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MicroRNA-493-5p inhibits proliferation and metastasis of osteosarcoma cells by targeting Kruppel-like factor 5

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Abstract

Osteosarcoma, including spinal osteosarcoma, has properties of high degree of malignancy, high rate of recurrence, and high incidence of metastasis. microRNAs can exert oncogenic or tumor suppressive roles in cancer cells. This study explored the effects of microRNA-493-5p (miR-493-5p) on osteosarcoma cell viability, migration, invasion, and apoptosis, as well as the underlying possible mechanism. First, the expression of miR-493-5p in osteosarcoma tissues and cells was detected using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Then, the effects of miR-493-5p overexpression (or suppression) on osteosarcoma cell viability, migration, invasion, and apoptosis, as well as Kruppel-like factor 5 (KLF5) expression, were assessed using the Cell Counting Kit-8 assay, two-chamber transwell assay, Annexin V-FITC/PI apoptosis detection kit, qRT-PCR, and western blotting, respectively. Finally, the roles of KLF5 in miR-493-5p suppression-induced U20S cell viability, migration, and invasion enhancement, as well as the PI3K/AKT pathway activation, were evaluated. We found that miR-493-5p had lower expression in tumor tissues of spinal osteosarcoma and osteosarcoma cells. Overexpression of miR-493-5p inhibited osteosarcoma U20S cell viability, migration, and invasion, but induced cell apoptosis. On the contrary, suppression of miR-493-5p-promoted U20S cell viability, migration, and invasion. KLF5 was a direct target gene of miR-493-5p, which participated in the effects of miR-493-5p on U20S cell viability, migration, invasion, and apoptosis. Furthermore, suppression of the miR-493-5p activated PI3K/AKT pathway in U20S cells by upregulating KLF5. In conclusion, we revealed that miR-493-5p exerted tumor suppressive roles in spinal osteosarcoma and osteosarcoma cells. Overexpression of miR-493-5p inhibited proliferation and metastasis of osteosarcoma cells by downregulating KLF5 and inactivating the PI3K/AKT signaling pathway.

HIGHLIGHTS

1. miR-493-5p has lower expression in tumor tissues of spinal osteosarcoma.
2. miR-493-5p exerts tumor suppressive roles in osteosarcoma cells.
3. KLF5 is a direct target gene of miR-493-5p in osteosarcoma cells.
4. KLF5 participates in the effects of miR-493-5p on osteosarcoma cells.
5. Suppression of miR-493-5p activates the PI3K/AKT pathway by upregulating KLF5.

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KEYWORDS
cell metastasis, cell proliferation, Kruppel-like Factor 5 (KLF5), microRNA-493-5p, PI3K/AKT signaling pathway, spinal osteosarcoma

1 | INTRODUCTION

Osteosarcoma is an aggressive bone tumor in skeletal system that frequently affects the long bones but also involves other bones in the body (Moore & Luu, 2014). Spinal osteosarcoma presents 3–5% of all osteosarcoma, which is characterized by low incidence, high malignant degree, and poor prognosis (Katonis et al., 2013). The main clinical symptom of spinal osteosarcoma is pain (Chan, Sun, Kam, Shing, & Poon, 2012). Owing to spinal osteosarcoma growth often within the spinal canal, most of the patients present some neurologic impairment (Katonis et al., 2013; Vogel, Chlan, Zebracki, & Anderson, 2011). The surgical treatment of spinal osteosarcoma is very difficult due to its specifically anatomic location, and its proximity to vital structures (Chung, Kim, Park, Jung, & Lee, 2012). Moreover, many patients present advanced stage cancer at diagnosis and the survival is very limited (Dekutoski et al., 2016). Therefore, more experimental and clinical research are urgently needed to find novel diagnostic and therapeutic strategies for spinal osteosarcoma.

microRNAs (miRNAs) are small, noncoding RNA transcripts in eukaryotic cells with 20–24 nucleotides (nt) in length (Kavitha et al., 2014). More than 2,000 miRNAs have been found in human cells and it is believed that they palay pivotal roles in the regulation of one-third of the genes in the genome (Hammond, 2015). miRNA-493 (miR-493), a type of miRNAs, has been discovered to exert tumor suppressive roles in a number of cancer cells, including breast cancer (Zhao et al., 2016), non-small-cell lung cancer (Liang et al., 2017), liver cancer (Wang, Fang, Han, Wang, & Huang, 2018; Zhao, Xu, Wang, Cai, & Chen, 2017), prostate cancer (Wang et al., 2017), and osteosarcoma (Qian et al., 2018). In terms of osteosarcoma, Qian et al. 2018 reported that miR-493 inhibited the proliferation and invasion of osteosarcoma HOS and U20S cells through directly targeting specificity protein 1 (SP1). More research is still needed to further explore the effects of miR-493 on spinal osteosarcoma and osteosarcoma cells.

