Original Article
SKA1 promotes the proliferation of osteosarcoma cell through AKT activation and regulates the expression of cyclins

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Abstract: Osteosarcoma is one of the most frequent malignant bone tumors, affecting the children and adolescents populations. Skal is a microtubule-associating protein, mediating binding of Ska complex to the microtubule. Here, we demonstrated that Skal, together with pAKT level, is up-regulated in human osteosarcomas. Skal promotes osteosarcoma cell lines growth in vitro and in vivo, which can be regulated by various AKT activities. Therefore, Skal, combined with pAKT/AKT may provide a novel therapeutic target for the treatment of osteosarcoma.

Keywords: Osteosarcoma, SKA1, pAKT, cyclins

Introduction

It has been well reported that osteosarcoma (OS) is one of the most malignant bone tumors among children and adolescents populations [1]. Although at present, the incidence of osteosarcoma is relatively lower compared with other malignant solid tumors, such as liver, breast and lung cancer, it also appeared to be of our interest for its high lethality and poor prognosis [2, 3]. The most obvious properties of osteosarcoma are its strongly invasive ability and high rate of early metastasis [4]. A complete radical, surgical, en bloc resection of the cancer, is the treatment of choice in osteosarcoma [5, 6].

Spindle and kinetochore-associated protein 1 (Skal) is a microtubule-associating protein, which mediates binding of Ska complex (Ska1/2/3) to the microtubule in plasma. Skal usually locates to the spindle microtubule and the outer kinetochore interface during mitosis, and the interaction with Ska complex is crucial for stabilizing kinetochore-spindle microtubule attachment [7-12]. Skal deficiency leads to severe chromosome segregation defects [8, 9], whereas over-expression leads to nucleation of interphase microtubules, suggesting a role in microtubule organization [9]. In spite of cellular dysfunctions caused by deregulated expression, few studies show the role of Skal plays in osteosarcoma development.

PI3K/Akt/mTOR pathway is important for proliferation, migration and invasion of many cancer cell lines [13-15]. As a major signaling cascade, it activates the receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and insulin-like growth factor-1 receptor (IGF-1R) [16, 17]. However, the relation of Akt pathway and Skal in osteosarcoma development is unknown.

In this study, we reported that Skal and pAKT is up-regulated in osteosarcoma and strongly associated with poor patient outcome. Collaborating with AKT, Skal inhibits osteosarcoma cell apoptosis, promotes osteosarcoma cell proliferation, migration, invasion and xenograft tumor growth.

Materials and methods

Patient samples

Thirty fresh samples of human osteosarcoma and paired normal tissues were obtained dur-
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ing surgery at the Second Affiliated Hospital of Guangzhou Medical University. All samples were collected with patients’ informed consent. A retrospective analysis of 30 cases of osteosarcoma tissue samples was performed.

**Cell lines and cell culture**

We specifically used the human osteosarcoma cell lines U2OS and Saos-2. All the cells were maintained in a Dulbecco’s Modified Eagle’s Medium (Gibco, Life Technologies, Carlsbad, USA), supplemented with 10% fetal bovine serum (FBS) (HyClone, Thermo, Fremont, USA), in a humidified 5% CO₂ atmosphere at 37°C; the medium was changed every other day.

**Cell cycle analysis by flow cytometry**

Apoptosis was measured by the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Invitrogen). After treatment with SKA1 transfection and AKT inhibitor for 24 h, cells were harvested and washed twice with ice-cold PBS, and fixed in 75% ethanol for overnight at 4°C. The fixed cells were again rinsed with PBS and resuspended in 0.5 mL PI staining solution containing 50 μg/mL PI, 0.25% Triton X-100, and 0.2 mg/mL DNase-free RNase for 30 min at room temperature in the dark. 10,000 events per sample were acquired with a FACS SCAN flow cytometer (Becton-Dickinson, San Jose, CA, USA) and the percentage of cells in G0/G1, S and G2/M phases of the cell cycle was determined using Modfit LT 3.0 (Becton-Dickinson).

