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Original Article

Overexpression of S100A1 in Osteosarcoma Inhibits Tumor Proliferation and Progression

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Abstract

Background: Osteosarcoma is the most common primary malignant tumor of bone. Abnormal expression of S100A1 protein is closely related to the occurrence and development of malignant tumors. However, S100A1 in osteosarcoma has not been studied.

Methods: All osteosarcoma tissues were collected from patients who received surgical therapy at the Affiliated Hospital of Inner Mongolia Medical University, China in 2020. QRT-PCR and western blot assays were used to detect the expression of S100A1 in osteosarcoma tissues and cells. The negative effect of S100A1 on osteosarcoma cell growth was confirmed by vitro and vivo experiments.

Results: S100A1 inhibited the growth of osteosarcoma cells in vitro. Overexpression of S100A1 may inhibit the proliferation of osteosarcoma cells by preventing the activation of AKT signaling pathway by western blot assay. Finally, animal experiments confirmed that overexpression of S100A1 could inhibit the proliferation of osteosarcoma cells. Overexpression of S100A1 obtained better survival benefit in mice.

Conclusion: Our findings provided a new insight to the treatment of osteosarcoma. It also raised the possibility that S100A1 could be used in targeted therapies for osteosarcoma.

Keywords: S100A1 protein; Osteosarcoma; Proliferation; Progression

Introduction

Osteosarcoma is the most common primary malignant tumor of bone. The primary site is mostly located in the epiphysis of the tibia, fibula, femur and humerus Ritter J Bielack SS (1). Osteosarcomas are highly malignant and invade surrounding soft tissues with distant metastasis. Clinically, osteosarcomas metastasize to the lung and other bone pathways, and some metastasize to lymph nodes and other organs in the abdominal cavity. Osteosarcoma occurs in adolescence and is the second leading cause of death in adolescents (2). In addition to an extremely high fatality rate, osteosarcomas have an extremely high disability rate. Even after systematic treatment, the disability rate of osteosarcoma remains high, and its prognosis has not been improved. This results in

a poor quality of life for patients and has a profound impact on their families and society (3). At present, the main treatment for osteosarcoma is surgery, accompanied by radiotherapy and chemotherapy. However, the clinical prognosis of patients with osteosarcoma has not been substantially improved in recent decades due to its high degree of malignancy, strong aggressiveness and easy early metastasis (4). Therefore, molecular targeted therapy has become the focus of current research.

The S100 calcium binding protein family is a small group of acidic calcium ion binding proteins that were first isolated from bovine brain tissue in 1965 (5). It is characterized by an EF hand structure, which is typically arranged in a helico-ring-helix pattern. Similar to human thumb and index finger in spatial arrangement, this structure can specifically bind to Ca2+ and undergo conformational changes, thus interacting with target proteins and participating in a variety of physiological and pathological processes in human body (6). The dysregulation of expression of multiple members of the S100 protein family is a common feature of human cancers (7). Multiple members of the S100 protein family have been proven to be used in early diagnosis, prediction of prognosis, determination of treatment options and detection of therapeutic effects(8). S100A1 protein is a member of the S100 protein family (9). Abnormal expression of S100A1 protein is closely related to the occurrence and development of malignant tumors under pathological conditions such as the occurrence of malignant tumors. For example, the expression of S100A1 is significantly increased in ovarian cancer tissues. Moreover, the overexpression of S100A1 enhanced the proliferation and migration of ovarian cancer cells (10). S100A1 is involved in the development and progression of a variety of diseases, such as endochondral ossification during skeletal development (11), cardiovascular disease (12), hepatocellular carcinoma (13), and chronic obstructive pulmonary disease (14). However, S100A1 in osteosarcoma has not been studied.

As a key regulator of cell survival under stress, the PI3K/Akt/mTOR signaling pathway is crucial for the growth and proliferation of tumor cells (15). The PI3K/Akt/mT0R signaling pathway is abnormally activated in a variety of cancer types, so the related proteins of this pathway can be used as a potential target for cancer therapy (16). The occurrence and development of osteosarcoma is related to the abnormality of PI3K/Akt/mTOR signaling pathway (17).

We aimed to study the role of S100A1 protein in osteosarcoma.

Materials and Methods

Tissues

All osteosarcoma samples were taken from patients of various grades of osteosarcoma treated at Affiliated Hospital of Inner Mongolia Medical University (Inner Mongolia, China) in 2020 and their consent was obtained.

