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Hypoxia-inducible factor-1 promotes cancer progression through activating AKT/Cyclin D1 signaling pathway in osteosarcoma

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ABSTRACT

Objective: Osteosarcoma is an aggressive malignant neoplasm, which commonly afflicts patients of 20–30 years of age, and its morbidity has increased markedly in recent years. Certain genes and signal pathways have been identified to exert key roles in osteosarcoma progression. Here, we set out to characterize in more detail of the role of HIF-1/AKT/Cyclin D1 pathway in the progression of osteosarcoma.

Methods: Immunohistochemistry, western blot and qPCR were used to test the protein or mRNA levels of HIF-1 in osteosarcoma tissues or adjacent nontumor tissues. MTT, clone formation, wound healing, Transwell, in vivo tumorigenesis, flow cytometry and western blot analysis were used to determine cell proliferation, clone formation ability, migration, invasion, tumorigenesis, and cell apoptosis in MG63 and U2OS cells, respectively. Immunoprecipitation and immunofluorescence assays were performed to investigate the protein-protein interaction between HIF-1α and proteins related to signal pathways.

Results: HIF-1 was overexpressed in osteosarcoma tissues and cell lines, which promoted cell proliferation, clone formation, migration, invasion and inhibited cell apoptosis. Results also demonstrated that HIF-1 combined with AKT, and there might be a positive loop between the two proteins of HIF-1 and AKT, then the protein-protein interaction up-regulated the expression of Cyclin D1 in protein level, but not mRNA level, made Cyclin D1 protein more stable, triggered cell proliferation, clone formation, tumorigenesis, but inhibited cell apoptosis.

Conclusions: The present study showed that HIF-1 modulated Cyclin D1 expression might through shaping a positive loop with AKT proteins. Additionally, HIF-1α promoted the tumor cells growth, migration and invasion in osteosarcoma through the activation of the AKT/Cyclin D1 signal cascade. We proposed that HIF-1 could be served as a marker for distinguishing osteosarcoma and an effective therapeutic target for osteosarcoma.

1. Introduction

Osteosarcoma is the most frequent primary malignant bone tumor, which always occurs in children and adolescents, for whom it represents more than 10% of solid cancers [1]. Currently, the standard treatment of osteosarcoma mainly consist of preoperative (neo-adjuvant) chemotherapy followed by surgical removal of all detectable disease (including metastases, that are yet present in 80% of patients at diagnosis), and postoperative (adjuvant) chemotherapy [2]. However, although great attention has been paid to the diagnosis and therapy of osteosarcoma, the overall survival rate has failed to improve over the past 20 years [3], mainly due to its recurrence and metastasis [4]. Therefore, exploring the molecular mechanisms involved in osteosarcoma tumorigenesis and development is urgently needed.

Intratumoral hypoxia is a typical profile of solid tumors, which mainly due to the aberrant formation of vasculature in the rapidly growing tumor. Tumor hypoxia has close relationship with increased tumor invasion, angiogenesis and distant metastasis [5–8]. The adaptation of tumor cells to hypoxia has given rise to tumor heterogeneity and the selection of resistant clones, evolving into a more aggressive phenotype [9]. Many studies suggest that the elevated hypoxia-inducible factor-1 (HIF-1) protein level has been discovered in numerous solid tumors and serves as an oncogene which can promote cancer metastases [10,11]. HIF-1 is detectable in osteosarcoma, and correlate with metastasis and poor prognosis of osteosarcoma patients [12–14], implying the potential importance of HIF-1 as a target to antagonize metastasis of osteosarcoma.

Cancer is a disease condition in which cell cycle phases is abnormal. Cyclin D1 plays a key regulatory role during the G1 phase and its gene is amplified and overexpressed in many cancers, such as non-small cell

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lung cancer [15], colorectal cancer [16], gastric cancer [17], mantle cell lymphoma [18], even in osteosarcoma [19]. However, the mechanism that would explain Cyclin D1 deregulation in osteosarcoma is yet to be discovered. Besides, the phosphoinositide 3-kinase (PI3K/AKT) pathway is well known to be a major cell survival pathway in osteosarcoma [20–22], which is a signal pathway not only can be activated by HIF-1[23] but also Cyclin D1 [24]. Nevertheless, if HIF-1 could regulate AKT or Cyclin D1 in osteosarcoma still remains unclear.

In the present study, experimental evidence showed that HIF-1 overexpressed in osteosarcoma tissues and cells, promoted cell growth, and inhibited cell apoptosis of osteosarcoma cells. In addition, there might be a positive loop in HIF-1 and AKT proteins, which induced the occurrence and progression of osteosarcoma through upregulating Cyclin D1 expression. This study provides a potential strategy to prevent osteosarcoma proliferation by targeting the HIF-1 oncogene.