Kruppel-like factor 5 (KLF5) is a DNA-binding transcriptional regulatory protein in eukaryotic cells that contributes to the regulation of multiple cell functions, such as proliferation, differentiation, migration, and apoptosis (Ma et al., 2017; Tarapore, Yang, & Katz, 2013). In addition, numerous reports provide evidence that abnormal expression of KLF5 is associated with many human diseases, including cancers (Dong & Chen, 2009; Pattison, Posternak, & Cole, 2016). A variety of miRNAs can exert oncogenic or tumor suppressive roles in cancer cells by regulating KLF5 expression (Jiang et al., 2017; Zhou et al., 2017).

Therefore, in the present study, we measured the expression of miR-493-5p in spinal osteosarcoma tumor tissues and osteosarcoma cells. Then, the effects of miR-493-5p on osteosarcoma cell viability, migration, invasion, and apoptosis, as well as KLF5 expression, were analyzed. Finally, the possible internal molecular mechanism and potential signaling pathway were also investigated. This study will be helpful for further affirming the effects of miR-493-5p on osteosarcoma and provides possible molecular targets for spinal osteosarcoma diagnosis and treatment.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens

Between May 2015 and October, 2017, a total of 20 patients (11 males and 9 females, mean age 22 years, range from 15 to 36 years) diagnosed with spinal osteosarcoma were recruited in this study at Linyi Central Hospital (Linyi, China) and China-Japan Union Hospital of Jilin University (Changchun, China). None of the patients received any therapy before surgery. Tumor tissues and corresponding normal tissues were collected from patients during the surgery. Written informed consents were obtained from patients or legal guardians of patients. This study was approved by the Ethics Committee of our local institution.

2.2 | Cell lines

Human osteoblast hFOB1.19 cells, human osteosarcoma MG63 and U20S cells, and human embryonic kidney HEK293 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). Human osteosarcoma OS732, HOS, and Saos-2 cells were obtained from Cell Culture Center of Peking Union Medical College (Beijing, China). All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Life Technologies) and 1% (v/v) penicillin-streptomycin-glutamine (100×, Gibco, Life Technologies). Cultures were placed at 37°C in a humidity incubator (Sanyo, Jencons, United Kingdom) with 5% CO_2.

2.3 | Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted to measure the expression levels of miR-493-5p and KLF5 in this study. Briefly, total RNA in patient's tissues and osteosarcoma cells were isolated using the Trizol™ Plus RNA Purfication kit (Invitrogen, Carlsbad, CA). Then, the expression of miR-493-5p was detected using the mirVana™ qRT-PCR miRNA Detection kit (Invitrogen). For the test of expression of KLF5, SuperScript™ III Platinum™ SYBR™ Green One-Step qRT-PCR kit (Invitrogen) was conducted according to the manufacturer's instruc-

tion. The expression levels of U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were acted as endogenous controls. The data were quantified using $2^{-\Delta\Delta Ct}$ method described previously (Ish-Shalom & Lichter, 2010).

2.4 | Cell transfection

Cell transfection was performed to change the expression levels of miR-493-5p and KLF5 in this study. miR-493-5p mimic, miR-493-5p inhibitor, and their negative controls (Scramble, NC) were designed and synthesized by GenePharma Corporation (Shanghai, China). si-KLF5 and siNC were designed and synthesized by Life Technologies. Cell transfection was conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol. Transfection efficiencies were verified using qRT-PCR.

2.5 | Cell viability assay

After relevant transfection, viability of U20S cells was assessed using Cell Counting Kit-8 (CCK-8) assay (Beyotime Biotechnology, Shanghai, China). Briefly, transfected or non-transfected U20S cells were seeded into 96-well plate (Corning Incorporated, New York, NY, USA) with 1×10^4 cells each well and placed at 37°C in humidity incubator for 24 hr. Then, 10 μl CCK-8 kit solution was added into the each well of the plate and the plate was placed at 37°C in humidity incubator for 1 hr. After that, the absorbance of each well at 450 nm was recorded using a Microplate Reader (ELx800, Bio-Tek Instruments, Winooski, VT). The cell viability (%) was calculated by average absorbance of transfected group/average absorbance of non-transfected group × 100%.

2.6 | Cell migration and invasion assay

After relevant transfection, migration, and invasion of U20S cells was determined using a modified two-chamber transwell assay (Corning Incorporated) with a pore size of 8 mm. For the cell migration assay, transfected or non-transfected U20S cells (5×10^3) were resuspended in serum-free DMEM (200 μl) and added into the upper compartment of chamber. Complete DMEM (600 μl) was added into the lower compartment of the chamber. After incubation at 37°C in humidity incubator for 48 hr, the cells were fixed with methanol, immediately. Non-migrated cells in the upper compartment were removed using cotton swab carefully and migrated cells in the lower compartment were counted under a microscope (Nikon, Tokyo, Japan). Relative migration (%) was calculated by the average number of migrated cells in the transfection group/average number of migrated cells in the non-transfection group × 100%.

The cell invasion assay was performed similarly with the cell migration assay, except that the transwell membrane was pretreated with Matrigel (BD Bioscience, Franklin Lakes, NJ). Relative invasion (%) was calculated by the average number of invaded cells in the transfection group/average number of invaded cells in the non-transfection group × 100%.

