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SIX1 reduces the expression of PTEN via activating PI3K/AKT signal to promote cell proliferation and tumorigenesis in osteosarcoma

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ABSTRACT

Objective: Osteosarcoma is the most common form of primary malignant bone cancer which is most prevalent in children and adolescents. Dysregulated expressions of SIX1 and PTEN/PI3K/AKT have been demonstrated in bone malignancies including osteosarcoma. However, the mechanism of SIX1/PTEN/PI3K/AKT on osteosarcoma progression remains unknown. Therefore, this study aims to investigate the molecular mechanism of SIX1 and PTEN/PI3K/AKT on osteosarcoma progression.

Methods: In this study, we first examined the expression of SIX1 and PTEN in human osteosarcoma tissues or blood samples and cell lines by immunohistochemistry, western blot analysis and qPCR. MTT, clone formation assay, wound healing assay, Transwell assay, in vivo tumorigenesis, flow cytometry and western blot were used to determine the function of SIX1/PTEN on cell proliferation, clone formation ability, migration, invasion, tumorigenesis, and cell apoptosis in SAOS2 and U2OS cells, respectively.

Results: Results showed that SIX1 was overexpressed in osteosarcoma tissues, blood samples and cell lines, whereas PTEN expression was reduced. SIX1 promoted cell growth, migration, invasion, and suppressed cell apoptosis. Up-regulation of SIX1 associated with reduced expression of PTEN and activation of PI3K/AKT signaling pathway. Down-regulated the expression of PTEN using gene transfer in U2OS and SAOS2 cells increased cell proliferation and inhibited cell apoptosis through activating PI3K/AKT signaling cascade. In addition, the tumorigenesis of U2OS and SAOS2 cells was suppressed when the cells were stably overexpressed SIX1 and PTEN simultaneously, compared with that in cells stably overexpressed SIX1 only.

Conclusions: SIX1 promoted the progression of osteosarcoma via regulating PTEN/PI3K/AKT signaling cascade, which might provide a new potent therapeutic target for osteosarcoma.

1. Introduction

Osteosarcoma is one of the most aggressive bone tumors in children and adolescents, characterized by high metastatic potential [1]. Approximately 2/5 of the patients appear with metastases at the moment of diagnosis, and only 1/5 patients could achieve long-term survival among them [2]. Even though a lot of effort has been made to improve outcome of patients with osteosarcoma, including neo-adjuvant chemotherapy and multi-modular therapies, the 5-year survival rate of patients with this disease remains poor [1,3]. Therefore, exploring the molecular mechanism of osteosarcoma occurrence and searching novel therapeutic strategies for osteosarcoma in order to improve the treatment outcomes is an urgent need.

Great achievements have been obtained in exploring the pathological mechanism of osteosarcoma development. Certain genes and signal pathways have been identified to exert key roles in osteosarcoma progression. Sineoculis homeobox homolog 1 (SIX1) is an evolutionary conserved transcription factor [4] and a critical regulator of embryonic development and implicated in tumor onset and progression [5]. SIX1 was frequently dysregulated in several cancers, such as pancreatic cancer [6], breast cancer [7], ovarian cancer [8], colorectal cancer [9,10], prostate cancer [11], as well as osteosarcoma [12,13], resulting in more aggressive and metastatic human cancer cell phenotype, and was closely associated with poor prognosis. In osteosarcoma, Liu et al. [13] verified that SIX1 was overexpressed in osteosarcoma cells and exert role in improvement of cell growth, proliferation, and migration of U2OS cells, as well as suppression of cell apoptosis. Additionally, Chao et al. [12] illuminated that SIX1 was

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differently expressed in cases of osteosarcomas with different clinicopathological features, showing positive correlations with Enneking stage and tumor size, which indicated poor prognosis in patients with positive SIX1 expression. But the specific molecular mechanism of SIX1 on osteosarcoma progression remains unknown.

The tumor suppressor phosphatase and tensin homolog deleted from chromosome ten (PTEN) is encoded by a 200 kb gene located on chromosome10q23, a genome region that suffers mutations or loss of heterozygosity in many human cancers [14]. Functional loss of PTEN is a major determinant, which influences a variety of cancer development, including colorectal cancer [15], gastric cancer [16], breast cancer [17] and osteosarcoma [18]. Inactivation of PTEN leads to loss of its lipid phosphatase activity and accumulation of phosphatidylinositol 3, 4, 5-trisphosphate (PIP3), and bring the activation of AKT. And PTEN/PI3K/AKT signaling pathway is primary positive or negative regulators in tumorigenesis for many solid cancers including osteosarcoma [19]. However, it's unclear that whether SIX1 is able to promote osteosarcoma progression through regulating PTEN/PI3K/AKT signal molecules.

In the present study, we set out to characterize in more detail the role of the SIX1/PTEN/PI3K pathway in the progression of osteosarcoma. Firstly, we detected the expression level of SIX1 and PTEN in osteosarcoma tissues, blood samples and cell lines. Then, we explored the function of SIX1 on osteosarcoma cell growth, clone formation, apoptosis, migration and invasion and investigated the relationship between SIX1 and PTEN/PI3K/AKT signal molecules. Finally, we studied the tumorigenesis of SIX1/PTEN in vivo.

