Original Paper

Effect of CXCR4 on Apoptosis in Osteosarcoma Cells via the PI3K/Akt/NF- κ B Signaling Pathway

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Key Words

Osteosarcoma • CXCR4 • Apoptosis • PI3K/Akt/NF- κ B signaling • ERS

Abstract

Background/Aims: Osteosarcoma, the most common primary bone malignancy, arises from primitive transformed cells of mesenchymal origin with the worldwide increasing morbidity and mortality. Previous studies found apoptosis of osteosarcoma cells was essential for an effective manner to improve the progress of osteosarcoma, and CXCR4 has been demonstrated to be relevant with various tumor progress and metastasis. **Methods:** The proliferation of cells transfected with CXCR4 shRNA and control shRNA were measured by BrdU assay. Apoptosis was detected by flow cytometry. Apoptotic protein expression levels were detected by Western blot. Caspase activity was detected by Colorimetric Assay Kits using microplate reader. Activation of NF- κ B signaling after CXCR4 down-regulation in osteosarcoma cells was examined by constructing NF- κ B promoter luciferase reporter plasmid. The expression and activation of NF- κ B Signaling relevant protein were analyzed to investigate the relationship between Akt and NF- κ B signaling after the down-regulation of CXCR4 in osteosarcoma cells. **Results:** Down-regulation of CXCR4 significantly reduced the cell proliferation, while remarkably increased the cell apoptosis and apoptotic protein expression levels in osteosarcoma cells. Furthermore, down-regulation of CXCR4 induced cell apoptosis was caspase dependent in osteosarcoma cells. This study also showed CXCR4 down-regulation induced apoptosis through inhibiting PI3K/Akt/NF- κ B signaling pathway. In addition, endoplasmic reticulum stress (ERS) activation was involved in cell apoptosis induced down-regulation of CXCR4. Knockdown of partial ERS relevant proteins followed down-regulation of CXCR4 significantly inhibited cell apoptosis and the apoptotic protein expression levels. **Conclusions:** Taken together, the results demonstrated that down-regulation of CXCR4 could induce apoptosis of human osteosarcoma cells through inhibiting PI3K/Akt/NF- κ B signaling pathway, indicating that CXCR4 could be vital for the clinical therapy of osteosarcoma.

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Introduction

Osteosarcoma, arising from primitive transformed cells of mesenchymal origin, is the most common primary bone cancer, which exhibits osteoblastic differentiation and produces malignant osteoid [1, 2]. Osteosarcoma has a high incidence of one to three per million every year in children and adolescents [3, 4]. Currently, the 5-year survival rate of osteosarcoma patients is quite low, especially in the patients insensitive to chemotherapy [4-6]. Although new targeting drugs have been developed in these years, side effects and poor response still disturb the osteosarcoma patients [7, 8]. Therefore, it is necessary to found new and effective strategies to improve osteosarcoma therapy.

It's well known that the induction of apoptosis is critical in the therapy of tumor [9]. The apoptotic process consists of extrinsic and intrinsic factors induced caspase-dependent classical apoptosis, which is regulated by transmembrane receptors and mitochondrial pathway respectively [10, 11]. Previous study found apoptosis of osteosarcoma cells was essential to improve the treatment of osteosarcoma [12]. NF- κ B is recognized as a crucial mediator in many steps of cancer initiation and progression, including the interaction of immune system, inflammation and down-regulation of apoptosis [13, 14]. I κ B α could suppress the activation of NF- κ B through binding to the dimmers of NF- κ B, whereas IKK α largely enhanced the activation of NF- κ B [15, 16]. In addition, phosphorylation of Akt, the downstream effector of PI3K, has been observed as a critical mediator in the NF- κ B signaling pathway promoting cell proliferation and protecting cancer cells against apoptosis in cancer cells [17]. Previous studies demonstrated that PI3K/Akt/NF- κ B signaling pathway was a crucial target in the mechanism investigation of therapeutic route in various cancer cells [18-20]. However, the detailed mechanisms of PI3K/Akt/NF- κ B signaling pathway and with the relationship to apoptosis of cancer are still unknown.