This experiment was approved by the Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University (Inner Mongolia, China) in 2020.

Cell Lines and culture conditions

Both human osteosarcoma cell lines, MG-63 (CLS Cat# 300441/p2938_MG-63, RRID: CVCL_0426) and U-2 OS (CLS Cat# 300364/p489_U-2_OS, RRID: CVCL_0042), were purchased from ATCC (American Type Culture Collection). The reagents used in the cell culture were all from Gibco (USA). The RPMI-1640 medium was consumed to culture MG-63 cells and McCoy's 5A medium was for U-2 OS cells. Both types of cells require 10% FBS (Fetal bovine serum) for culture. 1% penicillin/streptomycin is also added. All cells were grown in an incubator at a constant temperature (37 ° C) and humidity (70-80%), with a CO2 concentration of 5%.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using the Trizol reagent (Invitrogen, USA). The reverse transcription kit

(Invitrogen, USA) was used to reverse transcribe RNA into cDNA (complementary DNA). Real-time PCR procedure was operated on a Roche LightCycler 96 Real-Time PCR System with Applied Biosystems™ SYBR™ Green (ThermoFisher Scientific, USA). The primer sequences used are as follows: S100A1-F:5'-CCGCTCGAGAGCAGCCACATTT-GCAACCT-3';S100A1-R:5'-CGCGGATCCGCTGGTGAGAGA-3'.Akt-F:5'-TTGCTATGCCACAGTCAGGA-3', Akt-R:5'-AACAACCATCTCCGGCCTTC-3'.p-Akt(S473)-F:5'-CATGA-GAAGTATGACAACAGCCT-3',p-Akt(S473)-R:5'-AGTCCTTCCACGATACCAAAGT-3'.p-Akt(T308)-F:5'-AGCGGCGCATCTATGACATC-3',p-Akt(T308)-R:5'-GTCAACCCCTCAAGCCGTC-3',Akt-F:5'-AGCCAGATGACGACCCCAT-3'.Akt-R:5'-TGGCTCTTTCTCTGTCCAGT-3'.p-Erk-F:5'-ACGTCCCCAAGCCAGGCTC-3'Erk-R:5'-CTACTGTAGCTCAGGAATAA-3.

Western blot

Total protein of the cells was extracted and the protein concentration was determined (BCA Protein Assay Kit, Abcam, USA 6). Then the protein products were subjected to SDS-PAGE (polyacrylamide gel electrophoresis). After electrophoresis, the target bands were transferred to the PVDF (Polyvinylidene fluoride) membrane (ThermoFisher Scientific, USA) at 4 °C. After transferring, placed the membrane in 3% BSA (Bovine serum protein) (Sigma, USA) for blocking at room temperature for 2 hours. Then wash the membrane for 4 times with TBST (Sigma, USA), 5 minutes each time. Add the corresponding primary antibody (LSBio (LifeSpan) Cat# LS-C83526-200, RRID: AB_1652493) and incubate overnight at 4°C. Wash with TBST 4 times, 5 minutes each time. The corresponding secondary antibody (Thermo Fisher Scientific Cat# 618600, RRID: AB_11180737) was added and incubated at room temperature for 2 hours. Wash 4 times with TBST on a decolorizing shaker at room temperature for 5 minutes each time. Finally, the ECL reagent (ThermoFisher Scientific, USA) was added, and the target strip was developed and photographed. The antibody information used in this paper is as follows: S100A1 (abcam, ab109252), GAPDH(abcam, ab9484), p-ERK(Cell Signaling,#4376), ERK(Cell Signaling,#5013), p-Akt(S473)(Cell Signaling,#4070), p-Akt(T308)(Cell Signaling,#86758), Akt(abcam, ab18785).

Cell Counting Kit-8 (CCK 8) assay

The logarithmic phase cells were collected and the concentration was adjusted. Overall, 3000 cells per well were planted in a 96-well plate and drug was added after the cells adhered to the wall. Then the 96-well plate was incubated in 5% CO_2 at 37℃ for a certain time. Add 10μl CCK-8 solution (Abcam, USA) to each well, and cook for 1 hour. The 96-well plate was removed and placed on an enzyme-linked immunoassay to measure the absorbance at 450 nm.

