

SDF-1/CXCR4 promotes F5M2 osteosarcoma cell migration by activating the Wnt/ β -catenin signaling pathway

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Abstract Osteosarcoma (OS), the most common primary malignant bone tumor in children and adolescents, lacks an effective therapy. Stromal cell-derived factor (SDF-1) and its receptor, CXCR4, play multiple roles in migration, proliferation, and survival of different tumor cells. This study aimed to investigate whether the functional SDF-1/CXCR4 signaling mediates chemotaxis in F5M2 OS cells as well as the underlying mechanisms. Immunohistochemistry and immunofluorescence microscopy were used. RNA expression was detected by real-time quantitative polymerase chain reaction, and protein expression was examined by Western blotting. Migration assays were carried out in F5M2 cells. The results showed that the expression of CXCR4 and β -catenin mRNA and protein was significantly higher in OS tissues compared to the surrounding non-neoplastic tissues. SDF-1 promoted F5M2 cell migration by activating the AKT and Wnt/ β -catenin signaling pathway, which was abrogated by preincubation with AMD3100 and LY294002. In conclusion, SDF-1/

CXCR4 axis-promoted F5M2 cell migration was regulated by the Wnt/ β -catenin signaling pathway.

Keywords Osteosarcoma · SDF-1 · CXCR4 · Wnt/ β -catenin · Metastasis

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents [1]. The age distribution is bimodal: the first peak occurring during adolescence, and the second peak being observed in population older than 65 years of age [2]. The distal femoral, proximal tibial, and proximal humeral metaphyses are the most common sites for OS [3]. The use of neoadjuvant chemotherapy in combination with orthopedic surgery has increased the long-term survival rates that now approach 70 % in patients who have no metastatic OS at diagnosis [4, 5]. However, the outcome is more awful with a survival rate of <30 % in patients with metastatic disease at present [6]. Pulmonary metastasis is the primary site and the most common cause of death due to OS [3]. Unfortunately, survival has not improved in the last decade despite multiple attempts at increasing the intensity of chemotherapy and utilizing new agents in clinical trials [5]. Therefore, it is necessary to analyze the natural history and biology of OS to seek new therapeutic targets and approaches.

Chemokine 12 (CXCL12), also known as stromal cell-derived factor-1 (SDF-1), is secreted by stromal cells including fibroblasts and endothelial cells [7, 8]. SDF-1 is a member of the CXC chemokine subfamily, and it interacts specifically with the only known ligand for the seven-transmembrane G protein-coupled receptor CXCR4 [9]. It has been shown that the interaction between SDF-1 and

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CXCR4 results in progression of tumor, particularly breast cancer [10]. Recent studies have shown that SDF-1/CXCR4 axis is related to the pulmonary metastasis of OS [11, 12]. Although there are some studies that have demonstrated that SDF-1/CXCR4 axis is involved in the metastatic process of OS, the precise molecular mechanism has not been completely elucidated [13, 14].

β -catenin plays an important role in the classical Wnt/ β -catenin signaling pathway. Aberrant Wnt/ β -catenin signaling plays a role in multiple cancers such as colon, breast, prostate cancer, and OS [15–18]. It has been shown that upregulation of the Wnt/ β -catenin signaling pathway may contribute to the development of OS [19]. Moreover, SDF-1/CXCR4 can promote the progression of tumor by activating the canonical Wnt/ β -catenin signaling pathway including breast cancer and colon cancer [20, 21], but in OS such cooperation has not been elucidated.

Based on this premise, the expression of CXCR4 and β -catenin was confirmed in pathological samples of OS obtained after surgical resection. The objective of the present study was to observe whether SDF-1 can alter the expression of Wnt/ β -catenin in F5M2 OS cell line. Furthermore, by examining the phosphorylation of phosphatidylinositol 3-kinase (PI3K)/AKT and β -catenin, SDF-1/CXCR4 was found to be controlled by the Wnt/ β -catenin signaling pathway in an intricate manner. Evidence to examine the role of the Wnt/ β -catenin signaling pathway in SDF-1-promoted migration and survival of OS has been provided.

Materials and methods

Materials

RPMI-1640 medium was purchased from HyClone (Thermo Scientific, Waltham, MA). Enhanced chemiluminescence reagents, polyvinylidene fluoride membrane, and Immobilon Western reagents were from Millipore Corporation (Billerica, MA). Recombinant CXCL12 and monoclonal antibodies against p-AKT and p- β -catenin were purchased from Signalway Antibody LLC (Israel). AMD3100 was purchased from Sigma-Aldrich (USA), and LY294002 was purchased from Beyotime (China). Polyclonal antibodies against β -catenin and CXCR4 were bought from Cell Signaling Technology (Beverly, MA). Peroxidase-conjugated goat anti-rabbit immunoglobulin G and monoclonal antibodies against β -actin were purchased from Bioworld Technology (Minnesota, MN).

Cell culture

Human OS cell subline F5M2 was established and maintained in our laboratory as previously described [22], and it

was grown in RPMI 1640 supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μ g/mL), and glutamine (2 mM) at 37 °C in 5 % CO₂ and 95 % air. The cells were harvested for subsequent experiments after incubation for the desired period.