**Cell Migration assay**

Costar Transwell Migration Plates with 8 μm pore size were pre-coated with matrigel. Cells (1x10⁴) in 100 μl RPMI medium without FBS were placed in triplicate into the upper chamber. To the lower chamber, 500 μl medium containing 10% FBS were added. After 12 hours of incubation, the plate inserts were removed and washed with PBS buffer (phosphate buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, Ph7.4) several times to get rid of unattached cells. All the residual cells on the upper side were scraped with a cotton swab. Migrated cells on the lower side of the insert were fixed in 4% formalin for 15 minutes, washed with PBS twice, and stained with 0.1% crystal violet for 5 minutes.

**Crystal violet assay**

Cells were plated at a density of 1,500 cells/well in triplicate into 6-well plates using a two-layer soft agar setting. The bottom of each well was covered by a layer of 0.6% agarose reconstituted in complete DMEM medium. Cells were suspended in 1 ml of 0.3% agarose in complete DMEM medium and placed on the top of the bottom agar layer. After 2 weeks of culture (or otherwise indicated time), colonies were visualized by staining with 0.005% crystal violet (dissolved in methanol) for 1.5 hours.

**Matrigel invasion assay**

24-well BioCoat™ Matrigel chamber inserts (BD Bioscience) were used for this experiment. The upper surfaces of invasion chambers were coated with 30 μl of 0.5 mg/ml growth factor-reduced Matrigel (BD Bioscience), and lower surfaces were coated with 20 μl of 0.5 mg/ml fibronectin (Sigma-Aldrich). Before starting the experiment, coated inserts were rehydrated with RPMI1640 for 4 hours in a humidified 5% CO₂ incubator. Cells (5x10⁴) in 500 μl of media containing 0.1% FBS were plated into invasion chambers 36 hours after siRNA transfection. RPMI1640 medium containing 10% FBS (700 μl) was added to each well as a chemoattractant. After incubation for 18 or 80 hours, non-invading cells on upper insert surfaces were removed cleaned by scraping. Invaded cells in inserts were fixed and stained using 0.1% crystal violet for 5 minutes. Experiments were performed in triplicate. Pictures were taken under a light microscope.

**Western blot analysis**

After treatment with resveratrol for 24 h, the cancer cells were harvested and lysed. Equal amounts of cell lysates were resolved by SDS/PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with specific primary antibodies, washed with PBS containing 0.1% (v/v) Tween 20, and then incubated with horseradish peroxidase conjugated secondary antibodies followed by enhanced chemiluminescence (ECL). GAPDH was used for normalization of protein loading.

**In vivo xenograft assay**

Cell suspensions (1x10⁶ cells) of cancer cells in a total volume of 100 μl mixed with matrigel at
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A 1:1 ratio was injected subcutaneously into the right flanks of 4-week-old male BALB/C nude mice. The body weight and tumor volumes were measured and recorded every 10 days from 2 weeks after inoculation. Tumor volume was calculated with the following formula: volume = 0.5 × tumor length × tumor width². Tumors were collected and photographed at 50 days after inoculation. All mice were housed in the SPF animal facility in a pathogen-free environment with controlled temperature and humidity.

Statistical analysis

The data are expressed as means ± standard errors of means (SEM) of at least three independent experiments. Statistical analysis was performed with GraphPad Prism5.0 (GraphPad Software, San Diego, CA, USA). For in vitro assays, the significance of differences between control and treated cells was measured with the Student’s t test (P<0.05 was considered statistically significant).

Results

Ska1 is up-regulated in human osteosarcomas

To verify the microarray analysis results, we performed immunoblot experiments on human osteosarcoma specimens and their matched normal tissues. 30 tumor samples showed increased protein levels of Ska1 compared with their respective paired normal tissues (Figure 1). Interestingly, pAKT levels were also up-regulated in tumor samples while total AKT levels remained unchanged. Moreover, CyclinD1 and CylinB1 expressions in tumor samples were significantly higher than paired normal tissues. Combined with the expression of pAKT/AKT, CyclinD1/B1, these data implicated the clinical significance of SKA1-Akt-cyclinD1/cyclinB1 expression in osteosarcoma patients.

