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Original Article

HOXB5 mRNA is upregulated in osteosarcoma tissues and promotes osteosarcoma cell line proliferation *in vitro*

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Abstract: Objective: HOX (homeobox) genes is a gene family which regulates transcription and has important effects on morphogenesis and differentiation during embryonic development. This study aimed to compare the expression of HOXB5 mRNA in osteosarcoma and normal tissue/cell lines, as well as detect the biological roles of HOXB5 in osteosarcoma (OS) cell lines MG63 and U2OS. Method: Polymerase Chain Reaction (PCR) was performed to examine the expression of HOXB5 mRNA in osteosarcoma and normal tissue/cell lines. HOXB5 knock-down or over-expression was achieved in osteosarcoma cell lines MG63 and U2OS through transfection. MTT was utilized to detect the effects of HOXB5 knock-down or over-expression on MG63 and U2OS. Result: HOXB5 mRNA was significantly over-expressed in osteosarcoma tissues compared with adjacent normal tissues. The expression of HOXB5 was also higher in OS cells than in human osteoblast hFoB1.19 cells. Overexpression of HOXB5 promoted the proliferation and inhibited the apoptosis of osteosarcoma cells, knock-down of HOXB5 inhibited the proliferation and promote the apoptosis of OS cells. Moreover, knock-down the expression of HOXB5 decreased mRNA and protein levels of bcl-2, mTOR, 4EBP1 and p70s6k, over-expression of HOXB5 increased mRNA and protein levels of bcl-2, mTOR, 4EBP1 and p70s6k. Conclusions: HOXB5 mRNA was up-regulated in osteosarcoma tissues and cell lines, and it might play a role in osteosarcoma cell proliferation.

Keywords: Osteosarcoma, HOXB5, RT-PCR, siRNA, MTT

Introduction

Osteosarcoma (OS) is a common malignant bone tumor, which mainly affects children [1-4]. Chemotherapy and radiotherapy, this combined surgery has been utilized to treat the osteosarcoma, the overall survival and progression-free survival have been improved greatly [3, 5]. However, 15-20% of OS patients still suffered from recurrence at an early time and had a poor prognosis [6, 7]. Thus, there was still an urgent need to investigate the pathological mechanisms of OS development and progression.

HOX gene family is a huge gene family, which contains 39 genes. These genes could regulate many biological processes [8, 9]. Some research has shown that the HOX genes had a compact relationship with proliferation and differentiation, and the determination of cancer cells [10-13]. In the previous study, the sequencing technologies were performed in breast cancer tissue and cells, they found that HOXA13, HOXB7 and HOXB5 were overexpressed in tumor tissue and cells [14]. It is well known that the osteosarcoma occurred through a complicated process including gene mutation epigenetic regulation [15-18]. Although some investigations have been done in this field, the expression level of HOXB5 in OS tissue and the biological role of HOXB5 in MG63 and U2OS are still unknown.

The mammalian target of rapamycin (mTOR), a major gatekeeper of anabolism, is a protein serine/threonine kinase giving rise to two complexes (mTORC1 and mTORC2) with different sensitivity to rapamycin [19-21]. A main functional role of mTORC1 is the regulation of mRNA translation via its targets 4EBPs and S6Ks. Unphosphorylated 4EBP1 binds to the cap-binding complex and sequesters eIF4E away from it, resulting in a block of cap-dependent mRNA translation [22-24]. Therefore, the mTOR signal pathway plays an important role in occur-

rence and development of cancer, including OS, which is also one of the focuses of this study.

Material and methods

Collection of tissues

This study was certified by the Ethics Committee of the Department of Orthopedics, Children's Hospital of Nanjing Medical University, Nanjing (Jiangsu, China). All guardians signed an informed consent in this research, the specimens of primary OS tissues and matched non-tumor tissues were obtained from 50 patients, who were diagnosed as OS at the Department of Orthopedics, Children's Hospital of Nanjing Medical University from May 2013 to March 2016. No chemotherapy or radiotherapy was administered before the tissues were collected.