Tissue samples

Ethics Statement: This study was approved by the Ethics Committee of the Fourth Military Medical University, Xi'an, Shaanxi, China (approval ID: 2013158). Either the patients or their guardians provided written informed consent for participation in this study.

Tissue specimens were obtained from 21 patients who underwent surgical resection of OS or osteochondroma at TangDu Hospital, Fourth Military Medical University (Xi'an, China) between January 2013 and March 2014. As shown in Table 1, the investigated specimens included 15 samples of OS tissue and 6 samples of osteochondroma tissue. None of the patients underwent prior chemotherapy or radiotherapy. All the samples were evaluated for diagnosis by two similarly experienced pathologists and were classified according to the Enneking staging system. In the case of OS tissues, the adjacent normal tissue was collected at the time of surgery and, when suitable, the reverse transcription polymerase chain reaction was used to analyze the expression of CXCR4 and β -catenin RNA.

Immunohistochemistry

Immunohistochemistry was performed as previously described [23]. The dilution of CXCR4 and β -catenin antibody used for immunohistochemistry was 1:100. Phosphate-buffered saline (PBS) replaced the primary antibody in the negative control. The final scores of expression of CXCR4 and β -catenin were calculated according to a previous study [24] and classified as follows: –, (0); +, (1–3); ++, (4–8); and +++, (9–12). Scores of 0, 1–3, 4–8, and 9–12 were designated as negative, weak, moderate, and strong expression, respectively.

Real-time quantitative polymerase chain reaction

Real-time quantitative PCR (qPCR) was performed according to a previous study [25]. The primers for specific genes or for the housekeeping gene β -actin were designed and synthesized by Sangon Biotech (Shanghai, China). Relative gene expression was calculated using the C_t method using the $2^{-\Delta\Delta C_t}$. Primers were as follows: CXCR4, 5'-AAT AAAATCTTCTGCCCCACC-3' and 5'-CTGTACTTGTCC GTCATGCTTC-3'; β -catenin, 5'-TGAGCACCTGTTTGC CTGAA-3' and 5'-ATGAGCAGCACTCGGACCTT-3'; β -

Table 1 Clinical and histopathological features of human tissue specimens employed for the evaluation of CXCR4 and β -catenin expression

| Case no. | Gender | Age (years) | Diagnosis | β -Catenin | CXCR4 | Metastasis | Enneking |
|----------|--------|-------------|----------------|------------------|-------|------------|----------|
| 1 | M | 11 | OS | +++ | +++ | Yes | III |
| 2 | M | 13 | OS | + | ++ | Yes | III |
| 3 | M | 18 | OS | – | + | No | I |
| 4 | M | 19 | OS | ++ | + | Yes | III |
| 5 | F | 14 | OS | ++ | ++ | Yes | III |
| 6 | F | 16 | OS | + | ++ | Yes | II B |
| 7 | F | 25 | OS | + | + | Yes | III |
| 8 | F | 14 | OS | – | – | Yes | III |
| 9 | F | 26 | OS | + | – | No | II A |
| 10 | F | 43 | OS | ++ | + | Yes | III |
| 11 | M | 19 | OS | +++ | ++ | Yes | III |
| 12 | F | 15 | OS | ++ | ++ | Yes | III |
| 13 | M | 17 | OS | ++ | ++ | Yes | III |
| 14 | M | 15 | OS | ++ | ++ | Yes | III |
| 15 | F | 10 | OS | + | + | Yes | II B |
| 16 | M | 14 | Osteochondroma | – | – | No | 1 |
| 17 | M | 21 | Osteochondroma | – | – | No | 1 |
| 18 | M | 41 | Osteochondroma | – | + | No | 2 |
| 19 | M | 32 | Osteochondroma | – | – | No | 1 |
| 20 | F | 34 | Osteochondroma | + | – | No | 2 |
| 21 | F | 26 | Osteochondroma | – | – | No | 1 |

actin, 5'-TAGTTGCGTTACACCCTTTCTTG-3' and 5'-TCACCTTACCGTTCCAGTTT-3'. All experiments were performed in triplicate at least three times independently.

Migration assay

The migration potential of cells was assessed in a Transwell (Corning, NY) with 8.0-mm pore polycarbonate membrane insert. RPMI-1640 and different concentrations of SDF-1 (1, 10, 40, 100, and 200 ng/mL) were added to the lower chamber (0.6 mL per well), while inhibitors, if present, were added to the upper chamber with 1×10^4 cells. After the cells were incubated for 16 h, the cells migrated to the underside of the filters were stained according to a methodology described elsewhere [25]. The number of cells in the lower surface was mounted under a light microscope in six random fields at a magnification of 200 \times . All experiments were repeated three times on separate occasions.

Immunofluorescence and confocal microscopy

F5M2 cells were plated onto Millicell EZ SLIDE four-well glass (Millipore, Germany) for 12 h in RPMI-1640 medium in the absence of serum. Then, either PBS or specific CXCR4 antagonist AMD3100 (100 ng/mL) was added to

the glass slides. After 3-h incubation, CXCL12 (100 ng/mL) was added and incubated for 24 h. Serum-starved F5M2 cells were stained using an antibody against β -catenin (Cell Signaling Technology) according to a previous study elsewhere [26]. Images were captured using a confocal microscope (Olympus, Tokyo, Japan) with an objective lens 60 \times .

