

Original Article

Long-chain non-coding LSAMP-AS3 targets modulation of Mir-200a invasion and proliferation of osteosarcoma Via MAPK pathway

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Received March 20, 2017; Accepted November 5, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Objective: To investigate the expression of LSAMP-AS3 in osteosarcoma and the role of LSAMP-AS3 in the process of invasion and migration of osteosarcoma cells and its mechanism. Methods: The expression of LSAMP-AS3 in osteosarcoma and adjacent tissues and different osteosarcoma cells was detected by qPCR. Transwell invasion test was used to detect the invasion ability of osteosarcoma cells after silencing LSAMP-AS3. The interaction of LSAMP-AS3 with miR-200a was detected by double luciferase reporter gene; QPCR was used to detect the expression of miR-200a in osteosarcoma and adjacent tissues and different osteosarcoma cells; Transwell invasion assay was used to detect the effect of miR-200a on the invasion of osteosarcoma cells after silencing LSAMP-AS3; The effect of miR-200a on the size and volume of osteosarcoma after silencing LSAMP-AS3 was detected by subcutaneous tumor formation in nude mice; Western blotting was used to detect the expression of Notch pathway protein after silencing of LSAMP-AS3. Results: The expression of LSAMP-AS3 was significantly increased in osteosarcoma tissue compared with adjacent tissues, and the expression level of LSAMP-AS3 in osteosarcoma cells was the highest. Silencing LSAMP-AS3 could inhibit the invasion and migration of osteosarcoma cells. LSAMP-AS3 could specifically bind to 3'UTR of miR-200a; The expression of miR-200a in osteosarcoma tissue was significantly decreased compared with adjacent tissues; Inhibition of miR-200a could promote osteosarcoma cell invasion and migration ability after silencing LSAMP-AS3; The tumor volume and weight of LSAMP-AS3-siRNA+miR-200a-inhibitor group were significantly increased compared with LSAMP-AS3-siRNA group. The expression of MAPK pathway protein was down-regulated after silencing LSAMP-AS3. Conclusion: LSAMP-AS3 plays an important role in the development of osteosarcoma. LSAMP-AS3 can regulate the invasion and migration of osteosarcoma cells through MAPK signaling pathway.

Keywords: LSAMP-AS3, osteosarcoma, MiR-200a, transwell

Introduction

Osteosarcoma is one of the most common urological tumors in the world. According to the WHO International Agency for Cancer Research (Agency for Research on Cancer, IARC), osteosarcoma ranks seventh in male malignancies and ranks eleventh in female malignancies [1]. The most important treatment program is still surgical treatment program at present, but the treatment of advanced osteosarcoma is still ineffective [2]. Postoperative radiotherapy can only be a certain degree of improvement in the quality of life of patients. It is very important to investigate the molecular biological mecha-

nism of the occurrence and development of osteosarcoma with the development of tumor molecular biology, which may be helpful for the diagnosis and treatment of osteosarcoma.

MicroRNA (miRNAs) is an endogenous single-stranded small molecule RNA that regulates protein expression by inhibiting or degrading messenger RNA [3]. MiRNA plays an important role in the diagnosis, treatment and prognosis of tumors [4]. MiR-200a is a relatively small investigation in the field of miRNA, some investigations have shown that the expression of pancreatic cancer has a certain specificity, it has a certain anti tumor effect in glioma [5].

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Recent investigations have shown that the expression level of miR-200a in liver cancer, breast cancer and other malignant tumors may be down regulated, which may play an important role in inhibiting the invasion and migration of some tumors [6].