Cell clone formation assay

The monolayer cells of logarithmic growth phase were digested with 0.25% trypsin and blown into individual cells. The cells were suspended in the medium of 10% fetal bovine serum for later use. The cell suspension was divided into 100 cells in each dish with gradient density and inoculated into dishes containing 10mL of pre-heated culture solution at 37 ℃, respectively, and gently rotated to make the cells evenly dispersed. Place in the incubator for 2 weeks. When visual clones appeared in the dish, the culture was discontinued. Abandon the supernatant and carefully soak with PBS for 2 times. Add pure methanol and fix for 15 minutes. Then discard the fixed solution, add an appropriate amount of GIEMSA (Invitrogen, USA) and dye the solution for 30 minutes. Then wash the solution with running water slowly and dry it in the air. Finally, the number of clones greater than 10 cells was counted under a microscope (at low power).

Animal experiment

The Athymic BALB/C nude mice weighing 18-22 g aged 4-6 weeks were used for this experiment. The orthotopic tumor cell injection was performed as previously described (18). The suspension of both MG-63-OE S100A1 cells and (MG-63-OEC cells (25μL, about 1×10^5 cells) were injected into the upper medullary cavity of the tibia of nude mice. Each group contained 20 mice. The 4 mice of each group were sacrificed 28 days, the tumors were removed, weighed, fixed. The remaining mice were kept until they died of natural causes. Our animal experiments complied with the animal experiment ethics regulations (approval number NO.YKD20201214).

Statistical analysis

The data was analyzed by GraphPad Prism 8.0 statistical software (GraphPad Prism, RRID: SCR_002798). The expressed form of data was as mean ± standard deviation (SD). The differences between two groups were analyzed by Student's *t*-test, and $P < 0.05$ indicated statistical significance. The sample sizes were selected by the standard method to reduce the error. The data shown were the mean ± SD of the results of three independent experiments, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Results

S100A1 is low expressed in osteosarcoma tissues and cell lines

The results of qRT-PCR assay showed that the mRNA level of S100A1 decreased significantly in osteosarcoma, and the decrease became more obvious with the increase of malignant degree (* $P < 0.05$, ** $P<0.01$, *** $P<0.001$, Fig. 1A). In addition, we selected two osteosarcoma cell lines, MG63 and U-2 OS to detect the expression of S100A1. The qRT-PCR detection showed the expression level of S100A1 in osteosarcoma cell lines was down-regulated (** $P<0.01$, Fig. 1B). Meanwhile, we detected the expression of S100A protein in human osteosarcoma tissues by Western blot analysis, and the results showed that S100A1 protein expression was decreased in osteosarcoma tissues (*** $P<0.001$, Fig. 1C). S100A protein expression in normal osteoblasts was also higher than that in osteosarcoma cells (*** $P<0.001$, Fig. 1D). Immunohistochemistry of corresponding tissues also showed that the expression level of S100A1 was correlated with the degree of malignancy of osteosarcoma (Fig. 1E). These results suggest that S100A1 is highly expressed in normal human bone tissues, but is low in osteosarcoma. This phenomenon suggests that S100A1 may affect the occurrence and development of osteosarcoma.

S100A1 inhibits the proliferation of osteosarcoma cells

The transfection efficiency was successful (*** $P<0.001$, Fig. 2A). We then verified the effect of S100A1 on the proliferation of osteosarcoma cells. CCK8 assay showed that the proliferation rate of osteosarcoma was slowed down after S100A1 expression was overexpressed (*** $P<0.001$, Fig. 2B). Clonal formation assay indicated similar results, that is, the proliferation of osteosarcoma cells was significantly suppressed when S100A1 was in the high expression level (*** $P<0.001$, Fig. 2C). These results suggest that S100A1 may play a negative role in the proliferation of osteosarcoma cells.