Western blot

Cellular lysates were prepared using prechilled lysis buffer (1 % Triton X-100, 50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L ethylenediaminetetraacetic acid, 150 mmol/L NaCl, 2 mmol/L phenylmethanesulfonyl fluoride, and 1 mmol/L sodium orthovanadate). The lysates were clarified by centrifugation at 12,000 rpm for 20 min and resolved by sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis. The immunoblotting procedure was performed as previously described [27].

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences software (SPSS, Inc.) and represented as mean \pm standard deviation. Statistical analysis was

performed using one-way analysis of variance or two-tailed Student's *t* test for comparison between groups. $P < 0.05$ was considered statistically significant.

Results

Expression of CXCR4 and β -catenin in human OS or osteochondroma tissue specimens

The different levels of expression of the CXCR4 and β -catenin in OS and osteochondroma are described in Table 1. In the OS, the expression of CXCR4 was clearly detected in cell membrane and cytoplasm (Fig. 1a), and expression of β -catenin was predominantly detected in cell cytoplasm and/or nucleus (Fig. 1c). In contrast, the expression of CXCR4 and β -catenin was less evident in osteochondroma (Fig. 1b, d). The expression of CXCR4 was

absent with the exception of two cases (cases 8 and 9; Table 1) of 15 OS tissues. And β -catenin was not detected in cases 3 and 8 (Table 1) of 15 OS tissues. The clinical features of metastasis and Enneking staging are summarized in Table 1. Total RNA extracted from three individual OS cases with score ++ (cases 12, 13, and 14; Table 1) was detected for CXCR4 and β -catenin RNA expression using real-time qPCR so as to further demonstrate the contradiction in CXCR4 and β -catenin between the OS tissue and the surrounding non-neoplastic tissue. As shown in Fig. 1e, f, CXCR4 and β -catenin in OS tissue were remarkably higher compared to the surrounding non-neoplastic tissue.

SDF-1-induced F5M2 cell migration

SDF-1 promoted F5M2 OS cell migration within the range of 1–200 ng/mL, with a peak effect at 100 ng/mL (Fig. 2a, c).

Fig. 1 Expression of CXCR4 and β -catenin in human OS, osteochondroma, and the surrounding non-neoplastic tissues (CNT) using immunohistochemistry and/or real-time qPCR. **a** A diffuse membrane and cytoplasmic staining of CXCR4 were evident in OS tissues ($\times 40$). **b** Absence of expression of CXCR4 in osteochondroma ($\times 40$). **c** OS cells showed accumulation of nuclear β -catenin ($\times 40$). **d** Absence of expression of β -catenin in osteochondroma ($\times 40$). **e** The relative expression of CXCR4 mRNA in OS (cases 12, 13, and 14; Table 1) compared with CNT ($*P < 0.01$). **f** The relative expression of β -catenin mRNA in OS (cases 12, 13, and 14; Table 1) compared with CNT ($*P < 0.01$). OS, osteosarcoma; qPCR, quantitative polymerase chain reaction