CXCR4, also known as fusin, is a 45 kDa rhodopsin-like G protein-linked CXC chemokine receptor, mainly expressed in blood and tissue cells, including dendritic cells, endothelial cells, astrocytes, and lymphocytes [21]. CXCR4 is the receptor of chemokine SDF-1, which regulates cell migration and involved in cancer progress and immunodeficiency disorder diseases [22, 23]. Recent study reported that CXCR4 was involved in metastatic potential in osteosarcoma at bone marrow and the lungs [24]. Previous studies showed that cancer cells have higher CXCR4 expression levels compared to normal cells [25, 26]. In addition, over-expression of CXCR4 has been demonstrated to promote metastasis and associate with unfavorable prognosis in breast and lung cancer [27, 28]. Although CXCR4 has been reported to be relevant with various tumor progress and metastasis [29], the detailed molecular mechanisms of CXCR4 on osteosarcoma were still obscure.

This study aims to investigate a novel potential therapy strategy of osteosarcoma. We tested whether CXCR4 can be used as a target for osteosarcoma therapy. Here, we demonstrated that the protein expression of CXCR4 significantly increased in osteosarcoma cells and down-regulation of CXCR4 induced cell apoptosis in osteosarcoma cells. This study also showed CXCR4 down-regulation induced apoptosis through the inhibition of PI3K/Akt/NF- κ B signaling, which involved in endoplasmic reticulum stress (ERS) activation in osteosarcoma cells. In addition, Knockdown of ERS related proteins significantly inhibited cell apoptosis and the apoptotic protein expression levels induced by the down-regulation of CXCR4. These results provide CXCR4 as a novel target of potential therapy in osteosarcoma.

Materials and Methods

Medicines and reagents

LY294002 was acquired from Cell Signaling Technology (Beverly, MA, USA). The broad-spectrum caspase inhibitor (z-VAD-fmk) was acquired from Millipore (Billerica, MA, USA). Caspase-8 specific inhibitor (z-IETD-fmk) was obtained from BioVision (Mountain View, CA, USA). Caspase-9 specific inhibitor (z-LEHD-fmk) was obtained from BioVision (Mountain View, CA, USA).

Cell culture

The human osteosarcoma cell line MG63, Saos2, HOS, U2OS and Human Osteoblasts Hob were obtained from Shanghai Institute of Chinese Academy of Sciences (China). Cells were maintained in RPMI-1640 (Gibco, USA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum at 37 °C in a humid incubator with 5 % CO₂.

Proliferation Assays

The BrdU assay was used to detect cell proliferation. Cells were plated and treated with control or CXCR4 shRNA. CXCR4 shRNA: 5'-GATCCTGCCCACCATCTACTCCATTTCAGAGAATGGA GTAGATGGTGGGCATTTTTTGAAGCTAGCA-3'; Control shRNA: 5'-GATCCAATCGCATAGC GTATGCCGTTTCAAGAGAAACGGCATACTGCTATGCGATTTTTTGAAGCTAGCA-3'. BrdU (100 µM) was added and incubated for 2 h at 37°C. The cells were then fixed and incubated for 30 min at 37°C. Cells were washed with PBS and incubated with BrdU antibody conjugated with peroxidase for 60 min at 37°C. Substrate solution was added and the absorbance was measured at 370 nm with a microplate reader (Bio-Rad, Japan).

Apoptosis assay

The apoptosis detection kit (Biolegend, CA, USA) was used to detect apoptosis assay. Cells were incubated with indicated reagents, following treated with 5 µg/ml 7-aminoactinomycin D (7-AAD) and 2.5 µg/ml Annexin V-fluorescein isothiocyanate (FITC), and the results were analyzed by flow cytometry on the FACSCalibur (BD Biosciences, CA, USA).

