Original Article

BTG2 inhibits the proliferation and metastasis of osteosarcoma cells by suppressing the PI3K/AKT pathway

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Abstract: B cell translocation gene 2 (BTG2) has been reported to be a potential tumor suppressor in many types of tumors. However, the roles and molecular mechanisms of BTG2 in osteosarcoma progression are still unknown. In this study, we investigated the role of BTG2 in proliferation and metastasis of osteosarcoma and the underlying mechanism. BTG2 expression levels were measured in fresh osteosarcoma tissues and cell lines. The effects of BTG2 on cell proliferation, migration and invasion were explored by MTT, transwell assays, western blot, and in vivo tumorigenesis in nude mice. We found that BTG2 was down-regulated in human osteosarcoma tissues and cell lines. Overexpression of BTG2 inhibited the proliferation and migration/invasion of human osteosarcoma cells in vitro, it also markedly inhibited xenograft tumor growth in vivo. Furthermore, BTG2 significantly decreased the expression of phosphorylated PI3K and AKT in osteosarcoma cells. Taken together, our data indicate that BTG2 might suppress the tumor growth and metastasis via PI3K/AKT signaling pathway, implying that BTG2 may serve as a potential molecular target for the treatment of osteosarcoma.

Keywords: B cell translocation gene 2 (BTG2), osteosarcoma, proliferation, invasion, PI3K/AKT pathway

Introduction

Osteosarcoma is the most common primary bone tumor in children and adolescents. It is characterized by high malignant and metastatic potentials [1]. To date, despite rapid advancements of multimodal treatment, patients with metastatic disease and local relapse still have poor outcomes with survival rates of approximately 20%. Therefore, there is an urgent need to further understand the molecular mechanisms underlying the development and progression of osteosarcoma.

B cell translocation gene 2 (BTG2), also known as NGF-inducible anti-proliferative protein PC3 and NGF-inducible protein TIS21, belongs to the BTG/TOB family [2]. This family has been known to show anti-proliferative properties and comprises an emerging gene family that is involved in cell growth, death, differentiation and survival [3, 4]. The human BTG2 gene is located on band 2, region 3 of the long arm of chromosome 1 and encodes a 158 amino acid protein [5]. It has been reported that BTG2 plays an important role in regulation of cell cycle transition [6]. BTG2 overexpression impairs G1 to S progression by inhibiting cyclin D1 and cyclin E transcription through retinoblastoma (Rb)-dependent [7] and Rb-independent [4] mechanisms.

Recently, a growing body of evidence indicates that BTG2 is involved in many biological activities in cancer cells acting as a tumor suppressor. The expression level of BTG2 was significantly decreased during carcinogenesis in various human tissues, including gastric cancer [8], lung cancer [9], bladder cancer [10] and breast cancer [11]. The loss of BTG2 expression is related significantly with tumor grade, metastasis and resistance to cancer treatment [12]. Furthermore, BTG2 regulates cancer cell migration via regulations of reactive oxygen species (ROS) level [13]. However, the role of BTG2 in osteosarcoma is still unclear. Therefore, in this study, we investigated the role of BTG2 in osteosarcoma. Here, we report that BTG2 expression is decreased in human osteosarcoma tissues and cells lines. In addition, we show that BTG2
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reduces osteosarcoma cell proliferation, migration and invasion in vitro and markedly inhibits tumor growth in an allograft murine model in vivo. We also demonstrate that BTG2 inhibits the proliferation and metastasis of osteosarcoma cells by suppressing the PI3K/Akt pathway.

Materials and methods

Tissue specimens

This study was approved by the ethics committee of Tianjin First Central Hospital, and all patients provided informed consent. 17 paired OS and matched adjacent normal bone tissues were obtained from the Department of Orthopaedic Surgery, Tianjin First Central Hospital, and all the tissues were immediately stored in liquid nitrogen until use. Both osteosarcoma and normal bone tissue biopsies were histologically characterized by pathologists according to the criteria defined by the World Health Organization.