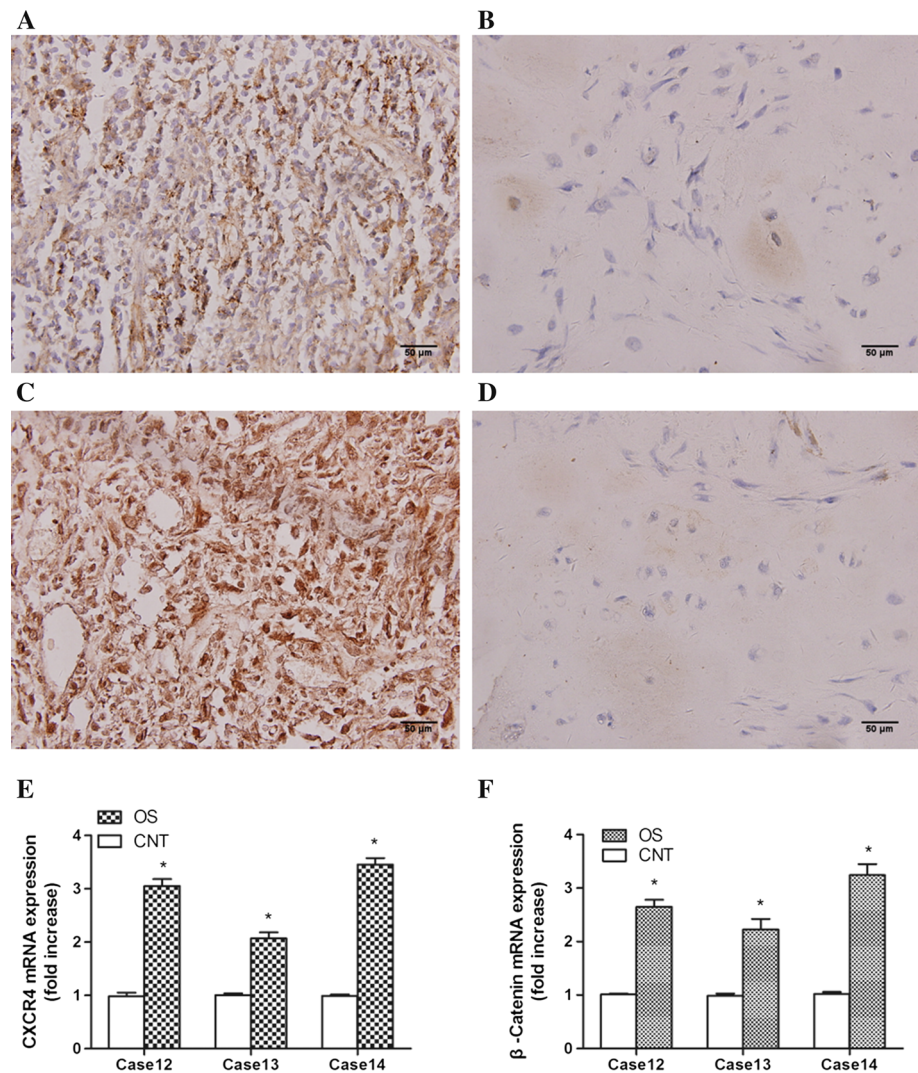
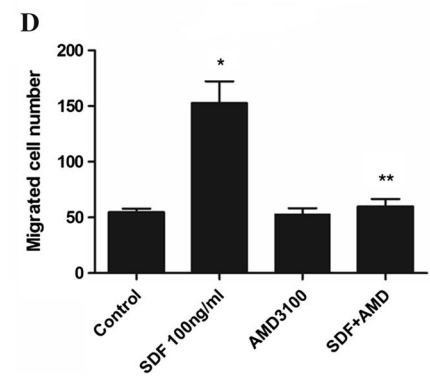
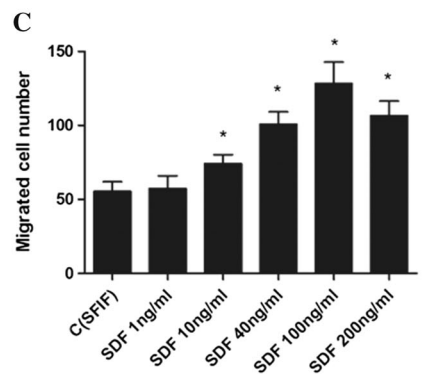
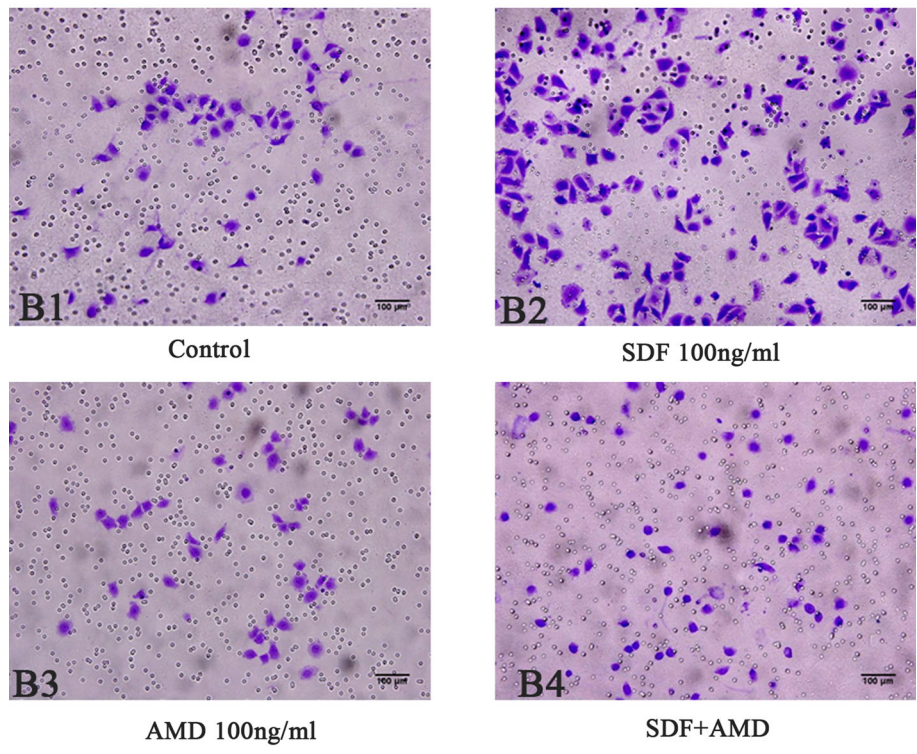
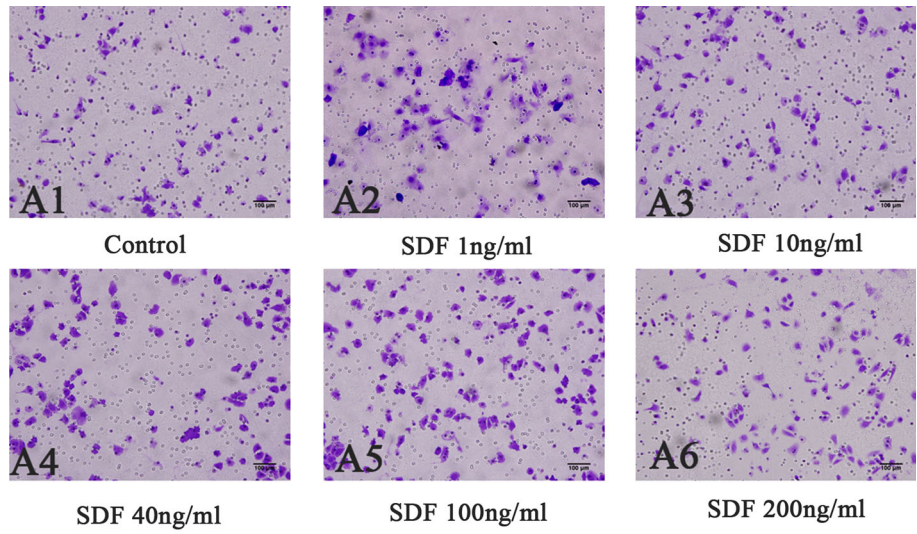


