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SPOCK1 promotes the growth of Osteosarcoma cells through mTOR-S6K signaling pathway

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ABSTRACT

SPOCK1 belongs to the SPARC family, which plays an important role in proliferation, invasion and migration of various tumour cells. However, the functions of SPOCK1 in osteosarcoma cell growth and proliferation have not been fully elucidated. In the present study, we found that SPOCK1 is significantly upregulated in osteosarcoma tissue. Moreover, overexpression of SPOCK1 was associated with tumour size, metastasis, Enneking stage and pathological degree. Furthermore, knockdown of SPOCK1 expression suppressed the growth of osteosarcoma cells *in vitro* and reduced tumourigenicity in nude mice *in vivo*. Additionally, our data suggest that inactivation of the mTOR-S6 K signaling pathway participated in inhibition of SPOCK1-mediated suppression of osteosarcoma cell growth. These findings represent a novel pathogenetic mechanism of osteosarcoma development that provides a potential target for therapeutic strategies for osteosarcoma.

1. Introduction

Osteosarcoma (OS) is one of the most common malignant tumours in the skeletal system [1]. The 5-year survival rate for OS is less than 45% in China [3]. The majority of OS patients die of metastasis or invasiveness rather than primary tumour lesions [4]. Thus, the development of a method for the early diagnosis of OS and the development of an understanding of the molecular mechanism underlying the metastasis of OS is urgently needed.

SPOCK1 belongs to the SPARC family, which is an important gene because it encodes matricellular glycoproteins. SPOCK1 has been demonstrated to consist of a C-terminus, a follistatin-like domain, and an N-terminus [5–8]. Recent studies have confirmed that the amplification of the SPOCK1 mRNA is involved in cell apoptosis and metastasis and has been found in lung cancer, colorectal cancer, and prostate cancer [8–10]. However, little information concerning the function and mechanism by which SPOCK1 contributes to OS cell growth and proliferation is available.

The mTOR-S6 K signaling pathway is a well-conserved pathway that participates in the regulation of cell growth and proliferation. Mammalian target of rapamycin (mTOR) consists of two distinct multiproteins, i.e., mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Many studies have demonstrated that mTORC1 plays an important role in the promotion of cell proliferation [11]. It is increasingly appreciated that mTOR1 induces the proliferation and survival of cancer cells through the phosphorylation of S6 K, which is the target kinase of mTOR1, and the accumulation of many tumourigenic products. A previous study reported that the mTOR/S6 K signal transduction pathway contributes to the progression of disease in osteosarcoma patients [12]. The apoptosis of osteosarcoma cells can be elicited through the inhibition of the Akt/mTOR/S6 K pathway [13]. It seems that the mTOR/S6 K signaling pathway may mediate the anti-apoptotic and metastatic properties of OS cells.

In the present study, we found that the expression level of SPOCK1 was upregulated in OS tissues. Moreover, SPOCK1 depletion effectively inhibited cell proliferation in *vitro* and suppressed tumour growth in *vivo*. More importantly, we also noted that the mTOR-S6 K signaling pathway was involved in the growth of SPOCK1 influenced OS cells. These findings indicate a novel pathogenesis of OS that provides a potential target for therapeutic strategies for OS.

2. Materials and methods

2.1. Specimen collection

OS samples and normal peritumour bone tissues were collected from eighty OS patients who underwent surgical treatment at the Liaoning Cancer Hospital from July 2009 to March 2011. None of these patients had received prior chemotherapy or radiotherapy. All of the patients provided informed consent prior to surgical treatment, and the study

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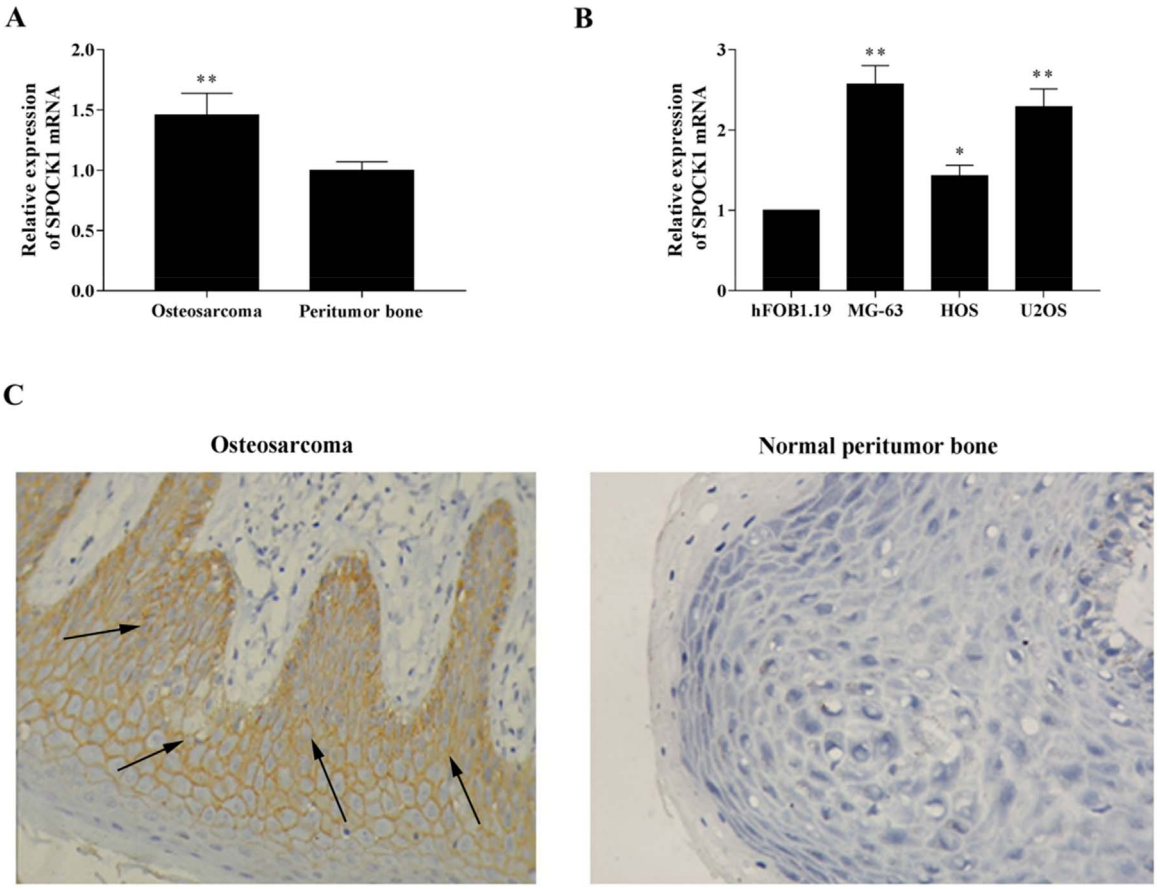