RNA extraction and quantitative real-time polymerase chain reaction analysis

TRIzol reagent (Invitrogen, Carlsbad, USA) was used to acquire Total RNA. Reverse-transcribed complementary DNA was synthesized using the Prime-Script® RT Reagent Kit (TaKaRa, Tokyo, Japan). qPCR analyses were using with LightCycler 480 SYBR Green I Master (Roche, Welwyn Garden, Swiss). CXCR4 forward primer (5'-CACTTCAGATAACTACACCG-3'); reverse primer (5'-ATCCAGACGCCAACATAGAC-3'), GAPDH: forward primer (5'-AATC CCATCACCATCTTCCA-3'); reverse primer (5'-TGGACTCCACGACGTACTCA -3').

Western blot

Protein was separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, USA). The membrane probed with antibodies against CXCR4, p-AKT, Bcl-2, BAX, PARP, caspase-3, caspase-8, caspase-9, DR5, Bid, IKKa, IkBa, CHOP, GRP78, XBP-1, P-eIF2a, eIF2a, GAPDH were purchased from Cell Signaling Technology, antibodies against P50, P52, P65, c-rel, relB were purchased from Santa Cruz Biotechnology. The membrane was then probed with horseradish peroxidase-conjugating (HRP) secondary antibody (1:10000; GE Healthcare, Tokyo, Japan).

Caspase activity

Caspase-3, Caspase-8 and Caspase-9 Colorimetric Assay Kits (Biovision, CA, USA) were used to detect the Caspase-3, -8 and -9 activities, respectively. The absorbance was detected at 405nm according to the manufacturer's protocol by using MR7000 microplate reader.

Luciferase Reporter assays

The promoter of NF-κβ was amplified and cloned in pGL 3.0 luciferase reporter plasmid. The cells were seeded and transfected with pRL-CMV renilla luciferase reporter and the pGL 3.0 luciferase reporter plasmid. The activities of firefly and Renilla luciferases were detected using a dual luciferase reporter assay system (Promega).

Flow cytometric analysis

To detect CXCR4 expression, cells were incubated in 6-well plates. After treatment with CXCR4 shRNA or control, cells were harvested and stained with CXCR4-PE (ebioscience) at 4°C for 30 min. Cells were analyzed with a flow cytometry (FACScan®; BD Biosciences).

Adenovirus vector construction, infection and transfection

Oligonucleotides of shRNA for CXCR4, CHOP, GRP78, XBP-1, P-eIF2a were obtained from Sangon Biotech (Shanghai). The sequences of Akt, IKKα and IκBα were cloned into shuttle vector. HEK-293T cells were cultured in dish, attached to the plate for 12 h. The linearized DNA, the shuttle vector and pacAd vector were cotransfected in cells by Lipofectamine 2000 (Invitrogen). Viral vectors were harvested, purified and tittered. The acquired adenovirus vectors were used to infect the cells.

Statistical analysis

Results are showed as the means $\pm$ S.D. from at least three independent experiments. The statistical differences were calculated by the Student's *t*-test or one-way analysis of variance (ANOVA) with Dunnett's test.

Results

Role of CXCR4 in osteosarcoma cells proliferation and apoptosis

The expression of CXCR4 was detected in osteosarcoma and Hob cells. As shown in Fig. 1a, the protein expression of CXCR4 in various osteosarcoma cells was significantly higher than Hob cells. Consistently, Higher mRNA level of CXCR4 was found in osteosarcoma cells compared with in Hob cells (Fig. 1b). To investigate the role of CXCR4 in osteosarcoma cells, we used the CXCR4 shRNA adenovirus to infect osteosarcoma cells, and the expression of CXCR4 was markedly decreased compared with control (Fig. 1c). Furthermore, CXCR4 shRNA also decreased CXCR4 expression on the membrane surface (Fig. 1i). The effect of CXCR4 shRNA on osteosarcoma cell proliferation was determined by BrdU assay. The proliferation of cell was significant decreased in a time-dependent manner with the CXCR4 shRNA treatment (Fig. 1f). We also performed apoptosis assay using double stained with 7AAD and Annexin V in osteosarcoma cell treated with CXCR4 shRNA. As shown in