Fig. 2 SDF-1-induced migration of OS cells. The number of migrated cells was quantified compared to control conditions. F5M2 cells incubated in serum-free overnight and then incubated for 16 h in different experimental conditions: **a, c** F5M2 cells incubated with SDF-1 at different concentrations (1, 10, 40, 100, and 200 ng/mL). * $P < 0.05$ versus control; **b, d** with or without SDF-1 pretreatment for 2 h with 100 ng/mL of AMD3100. * $P < 0.05$ versus control, ** $P < 0.05$ versus SDF-1. Data are presented as mean \pm SD of three independent experiments. OS osteosarcoma, SDF-1 stromal cell-derived factor



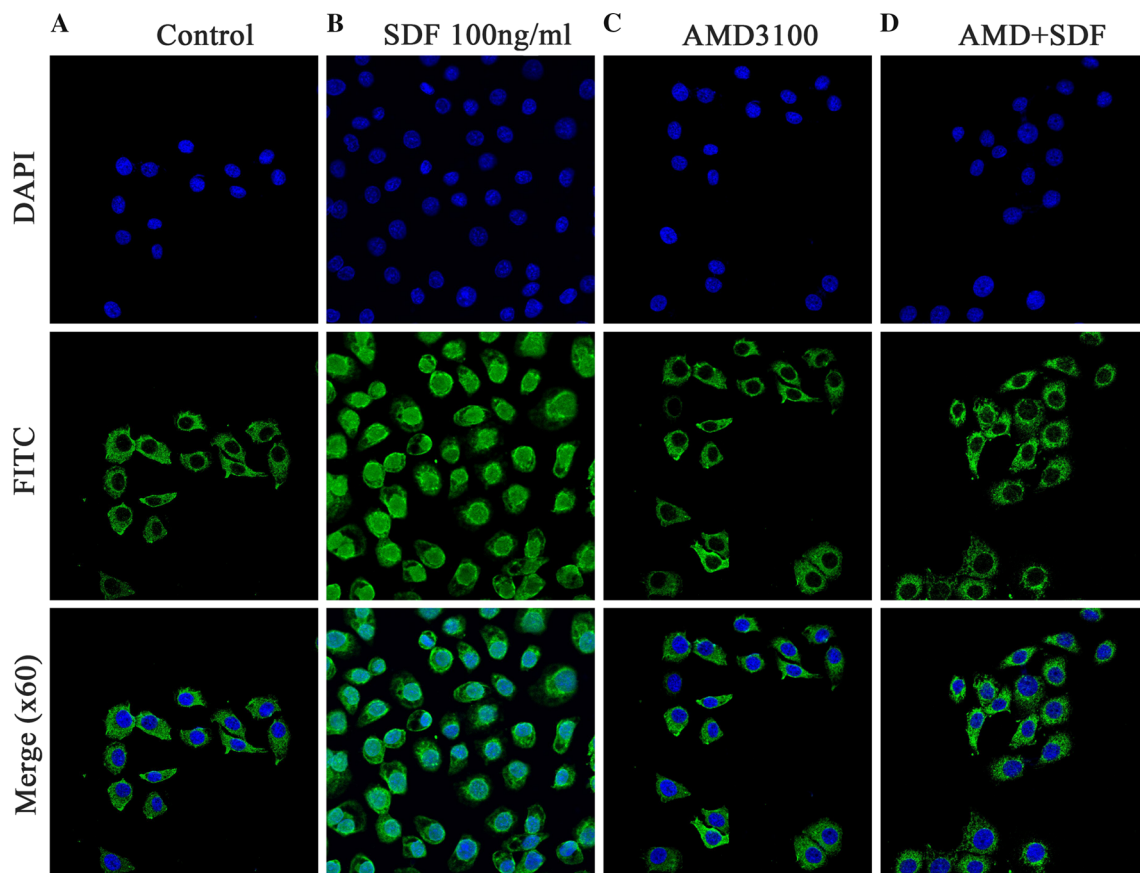


Fig. 3 SDF-1 upregulated the expression of β -catenin in the cell nucleus using immunofluorescence. **a** Expression of β -catenin was lowly stained in F5M2 cell nucleus incubated without SDF-1. **b** Level of expression of β -catenin was significantly higher at 24 h after incubation with SDF-1. **c** The expression of β -catenin did not alter

with incubation with AMD3100. **d** The expression of β -catenin slightly increased after incubation with SDF-1 and AMD3100. *DAPI* 4',6-diamidino-2-phenylindole, *FITC* fluorescein isothiocyanate, *SDF-1* stromal cell-derived factor