2.7 | Cell apoptosis assay

Cell apoptosis was detected using the Annexin V-FITC/PI apoptosis detection kit (Invitrogen, Carlsbad, CA). Briefly, transfected or non-transfected U20S cells were seeded into six-well plate (Corning Incorporated) with 1×10^5 cells each well and placed at 37°C in humidity incubator for 24 hr. Then, the cells in each group were harvested and disposed as the following step: washed with phosphate buffered saline (PBS) for twice, mixed with the kit solution for 30 min at room temperature in the dark, washed with PBS twice, and subjected to the flow cytometry analysis (Beckman Coulter, Fullerton, CA). The data were analyzed using FlowJo software (Tree Star Incorporation, Ashland, OR).

2.8 | Dual-luciferase activity assay

The 3′-untranslated region fragment of KLF5, containing the potential miR-493-5p binding site, was amplified by PCR and constructed into pmirGLO vector (Promega, Madison, WI), which was referred as KLF5-wild type (KLF5-wt). To mutate the potential miR-493-5p binding site, the potential binding site was replaced, amplified, and also constructed into pmirGLO vector, which was referred as KLF5-mutated type (KLF5-mt). Then, miR-493-5p mimic and KLF5-wt (or KLF5-mt) were cotransfected into HEK293 cells, simultaneously. The relative luciferase activity was detected using the dual-luciferase reporter assay (Promega).

2.9 | Western blot analysis

Total proteins used for western blotting were isolated from U20S cells using RIPA Lysis Buffer (Beyotime Biotechnology) supplemented with protease inhibitors (Roche, Basel, Switzerland). The concentration of total proteins was quantified using the BCA Protein Assay kit (Beyotime Biotechnology). Then, proteins in equal concentration were electrophoresed in polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO), nitrocellulose membranes were incubated with primary antibodies against Bcl-2 (ab32124), Bax (ab32503), Pro-caspase 3 (ab32150), Cleaved-caspase 3 (ab49822), KLF5 (ab24331), p-phosphatidylinositil 3-kinase (PI3K, ab182651), PI3K (ab227204), p-protein kinase 3 (AKT, ab38499), AKT (ab8805), and GAPDH (ab8245, Abcam Biotechnology, Cambridge, MA) for 12 hr. All primary antibodies were diluted in 1% BSA solution at a dilution of 1:1,000. Then, nitrocellulose membranes were washed with Tris-Buffered Saline and Tween (Sigma-Aldrich) for twice and incubated with Goat Antimouse (or Antirabbit) IgG H&L (HRP) secondary antibodies (ab205718, ab205719) for 1.5 hr at room temperature in the dark. Followed by adding 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore) to the surfaces of nitrocellulose membranes, the signals of proteins were recorded using the Bio-Rad ChemiDoc™ XRS system (Bio-Rad Laboratories, Hercules, CA).

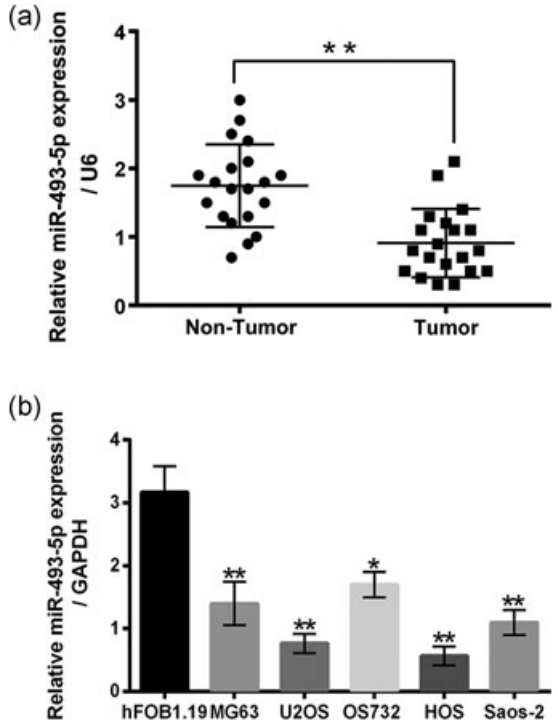

FIGURE 1 miR-493-5p had lower expression in tumor tissues of spinal osteosarcoma and osteosarcoma cells. (a) The expression of miR-493-5p in tumor tissues and corresponding normal tissues of 20 patients with spinal osteosarcoma was detected using qRT-PCR. (b) The expression of miR-493-5p in human normal osteoblast hFOB1.19 cells and human osteosarcoma MG63, U20S, O732, HOS, and Saos-2 cells was measured using qRT-PCR. $^{*}p < 0.05$; $^{**}p < 0.01$. miR-493-5p: microRNA-493-5p; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR: quantitative reverse transcription polymerase chain reaction

2.10 | Statistical analysis

All above experiments were repeated three times. Values of multiple experiments were expressed as mean ± standard deviation (SD). A statistical analysis was conducted using GraphPad 6.0 software (Graphpad, San Diego, CA). Statistical comparison was calculated using one-way analysis of variance. $p < 0.05$ was considered to be significantly different; $p < 0.01$ and $p < 0.001$ were considered to be extremely significantly different.