Fig. 1. CXCR4 down-regulation induced cell apoptosis in osteosarcoma cells. (a) The protein expression of CXCR4 was detected by Western Blot in osteosarcoma and Hob cells. (b) CXCR4 mRNA was measured by Real-time PCR analysis in osteosarcoma and Hob cells. **P*<0.05. (c) The protein expression of CXCR4 was detected in MG63 osteosarcoma cells following treatment with CXCR4 shRNA or control shRNA for 24 h. (d) Cells were treated with CXCR4 shRNA or control shRNA for 48 h. Cell apoptosis was detected with annexin V-FITC/7-AAD and analyzed by flow cytometry. ****P*<0.001 VS Blank. (e) Western blotting analysis for protein expressions of PARP, caspase-3, 8, 9 and Bid were measured in osteosarcoma cells after down-regulation with CXCR4. (f) The proliferative effect of CXCR4 on osteosarcoma cells. **P*<0.05 VS Blank. (g) Caspase activities in osteosarcoma cells. **P*<0.05 VS Blank (h) Cell apoptosis was detected for osteosarcoma cells treated with CXCR4 shRNA followed by the pretreatment with caspase inhibitors for 1 h. **P*<0.05 VS CXCR4 shRNA treatment. (i) The expression of CXCR4 was detected by flow cytometry after treatment with CXCR4 or control shRNA in MG63 osteosarcoma cells.

Fig. 2. CXCR4 down-regulation induced Akt and NF- κ B inhibition in osteosarcoma cells. (a) The protein expression of p-AKT was detected in MG63 osteosarcoma cells after CXCR4 down-regulation. (b) The protein expression of p-AKT was detected in osteosarcoma cells after treatment with CXCR4 down-regulation and/or Akt overexpression. (c) The protein expression of p-AKT was detected in osteosarcoma cells after treatment with CXCR4 down-regulation and/or LY294002. (d) Cell apoptosis was detected for osteosarcoma cells treated with CXCR4 shRNA followed transfection with Akt or LY294002. (e) CXCR4 shRNA decreased the transcriptional activity of NF- κ B promoter. After incubating with or without CXCR4 shRNA, MG63 cells were prepared for luciferase assays. Values were normalized as the arbitrary luciferase activity. (f) The protein expressions of P50, P52, P65, c-rel, relB were detected in MG63 osteosarcoma cells after CXCR4 down-regulation. (g) The protein expression of IKKa was detected in MG63 osteosarcoma cells

after treatment with CXCR4 down-regulation and/or IKKa overexpression. (h) The protein expression of IkBa was detected in MG63 osteosarcoma cells after treatment with CXCR4 down-regulation and/or IkBa overexpression. (i) Cell apoptosis was detected for osteosarcoma cells treated with CXCR4 shRNA followed transfection with IKKa or IkBa. * $P < 0.05$ VS CXCR4 shRNA treatment, ** $P < 0.01$ VS Blank.

Fig. 1d, apoptotic cells were significantly increased with CXCR4 shRNA treatment ($P < 0.001$). Moreover, apoptosis related proteins, including PARP, caspase-3, caspase-8, caspase-9 and Bid, were detected by western blot. The expression levels of PARP, caspase-3, caspase-8, caspase-9 were obviously increased after CXCR4 shRNA treatment, whereas the expression level of Bid was expectedly decreased with CXCR4 shRNA treatment in osteosarcoma cells (Fig. 1e). Furthermore, we detected caspase activities of caspase-3, -8, and 9 after CXCR4 shRNA treatment. The caspase activities of caspase-3, -8, and 9 were markedly enhanced with CXCR4 shRNA treatment (Fig. 1g). To further confirm these results, caspase inhibitors such as z-VAD-fmk, z-IETD-fmk and z-LEHD-fmk were used to pretreat osteosarcoma cells followed treatment with CXCR4 shRNA. Apoptosis induced by CXCR4 shRNA treatment was significantly decreased with z-IETD-fmk, z-LEHD-fmk or z-VAD-fmk pretreatments (Fig. 1h).