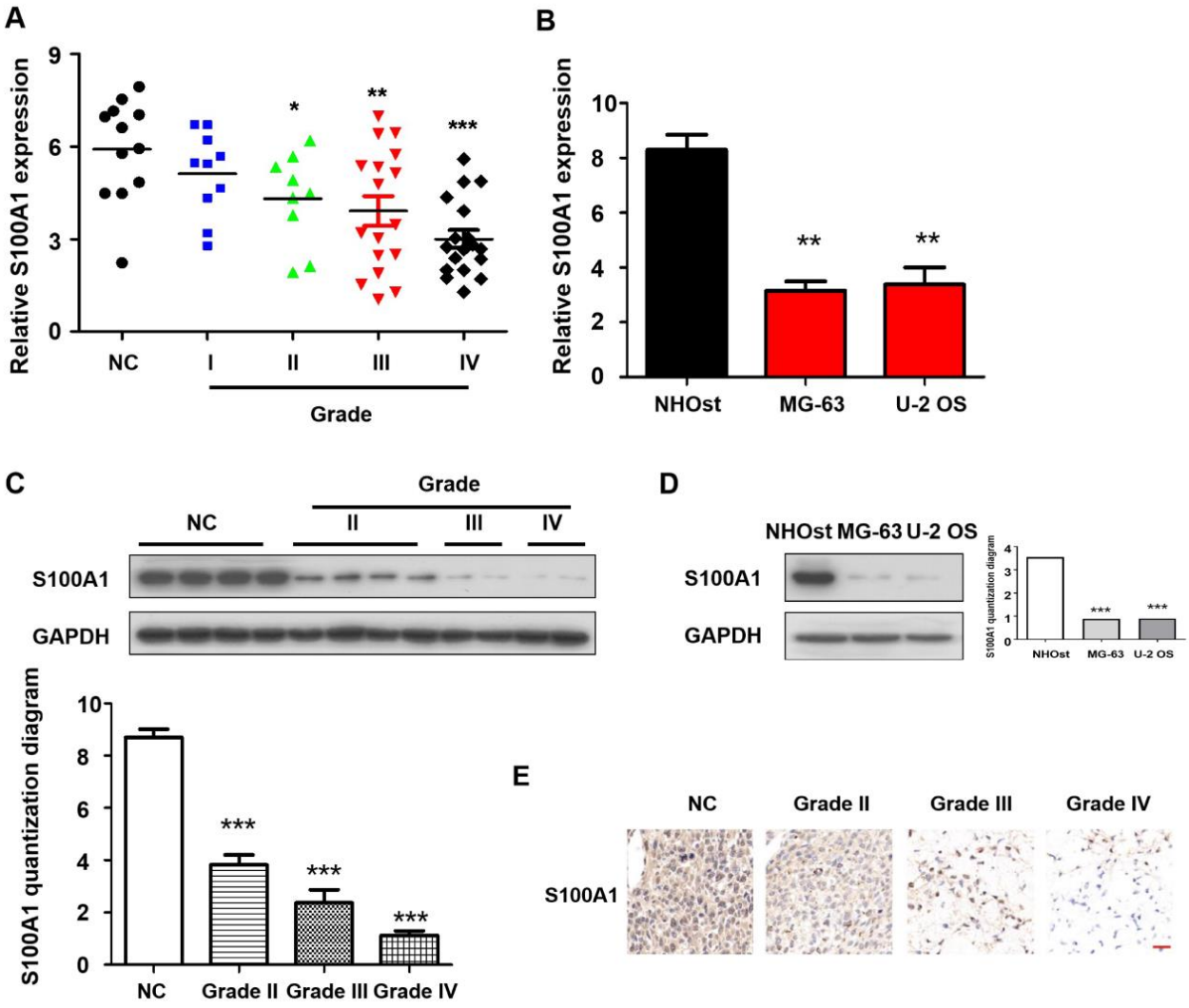

Fig. 1: Low expression of S100A1 was observed in osteosarcoma tissue and cell lines. A.QRT-PCR assay examined the expression of S100A1 in different grades of osteosarcoma tissues and normal bone tissues. B. The expression level of S100A1 in human osteosarcoma cells (MG-63, U-2 OS cells) and control cells was measured by qRT-PCR assay. C. Western blot analysis analyzed the expression of S100A1 in different grades of osteosarcoma tissues and normal bone tissues. D. The expression of S100A1 in human osteosarcoma cells (MG-63, U-2 OS cells) and normal human osteoblasts cells (NHOst) were measured by western blot analysis. E. Immunohistochemical analysis analyzed the expression of S100A1 in different grades of osteosarcoma tissues and normal bone tissues. **P* < 0.05, ***P*<0.01, ****P*<0.001