RT-PCR for HOXB5

Semi-quantitative real-time RT-PCR was carried out by SYBR Green Kit (life Invitrogen USA) with the ABI 7500 real-time rotary analysis (ABI Life Science) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Real-time PCR primers were shown as follows: HOXB5, forward 5-TGTAAACTCCTTCTCGGGG-CG-3 and reverse 5-G TCTATTTCGGTGAAATT-GG-3; GAPDH, forward 5-CGGCGACGACC CA-TTCGAAC-3 and reverse 5-GAATCGAACCC-TGATTCCCCGTC-3. For each run, the reaction was repeated independently at least three times.

Cell culture

The human OS cell lines MG63 and U2OS, and normal human osteoblast hFoB1.19 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in RPMI-1640 medium (Sigma, USA). The medium was replenished with 10% fetal bovine serum (FBS), two antibiotic penicillin and streptomycin (all from Sigma, USA). The cells were cultured at 37°C with 5% CO_2 atmosphere.

Plasmid construction and cell transfection

Cells were transfected with 100 nM of HOXB5-siRNA (GAGTATGTCGATGCTATAGC), negative control (NC) siRNA, pc-DNA3.1-HOXB5 expression plasmid or pc-DNA3.1 vector (all purchased from Yearthbio, Changsha, China) using Lipofectaminemax and Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. PCR was performed to validate whether the HOXB5-siRNA and pc-DNA3.1-HOXB5 can be transfected into the cells.

Cell viability assays

MG63 cells were seeded into 96-well plates and cultured for 0, 24, 48, 72 h or 96 h using RPMI-1640 medium with 10% FBS at 37°C. The viability of MG63 cells was determined using MTT assay. Briefly, after cell culture, 20 μl CCK (Sigma USA) was added to each well and the 96 well plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 h. The resulting formazan product was dissolved with 100 μl isopropanol and evaluated by absorbance at 490 nm by FlexStation 3 (Molecular Device, USA). Each experiment was repeated for at least three times.

Apoptosis assay

MG63 cells were seeded onto six wells after transfected with siRNA. MG63 cells were treated according to manufacturer's instructions, stained cells with AnnexinV-APC and PI, then analyzed with BD FACSC auto II.

Western blotting

Cells were lysed by using radio immune precipitation assay (RIPA) buffer and protein was obtained. The concentration of protein was determined by the BCA protein assay kit (Thermo, USA). The protein was separated by 8-15% SDS-PAGE and the separated bands were electrotransferred to PVDF membrane (Amersham Pharmacia, Piscataway, NJ, USA). PVDF membrane was blocked with 5% non-fat skimmed milk at a room temperature, the membrane was probed with antibodies, HOXB5 (1:1000), mTOR (1:1000), 4EBP1 (1:1000), ACTB (1:5000), P70S6K (1:1000) (Abcam, Cambridge, MA, USA). The diluted corresponding secondary antibody was incubated at room temperature for 1 h, rinsed with TBS for 5 minutes for a total of 3 times and analyzed by chemiluminescence method. After the protein bands were scanned by the scanner, the relative quantification of the gray bands of the protein bands was carried out by Imagaquent 5.1 software. The β-actin band was used as a refer-