Ska1 promotes osteosarcoma cell lines growth in vitro

We then examined the biological function of Ska1 in osteosarcoma using a lentivirus-mediated knockdown and overexpression system. We performed immunoblot experiments on transfected cell lysates (Figure 2A). As shown in Figure 2B, cell apoptosis was slightly enhanced by Ska1 RNA interference in U2OS and Saos-2 cells, and it was significantly enhanced by administration of AKT inhibitor in U2OS and Saos-2 cells (Figures 2B, 3A). In addition, expression of Ska1 had the opposite effect on cell apoptosis.

To assess the impact of Ska1 combined with various AKT activities on migration capability of U2OS and Saos-2 cells was detected with Cell Migration assay (Costar Transwell Migration Plates). Although Ska1 knockdown attenuated the migration capability of U2OS and Saos-2 cells, Ska1 overexpression promoted clone formation in U2OS and Saos-2 cells because the number of soft agar clones was increased markedly (Figures 2C and 3B).

We further assessed the impact of Ska1 on cell invasion using 24-well BioCoat™ Matrigel chamber inserts assays. As shown in Figures 2D and 3C, TRIM59-short hairpin RNA (shRNA) transfection hindered U2OS and Saos-2 cells invasion, which could be recovered by AKT activator; whereas forced expression of Ska1 had the opposite effect on invasion, which could be attenuated by AKT inhibitor. These data were consistent with our finding that increased invasion is associated with a high degree of human osteosarcoma infiltration (Figure 1).

Ska1 promotes osteosarcoma cell lines growth in vivo

To verify the positive role of Ska1 in osteosarcoma progression in vivo, we performed xenograft tumor assays using U2OS and Saos-2
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Figure 2. Ska1 promotes osteosarcoma cell lines growth in vitro. A. Immunoblot shows overexpression and knockdown of Ska1 and the protein levels of pAKT/AKT in U2OS and Saos-2 cells. Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) is used as a loading control. B. Cell apoptosis analysis. C. Cell migration assay. D. Cell invasion assay.
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cells stably transfected by Ska1-shRNA lentiviruses plus AKT activator or Ska1-overexpression plus AKT inhibitor, respectively. We found that Ska1 knockdown plus AKT activator significantly inhibited xenograft tumor growth in nude mice (Figure 4). On the other hand, lentiviral expression of Ska1 plus AKT inhibitor resulted in accelerated xenograft tumor growth (Figure 4). These data collectively indicate that Ska1 acted as a novel tumor-promoting molecule and positively regulates osteosarcoma growth collaborating with AKT.

Discussion

Osteosarcoma is the most common malignant tumor of skeletal system in childhood. In many cases, surgery in combination with routine chemotherapy is the standard treatment. However, the 5-year survival rate of osteosarcoma patients is still low [1]. Although more and more progress in identifying various molecular targets has showed that gene-based target therapy is a novel approach, therapeutic effects remain unsatisfactory due to the uncontrollable gene expression level and effective regions [6]. Therefore, it’s necessary to develop novel approaches to treat patients diagnosed with OS.

In the present study, we investigated the functional association between Ska1, and osteosarcoma cell apoptosis, proliferation, migration, invasion and xenograft tumor growth in-
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Figure 4. Ska1 promotes osteosarcoma cell lines growth in vivo. Ska1 gene silencing by shRNA resulted in suppressed tumorigenicity of U2OS and Saos-2 cells in vivo. Six of 10 mice inoculated with lentivirus-infected cells form a tumor.

In conclusion, our results suggest that Ska1 expression is associated with OS patients, and correlated with the proliferation, migration, invasion and xenograft growth. Furthermore, Ska1 can promote OS progression by promoting OS cell migration and invasion through the Akt signaling pathway. Our research suggests Ska1 play an important role in the progression and metastasis of OS and Ska1, together with pAKT, might be a promising target for the treatment of OS.

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Disclosure of conflict of interest

None.

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