CXCR4 down-regulation inhibited Akt activation in osteosarcoma cells

To investigate the effect of CXCR4 on survival signaling in osteosarcoma cells, we analyzed the effect of CXCR4 shRNA on AKT activation. Treatment of osteosarcoma cells with CXCR4 shRNA could markedly reduce P-AKT protein expression compared with control (Fig. 2a). We further investigated the effect of Akt signaling in CXCR4 down-regulation induced apoptosis using Akt over-expression and PI3K-AKT inhibitor LY294002 (Fig. 2b). The p-AKT protein expression was obviously enhanced in the treatment with CXCR4 shRNA and Akt overexpression compared with CXCR4 shRNA treatment alone. In addition,

Fig. 3. Relationship between Akt and NF- κ B signaling followed CXCR4 down-regulation in osteosarcoma cells. (a, c) The protein expressions of P50, P52, P65, c-rel, relB were detected in MG63 osteosarcoma cells after CXCR4 down-regulation followed Akt or LY294002 treatment. (b) Akt enhanced the transcriptional activity of NF- κ B promoter. After incubating with or without CXCR4 shRNA followed Akt or LY294002 treatment, cells were prepared for luciferase assays. Values were normalized as the arbitrary luciferase activity. * $P < 0.05$ VS CXCR4 shRNA treatment, ** $P < 0.01$ VS CXCR4 shRNA treatment.

exposure of osteosarcoma cells to CXCR4 shRNA and LY294002, could remarkably down-regulate the protein level of p-AKT (Fig. 2c). However, the effect of Akt over-expression and PI3K-AKT inhibitor LY294002 on osteosarcoma cell apoptosis need further study. The results showed treatment with LY29400 could significantly enhance the apoptosis of CXCR4 shRNA (Fig. 2d), whereas treatment with Akt vectors markedly decreased the apoptosis activity of CXCR4 shRNA in osteosarcoma cells (Fig. 2d).

CXCR4 down-regulation induced apoptosis via inhibiting NF- κ B signaling in osteosarcoma cells

To further elucidate the role of CXCR4 down-regulation in the apoptosis of osteosarcoma cells, we constructed NF- κ B promoter luciferase reporter plasmid to test whether the activation of NF- κ B signaling was involved. The results demonstrated that CXCR4 shRNA considerably decreased luciferase expression compared with control in osteosarcoma cells (Fig. 2e). Moreover, the protein expressions of P50, P52, P65, c-rel, relB were obviously decreased in osteosarcoma cells after treatment with CXCR4 shRNA (Fig. 2f). We subsequently investigated the NF- κ B signal in osteosarcoma cells after treatment with CXCR4 shRNA using IKK α and IkB α vectors. The IKK α protein expression was markedly enhanced in the treatment with CXCR4 shRNA and IKK α vectors compared with CXCR4 shRNA alone treatment (Fig. 2g). In addition, exposure of osteosarcoma cells to CXCR4 shRNA and IkB α vectors, could remarkably promote the protein expression level of IkB α (Fig. 2h). The effect of IKK α and IkB α vectors on osteosarcoma cell apoptosis was further investigated. The results showed treatment with IKK α vector could significantly decrease the apoptosis induced by CXCR4 shRNA (Fig. 2i), whereas treatment with IkB α vectors could markedly increased the apoptosis activity of CXCR4 shRNA in osteosarcoma cells (Fig. 2i).

CXCR4 regulates NF- κ B signaling through Akt inactivation in osteosarcoma cells

To investigate the relationship between Akt and NF- κ B signaling followed CXCR4 down-regulation in osteosarcoma cells, we evaluated the expression of NF- κ B signaling proteins after CXCR4 down-regulation followed treatment with Akt vectors and PI3K-AKT inhibitor LY294002. The protein expressions of P50, P52, P65, c-rel, relB were detected in osteosarcoma cells after CXCR4 down-regulation followed Akt vectors or LY294002 treatment. The results showed Akt over-expression could promote the protein expressions of P50, P52, P65, c-rel, relB, whereas LY294002 obviously down-regulated the protein expressions of P50, P52, P65, c-rel, relB compared with CXCR4 shRNA treatment alone (Fig. 3a and c). Subsequently, we testified the transcriptional activity of NF- κ B promoter followed treatment with CXCR4 shRNA and Akt vectors or LY294002. The results demonstrated that Akt over-expression could significantly increase luciferase expression driven by NF- κ B promoter, whereas