3 | RESULTS

3.1 | miR-493-5p had lower expression in tumor tissues of spinal osteosarcoma and osteosarcoma cells

First, we assessed the expression of miR-493-5p in tumor tissues and corresponding normal tissues of 20 patients with spinal osteosarcoma. Figure 1a presented that miR-493-5p had lower expression in tumor tissues, compared with corresponding normal tissues ($p < 0.01$). Moreover, the expression of miR-493-5p in human normal osteoblast hFOB1.19 cells and human osteosarcoma MG63, U20S, OS732, HOS, and Saos-2 cells were also detected. Figure 1b displayed that compared with human normal osteoblast hFOB1.19 cells, miR-493-5p had lower expression in human osteosarcoma MG63, U20S, OS732, HOS, and Saos-2 cells ($p < 0.05$ or $p < 0.01$). These results indicated that miR-493-5p had lower expression in tumor tissues of spinal osteosarcoma and osteosarcoma cells, which suggested that miR-493-5p might exert tumor suppressive roles in spinal osteosarcoma and osteosarcoma cells. U20S cells were chosen for subsequent experiments.

3.2 | miR-493-5p exerted tumor suppressive roles in osteosarcoma cells

To investigate the effects of miR-493-5p on U20S cell viability, migration, invasion, and apoptosis, miR-493-5p mimic and miR-493-5p inhibitor were transfected into U20S cells, respectively. Figure 2a showed that the expression of miR-493-5p in U20S cells was dramatically enhanced after miR-493-5p mimic transfection ($p < 0.001$) and obviously reduced after miR-493-5p inhibitor transfection ($p < 0.01$). The results of Figure 2b–d presented that miR-493-5p mimic transfection significantly inhibited the viability, migration, and invasion of U20S cells ($p < 0.05$ or $p < 0.01$). On the contrary, miR-493-5p inhibitor transfection remarkably promoted the viability, migration, and invasion of U20S cells ($p < 0.05$). Furthermore, Figure 2e displayed that miR-493-5p mimic transfection notably induced U20S cell apoptosis ($p < 0.001$) and miR-493-5p inhibitor transfection had no significant effect on U20S cell apoptosis. Western blotting illustrated that the expression levels of Bax and Cleaved-caspase 3 in U20S cells were enhanced, as well as the expression level of Bcl-2 was reduced after miR-493-5p mimic transfection (Figure 2f). These above findings confirmed that miR-493-5p exerted tumor suppressive roles in osteosarcoma U20S cells.

3.3 | KLF5 was a direct target gene of miR-493-5p in osteosarcoma cells

The messenger RNA (mRNA) and protein expression of KLF5 in U20S cells after miR-493-5p mimic or miR-493-5p inhibitor transfection were assessed using qRT-PCR and western blotting, respectively. As displayed in Figure 3a,b, miR-493-5p mimic transfection downregulated the mRNA and protein expression of KLF5 in U20S cells ($p < 0.05$ in mRNA level) and miR-493-5p inhibitor transfection upregulated the mRNA and protein expression of KLF5 in U20S cells ($p < 0.01$ in mRNA level). In addition, Figure 3c showed that the relative luciferase activity was significantly decreased after cotransfection with miR-493-5p mimic and KLF5-wt ($p < 0.05$). These results indicated that miR-493-5p negatively regulated the expression of KLF5 in U20S cells and KLF5 was a direct target gene of miR-493-5p in osteosarcoma cells.