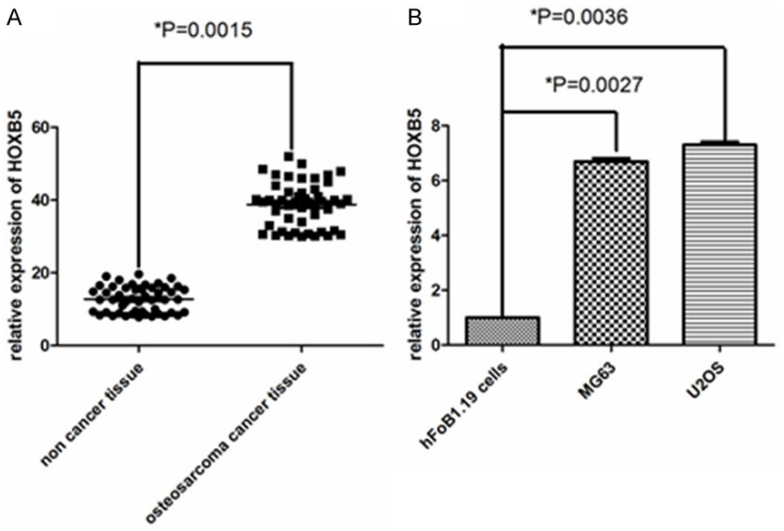

Figure 1. A. Relative expression levels of HOXB5 mRNA in OS tissues (n=50) compared with corresponding non-tumor tissues (n=50). HOXB5 expression was examined by PCR and normalized to ACTB expression. The ΔCt value was determined by subtracting the ACTB Ct value from the HOXB5 Ct value (relative to a single reference value) compared with non-cancer tissues. The expression of HOXB5 was higher in cancer tissues (P<0.05). B. Relative expression levels of HOXB5 mRNA in U2OS, MG63 and hFoB1.19 cells. Compared with hFoB1.19 cells, the expression of HOXB5 was higher in MG63 and U2OS cells (P<0.05).

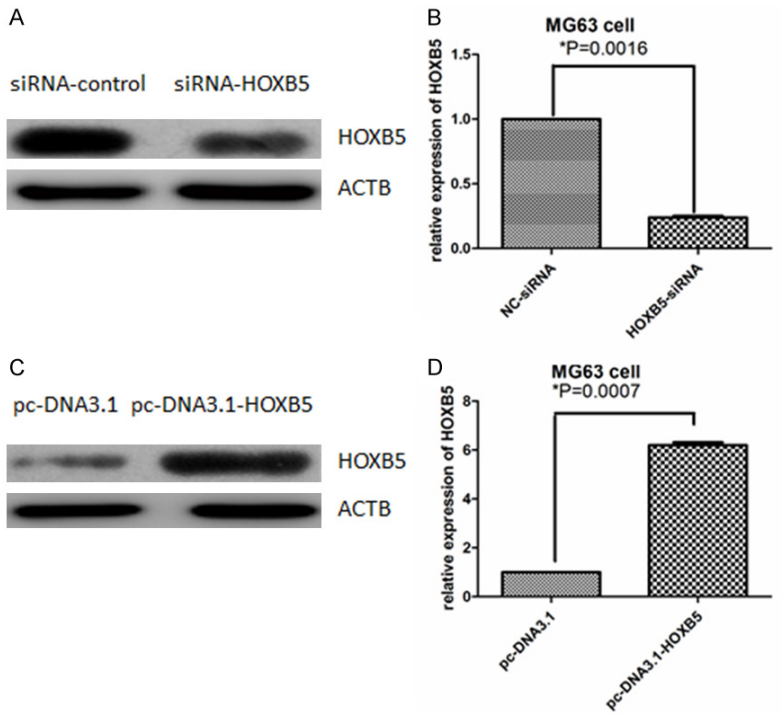

Figure 2. MG63 were transfected with siRNA, WB (A) and PCR (B) were performed to detect the levels of HOXB5 in two groups. Compared with the siRNA-control group, the level of HOXB5 was lower in the siRNA-HOXB5 group (P<0.05). MG63 were transfected with pc-DNA3.1, WB (C) and PCR (D) were performed to detect the levels of HOXB5 in two groups. Compared with the pc-DNA3.1 group, the level of HOXB5 was higher in the pc-DNA3.1-HOXB5 group (P<0.05).

ence. The target protein bands were utilized to compare the relative gray-scale ratios of β-actin.