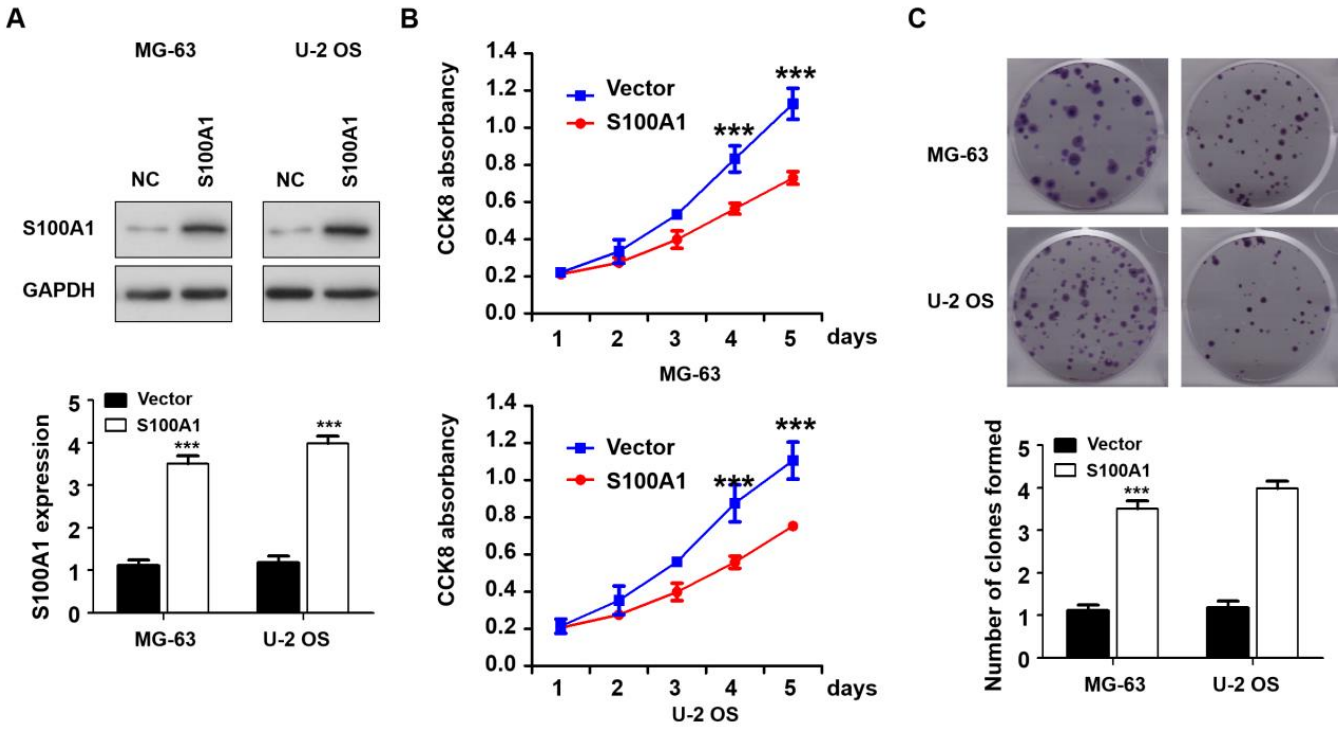

Fig. 2: Overexpression of S100A1 inhibits the growth of osteosarcoma. The overexpression plasmids of S100A1 were transfected into human osteosarcoma cells (MG-63, U-2 OS cells). A. Western blot analysis detected the transfection efficiency and the gray value was calculated and quantified. B. CCK8 assay examined the effect of overexpression of S100A1 on proliferation of human osteosarcoma cells. C. Clone formation assay confirmed the effect of overexpression of S100A1 on growth of human osteosarcoma cells. ****P*<0.001

Overexpression of S100A1 blocks the Akt pathway

Altering the expression of S100A1 could affect the activation level of the Akt pathway. As shown in Fig. 3A, after the overexpression of S100A1, the total amount of Akt remained unchanged, while the phosphorylation of Akt at both sites (S473 and T308) was significantly decreased, and accordingly, the phosphorylation level of ERK was also significantly decreased (****P*<0.001). The quantitative analysis was shown (Fig. 3B). Dihydrocapsaicin(DHC), an AKT pathway activator, was used to determine the effect of S100A1 on osteosarcoma due to phosphorylation of Akt pathway. As shown in Fig. 3C, the inhibitory effect of S100A1 overexpression on osteosarcoma cells was reversed by DHC (****P*<0.001). Therefore, we believe that the overexpression of S100A1 might dephosphorylate the two-phosphorylation sites in the Akt signaling pathway and inhibit the proliferation of osteosarcoma cells.