Fig. 4. CXCR4 down-regulation induced apoptosis involved ERS signaling in osteosarcoma cells. (a) The protein expressions of CHOP, GRP78, XBP-1, P-eIF2 α , eIF2 α were detected in MG63 osteosarcoma cells after CXCR4 down-regulation. (b) The protein expressions of CHOP, GRP78, XBP-1, P-eIF2 α , eIF2 α were detected in MG63 osteosarcoma cells after CXCR4 down-regulation followed CHOP, GRP78, XBP-1, P-eIF2 α ShRNA treatment. (c, d) The protein expressions of DR5, Caspase-3, Bcl-2, BAX were detected in MG63 osteosarcoma cells after CXCR4 down-regulation followed CHOP, GRP78, XBP-1, P-eIF2 α ShRNA treatment. (e) Cell apoptosis was detected for osteosarcoma cells treated with CXCR4 shRNA followed transfection with CHOP, GRP78, XBP-1, P-eIF2 α ShRNA. * $P < 0.05$ VS CXCR4 shRNA treatment, ** $P < 0.01$ VS Blank. *** $P < 0.001$ VS CXCR4 shRNA treatment.

LY294002 markedly decreased the transcriptional activity of NF- κ B promoter compared with CXCR4 shRNA treatment alone (Fig. 3b).

CXCR4 down-regulation induced apoptosis involved ERS signaling in osteosarcoma cells

Whether apoptosis induced by CXCR4 down-regulation in osteosarcoma cells was associated with ERS signaling need to be further investigated. To detect whether ERS signaling was involved in CXCR4 down-regulation induced apoptosis, the expression of ERS relevant markers including CHOP, GRP78, XBP-1, P-eIF2 α , eIF2 α were determined by western blot. The results showed that CHOP, GRP78, XBP-1, P-eIF2 α protein expression levels were significantly increased with exposure to CXCR4 shRNA, while not the eIF2 α (Fig. 4a). Furthermore, we determined the knockdown efficiency of CHOP, GRP78, XBP-1 or P-eIF2 α after treatment with CXCR4 shRNA (Fig. 4b). Interestingly, knockdown of CHOP and P-eIF2 α could downregulate the protein levels of DR5, Caspase-3 and BAX and increased Bcl-2 protein level, while knockdown of GRP78 could remarkably downregulate the protein levels of DR5 and increased Bcl-2 protein level combined with CXCR4 shRNA treatment compared with CXCR4 shRNA treatment alone (Fig 4c and d). Consistently, downregulation of CHOP, GRP78, P-eIF2 α instead of XBP-1 could significantly inhibit apoptosis followed treatment with CXCR4 shRNA compared with CXCR4 shRNA alone treatment (Fig. 4e).

Discussion

Osteosarcoma oncogenesis is a multistep process that involves multiple process including oncogenes and cell invasion and metastasis, detached from the primary tumor site and adhere to local migration matrix through stromal tissue, which depend on cell communication and cell to matrix interactions [30, 31]. Clinical studies demonstrated that CXCR4 was up-regulated in various cancers and involved in cancer cells angiogenesis and metastasis [32, 33]. Previous studies revealed that CXCR4 inhibitor could induce apoptosis and inhibit proliferation of osteosarcoma cells *in vitro* [34]. Oda et al. also showed high expression of CXCR4 was detected in metastatic and primary sites, which correlated with activation of vascular endothelial growth factor signaling pathway [35]. However, the detailed mechanisms of how CXCR4 regulating the death of osteosarcoma cells need to be further elucidated, especially the influence on the downstream signaling pathways.