Statistical analysis

The experimental data were expressed as the mean ± standard deviation of at least three independent experiments. SPSS 19.0 statistical software (IBM Corp, Armonk, NY, USA) was utilized for statistical analysis. Significance differences were analyzed using a Student's t-test or one-way analysis of variance (ANOVA), and *P* values less than 0.05 were considered to indicate a statistically significant difference.

Results

The expression of HOXB5 mRNA in OS was significantly higher compared with non-cancerous tissues (**Figure 1A**). The expression of HOXB5 was also examined in OS cells (MG63 and U2OS) and human osteoblast hFo B1. 19 cells by RT-PCR. The result indicated that the HOXB5 mRNA expression was higher in OS cell lines compared with human osteoblast hFoB1.19 cells (**Figure 1B**).

In order to detect biological roles of HOXB5 in MG63 cells, WB and PCR were utilized to characterize the expression levels of HOXB5 in NC-siRNA and NOXB5-siRNA groups. The data showed that the HOXB5 mRNA expression in MG63 cells was inhibited by specific siRNA against HOXB5. The expression of HOXB5 was down-regulated in the siRNA transfected cells compared with NC-siRNA cells at 48 h

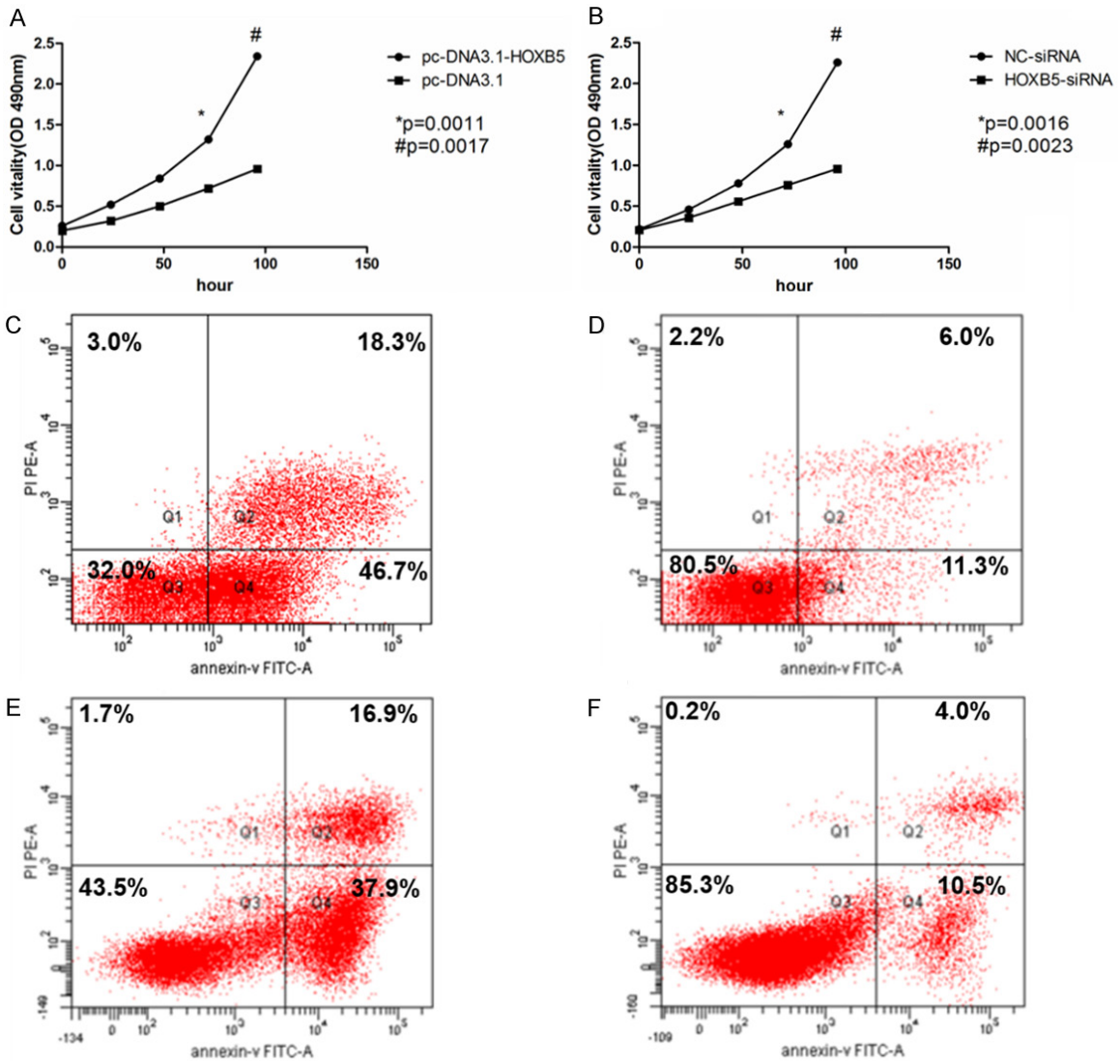

Figure 3. A. Overexpression of HOXB5 promoted the proliferation of MG63 cells at 72 and 96 hours ($P<0.05$). B. Knock down expression of HOXB5 inhibited the proliferation of MG63 cells at 72 and 96 hours ($P<0.05$). C, D. Overexpression of HOXB5 inhibited the apoptosis of MG63 cells ($P<0.05$). E, F. Knock down the expression of HOXB5 promoted the apoptosis of MG63 cells ($P<0.05$).