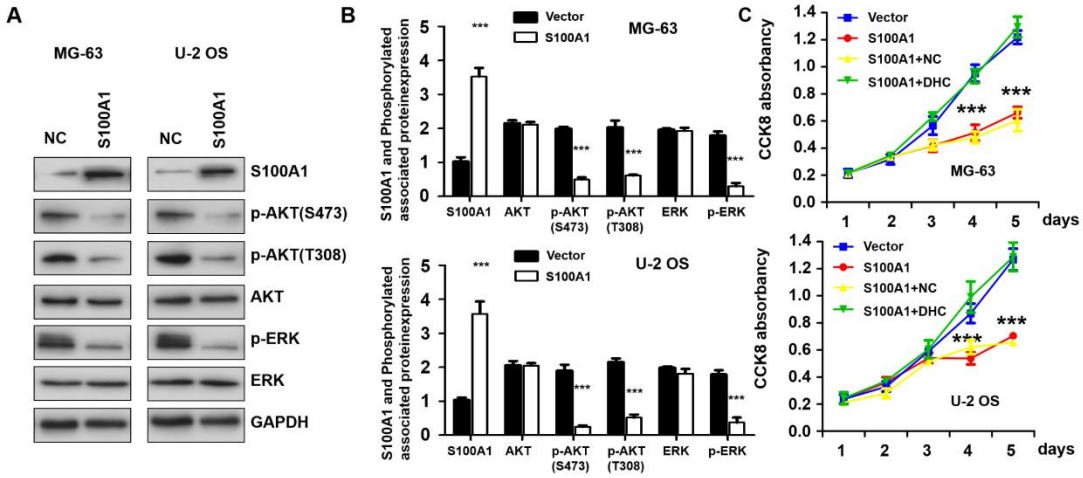

Fig. 3: S100A1 inhibited the proliferation of osteosarcoma cells by preventing the activation of Akt signaling pathway. A. Western blot analysis assessed the effect of overexpression of S100A1 on activation of the Akt pathway. B. Quantitative analysis of western blot analysis. GAPDH as an internal reference.C. CCK8 assay examined the effect of overexpression of S100A1with or not with DHC on proliferation of human osteosarcoma cells. ***P<0.001

Overexpression of S100A1 inhibited the proliferation of osteosarcoma in vivo and obtained better survival benefit

MG-63 cell lines with stable S100A1 overexpression (MG-63-OE S100A1) and corresponding control cells (MG-63-OEC) were established (Fig. 4A). The tumor volume of mice overexpressing S100A1 was significantly smaller than that of the control group (***P<0.001, Fig. 4A). S100A1 was highly expressed in osteosarcoma tissues (Fig. 4B). In addition, the S100A1 overexpressed group had a longer survival time than the control group (Fig. 4C). To sum up, overexpression of S100A1 inhibited the growth of osteosarcoma cells in vivo.

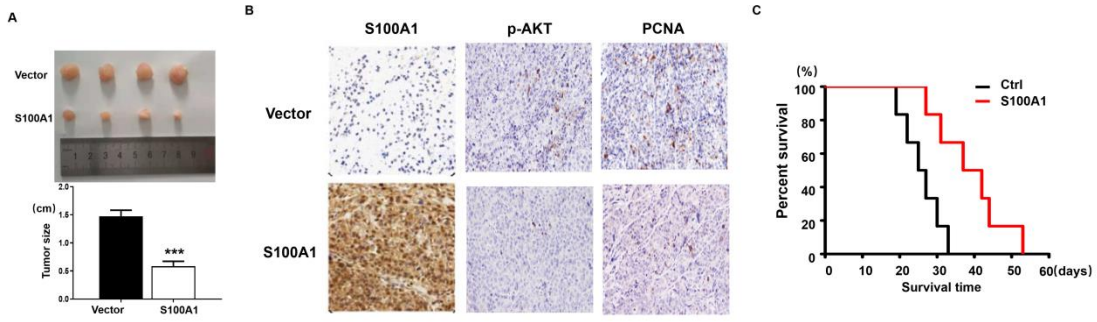

Fig. 4: Overexpression of S100A1 inhibited the growth of osteosarcoma in vivo and resulted in longer survival. A. The volume of osteosarcoma in mice which were sacrificed after 8 weeks of feeding. On the right is the quantitative analysis. B. Immunohistochemical experiments detection the expression of S100A1, p-Akt and PCNA in osteosarcoma tissue and normal bone tissue of mice. C. Survival curves of mice injected with overexpressed S100A1 and control group. ***P<0.001