2. Materials and methods

2.1. Tissues and blood samples collection

This study involving human and animals was approved by the ethics committee of Xi'an Jiaotong University Health Science Center, and have been performed in accordance with the Helsinki Declaration. A total of 25 primary osteosarcoma tissues and their paired adjacent non-tumor tissues were collected at our hospital from April 2015 to March 2016. None of these osteosarcoma patients received any radiation therapy or chemotherapy before surgery. Histological slides were reviewed by two experienced pathologists without consideration for the clinical data. Then the tissues were immediately frozen in liquid nitrogen after surgical resection for further study.

The blood samples were collected from 25 patients with osteosarcoma and 25 healthy at our hospital from April 2015 to March 2016. All participators signed informed consent before the study.

2.2. Immunohistochemistry

The human tissue samples were embedded in paraffin and sectioned. Sections (4 μm thick) were deparaffinized, rehydrated with PBS (pH 7.4), incubated with 3% H_2O_2 for 10 min, and then the antigen was retrieved in 0.1% trypsin (M/V) for 10 min at 37 °C. Samples were blocked with 5% BSA for 30 min at room temperature, then monoclonal antibody of rabbit anti-SIX1 and rabbit anti-PTEN were applied at the concentration of 1:150, at 4 °C overnight. Next, they were incubated with second antibody conjugated Diaminobenzidine (DAB) at room temperature for 15 min and then washed with PBS. DAB was applied for 5 min and the cell nucleus was dyed with Harri's hematoxylin solution. Imagine were obtained by imagine autoanalysis system (Olympus BX50) and one representative area was shown in Fig. 1.

2.3. Cell culture

Human osteoblast cell line hFOB and osteosarcoma cell lines (U2OS, HOS, MG63, SAOS2 and SJSA-1) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies), penicillin (100 units/ml), and streptomycin (100 μg/ml), in a 37 °C humidified atmosphere of 5% CO_2.

2.4. Construction of stable U2OS and SAOS2 cell lines up/down-regulated PTEN or SIX1

Twenty-four hours before transfection, U2OS and SAOS2 cells were plated at 70–90% confluence in 6-well plates. The SIX1 specific siRNAs (siRNAs-SIX1) (No. AM16708; 5 nM) and the scrambler oligonucleotides as control (siRNA-NC) (5 nM) were purchased from Thermo Fisher (USA), pCMV6-SIX1 plasmid (No. SC123830), pCMV6-PTEN (No. SC119965), siRNAs-PTEN (No. SR321496) and their negative vector all were purchased from OriGene (USA), and were transfected into U2OS and SAOS2 cells with Opti-MEM containing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To establish cell lines, 400 μg/ml G418 (Life Technologies) was added to the culture medium 48 h after transfection with the expressing plasmids of PTEN (No.13039, Addgene, USA) and shRNA-PTEN (purchased from Shanghai GenePharma Co.,Ltd, China) and their control vector; Puromycin was used for selection of expressing plasmids of SIX1 (No. SC123830, OriGene, USA) and shRNA-SIX1 (No. TL309429, OriGene, USA).The resulting stable transfectants were screened by western blotting and positive colonies were maintained in G418/puromycin selection.

2.5. Reverse transcription polymerase chain reaction and real-time quantitative polymerase chain reaction (RT-qPCR)

Cells total RNA was isolated using TRIZOL reagent (Invitrogen, USA) and blood DNA was extracted using GenElute™ Blood Genomic DNA Kit (Sigma, USA), according to the manufacturer's description. The first-strand cDNA was produced from 1 μg total RNA with M-MLV Reverse Transcriptase (Promega, USA). The RT-qPCR was performed using the SYBR Premix Ex Taq II (Tli RNaseH Plus) (TAKARA, Japan) with the cDNA template and detected using the ABI 7500 Real-time PCR System (Life Technologies, USA). The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers for RT-qPCR were listed as follows: SIX1 (F): 5'-AGGGAGAACACCGAAAACA ATAACT-3', SIX1 (R): 5'-AATTCCTCTTCTGAGCTGGACATGA-3'; PTEN (F):5'- ACAGCCATCATCAAAGAGATCGT-3', PTEN (R): 5'- TGCTTTGA ATCCAAAAACCTTACT-3'; GAPDH (F): GAAGGTGAAGGTCGGAGT; GAPDH (R): GAAGATGGTGATGGGATTTC.

2.6. Western blotting analysis and immunoprecipitation (IP) assay

For western blotting analysis, cell lysate with equal amount of protein was resolved by SDS-PAGE, and then transferred to PVDF membrane (Millipore, USA). After being blocked by 5% nonfat milk,the membrane was incubated with primary and secondary antibodies sequentially. Signals were developed by Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA) on films.

The procedures for the IP experiments were adapted from previous report [20], the cells were washed with cold PBS and then lysed in IP lysis buffer (10 mM Tris, pH7.4, 140 mM NaCl, 5 mM EDTA, 25 mM NaF, 10 mM sodium pyrophosphate, and 1% NP-40 freshly supplemented with 1 mM sodium orthovanadate, 5 mM sodium glycerophosphate and one complete tablet/50 ml). The cell lysate (200 μg total proteins) were precleared for 1 h with Dynabeads® protein G, incubated with 2 μg of Ab overnight at 4 °C, and then incubated with Dynabeads® protein G for an additional 1 h of incubation. The beads were washed four times with IP lysis buffer before being loaded onto gels.

Soft agar clone formation assay

Soft agar clone formation assay was used to determine cell clone formation abilities. The procedures for colony formation assay were adapted from previous reports [21]. Briefly, 1×10^3 cells were