after transfection (**Figure 2A** and **2B**). In another experiments that were also characterized by WB and PCR, a stable expression plasmid of HOXB5 (pc-DNA3.1-HOXB5) was constructed and transfected into MG63 cells. The expression of HOXB5 mRNA in the MG63 cells was increased compared with the control pc-DNA3.1 cells (**Figure 2C** and **2D**).

Cell viability and apoptosis assays were utilized to detect cell proliferation and apoptosis. The results showed that over-expression of HOXB5 promoted the proliferation of MG63 cells at 72 and 96 hours, while knock down the expression of HOXB5 inhibited the proliferation of MG63 cells at 72 and 96 hours (**Figure 3A** and **3B**). For the cell apoptosis results, the data showed that over-expression of HOXB5 inhibited the apoptosis of MG63 cells, while knock down the expression of HOXB5 promoted the apoptosis of MG63 cells (**Figure 3C-F**).

The mRNA and protein levels of bcl-2, mTOR, 4EBP1 and p70s6k were also measured after knockdown or over-expressed HOXB5. The result showed that silencing the expression of HOXB5 decreased mRNA levels of bcl-2, mTOR, 4EBP1 and p70s6k, while over-expression of HOXB5 increased mRNA levels of bcl-2, mTOR, 4EBP1 and p70s6k (**Figure 4**). Similar to the mRNA levels, after WB abd PCR characterization, protein levels of bcl-2, mTOR, 4EBP1 and