This peak dose of SDF-1 was used in all the following experiments according to the above results. SDF-1 chemotaxis experiments in F5M2 cells were performed to reveal the involvement of CXCR4 in SDF-1-induced migration using AMD3100, a highly selective CXCR4 receptor antagonist to potentially inhibit the binding of SDF-1 to CXCR4. Preincubation with 100 ng/mL of SDF-1 promoted SDF-1-induced cell migration (Fig. 2b, d). Preincubation with 100 ng/mL of AMD3100 abrogated SDF-1-induced cell migration, suggesting that the process of SDF-1-induced cell migration was involved in CXCR4 (Fig. 2d).

SDF-1 upregulated the expression of β -catenin in the cell nucleus

Confocal immunofluorescent microscopy revealed that β -catenin was lowly stained in F5M2 cell nucleus incubated without SDF-1 (Fig. 3a). In contrast, the level of expression of β -catenin was significantly higher at 24 h after incubation with SDF-1 (Fig. 3b). The expression of β -catenin did not alter with the incubation with AMD3100 (Fig. 3c).

Moreover, the expression of β -catenin slightly increased after incubation with SDF-1 and AMD3100 (Fig. 3d).

SDF-1 induces F5M2 cell migration through activation of AKT kinase and Wnt/ β -catenin signaling pathway

Chemotaxis assays were performed in cells pretreated with LY294002 (inhibitor of PI3K/AKT) and FH535 (inhibitor of Wnt/ β -catenin) to reveal the possible mechanism of AKT kinase and Wnt/ β -catenin, which are key signaling pathways supporting SDF-1-induced F5M2 cell migration (Fig. 4a, b). All the inhibitors showed a significant reduction of F5M2 cell migration, which suggested that SDF-1-induced F5M2 cell migration is related to the AKT kinase and Wnt/ β -catenin signaling pathway. Accordingly, to observe whether phosphorylation is involved in F5M2 cell migration and downregulation of β -catenin, the phosphorylation of AKT and β -catenin was detected, which demonstrated that SDF-1 induced the phosphorylation of AKT and β -catenin. The AKT and β -catenin were evidently activated at 1 min after