2. Materials and methods

2.1. Tissue sample 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.

2.2. Immunohistochemistry

Paraffin sections of tumor and adjacent tissues were sliced into sections of 4 mm thickness by a slicing machine, and then a routine 3-step immunohistochemical stain was used: sectioning, dewaxing and hydrating the tissues; incubation with 3% H_2O_2 at room temperature for 10 min; antigen repairing with Tris-EDTA; sealing with 5% goat serum (diluted in PBS); incubating overnight at 4 °C with primary antibody against HIF-1 at a 1:150 dilution (Abcam, USA); and then incubating with secondary antibody and rinsing with PBS. Chromogen 3, 3′-diaminobenzidine tetrachloride (DAB) (Serva, Heidelberg, Germany) was used as a substrate and the cell nucleus was dyed with Harri's hematoxylin solution.

2.3. Cell culture

Human osteoblast cell line hFOB and osteosarcoma cell lines (MG63 and U2OS) were all purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies), penicillin (100 units/ml), and streptomycin (100 g/ml), in a 37 °C humidified atmosphere with 5% CO_2.

2.4. RNA interference and cell transfection

Lipofectamine 2000 (Life Technologies, USA) was used to perform cell transfection according to the manufacturer's instruction. The small interfering RNAs (siRNAs) targeting the human HIF-1α (No. SR300295), AKT-1 (No.SR300143) and Cyclin D1 (No.SR300410) genes and their control siRNAs were all purchased from OriGene Technologies, Inc (USA).

For HIF-1 and AKT overexpression, pCMV6-HIF-1 (No.SC119189) and pCMV6-AKT1 (No.SC116883) plasmids (OriGene, USA) were used to transfect U2OS and MG63 cells, and pCMV6-vector (vector-NC) was used as control. After transfection for 48 h, RT-PCR or Western blot assay was conducted to examine the expression level of HIF-1, AKT or Cyclin D1.

2.5. Immunoprecipitation (IP) assay

The procedures for the IP experiments were adapted from previous report [25], 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 pre-cleared for 1 h with Dynabeads® protein G, incubated with anti-HIF-1α antibody 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.

2.6. Western blot analysis

The total proteins were extracted from tissues and cells. The supernatants of cells and tissues lysate were collected after centrifuging at 12,000*g* and 4 °C for 15 min. The protein concentration was measured by the bicin choninic acid (BCA) method (Thermo Fisher Scientific, USA). To determine the levels of protein expression, 20 μg proteins from each simple were separated by 10% SDS-PAGE and transferred to PVDF membranes, incubated with primary antibodies at 4 °C overnight, respectively, and detected using the SuperSignal Chemiluminescent HRP Substrate after incubation with peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immune complexes were examined by ECL detection (Millipore, USA). For quantification, the western blotting bands were quantified by ImageJ software (National Institutes of Health) after background subtraction. The primary antibodies were as follows: anti-HIF-1α (ab113642), anti-Caspase3 antibody (ab13847), anti-Caspase7 (ab32522), anti-MAPK (ab197348), anti-YAP (ab52771), anti-NF-κB (ab16502), anti-p53 (ab26), anti-PI3K (ab86714), anti-AKT (ab8805), anti-Ub (ab7780), anti-GAPDH (ab37168), and all antibodies were purchased from Abcam (USA).

2.7. RNA extraction and real-time PCR

Total RNA from cells and tissues was extracted with TRIzol reagent (Life Technologies, USA) according to the manufacturer's instruction. RNA was then converted into cDNA using PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Tokyo, Japan). For mRNA expression detection, SYBR Green I Real-Time PCR Kit (Biomics, Nantong, Jiangsu. China) was used to perform real-time PCR. GAPDH was used as an internal control. The reaction condition was 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The relative expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. The sequences were as follows: HIF-1 forward primer:5′- CTCCATTACCCACCGCTGAA -3′, HIF-1α reverse primer:5′- GTAGCTGCATGATCGTCTGG -3′; CyclinD1 forward primer:5′- GGGACTTGGCATCTGTGACC -3′, CyclinD1 reverse primer:5′- TACAAGTGTCCAGAAGGTGTGA -3′; GAPDH forward primer: 5′- AGTCAGCTCTCTCCTTTCAGG -3′, GAPDH reverse primer: 5′- TCCACCACCCTGTTGCTGTA -3′.

2.8. Cell activity detection

MTT assay was used to examine cell proliferation. U2OS and MG63 cells (1×10^4 cells/per well) in each group were plated in a 96-well plate and then incubated at 37 °C, 5% CO_2 for 0, 24, 48, or 72 h, respectively. Then 10 μl of MTT (5 mg/ml) was added to each well and incubated at 37 °C, 5% CO_2 for 2 h. The absorbance was detected at 570 nm with a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA).

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