Cell culture

The human osteosarcoma U2OS, SaOS₂, MG-63 and 143 B cell lines were purchased from American Type Culture Collection (ATCC, USA). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% FBS, 100 mg/ml streptomycin, and 100 IU/ml penicillin at 37°C under a humidified 5% CO₂ atmosphere.

Construction of plasmids and transfection

All recombinant adenovirus were constructed as previously described [14]. Briefly, full-length BTG2 cDNA was amplified and subcloned into pAdTrack-cytomegalovirus (CMV), whereas green fluorescent protein (GFP) was used as a non-specific control. Then, the recombinant shuttle plasmids pAdTrack-CMV and pAdEasy-1 were then homologously recombined in Escherichia coli strain BJ5183. The obtained recombinant plasmids were transfected into 293 cells to generate recombinant adenovirus. The recombinant adenoviruses were harvested and the titers were determined using the p24 ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA).

For in vitro transfection, osteosarcoma cells were seeded in each well of 24-well microplates, grown for 24 h to reach 50% confluence, and transfected with Ad-BTG2 or Ad-GFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

MTT assay

Cell proliferation was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay. Cells transfected with Ad-BTG2 or Ad-GFP were seeded into 96-well plates (5×10³ cells/well) and incubated for 24 h, 48 h, 72 h and 96 h, respectively. After incubation with 25 µl of MTT (5 mg/ml) (Sigma, St. Louis, MO, USA) at 37°C for 4 h, the supernatants were removed, and 150 µl of dimethyl-sulfoxide (DMSO; Sigma, St. Louis, MO, USA) was added to each well. The absorbance value (OD) of each well was measured at 450 nm. Experiments were repeated at least three times.

Cell migration assay

Migration assay with MG63 and SaOS₂ cells transfected with Ad-BTG2 or Ad-GFP were examined in transwell cell-culture chambers (Costar, Cambridge, MA). The lower chamber of the transwell plates were filled with 500 µl of RPMI medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). MG63 and SaOS₂ cells (5×10⁴/ml) suspended in RPMI medium were added to the upper chamber, and the plate was incubated with 5% CO₂ for 12 h at 37°C. Cells on the upper surface of the filters were removed using cotton swabs. Migrated cells to the lower surface of the filters were washed, fixed with Giemsa, and counted under a microscope. Experiments were repeated at least three times.

Cell invasion assay

The cell invasion assay was performed using a Boyden chamber coated with matrigel (8 µm pore; BD Biosciences, France), according to the manufacturer’s protocol. Cells transfected with Ad-BTG2 or Ad-GFP (5×10⁴/ml) suspended in 0.1% FBS medium were seeded in the upper compartment. The medium including 1% FBS was added into the lower compartment. Forty-eight hours later, cells that did not migrate through the filter were removed by wiping out with a cotton swab. The cells migrated to the underside were fixed in 3.7% paraformaldehyde.
in PBS at 4°C, washed in PBS and stained with Giemsa. Invasive cells in the bottom of the chamber were counted in high-power fields under an microscope. Three independent assays were performed.

Quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted from frozen tissues and cell lines using the Trizol reagent (Invitrogen, 

Figure 1. BTG2 was decreased in osteosarcoma tissues and cell lines. A. The expression levels of BTG2 proteins were significantly decreased in osteosarcoma tissues compared with that in the corresponding normal tissues. B. The expression levels of BTG2 mRNA were significantly decreased in osteosarcoma tissues compared with that in the corresponding normal tissues. C. Representative Western image of BTG2 protein in osteosarcoma cell lines. BTG2 was significantly decreased in four osteosarcoma cell lines, U2OS, SaOS2, MG-63 and 143B compared with that in human osteoblast cell line hFOB 1.19 cells. D. Representative mRNA expression of BTG2 in osteosarcoma cell lines; Data is expressed as mean ± SD. Experiments were performed in triplicate. *P<0.05 compared with the control group.
Carlsbad, CA, USA). cDNA was amplified with 1 µg of total RNA using a Primer Script Kit (TaKaRa, Dalian, China). qRT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, USA) with Fast Start Universal SYBR Green Master (Roche, USA). The specific primers were as follows: BTG2, forward: 5’-CATCATCAGCAGGGTGGC-3’, reverse: 5’-CCCAATGCGGTAGGACAC-3’; and β-actin, forward: 5’-AGAAAATCTGGCACCACACC-3’, reverse: 5’-TAGCACAGCCTGGATAGCAA-3’. All procedures were performed in triplicate.