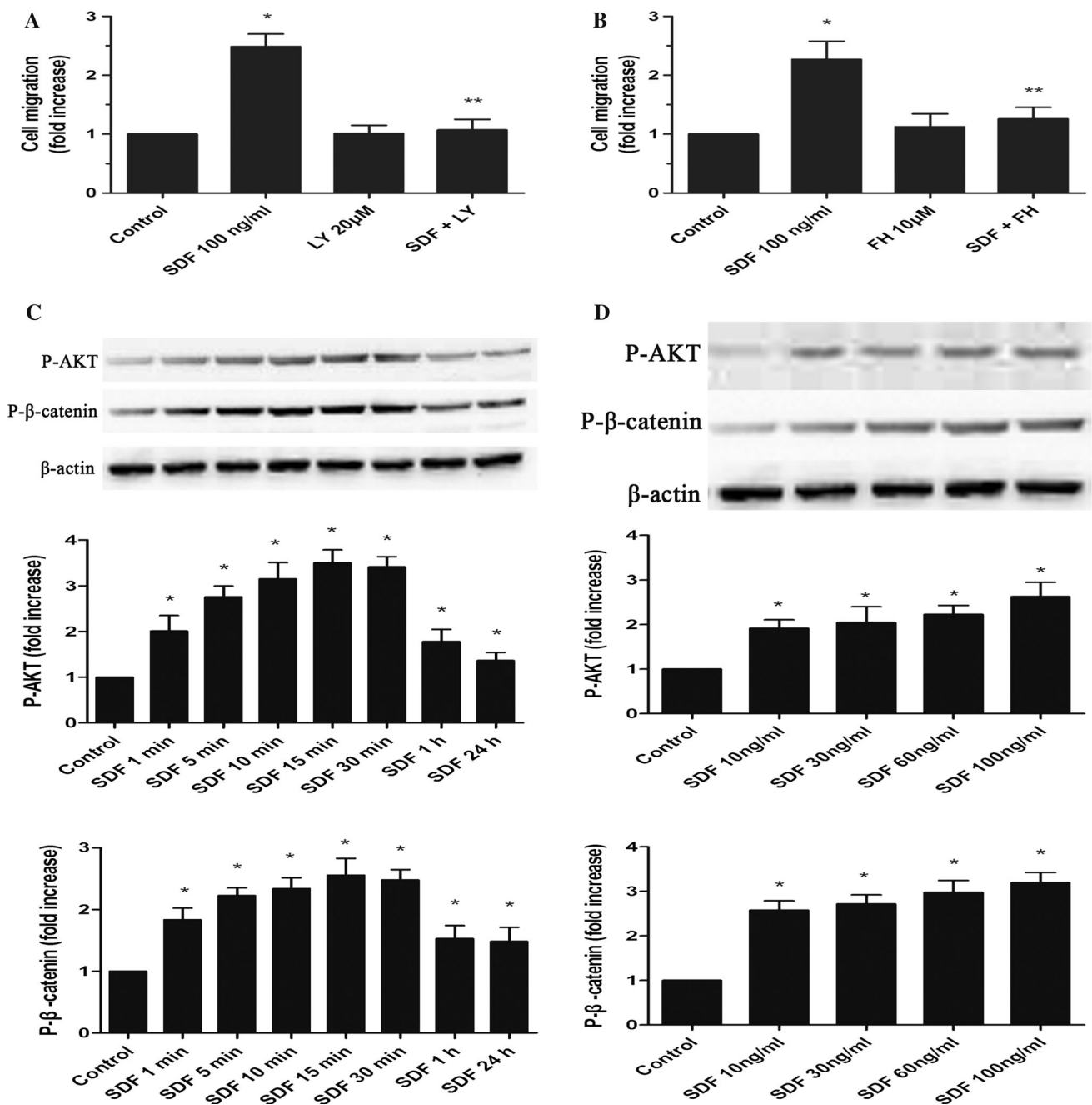


Fig. 4 Involvement of p-AKT and p-β-catenin in SDF-1/CXCR4-induced migration of F5M2 cells. The number of migrated cells is shown as fold increase compared to control conditions. Migration of F5M2 cells was assayed according to the protocol described in Fig. 2. **a** Cells were incubated with 100 ng/mL of SDF-1 with or without 2 h pretreatment with 20 μM of LY294002, an inhibitor of AKT; **P* < 0.05 versus control, ***P* < 0.05 versus SDF-1 alone. **b** Cells were incubated with 100 ng/mL of SDF-1 with or without 2 h pretreatment with 10 μM of FH535, an inhibitor of Wnt; **P* < 0.05

versus control, ***P* < 0.05 versus SDF-1 alone. **c** Phosphorylation of AKT and β-catenin after incubation with 100 ng/mL of SDF-1 at different time points (1, 5, 10, 15, 30 min and 1, 24 h); **P* < 0.05 versus control. **d** Phosphorylation of AKT and β-catenin after incubation with SDF-1 at different concentrations (10, 30, 60, and 100 ng/mL) for 30 min; **P* < 0.05 versus control. Data are presented as mean ± SD of three independent experiments. *SD* standard deviation, *SDF-1* stromal cell-derived factor

incubation with SDF-1. The phosphorylation reached its peak at 15 min for both the proteins after incubation with SDF-1, followed by different time points (Fig. 4c). To detect

the dose-dependent effect of SDF-1-induced phosphorylation of AKT and β-catenin, F5M2 cells were treated with SDF-1 at different concentrations (0, 10, 30, 60, and 100 ng/

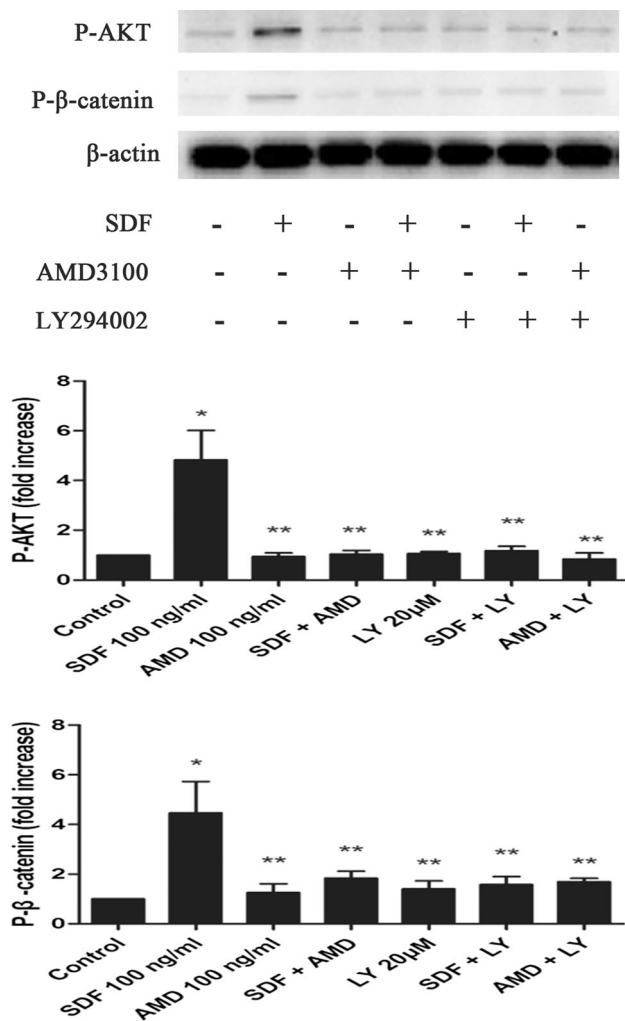


Fig. 5 AMD3100 and LY294002 inhibit SDF-1-induced phosphorylation of AKT and β -catenin. AMD3100 or LY294002 alone has no effect on the phosphorylation of AKT and β -catenin. * $P < 0.05$ versus control, ** $P < 0.05$ versus SDF-1 alone. Data are presented as mean \pm SD of three independent experiments. *SD* standard deviation, *SDF-1* stromal cell-derived factor