Mitogen-activated protein kinase (MAPK) signal transduction pathway is also known as extracellular signal-regulated kinase (ERK) cascade pathway [7]. The structure of MAPK family members is highly conserved, which plays an important role in cell proliferation and differentiation, and plays a different role in the development and development of different tumors. The pathway is the cell surface of the stimulus signal to the inside of the nucleus and mediate cell response to the signal transduction pathway in the most important one transmission pathway, such as growth and development, differentiation, division and apoptosis are subject to MAPK signal transduction pathway regulation [8]. This research intends to analyze the expression of LSAMP-AS3 in osteosarcoma, and to further investigate the interaction between LSAMP-AS3 and miR-200a, and to investigate the role and mechanism of MAPK pathway in the migration and invasion of osteosarcoma.

Materials and methods

Samples collection

30 cases of osteosarcoma patients and 30 cases of adjacent normal tissues patients were collected admitted to our hospital from March 2015 to May 2016. All patients had no chemotherapy or radiotherapy before surgery, pathological sections were confirmed by two pathologists, the tumor tissue was put into RNA preservation solution quickly.

Cell lines

Human cells OS-U2, MG63, Saos-2, HOS were purchased from Shanghai Zhongshan cell bank, which were cultured and passaged in DMEM 37°C, 5% CO₂ culture medium containing of 10% fetal bovine serum. Fetal bovine serum, RPMI 1640 medium were purchased from Hyclone Corporation (Hyclone, Logan, UT). Transwell chamber was purchased from Millipore (Millipore, Billerica, MA), Matrigel was purchased from Bio-Rad (Bio-Rad, Madrid,

Spain). Lipofectamine 2000, miR-200a-inhibitor was purchased from (Gnenpharma Co., Shanghai, China). Trierol was purchased from Ambion (Ambion Inc., Austin, TX, USA), reverse transcription kit (FSQ-101) was purchased from Japan TOYOBO Corporation (TOYOBO, FSQ-101, Japan), PCR kit was purchased from Sigma (KapaBiosystems Inc., Boston, US). The luciferase activity assay kit was purchased from Promega Company (Promega Biotech Co., Beijing, China). The luciferase reporter vector was synthesized by Promega Corporation (Promega Biotech Co., Beijing, China).

Quantitative real-time polymerase chain reaction

Detection of LSAMP-AS3 expression by qPCR method: Different groups of cells were inoculated in the culture flask, the inoculation density was $1 \times 10^6/L$, and the total RNA was extracted according to Trizol instructions after 36 hours of culture. The concentration and purity of the nucleic acid were detected by ultraviolet spectrophotometer. The total RNA was diluted to the same concentration with DEPC, the reagents were added as described, and the cDNA was stored at -20°C after completion of the reaction. The reaction system was prepared according to the operating instructions of the American Sigma Quantitative Kit (GoTaq @ qPCRMaster Mix). The volume of each reaction system was 20 μ L, and each of the three wells was set. Each group of cDNAs was required to amplify the target RNA and GAPDH fluorescence quantitative PCR. The reaction conditions were as follows according to the $\Delta\Delta$ CT method: 37°C 15 min, 98°C 5 min. PCR was performed according to the PCR kit instructions to obtain the mRNA expression level with $RQ = 2^{-\Delta\Delta CT}$.

Cell transfection

The MOI value was set at 0, 20, 50, 200, the ratio of fluorescent cells was observed, the MOI value with the highest percentage of fluorescence cells was selected and the minimum MOI value was set to the best MOI value, and the best value was 50. The cells were cultured in 24-well plates at 36 h before transfection, and the cell density reached 50% to 80% at the second day. The virus was diluted by the culture medium according to the best MOI value after it is frozen on ice, a final concentra-

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tion of 5 ng/mL of polybrene was added, gently mixed and added to the cells, LSAMP-AS3-siRNA and negative control group were transfected into cells; The siRNA sequence of LSAMP-AS3 is: 5'-CAGAGCTCTCTCCCCCTC-CTC-3'; The negative control sequence is: 5'-CAGCACGCCGAGAGACGCACAG-3'. The miR-200a-inhibitor and negative control cells were transfected according to the Lipofectamine 2000 transfection kit. The complete medium was replaced after incubation in the incubator for 12 h. The fluorescent cells were observed under fluorescence microscope after the virus was infected for 24 h.