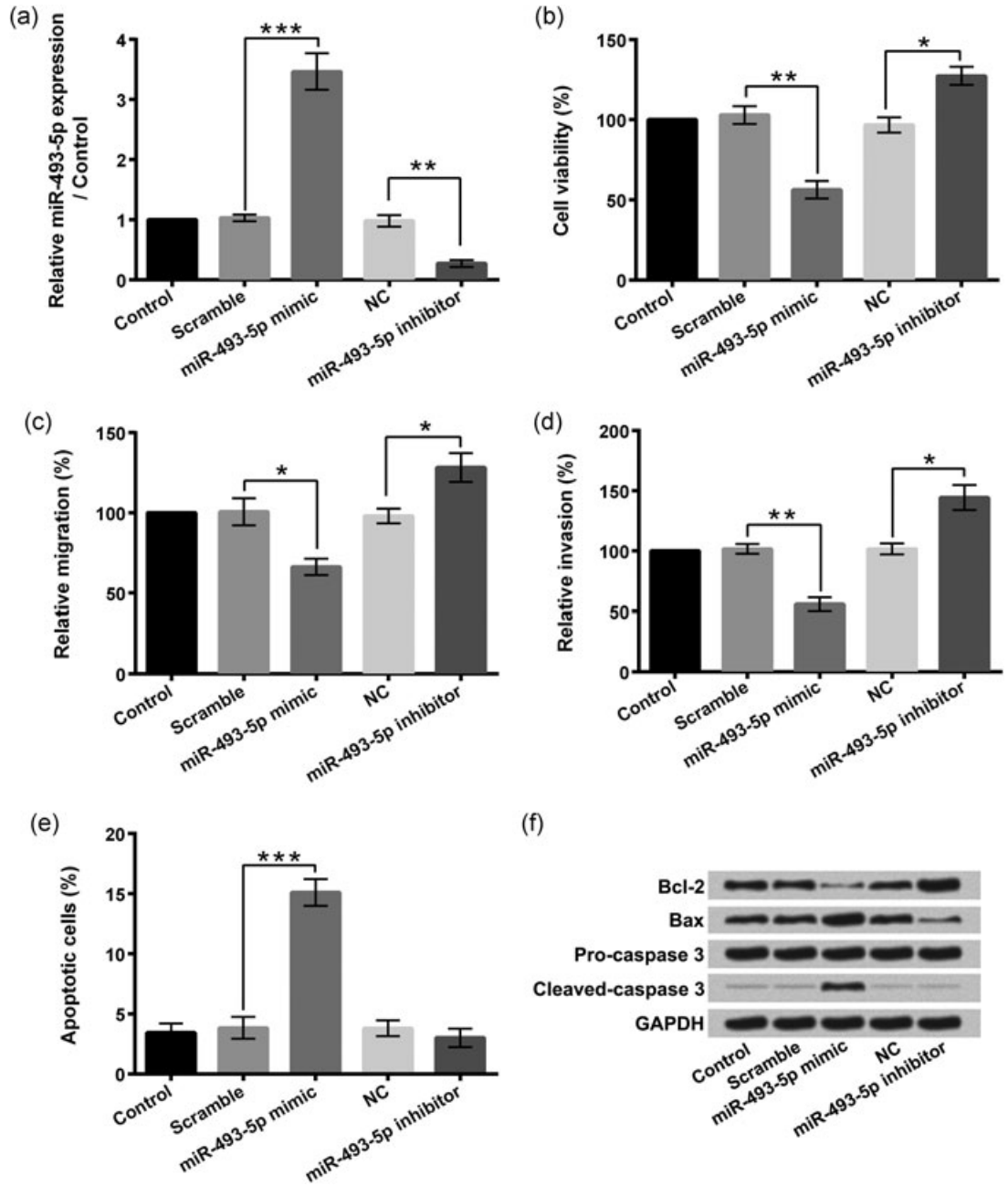

FIGURE 2 miR-493-5p exerted tumor suppressive roles in osteosarcoma cells. After miR-493-5p mimic or miR-493-5p inhibitor transfection, (a) the expression of miR-493-5p in osteosarcoma U20S cells, (b) the viability of U20S cells, (c) the migration of U20S cells, (d) the invasion of U20S cells, (e) the apoptosis of U20S cells and (f) the expression levels of Bcl-2, Bax, Pro-caspase 3, and Cleaved-caspase 3 in U20S cells were assessed using qRT-PCR, the Cell Counting Kit-8 (CCK-8) assay, the two-chamber transwell assay, the Annexin V-FITC/PI apoptosis detection kit, and western blotting, respectively. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{**}p < 0.001$. miR-493-5p: microRNA-493-5p; NC: negative control; qRT-PCR: quantitative reverse transcription polymerase chain reaction

3.4 | Suppression of miR-493-5p promoted osteosarcoma cell viability, migration, and invasion by upregulating KLF5

To analyze the roles of KLF5 in miR-493-5p suppression-induced U20S cell viability, migration, and invasion enhancement, si-KLF5 was transfected into U20S cells. Figure 4a showed that compared with miR-493-5p inhibitor transfection, the mRNA and protein expression of KLF5 in U20S cells were decreased after miR-493-5p inhibitor + si-KLF5 cotransfection ($p < 0.001$ in mRNA level). Figure 4b–d displayed that si-KLF5 transfection remarkably reversed the miR-493-5p inhibitor transfection-induced U20S cell viability, migration, and invasion enhancement ($p < 0.01$). Furthermore, Figure 4e presented that miR-493-5p inhibitor and si-KLF5 cotransfection dramatically induced U20S cell apoptosis ($p < 0.001$). Western blotting illustrated that the expression levels of Bax and Cleaved-caspase 3 were enhanced, as well as the expression level of Bcl-2 was reduced, in U20S cells after cotransfection