mL) for 15 min. This effect showed that SDF-1 treated for 15 min increased the phosphorylation of AKT and β -catenin in a dose-dependent manner (Fig. 4d). The phosphorylation of AKT and β -catenin evidently increased after incubation with 10 ng/mL of SDF-1 and reached its peak after incubation with 100 ng/mL of SDF-1 (Fig. 4d).

Inhibition of AKT prevented phosphorylation of SDF-1-induced β -catenin

To reveal the relationship between AKT and β -catenin, phosphorylation assays were performed in F5M2 cells pretreated with AMD3100 and LY294002, which showed that AMD3100 induced a decrease in SDF-1-induced phosphorylation of AKT and β -catenin (Fig. 5). Further research demonstrated that preincubation with 20 μ M

LY294002 also inhibited a significant phosphorylation of AKT and β -catenin induced by SDF-1, suggesting that β -catenin is phosphorylated through AKT, and may be the downstream signaling molecule of AKT.

Discussion

The interaction between SDF-1 and CXCR4 has been shown to be important in mediating tumor migration, proliferation, angiogenesis, and survival in various types of cancer including OS [11, 12]. The mechanism underlying the effect of chemotaxis remains unclear. In this study, the relationship between SDF-1/CXCR4 and activation of Wnt/ β -catenin in OS was observed. To demonstrate the potential correlation between SDF-1/CXCR4 axis and Wnt/ β -catenin in human OS, the immunohistochemical expression of CXCR4 and β -catenin was estimated in 15 individual surgical specimens of human OS in comparison with osteochondroma tissue. It supports that the expression of CXCR4 and β -catenin is a constant feature of OS cells when compared with osteochondroma. This is confirmed by the significant upregulation of CXCR4 and β -catenin mRNA expression in OS tissue when compared to the adjacent normal tissue. These results confirmed the previously established role of high expression of CXCR4 and the aberrant expression of β -catenin in OS [19, 28]. Thus, the relationship between SDF-1/CXCR4 and activation of Wnt/ β -catenin signaling in OS has been put forward. These results showed that SDF-1 upregulated the expression of β -catenin in the cell nucleus. Indeed, the expression of β -catenin in cell nucleus has been considered a hallmark of activation of the Wnt/ β -catenin signaling pathway [29, 30].

However, it is unknown whether SDF-1-induced signaling in OS is involved in regulating the Wnt/ β -catenin signaling pathway and influences the metastasis. In the present study, it was found that SDF-1 chemotaxis in F5M2 is mediated by the activation of the AKT and β -catenin signaling pathway. This result is in agreement with what was reported in other cancer cell types [20, 31]. Interestingly, it was found that the PI3K/AKT signaling pathway inhibitor LY294002 suppressed the activation of the PI3K/AKT signaling pathway, and the Wnt signaling pathway inhibitor FH535 inhibited the activation of the canonical Wnt/ β -catenin signaling pathway, abolishing F5M2 cell migration induced by SDF-1/CXCR4. Furthermore, the phosphorylation of Akt and β -catenin was observed to analyze SDF-1 signaling in F5M2, similar to that observed in other cancer cell lines [32]. Finally, the mechanism by which SDF-1 induces the phosphorylation of β -catenin via the AKT signaling pathway was also observed. Both AMD3100 and LY294002 inhibited the phosphorylation of the AKT signaling pathway, and β -catenin may be the

downstream signaling molecule of the AKT pathway, suggesting that the upregulation of cell nuclear β -catenin may be involved in the phosphorylation of β -catenin. The phosphorylation of β -catenin may take effect via the AKT signaling pathway.

In this study, the data show that SDF-1-induced F5M2 cell migration is partly dependent on the expression of CXCR4, similar to that described in other cancer cell lines [33]. AMD3100 was used to block the activation of the SDF-1/CXCR4 pathway. And, this result further confirmed the role of the SDF-1/CXCR4 pathway in the progression of OS. AMD3100, which was able to increase the mobilization of bone marrow hematopoietic stem cells quickly, inhibited tumor growth and metastasis in a previous study [34]. Thus, the data suggest that AMD3100 may provide a new way for the treatment of OS.

In summary, the data show that CXCR4 and β -catenin have abnormal expression in OS tissue. SDF-1 induced the F5M2 cell migration via binding to CXCR4. SDF-1/CXCR4 axis activates the AKT and Wnt/ β -catenin pathway via the phosphorylation of AKT and β -catenin. LY294002 inhibits SDF-1-induced phosphorylation of AKT and β -catenin, suggesting that β -catenin may be the downstream signaling molecule of the AKT signaling pathway. These trials may significantly reveal the mechanism of metastasis in OS and other malignancies.

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Conflict of interest The authors declare that there is no conflict of interest in this study.

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