Western blot

For Western blot analysis, the proteins were extracted from tissues and cells using RIPA lysis buffer (Beyotime, Nantong, China). The protein concentration of the lysates was measured using a BCA Protein Assay Kit (Pierce, Rockford, USA). Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline. After blocking, the target proteins were probed with primary antibodies (anti-BTG2, anti-phospho-PI3K Tyr607, anti-PI3K, anti-phospho-AKT Ser473, anti-AKT or GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Then, the blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence (Millipore, Boston, MA, USA). GADPH was used as the loading control.

In vivo xenograft tumor assay

MG63 cells (2×10⁶ cells) transfected with Ad-BTG2 or Ad-GFP diluted in 200 µl of PBS were inoculated subcutaneously into the right flank of nude mice. The tumor volume was measured every 7 days by a vernier caliper and calculated using the formula: Volume = length × width² × π/6. About 5 weeks after inoculation, mice were euthanized by subcutaneous injection with sodium pentobarbital (40 mg/kg) and
the tumors were weighed. This study was performed with approval from the Animal Ethics Committee of Tianjin First Central Hospital. All surgeries were performed under sodium pentobarbital anesthesia (Sigma, St. Louis, MO, USA), and all efforts were made to minimize suffering.

**Statistical analysis**

Data were processed as mean ± SD. The differences were analyzed by the Student’s t test or one-way analysis of variance and Student’s t test. A P value of <0.05 was considered to be statistically significant.

**Results**

*BTG2 is down-regulated in osteosarcoma tissues and cell lines*

To investigate the role of BTG2 in osteosarcoma, the expression levels of BTG2 in osteosarcoma tissues were examined by qRT-PCR and western blot. These results demonstrated that BTG2 was significantly down-regulated in tumors compared with their adjacent non-tumorous tissues (Figure 1A and 1B). Furthermore, we investigated the expression of BTG2 in several osteosarcoma cell lines, including U2OS, SaOS2, MG-63 and 143B. As shown in Figure 1C and 1D, as compared with normal bone cells, the expression levels of BTG2 were significantly decreased in osteosarcoma cell lines. These results indicate that BTG2 is down-regulated in both osteosarcoma tissues and cancer cell lines.

*BTG2 suppresses the growth of osteosarcoma cells*

To gain further insight into the role of BTG2 in the tumorigenesis of osteosarcoma, the effect of BTG2 overexpression on the growth of MG63 and SaOS2 osteosarcoma cells was examined by MTT assay. The expression levels of BTG2 protein were obviously increased in MG63 (Figure 2A) and SaOS2 (Figure 2B) cells. In
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addition, BTG2 significantly inhibited the growth of MG63 (Figure 2C) and SaOS2 (Figure 2D) in a time-dependent manner.

**BTG2 suppresses the migration and invasion of osteosarcoma cells**

Cell migration and invasion play important roles in the development of tumorigenesis. Therefore, we investigated the effects of BTG2 on the migration and invasion of osteosarcoma cells. As shown in Figure 3, we found that BTG2 markedly reduced the migration of MG63 and SaOS2, as compared with control cells. In addition, BTG2 also inhibited the invasion of MG63 and SaOS2 compared to control cells. These data show that BTG2 reduces osteosarcoma cell invasion and migration in vitro, suggesting that BTG2 is involved in osteosarcoma cell invasiveness in vitro.

**BTG2 suppresses the growth of osteosarcoma in vivo**

Furthermore, to assess the effects of BTG2 on osteosarcoma growth in vivo, transfected cells were injected into the flanks of nude mice and tumor growth was determined after 5 weeks. We found that overexpression of BTG2 in MG63 cells significantly reduced the weight of tumors compared to control mice (Figure 4A). In addition, the analysis of tumor size demonstrated that overexpression of BTG2 also obviously reduced tumor volume, as compared with control tumors (Figure 4B).