Transwell invasion assays

The two transwell invasion chambers with Matrigel (1 mg/ml) (Becton-Dickinson, New Jersey, USA) were used in assay invasion assays of cells in vitro. Firstly, 200 μ l serum-free medium containing 1×10^5 cells/well was added into the upper chamber, and the lower chamber contained 0.6 ml medium containing 20% FBS. After incubation at 37°C for 24 h, non-invading cells on the upper membranes were removed with a cotton swab. The migrated or invaded cells were fixed in 95% ethanol, stained with hematoxylin. The cell numbers were counted by ImageJ software and photographed under an inverted microscope on 10 random fields in each well. Each experiment was independently repeated in triplicate.

Colony formation assay

The cell density was adjusted to 5×10^5 cells/ml, inoculated into 6-well plates, placed in 5% CO₂ incubator overnight, planted in the plate, keep the cells as much as possible alone suspended. Serum-free media was added and placed in 5% CO₂ at 37°C incubator for incubation every two days. Followed the time point in the test design and took a picture under the microscope. Measured the size of cells into groups and took pictures to calculate the cell proliferation rate.

Luciferase activity assays

The luciferase reporter vector was co-transfected with LSAMP-AS3-siRNA to HOS cells. The transfected pRL-TK was used as standard internal control. The cells were harvested after transfection for 36 h. The luciferase activity of

HOS cells was detected by Promega's luciferase activity assay kit. Calculate relative luciferase activity = firefly luciferase activity value/bloody luciferase activity value.

Osteosarcoma xenografts

Nude mice were selected from 4 to 6 weeks old to establish a tumor model. Logarithmic growth of osteosarcoma LSAMP-AS3-siRNA cells and LSAMP-AS3-siRNA+miR-200a-inhibitor cells were selected. The cell concentration was adjusted to 2×10^8 /ml. 0.1 ml cell suspension was injected in each nude mouse left forelimb armpit subcutaneous, a total of 10. The survival, weight, and survival status of the mice were monitored, and the size and weight of the tumor in the immediately-dead mice were measured within 4 weeks after injection.

Statistical analysis

SPSS 13.0 software was used for statistical analysis. The data were expressed as mean \pm standard deviation, metrological data were analyzed by one-way ANOVA, variances were handled with the Karuskal-wallis method. The difference was considered statistically significant at $P < 0.05$.

Results

Increased expression of LSAMP-AS3 mRNA in osteosarcoma tissues, adjacent tissues and osteosarcoma cells

QPCR results showed that (**Figure 1A**) the expression of LSAMP-AS3 mRNA in osteosarcoma was significantly higher than that in adjacent tissues [(0.86 \pm 0.05) vs (0.22 \pm 0.03), $P < 0.05$], the difference was statistically significant. The expression level of LSAMP-AS3 in HOS cells was the highest in different osteosarcoma cells (**Figure 1B**), HOS was selected as a further experimental cell line combined with the above results and considering the role of LSAMP-AS3 in osteosarcoma.

Detection of LSAMP-AS3 and miR-200a by luciferase reporter gene

Bioinformatics was used as a predictive tool in order to clarify the miRNA associated with LSAMP-AS3, and LSAMP-AS3 may interact directly with miR-200a. LSAMP-AS3-siRNA and

