mechanisms of CXCR4-mediated inhibition of laryngeal squamous-cell carcinoma tumor cells.

The available studies showed that CXCR4 was able to act on the AKT and MAPK signaling pathways to promote the growth and survival of tumor cells [11, 12]. Accordingly, we used WB technique to determine the intracellular phosphorylation levels of AKT and MAPK after CXCR4 silencing. The results showed that the phosphorylation of both AKT and MAPK were inhibited, indicating inhibition of both signaling pathways, which was consistent with the literature reports. Some recent studied showed that CXCR4 could promoting tumor cell metastasis by regulating VEGF and MMP-9 expression in prostate cancer and breast cancer [13]. We found in WB assay that more VEGF and MMP-9 were expressed in Hep-2 than Hep-2/shRNA. After silencing CXCR4, VEGF and MMP-9 expression was also inhibited with declined invasion and migration capability, suggesting that CXCR4 may also regulate VEGF and MMP-9 expression to facilitate metastasis in laryngeal squamous-cell carcinoma cells. VEGF is an important regulator of vascular endothelial cell growth and the formation of new blood vessels, thereby playing a key regulatory role in tumor angiogenesis [18]. The available studies showed that VEGF was over-expressed in laryngeal squamous-cell carcinoma tissue and its expression was related to poor prognosis of patients [15, 19]. MMP-9 can hydrolyze extracellular matrix to assist the tumor cells in invasion and angiogenesis, consequently involved in the progression of a variety of cancers [20]. The available studies also showed that MMP-9 was over-expressed in laryngeal squamous-cell carcinoma cells and related to disease progression [16, 17]. Because CXCR4 can regulate NF-κB activity while NF-κB, in turn, can regulate VEGF and MMP-9 expression [21, 22], NF-κB may be the key regulatory factor connecting CXCR4 with VEGF and MMP-9. Our WB results showed that the IκB phosphorylation level decreased, suggesting a possible inhibition of NF-κB activity. We confirmed our hypothesis using reporter gene assay.

In summary, this study showed that CXCR4 played a role in the metastasis of Hep-2 cells; silencing CXCR4 can inhibit the in vitro growth, invasion and migration of these tumor cells. In addition to regulating the activity of AKT and MAPK signaling pathways, its mechanisms of action may include regulation of the transcriptional activity of NF-κB to further down-regulate VEGF and MMP-9 expression.

Acknowledgements

This study was suported by the Science. Foundation of Tianjin Medical University (2014KYQ29).

Disclosure of conflict of interest

None.

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