**BTG2 inhibits the proliferation and metastasis of osteosarcoma cells by suppressing the PI3K/Akt pathway**

PI3K/AKT pathway is one of the major signaling pathways associated with cancer progression and invasion [15, 16]. Therefore, we investigated the effect of BTG2 on the PI3K/AKT signaling pathway. As shown in Figure 5, the levels of phosphorylated PI3K (Tyr607) and Akt (Ser473) were decreased in BTG2-transfected cells, compared with control cells. While their total protein levels were unaffected. These results suggest that BTG2 inhibits the proliferation and metastasis of osteosarcoma cells by suppressing the PI3K/Akt pathway.

**Discussion**

In this study, we found that BTG2 was down-regulated in osteosarcoma tissues and cell lines, and its ectopic expression inhibited cell proliferation, migration/invasion and tumor growth. In addition, BTG2 also decreased the levels of phosphorylated PI3K (Tyr607) and Akt (Ser473) in Ad-BTG2-transfected cells.

BTG2 has been reported to be lowly expressed in several cancers and plays a critical role in cancer development. For instance, a recent study showed that the expression level of BTG2 was significantly decreased in breast cancer cell lines, and low BTG2 expression was correlated with tumor grade, size, metastasis, recurrence, and poor survival in breast cancer [12]. Another study reported that BTG2 was down-regulated in renal cell carcinoma [17]. In line with these findings, in this study, we found that BTG2 is down-regulated in both osteosarcoma tissues and cell lines. All these findings indicated that BTG2 is a tumor suppressor in osteosarcoma.

Cell proliferation is one of the most important features of malignant cell behavior [18]. Cancer
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Cell migration and invasion are the most subtle and critical steps for cancer progression and metastasis [19]. A report has shown that BTG2 significantly inhibited the proliferation and invasiveness of lung cancer cells, and it also inhibited the protein expression of cyclinD1, matrix metalloproteinase 1 (MMP-1) and MMP-2 in lung cancer cells [9]. Zhang et al. reported that BTG2 suppressed the proliferation and invasion of human triple-negative breast cancer cells [20]. In line with these results, our data showed that BTG2 obviously inhibited the proliferation and invasion of osteosarcoma cells, suggesting that BTG2 plays an important role in promoting the cell proliferation and invasion of osteosarcoma.

The mouse homolog of BTG2 is designated as 12-O-tetradecanoylphorbol-13-acetate (TPA) inducible sequences 21 (TIS21). It was recently reported that TIS21-/- mice show enhanced hepatocellular carcinoma development at the late stage of diethylnitrosamine-induced hepatocarcinogenesis [21]. Consistent with above results, we demonstrated that BTG2 suppresses the growth of osteosarcoma in vivo. Collectively, these results obtained from both in vivo and in vitro experiments strongly support BTG2 as tumor suppressor.

PI3K/AKT is a major pathway involved in the malignant progression of various tumors, mediates the proliferation, migration and invasion of cancer cells [15, 22, 23]. PI3K is activated by oncogenes, and activated PI3K promotes cancer cell growth and survival [24]. AKT, a member of the AGC serine-threonine kinase family, is a major downstream effector involved in the oncogenic activity of PI3K [25]. Akt is downregulated in many types of cancer, including osteosarcoma [26]. Previous studies demonstrate that its activation requires phosphorylation of S473 by mTORC2 [27], and activated AKT induces cell growth, promotes EMT, and stimulates Bax-mediated signaling for apoptosis progression [28-30]. To further clarify the underlying mechanism involved in BTG2-inhibited osteosarcoma cell proliferation and invasion, we detected the levels of phosphorylated PI3K (Tyr607) and Akt (Ser473) after BTG2 transfection. Our results showed that BTG2 decreased the phosphorylation of PI3K and AKT in MG63 cells. These results suggest that BTG2 inhibits the proliferation and metastasis of osteosarcoma cells by suppressing the PI3K/Akt pathway.

In conclusion, these results demonstrate that BTG2 may play important roles in tumor growth and metastasis and that BTG2 may be a potential therapeutic target for the treatment of osteosarcoma.

Disclosure of conflict of interest

None.
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