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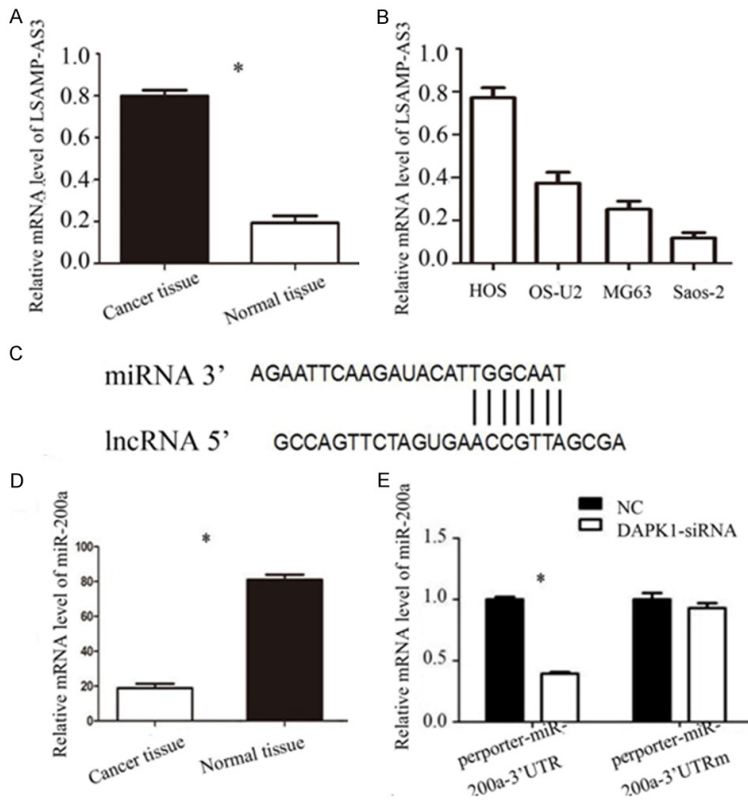


Figure 1. A. The mRNA expression of LSAMP-AS3 were detected by qPCR in osteosarcoma tissues and normal tissues. B. The mRNA expression of LSAMP-AS3 were detected by qPCR in different osteosarcoma cells. C. LSAMP-AS3 binds to miR-200a. D, E. Luciferase report gene detects miR-200a the direct target of LSAMP-AS3-siRNA. Error bars represent standard error. *P<0.05.

miR-200a were co-transfected into osteosarcoma cells HOS in order to demonstrate whether LSAMP-AS3 binds to miR-200a 3'UTR. The luciferase reporter gene results showed that (**Figure 1D, 1E**) LSAMP-AS3-siRNA significantly inhibited luciferase activity in miR-200a, indicating that LSAMP-AS3-siRNA can bind specifically to the 3'UTR of miR-200a.

LSAMP-AS3-siRNA reduced HOS proliferation and invasion ability in human osteosarcoma cells

The ability of cells to penetrate through Matrigel could reflect the ability of cells to invade. The results of Transwell showed that (**Figure 2A**) the number of cells in Matrigel matrix was (179.2±7.4), which was significantly higher than that in LSAMP-AS3-siRNA group (44.8±2.9), the difference was statistically significant (P<0.01). It indicated that LSAMP-AS3-siRNA could inhibit the invasion of HOS in

human osteosarcoma cells. The results of clonal formation showed that (**Figure 2B**) the proliferation rate of LSAMP-AS3-siRNA group was significantly lower than that of NC group [(173.4±12.8)% vs (22.1±5.0)%, P<0.01], the difference was statistically significant. It indicated that LSAMP-AS3-siRNA could inhibit the proliferation and invasion of HOS in human osteosarcoma cells.

Silencing LSAMP-AS3 inhibited proliferation and invasion of human osteosarcoma cells via targeting miR-200a

The results of Transwell showed that (**Figure 3A**) the number of cells in the LSAMP-AS3-siRNA group was (63.4±5.3), which was significantly lower than that of LSAMP-AS3-siRNA+miR-200a-inhibitor group (187.2±6.9), the difference was statistically significant (P<0.01), indicating that silencing of LSAMP-AS3, inhibition of miR-200a can promote

the invasion of human osteosarcoma cells HOS ability. The results of clonal formation showed that (**Figure 3B**) the mobility of LSAMP-AS3-siRNA+miR-200a-inhibitor group was significantly higher than that of LSAMP-AS3-siRNA group [(154.5±15.1)% vs (41.4±4.8)%, P<0.01], the difference was statistically significant, indicating that inhibition of miR-200a after silencing LSAMP-AS3 can promote the proliferation and invasion of HOS in human osteosarcoma cells.