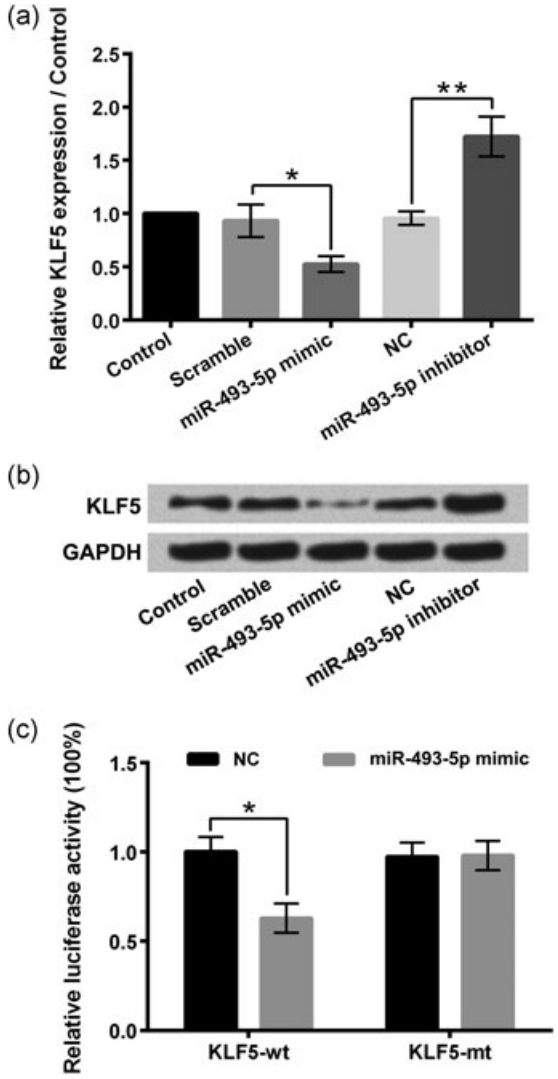

FIGURE 3 KLF5 was a direct target gene of miR-493-5p in osteosarcoma cells. (a and b) After miR-493-5p mimic or miR-493-5p inhibitor transfection, the mRNA and protein expression of KLF5 in U20S cells was detected using qRT-PCR and western blotting, respectively. (c) After cotransfection with miR-493-5p mimic and KLF5-wt (or KLF5-mt), the relative luciferase activity was determined using the dual-luciferase reporter assay. $^{*}p < 0.05$; $^{**}p < 0.01$. KLF5: Kruppel-like factorFactor 5; miR-493-5p: microRNA-493-5p; mt: mutated type; NC: negative control; qRT-PCR: quantitative reverse transcription polymerase chain reaction; wt: wild type

with miR-493-5p inhibitor and si-KLF5 (Figure 4f). These above findings suggested that KLF5 was involved in the effects of miR-493-5p on U20S cells and suppression of miR-493-5p promoted U20S cell viability, migration, and invasion by upregulating KLF5.

3.5 | Suppression of the miR-493-5p activated PI3K/AKT pathway in U20S cells by upregulating KLF5

Finally, we explored the regulatory effects of miR-493-5p and KLF5 on the PI3K/AKT pathway in U20S cells. As displayed in Figure 5, miR-493-5p mimic transfection downregulated the expression levels of p-PI3K and p-AKT in U20S cells, which suggested that the overexpression of miR-493-5p inactivated the PI3K/AKT pathway in U20S cells. On the contrary, miR-493-5p inhibitor transfection upregulated the expression levels of p-PI3K and p-AKT in U20S cells, which suggested that suppression of miR-493-5p activated the PI3K/AKT pathway in U20S cells. Moreover, compared with the miR-493-5p inhibitor group, the expression levels of p-PI3K and p-AKT in U20S cells were decreased in miR-493-5p inhibitor + si-KLF5 group. These results suggested that suppression of the miR-493-5p activated PI3K/AKT pathway in U20S cells by upregulating KLF5.

4 | DISCUSSION

Osteosarcoma, including spinal osteosarcoma, has properties of high degree of malignancy, high rate of recurrence, and high incidence of metastasis (Cao et al., 2018; Katonis et al., 2013). In this study, we revealed that miR-493-5p had lower expression in tumor tissues of spinal osteosarcoma and osteosarcoma cells. Further results indicated that miR-493-5p exerted tumor suppressive roles in osteosarcoma U20S cells by inhibiting cell viability, migration, and invasion, as well as promoting cell apoptosis. KLF5 was a direct target gene of miR-493-5p, which participated in the effects of miR-493-5p on U20S cell viability, migration, invasion, and apoptosis. Moreover, we also found that suppression of miR-493-5p activated the PI3K/AKT pathway in U20S cells by upregulating KLF5.