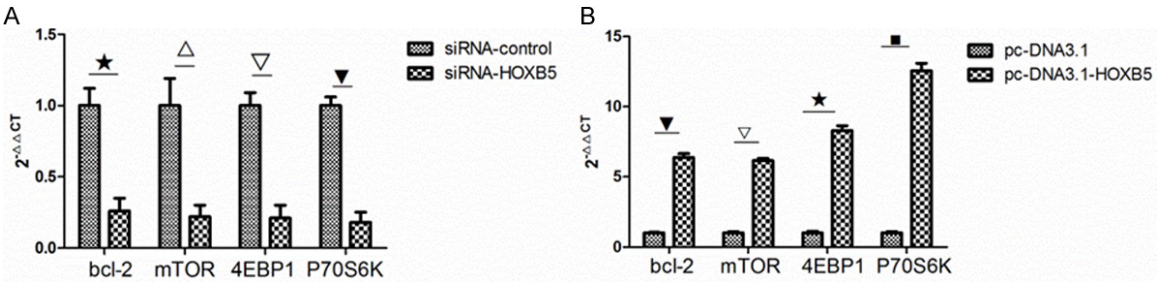

Figure 4. A. Compared with the siRNA-control group, mRNA levels of bcl-2, mTOR, 4EBP1 and P70S6K were lower in the siRNA-HOXB5 group (★Δ▽▼P<0.05). B. Compared with the pc-DNA3.1 group, mRNA levels of bcl-2, mTOR, 4EBP1 and P70S6K were higher in the pc-DNA3.1-HOXB5 group (▼▽★■P<0.05).

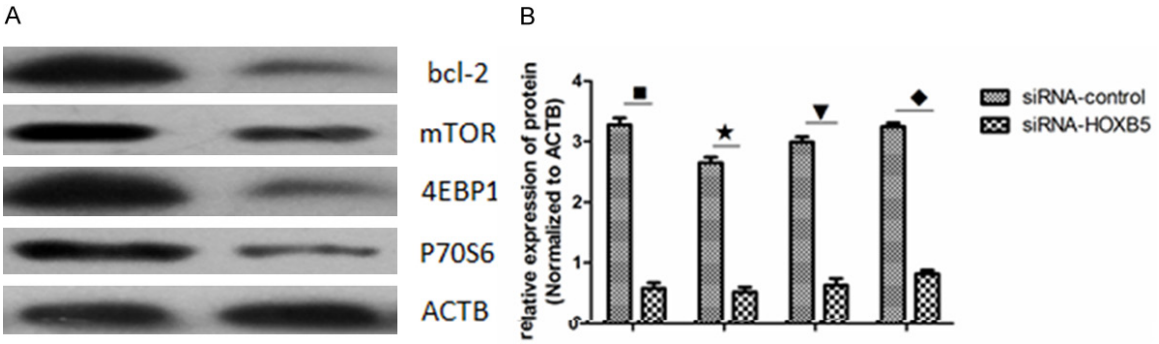

Figure 5. WB (A) and PCR (B) characterization results of bcl-2, mTOR, 4EBP1 and P70S6K protein levels for siRNA-control and siRNA-HOXB5 groups. Compared with the siRNA-control group, protein levels of bcl-2, mTOR, 4EBP1 and P70S6K were lower in the siRNA-HOXB5 group (■★▼◆P<0.05).

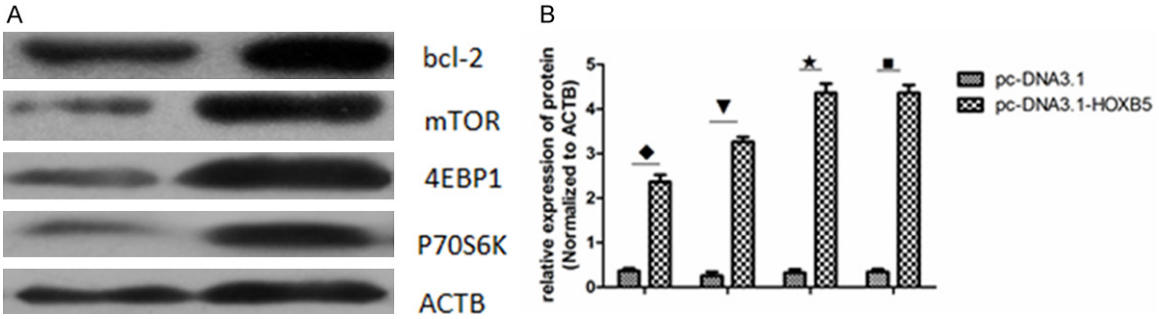

Figure 6. WB (A) and PCR (B) characterization results of bcl-2, mTOR, 4EBP1 and P70S6K protein levels for pc-DNA3.1 and pc-DNA3.1-HOXB5 groups. Compared with the pc-DNA3.1 group, protein levels of bcl-2, mTOR, 4EBP1 and P70S6K were higher in the pc-DNA3.1-HOXB5 group (◆▼★■P<0.05).