Silencing LSAMP-AS3 inhibited subcutaneous tumorigenesis in nude mice via targeting miR-200a

The survival time of tumor-bearing mice was 4-9 weeks, the median survival time was 6.5 weeks. The autopsy showed that all the tumors in the left armpit were growing, and the tumors were gray white, solid, round or oval,

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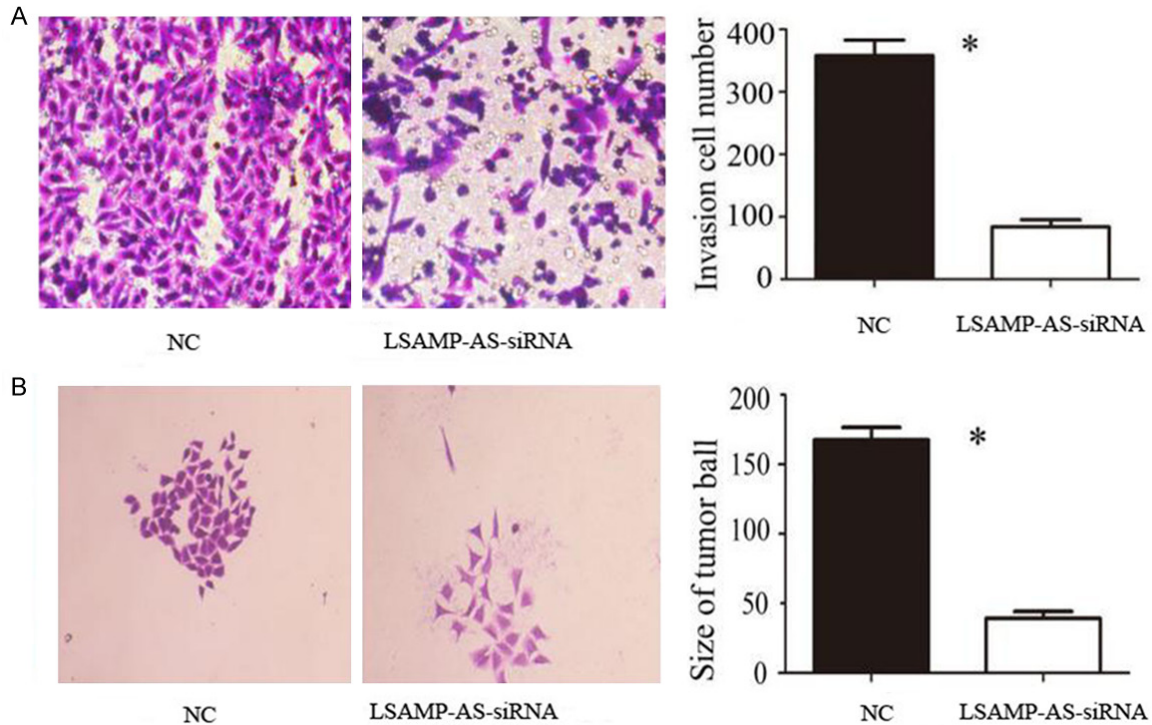


Figure 2. A, B. Effect of LSAMP-AS3-siRNA on the invasion ability of HOS cells were detected by Transwell matrigel invasion assays. Error bars represent standard error. * $P < 0.05$.

with nodular protrusions on the surface and fish in the profile. The tumor formation rate was 100%. Tumor growth in nude mice (**Figure 4A**): LSAMP-AS3-siRNA+miR-200a-inhibitor group was significantly larger than LSAMP-AS3-siRNA group. Comparison of tumor weight and volume (**Figure 4B, 4C**): the tumor volume and weight of LSAMP-AS3-siRNA+miR-200a-inhibitor group were significantly higher than those of LSAMP-AS3-siRNA group [Volume (2.99 ± 0.35) cm^3 vs (0.29 ± 0.11) cm^3 , $P < 0.05$; Weight (3.12 ± 0.34) g vs (0.36 ± 0.07) g, $P < 0.05$], indicating that silencing LSAMP-AS3, inhibition of miR-200a can promote osteosarcoma tumorigenesis.