miRNAs do not encode proteins, but participate in the regulation of the gene expression in eukaryotic cells (Fang, Kong, & Xu, 2018; Hammond, 2015). As important regulatory factors in the cells, miRNAs can act as oncogenes or tumor suppressors to play pivotal roles in controlling tumor cell proliferation, migration, invasion, and apoptosis (Fonseca-Sanchez et al., 2013; Zhao et al., 2018). Previous studies reported that miR-493-5p exerted tumor suppressive roles in a variety of cancer cells (Liang et al., 2017; Wang et al., 2017, 2018; Zhao et al., 2016, 2017). More importantly, Qian et al. (2018) proved that miR-493 suppressed the proliferation and invasion of osteosarcoma cells. In consistent with the previous studies, we found that miR-493-5p had lower expression in tumor tissues of spinal osteosarcoma and osteosarcoma cells. In addition, overexpression of miR-493-5p significantly promoted osteosarcoma U20S cell viability, migration, and invasion, but induced cell apoptosis. While suppression of miR-493-5p obviously promoted U20S cell viability, migration, and invasion. These results suggested that miR-493-5p also acted as a tumor suppressor in spinal osteosarcoma and osteosarcoma cells. Considering that each miRNA, including miR-493-5p, can regulate the expression of a number of genes in the genome (Hammond, 2015). miRNA-373 (miR-373) and miRNA-132 (miR-132) were also found to be related to proliferation, migration, and invasion of spinal osteosarcoma cells (Hou et al., 2016; Liu, Cheng, Pan, & Yan, 2017), we could propose that many miRNAs might participate in the regulation of spinal osteosarcoma growth and metastasis.

One of the most important findings in this study was that KLF5 participated in the tumor suppressive effects of miR-493-5p on