Fig. 1. Up-regulation of SPOCK1 in OS clinical specimens. (A) The expression of SPOCK1 in 80 pairs of OS tissue samples determined by qRT-PCR. (B) The expression of SPOCK1 in hFOB1.19, MG-63, HOS and U2OS cells determined by qRT-PCR. (C) The expression of SPOCK1 in 80 pairs of OS tissue samples determined by immunohistochemistry assay (indicated by arrows). $^{*}P < 0.05$, $^{**}P < 0.01$. Data are expressed as mean ± SD. All experiments were performed at least three times.

was approved by the Committees for Ethical Review of Research Involving Human Subjects at the Liaoning Cancer Hospital. Resected tumour and normal peritumour bone tissue were collected and immediately stored in liquid nitrogen. The grades and histological types of the samples were independently confirmed by two professional pathologists. The clinical stages of the osteosarcoma cases were graded according to the Enneking criteria [14] as follows: I, low grade; II, high grade; and III, the presence of metastases. The pathological degrees of osteosarcomas were graded according to the standard of Price's grade [15] as follows: G_1, low-grade malignancy; G_2,high-grade malignancy.

2.2. Cell lines and culture

The immortalized human osteoblast cell line hFOB1.19 and three other OS cell lines i.e., MG-63, HOS and U2OS, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at 37 °C in 5% humidified CO_2. The MG-63, HOS and U2OS cell were maintained in the medium and passaged 2–3 days.

2.3. Quantitative real time quantitative PCR

Total RNA extraction and quantitative real-time PCR were performed as previously described [10]. Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, CA), and 0.5 μg total RNA was reverse transcribed with a PrimeScript RT reagent kit (Takara Bio, Tokyo, Japan) according to the manufacturer's instructions. The quantification of the mRNA was performed using SYBR Premix Ex Taq (Takara Bio, Tokyo, Japan) and a StepOne PCR system (Applied Biosystems, UK). The relative expression of the target gene was normalized to GAPDH using the $^{\triangle\triangle}$Ct comparative method. The specific forward and reverse primers were as following: SPOCK1, F: 5′-ATGCCTGTAACAAAGAAGCCCC-3′, R: 5′-CACACAGTTGCCCCCGTTTTTAC-3′; s6 K, F: 5′-AAGGGGGCTATGGAAAGGCAA-3′, R: 5′- AATCCACGATGAAGGGATGCT-3′; mTOR, F: 5′-GAACCTCAGGGCAAGATGCT-3′, R: 5′-CTGGTTTCCTCATTCCGGCT-3′. GAPDH F: 5′-CACCATTGGCAATGAGGGGTTC-3′, R: 5′-AGGTCTTTGCGGATGTCCACGT-3′.

2.4. Western blot

Total proteins were extracted and determined by BCA protein assay (Beyotime, Beijing, China). Western blotting was performed as described previously [8]. The antibodies were employed as following: SPOCK1 (1:100; BOSTER, Wuhan, China), mTOR (1:1000; Beyotim, Haimen, China), *p*-mTOR (1:200; Bioworld, Nanjing, China), S6 K and *p*-S6 K (1:200; Bioworld, Nanjing, China), GAPDH (1:200; Sangon, Shanghai, China). The chemiluminescence reagent was obtained from Millipore (Danvers, MA, USA).

2.5. Immunohistochemistry staining

Immunohistochemical staining was performed with an Envision Plus System according to the manufacturer's protocol. The staining for SPOCK1 in the samples was examined in a blinded manner; the cell staining intensity and proportion of positive cells were examined in three randomly selected fields, according to the immunoreactive score (IRS), IHC staining was evaluated by cell staining intensity and the