LSAMP-AS3-siRNA down-regulated MAPK signal pathway protein level

Western blotting showed that (**Figure 4D**) the expression of MAPK protein (MAP2K, ERK) in LSAMP-AS3-siRNA group was significantly lower than that in NC group [(79.2 ± 8.3) vs (18.6 ± 3.3), $P < 0.05$; (73.4 ± 6.8)% vs (15.9 ± 2.6)%, $P < 0.05$], the difference was statistically significant ($P < 0.01$), indicating that LSAMP-AS3-siRNA can down-regulate the protein expression level of MAPK signal pathway.

Discussion

Osteosarcoma is one of the most common malignant tumors of the skeletal system. Its malignant degree is high, the early diagnosis rate is low, and the treatment of advanced osteosarcoma is less effective. The early diagnosis rate is low, the treatment of advanced osteosarcoma surgery is poor, and the recurrence rate and mortality after surgery is high, the prognosis of patients is extremely unsatisfactory, which is the world's very difficult malignant tumor disease [9]. Early diagnosis and prognosis of osteosarcoma patients and predictions of chemotherapy are clinically relevant. lncRNA abnormalities can lead to microRNA and related DNA and other tumor gene function changes in the occurrence and development of tumor, and thus cause oncogene activation and tumor suppressor gene inactivation [10]. Therefore, the relationship between lncRNA and its downstream miRNA may be related to the diagnosis and prognosis of osteosarcoma.

Many genes do not play a role through the translation of protein, a large part of the other will be transcribed as non-coding RNA with

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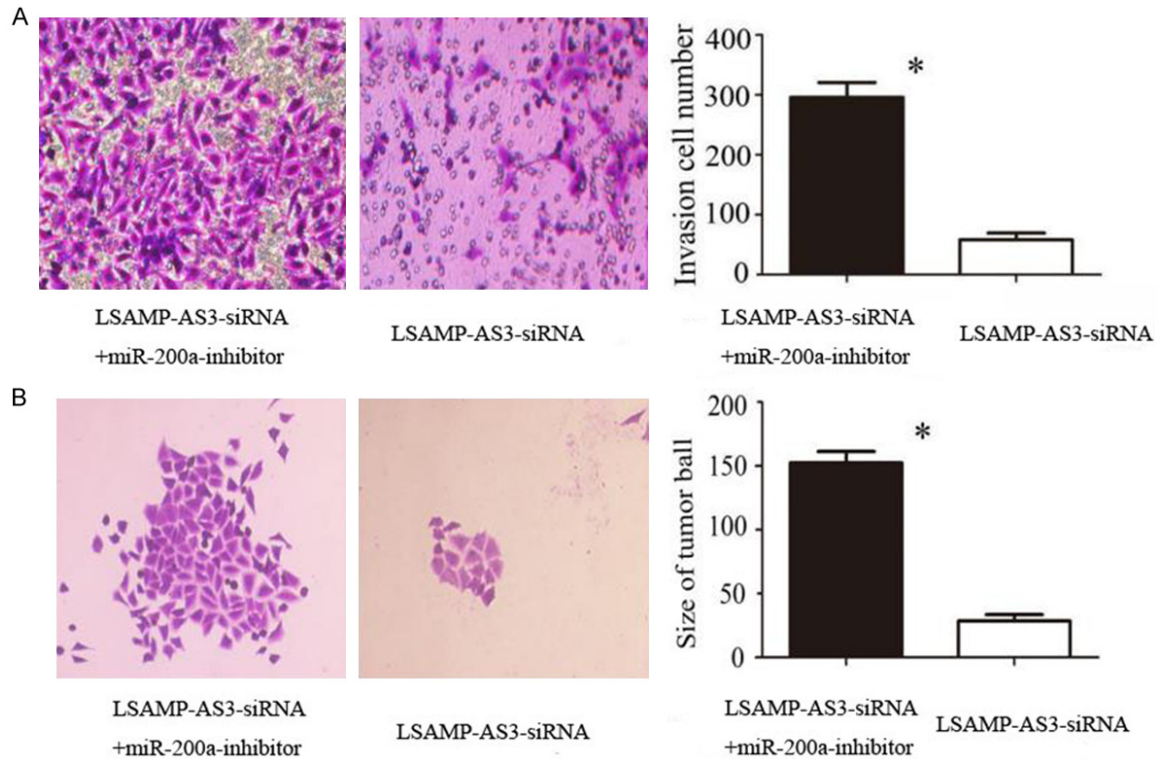