Discussion

Osteosarcoma is a malignant primary tumor with a high incidence in adolescents (2). In the development of osteosarcoma, accompanied by chromosomal increase or deletion, some tumor suppressor genes are mutated and genetically modified. Despite advanced strategies such as surgery,

adjuvant chemotherapy, and radiation therapy, the prognosis of osteosarcoma remains poor (4). At present, the pathogenesis of osteosarcoma has not been fully understood. Therefore, the study of methods related to the diagnosis, treatment and prognosis of osteosarcoma is beneficial to improve the survival rate of patients. Molecular targeted therapy has become the focus of current research.

S100A1 has been implicated in the differentiation of human articular chondrocytes (19). This protein is uniformly expressed in all chondrocyte regions. Its expression is reduced during chondrocyte dedifferentiation and can be induced under conditions that promote redifferentiation (19, 20). Although S100 proteins have a wide range of extracellular and intracellular roles, functional studies of S1001 proteins expressed in chondrocytes have focused on their extracellular roles in relation to catabolic processes. The role of S100A1 in chondrocytes has not been well explored, but studies on its intracellular activity suggest that S100A1 may play an important role in chondrocyte biology (20). In addition, it has been reported that S100A1 interacts with a variety of calcium regulatory proteins to promote calcium circulation (21). S100A1 plays an important role in energy balance, myofilament gliding, myofilament calcium sensitivity, actin interaction, apoptosis, and cardiac remodeling (22). There is no change in S100A1 expression in normal or cancerous cervix, myometrium, endometrium, uterus, intestine, colon, liver, or lung. However, it was highly expressed in normal skeletal muscle and normal and diseased thyroid (10). S100A1 can affect the p53-MDM2 interaction, thereby blocking the proliferation of cancer cells (23). Abnormal expression of S100A1 protein is closely related to the occurrence and development of malignant tumors under pathological conditions such as the occurrence of malignant tumors. For example, the expression of S100A1 is significantly increased in ovarian cancer tissues, and the overexpression of S100A1 enhances the proliferation and migration of ovarian cancer cells (10). However, S100A1 in osteosarcoma has not been studied.

The content of S100A1 decreased significantly in osteosarcoma compared to normal bone tissue, and the decrease became more obvious with the increase of malignant degree. In addition, we selected two osteosarcoma cell lines, MG63 and U-2 OS for further research. QRT-PCR was used to detect the expression of S100A1 in normal bone tissue and two kinds of osteosarcoma cells, and the results were consistent with the former. Serine/threonine kinase Akt (also known as protein kinase B or PKB), a proto-oncogene, has become a major focus of attention in the medical community because of its important role in regulating a variety of cellular functions, including metabolism, growth, proliferation, survival, transcription, and protein synthesis (24). Besides, S100A1 inhibited the growth of osteosarcoma cells in vitro. We found that Overexpression of S100A1 might inhibit the proliferation of osteosarcoma cells by preventing the activation of AKT signaling pathway by western blot assay. Finally, animal experiments confirmed that overexpression of S100A1 could inhibit the proliferation of osteosarcoma cells. Overexpression of S100A1 obtained better survival benefit in mice. These results provided a new insight to the treatment of osteosarcoma. These experimental results further confirm our conjecture. S100A1 might play a negative role in the pathogenesis of osteosarcoma, and it may become a new therapeutic target for osteosarcoma.

However, our study still has some limitations. We have only preliminarily explored the mechanism of S100A1 inhibiting the growth of osteosarcoma cells and further studies are needed to explore further the mechanism. In addition, whether S100A1 also has an inhibitory effect on the progression of osteosarcoma remains to be further explored. We are looking forward to more data to support our research.

Conclusion

S100A1 might play a suppressive role in osteosarcoma by preventing the activation of Akt signaling pathway. Our findings provided a new in-

sight to the treatment of osteosarcoma. It also raised the possibility that S100A1 could be used in targeted therapies for osteosarcoma.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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