This study demonstrated that the expression level of CXCR4 in osteosarcoma cells was higher than in normal osteoblast cells. To investigate the effect of CXCR4 on osteosarcoma cells, we found down-regulation of CXCR4 significantly reduced the cell proliferation and remarkably increased the cell apoptosis in osteosarcoma cells. Previous study revealed the enhanced CXCR4 expression was involved in distant metastasis of human osteosarcoma cells [36]. In addition, the CXCR4 blocking antibody eliminated pulmonary lesions from human osteosarcoma cells in a lung-seeding tumor model [37]. Consistently, the apoptotic protein expression levels including PARP, caspase-3, caspase-8, and caspase-9 were obviously enhanced after CXCR4 shRNA treatment, whereas Bid protein expression level was expectedly decreased with CXCR4 shRNA treatment in osteosarcoma cells. Furthermore, the results demonstrated that down-regulation of CXCR4 induced cell apoptosis was caspase dependent evidenced by z-VAD-fmk, z-IETD-fmk and z-LEHD-fmk caspase inhibitors treatment blocked osteosarcoma cells apoptosis induced by CXCR4 shRNA treatment. Previous study showed that silencing of CXCR4 could induce apoptosis of triple-negative breast cancer cells, involving the promoted protein level of caspase-3 [38]. To further explore the molecular mechanisms of CXCR4 shRNA in osteosarcoma cell apoptosis, PI3K-AKT and NF- κ B signaling pathway were detected and the results showed that the cell apoptosis of osteosarcoma induced by down-regulation of CXCR4 through inhibition of PI3K-AKT and NF- κ B signaling pathway. The results showed LY29400 could significantly increase the cell apoptosis induced by down-regulation of CXCR4. However, treatment with Akt vectors could markedly decrease the apoptosis proportion induced by down-regulation of CXCR4 in osteosarcoma cells. Interestingly, similar with LY29400, transfection with I κ B α vectors could prominently increase the apoptosis proportion induced by CXCR4 shRNA, whereas treatment with IKK α vectors could significantly decrease the cell apoptosis induced by down-regulation of CXCR4 in osteosarcoma cells. The roles of PI3K-AKT and NF- κ B in the regulation of cell growth, proliferation and apoptosis had been extensively investigated [39, 40]. Previous studies revealed that PI3K/AKT/NF- κ B signaling pathway played a vital role in regulating apoptosis in various cancer cells [41-44]. Yu et al. indicated that celastrol induced apoptosis and decreased cell invasion and migration via inhibition of PI3K/AKT/NF- κ B signaling pathway in human osteosarcoma cells [45]. Thus, we further investigated the relationship between Akt and NF- κ B signaling followed CXCR4 down-regulation treatment in osteosarcoma cells. As expected, the results indicated that Akt over-expression could promote the activation of NF- κ B, while LY294002 treatment reversed outcome in osteosarcoma cells, which determined by the protein expression levels of P50, P52, P65, c-rel, relB and the transcriptional activity of NF- κ B promoter. Therefore, our results suggested that down-regulation of CXCR4 induced apoptosis via inhibiting PI3K/Akt/NF- κ B signaling pathway in osteosarcoma cells. In addition, we also investigated whether ERS signaling was involved in down-regulation of CXCR4 induced apoptosis in osteosarcoma cells. The results showed that ERS related protein expression levels were obviously increased with down-regulation of CXCR4. Knockdown of ERS related proteins could significantly inhibit cell apoptosis and the apoptotic protein expression levels induced by the down-regulation of CXCR4. These results suggested that down-regulation of CXCR4 induced apoptosis was involved in ERS activation in osteosarcoma cell.

Conclusion

These findings showed that down-regulation of CXCR4 induced apoptosis via inhibiting PI3K/Akt/NF- κ B signaling pathway in osteosarcoma cells. Meanwhile, ERS activation was involved in down-regulation of CXCR4 induced osteosarcoma cell apoptosis. We further demonstrated knockdown of ERS related proteins followed down-regulation of CXCR4 could significantly inhibit cell apoptosis. These findings provided a novel mechanism of osteosarcoma treatment by down-regulation of CXCR4 induced apoptosis through PI3K/

Akt/NF- κ B signaling pathway. This newly mechanism could be a new potential therapeutic manner and improve the outcome of treatment in osteosarcoma.

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Disclosure Statement

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