Figure 3. A. Effect of miR-200a on the invasion ability of HOS cells were detected after silencing LSAMP-AS3 by Transwell matrigel invasion assays. B. Effect of miR-200a on the HOS cells migration ability were detected after silencing LSAMP-AS3 by Clone formation assays. Error bars represent standard error. *P<0.05.

the recent advances in molecular biology and molecular oncology research and genomics research reports [11]. Non-coding RNAs are classified as lncRNA and small non-coding RNAs, depending on the relevant sequences of the transcribed genes. lncRNA is a class of about 200 nucleotides of RNA molecules which has no relatively complete open reading frame and has been a lot of scholars with no biological function. However, many recent studies have reported that lncRNA is closely related to a variety of diseases, including malignancies, digestive diseases, neurodegenerative diseases, autoimmune diseases, etc. [12]. LSAMP-AS3 was originally found in breast cancer, and its overexpression may affect breast cancer proliferation and invasion [13]. Researches have shown that LSAMP-AS3 expression in the liver, bone, lung and ovarian and other normal tissues is relatively high, but in a variety of malignant tumor cells show the phenomenon of down-regulation or deletion [14, 15]. The current investigation confirmed that LSAMP-AS3 its anti-cancer effect and wnt signal pathway have a certain association, Chen

et al. [16] found that in pancreatic cancer tissue and cell lines, LSAMP-AS3 expression were correspondingly reduced, the protein activity of wnt signaling pathway was increased by increasing the expression of LSAMP-AS3, suggesting that LSAMP-AS3 could inhibit the proliferation and invasion of pancreatic cancer cells through wnt signaling pathway and accelerate the process of autophagy. Subsequent studies have confirmed that LSAMP-AS3 can play a role in cancer prevention by a variety of ways, and its mechanism is related to DNA methylation, wnt pathway, Rb pathway and angiogenesis [17-19]. The expression of lncRNA LSAMP-AS3 in osteosarcoma was significantly down-regulated compared with normal tissue in this investigation. A functional experiment of LSAMP-AS3 inhibition was performed in osteosarcoma cell lines in order to verify the role of LSAMP-AS3 expression in the development of osteosarcoma. The results showed that down-regulation of LSAMP-AS3 could promote the proliferation and invasion of osteosarcoma cells. These findings suggest that lncRNA LSAMP-AS3 osteosarcoma plays a role in the development