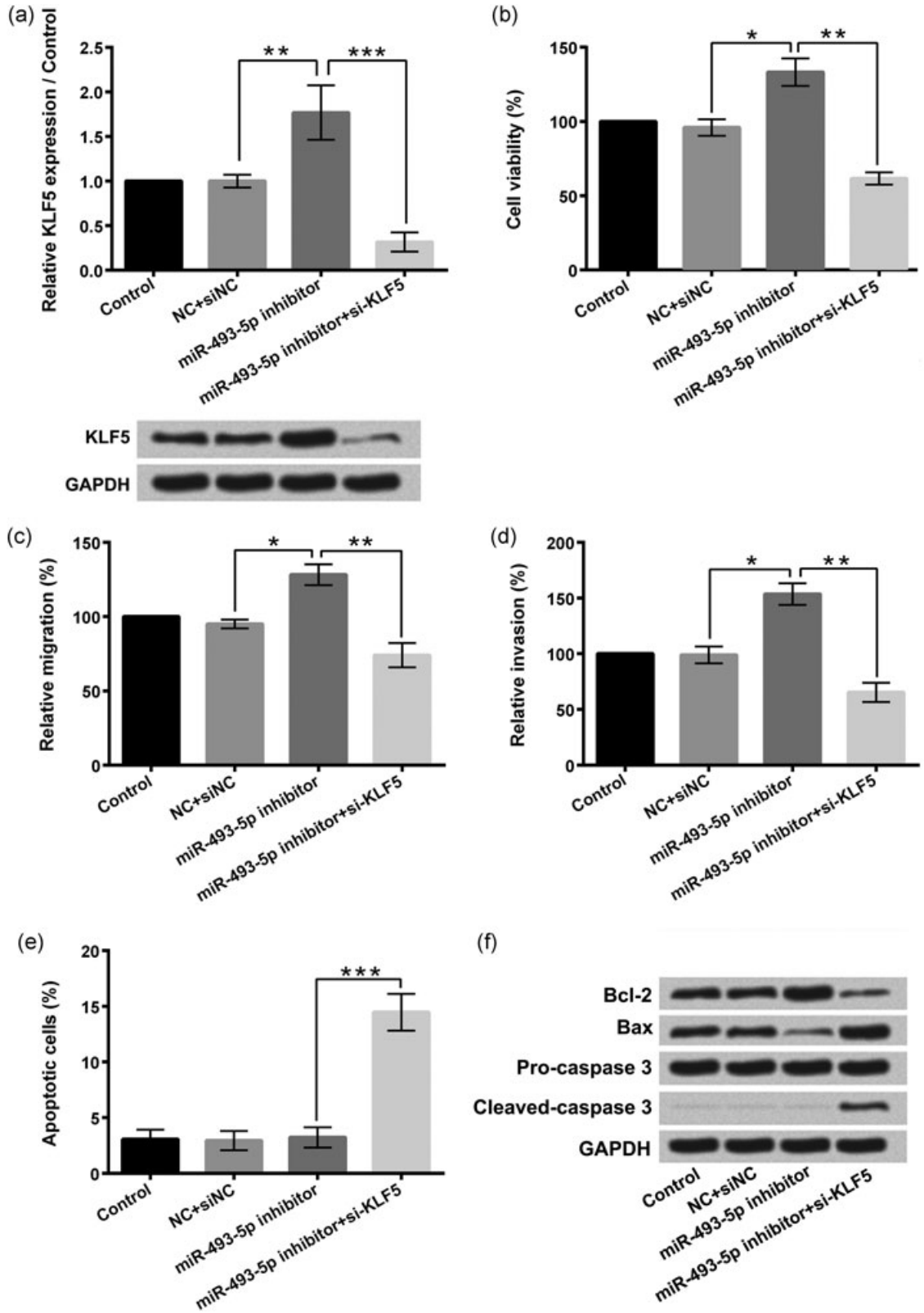

FIGURE 4 Suppression of miR-493-5p promoted osteosarcoma cell viability, migration, and invasion by upregulating KLF5. After miR-493-5p mimic and/or si-KLF5 transfection, (a) the mRNA and protein expression of KLF5 in U20S cells, (b) the viability of U20S cells, (c) the migration of U20S cells, (d) the invasion of U20S cells, (e) the apoptosis of U20S cells, and (f) the expression levels of Bcl-2, Bax, Pro-caspase 3, and Cleaved-caspase 3 in U20S cells were assessed using qRT-PCR, western blotting, cell counting kit-8 (CCK-8) assay, two-chamber transwell assay, and Annexin V-FITC/PI apoptosis detection kit, respectively. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{**}p < 0.001$. miR-493-5p: GAPDH: glyceraldehyde 3-phosphate dehydrogenase; microRNA-493-5p; KLF5: Kruppel-like factorFactor 5; NC: negative control; qRT-PCR: quantitative reverse transcription polymerase chain reaction

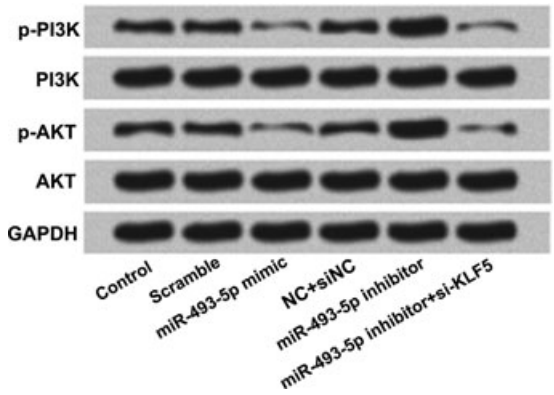

FIGURE 5 Suppression of miR-493-5p activated the PI3K/AKT pathway in U20S cells by upregulating KLF5. After miR-493-5p mimic or miR-493-5p inhibitor and/or si-KLF5 transfection, western blotting was performed to measure the expression of p-PI3K, PI3K, p-AKT, and AKT in U20S cells. AKT: protein kinase 3; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; KLF5: Kruppel-like Factor 5; miR-493-5p: microRNA-493-5p; NC: negative control; PI3K: phosphatidylinositil 3-kinase

osteosarcoma U20S cells. KLF5 is a member of zinc-finger-containing transcription factors, which plays critical roles in regulating expression of a wide range of genes (Gao, Ding, Chen, Chen, & Zhou, 2015; Tarapore et al., 2013). The aberrant activation of KLF5 has been found to associate with the occurrence of many tumors (Shi, Yang, Kong, & Zhou, 2016; Wei et al., 2017). In the present study, we revealed that miR-493-5p overexpression downregulated the mRNA and protein expression of KLF5 in U20S cells and miR-493-5p suppression upregulated the mRNA and protein expression of KLF5 in U20S cells. The relative luciferase activity was significantly decreased after cotransfection with miR-493-5p mimic and KLF5-wt. In addition, knockdown of KLF5 significantly reversed the miR-493-5p suppression-induced U20S cell viability, migration and invasion enhancement, as well as induced U20S cell apoptosis. These above findings suggested that KLF5 was a direct target gene of miR-493-5p and miR-493-5p exerted tumor suppressive roles in osteosarcoma U20S cells at least in part by upregulating KLF5.

The PI3K/AKT signaling pathway is thought to be an important oncogenic signaling pathway in human cancer cells, including osteosarcoma (Niu et al., 2015; Ramakrishnan & Kumar, 2018). Numerous studies have demonstrated that the PI3K/AKT signaling pathway is related to the proliferation, migration, invasion, and apoptosis of osteosarcoma cells (Keremu et al., 2017; Kim et al., 2017). Liu et al. (2017) reported that miR-373 promoted osteosarcoma cell growth and invasion by activating the PI3K/AKT signaling pathway. So, we investigated the effects of miR-493-5p and KLF5 on the PI3K/AKT signaling pathway in U20S cells. The results revealed that miR-493-5p overexpression inactivated the PI3K/AKT signaling pathway in U20S cells. On the contrary, miR-493-5p suppression activated the PI3K/AKT signaling pathway in U20S cells. More importantly, knockdown of KLF5 reversed the miR-493-5p suppression-induced activation of the PI3K/AKT signaling pathway in U20S cells. These above results indicated that miR-493-5p exerted tumor suppressive roles in osteosarcoma cells might via downregulating KLF5 and then inactivating the PI3K/AKT pathway.

To conclude, our research verified the tumor suppressive effects of miR-493-5p on spinal osteosarcoma and osteosarcoma cells. Overexpression of miR-493-5p inhibited proliferation and metastasis of osteosarcoma cells by downregulating KLF5 and inactivating the PI3K/AKT signaling pathway. We proposed that miR-493-5p and KLF5 could be as the possible internal molecular targets for spinal osteosarcoma diagnosis and treatment. Moreover, targeting molecule medicines that can enhance the expression of miR-493-5p in spinal osteosarcoma tissues may be useful for spinal osteosarcoma treatment. Further in vitro and in vivo research are still needed to confirm our proposal.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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