P70S6K were lower in the siRNA-HOXB5 group compared to siRNA-control group (**Figure 5**). Moreover, compared with control group pc-DNA3.1, protein levels of bcl-2, mTOR, 4EBP1 and P70S6K were higher in HOXB5 over-expression group pc-DNA3.1-HOXB5 (**Figure 6**).

Discussion

In this study, the expression of HOXB5 was significantly up-regulated in osteosarcoma (OS) tissues and cell lines; knock down of HOXB5 can inhibit the proliferation while over-expression of HOXB5 can promote the proliferation of OS cells.

Osteosarcoma is a very common bone cancer among the bone diseases, according to world health organization, osteosarcoma robs 600000 lives of people every year. Although the incidence of OS is relatively low (about 6% of all childhood cancers) compared with other

malignant tumors, the five-year survival rate is still very poor. There are many reasons for this scenario such as gene mutation, cytokines and epigenetic regulation of the gene, which all could lead to OS.

Aberrant expression of the class I HOX, a group of genes crucial in embryogenesis, has been reported in a variety of malignancies including solid tumors [25]. For example, in the non small cell lung cancer, the expression of HOXB5 is higher in lung cancer tissues and knock down of HOXB5 inhibited the proliferation of lung cancer cells [26]. In human gastric cancer. HOXB5 mRNA was significantly over-expressed in gastric cancer tissues compared with adjacent normal tissues [11]. Moreover, in the breast cancer, knock down the expression of HOXB5 also could inhibit the proliferation of MCF-7 breast cancer cells [10].

There were limited papers about exact biological roles of HOXB5 in the growth of OS. This research has found that the expression levels of HOXB5 were significantly higher in OS tissues and cell lines compared with adjacent non-tumor of OS. After that, MG63 cells were selected for further mechanism investigation. The experimental data demonstrated that knockdown the expression of HOXB5 could inhibit the proliferation and promote the apoptosis of MG63 cells, while over-expression of HOXB5 could promote the proliferation and inhibit the apoptosis of OS cells. Moreover, bcl-2 mRNA and protein levels after knock down and overexpressed HOXB5 were also measured. The result indicated that knock down the expression of HOXB5 decreased the mRNA and protein levels of bcl-2, overexpression of HOXB5 increased the mRNA and protein levels of bcl-2. Therefore, we speculated that silencing HOXB5 expression could inhibit the proliferation and promote the apoptosis of OS cells by decreasing mRNA and protein levels of bcl-2.

In most solid tumors, the mTORC1 pathway has been found to be up-regulated. These increasing was consistent with the increasing of tumor suppressors that related to the mTORC1 signaling pathway. The emerging network of signaling pathways by which mTORC1 is an intricate complex of multiple signaling components that integrate extracellular signals and intracellular cues together. Growth factors such as IGF and insulin, which signal through either PI3K and/or RAS, eventually converge on and inhibit the negative regulator of mTORC1 through the Tuberous Sclerosis Complex (TSC1/2-TBC1D7) [20, 23, 27]. In this study, silencing the expression of HOXB5 decreased mRNA and protein levels of mTOR, 4EBP1 and p70s6k, overexpression of HOXB5 increased mRNA and protein levels of mTOR, 4EBP1 and p70s6k. Thus, we speculated that HOXB5 may interact with mTOR then up-regulate the expression of mTOR, 4EBP1 and p70s6k.

There still exist some drawbacks in this study. For example, the protein levels of HOXB5 in tumor tissue and relationship between HOXB5 levels and clinical stage were not investigated due to several limited conditions. In conclusion, this study showed that the HOXB5 is overexpressed in osteosarcoma cells and could regulate their proliferation and apoptosis.

Disclosure of conflict of interest

None.

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