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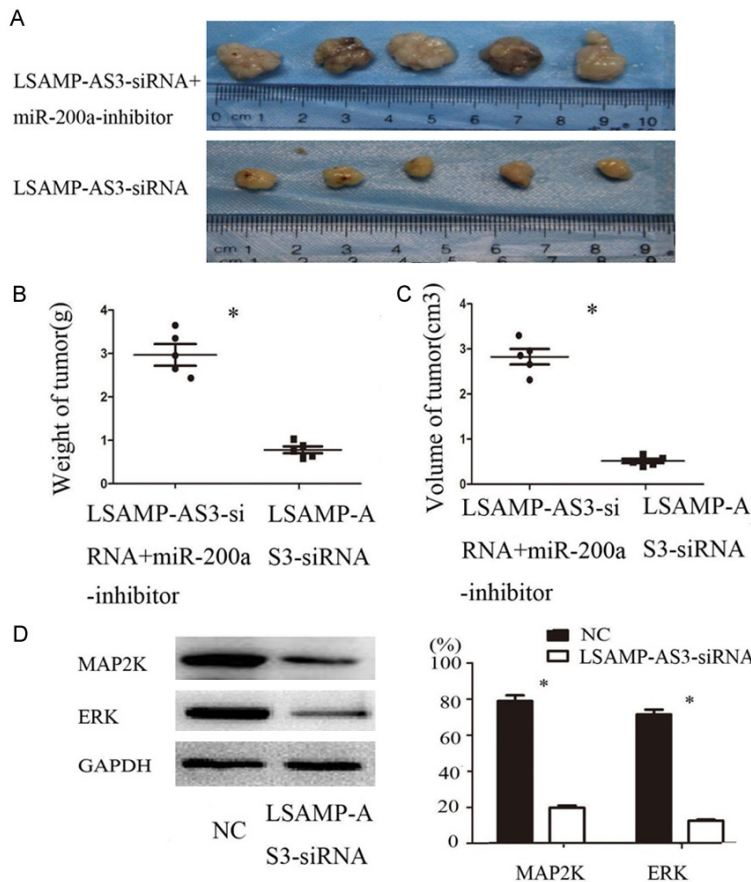


Figure 4. Effect of miR-200a on tumorigenesis after silencing LSAMP-AS3 in vivo. A. Comparison of the tumor size of nude mice. B, C. Compare the volume and weight of the tumor in nude mice. D. Expression of MAPK pathway protein.

of a tumor suppressor gene, and its deletion or down-regulation is involved in the development and development of osteosarcoma. Therefore, LSAMP-AS3 plays an important role in osteosarcoma and serves as a target for future treatment of osteosarcoma and an indicator of prognosis.

Mature miR-200a is generally expressed in breast, pancreatic and ovarian cancers. Eades et al. [20] examined the expression of miR-200a in breast cancer, and the level of miR-200a expression in breast cancer was down-regulated relative to normal tissue and was associated with migration and invasion of breast cancer. The expression of miR-200a in osteosarcoma and normal lung tissue was detected by qPCR in this investigation. The expression of miR-200a in osteosarcoma was significantly down-regulated, indicating that

miR-200a played an anti-cancer effect in osteosarcoma. The effect of miR-200a on the invasion and migration of osteosarcoma cells was further studied by silencing LSAMP-AS3 and inhibiting the expression of miR-200a in combination with the previous experimental results. The results showed that inhibition of miR-200a expression after silencing LSAMP-AS3 could promote the invasion and migration of osteosarcoma cells. Similar results were also found in nude mice in vivo, and the relative expression of MAPK signaling protein was decreased after silencing LSAMP-AS3.

QPCR was used to detect the expression of LSAMP-AS3 and miR-200a in osteosarcoma and normal tissues in this investigation. The interaction between LSAMP-AS3 and miR-200a was further investigated and the role of LSAMP-AS3 and miR-200a in the process of invasion and migration of osteosarcoma cells was further studied. The results

showed that LSAMP-AS3 was up-regulated in osteosarcoma, and miR-138 was down-regulated in osteosarcoma. LSAMP-AS3 interacted directly with miR-200a. LSAMP-AS3 can target the invasion and migration of osteosarcoma by miR-200a. miR-200a regulates the invasion and migration of osteosarcoma. It is suggested that LSAMP-AS3 and miR-200a may be involved in the invasion and migration of osteosarcoma cells through MAPK signaling pathway, which may be a marker for predicting the progress, prognosis and monitoring of osteosarcoma.

Disclosure of conflict of interest

None.

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