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Original article

Upregulation of miR-95-3p inhibits growth of osteosarcoma by targeting HDGF

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ABSTRACT

Osteosarcoma is the most common bone malignancy and miR-95-3p plays an important role in multiple cancers. The purpose of this study was to explore the effect and potential mechanism of miR-95-3p on the growth of osteosarcoma. *In vitro*, the osteosarcoma cell lines, SAOS-2 and U2OS cells, were transfected with miR-95-agomir to assess the role of miR-95-3p in proliferation and apoptosis of osteosarcoma cells. We determined that overexpression of miR-95-3p significantly attenuated cell proliferation but enhanced apoptosis in SAOS-2 and U2OS cells. We also found that overexpression of miR-95-3p in osteosarcoma cells downregulated the expression of hepatoma-derived growth factor (HDGF). Next, knockdown of HDGF by siRNA targeting HDGF clearly inhibited cell proliferation and induced apoptosis in U2OS cells. *In vivo*, a tumor formation assay in BALB/c nude mice was conducted by injecting the pre-miR-95 or control vector lentivirus-infected U2OS cells to determine the effect of miR-95-3p on the growth of osteosarcoma. Results showed miR-95-3p overexpression inhibited the osteosarcoma growth and downregulated the HDGF expression in xenografted tumor. For mechanism study, we co-transfected HDGF/pcDNA3.1 plasmid and miR-95-agomir to U2OS cells, and we demonstrated that overexpression of HDGF could attenuate the effects of miR-95-3p on U2OS cell proliferation, apoptosis and migration. These findings indicated that miR-95-3p might act as a potential tumor suppressor in osteosarcoma by targeting HDGF. Thus, miR-95-3p may become a potential therapeutic in treatment of osteosarcoma.

1. Introduction

Osteosarcoma is the most common primary malignancy of bone in adolescents and children [1]. Osteosarcoma easily develops metastases to lung, pleura, and heart with high rates (40%–50%) and the common treatment strategies including surgery, neoadjuvant chemotherapy and radiotherapy could improve a of 5-year overall survival rate of approximately 50% in patients [2–4]. It is therefore very necessary to uncover the molecular mechanisms for the progression of osteosarcoma and new therapeutic targets for osteosarcoma treatment. Many studies have focused on the development and metastases of osteosarcoma. Studies found that a number of biological proteins such as dopamine D1 receptors, CXCR4, and PTEN regulate the proliferation, apoptosis, migration and invasion of osteosarcoma through PI3K/Akt related signaling pathways [5–7]. Additionally, Jin et al. reported that the DNA-dependent protein kinase complex catalytic subunit (DNA-PKcs) is associated with restoring DNA damage in osteosarcoma and silencing DNA-PKcs is able to suppress cell proliferation, migration and invasion but induce apoptosis of osteosarcoma MG-63 cells [8].

MicroRNAs (miRNAs), a class of short noncoding RNAs with 18–25 nucleotides in length, play an important role in inducing mRNA degradation or translation repression by interacting with the 3′-UTR or coding region of mRNAs [9,10]. A large number of studies showed that multiple miRNAs such as miR-192, miR-330-3p, MiR-451 and miR-30a are implicated in malignant behaviors of osteosarcoma cells including cell proliferation, apoptosis, invasion and migration by targeting different bioactive proteins [11–14]. Studies also have shown that miR-95 is an important regulator involved in cancer progression. It's found that upregulation of miR-95 promotes hepatocarcinogenesis, enhances cell proliferation and migration, and promotes cancer cell growth in colorectal carcinoma [15–17]. However, Wei Zhang et al. found that downregulation of miR-95 could improve EMT in gastric cancer, which

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will help promote gastric cancer cell migration and invasion [18]. In osteosarcoma patients, serum miR-95-3p level is clearly decreased and could be used as a marker for the diagnosis and prognosis of osteosarcoma, but the effect of miR-95-3p on osteosarcoma growth has not yet been fully understood [19].

HDGF (hepatoma-derived growth factor) has been considered as a growth factor and anti-apoptotic factor for development of multiple malignant tumors by promoting cellular proliferation and invasion and inhibiting apoptosis of cancer cells [20–22]. It has reported that HDGF has highly expressed in osteosarcoma tissues and cells and promotes osteosarcoma cell proliferation through Akt and MAPK signaling pathways [23]. However, the direct connection between miR-95-3p and HDGF in the growth of osteosarcoma didn't investigate yet. In this study, the effects of miR-95-3p and HDGF on cell proliferation and apoptosis in osteosarcoma cells was determined. In addition, whether miR-95-3p regulating the development of osteosarcoma by targeting HDGF was explored both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and transfection

The osteosarcoma cell lines SAOS-2, U2OS, MG-63, 143B were purchased form Cell Research (Shanghai, China) and normal osteoblast cell line hFOB 1.19 was purchased from Procell (Wuhan, China). SAOS-2, U2OS and hFOB 1.19 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) and MG-63 and 143B were cultured in Eagle's Minimal Essential Medium (Cell Research) supplemented with 10% FBS (Hyclone, South Logan, UT, USA) in a humidified incubator at 37 °C with 5% CO_2. The miR-95-agomir or negative control (NC) was transfected into SAOS-2 and U2OS cells. Briefly, cells were seeded in 6-well plates and cultured till a density of approximately 70% confluent. Then the cells were transfected with 100 pmol of miR-95-agomir or NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

For small interfering RNA (siRNA) transfection, a siRNA targeting human HDGF (HDGF siRNA) was synthesized by Sangon Biotech (Shanghai, China). 100 pM of HDGF siRNA or negative control siRNA (NC) was transfected into U2OS cells using Lipofectamine 2000. The sequences of HDGF siRNA were as following: HDGF siRNA-Sense, 5′-AACCGGCAGAAGGAGUACAAATT-3′; HDGF siRNA- Antisense, 5′-UUUGUACUCCUUCUGCCGGUUTT-3′. For mechanism study, U2OS cells were co-transfected with constructed HDGF/pcDNA3.1 plasmid or pcDNA3.1 vector (Invitrogen) and miR-95-agomir or NC using Lipofectamine 2000. The HDGF primers for PCR amplification were obtained from GenScript (Nanjing, China) and the sequences were as following: HDGF Forward, 5′-CAAGGATCCATGT CGCGATCCAACCGG CAGAA-3′; HDGF Reverse, 5′- CACCTCGAGCTACAGGCTCTCATG ATC TCTG-3′. After transfection, cells were incubated in DMEM medium with 10% FBS before subjecting to the required experiments.

2.2. CCK-8 assay

Cell proliferation assay was measured with a Cell Counting Kit-8 (CCK-8) (KeyGen Biotech, Nanjing, China). The SAOS-2 and U2OS cells (5×10^3) were seeded into 96-well plates with 5 replications per group and 50 pmol of miR-95-agomir, HDGF siRNA or their controls were transfected to cells. After transfection, the cells were cultured subsequently for 0, 24, 48, 72, 96 and 120 h, respectively. U2OS cells were also co-transfected with HDGF/pcDNA3.1 plasmid (or pcDNA3.1 vector) and miR-95-agomir (or NC) and cultured for 48 h. The 10 μl CCK-8 solution was added to each well and the cells were incubated for 2 h at 37 °C. The optical density at 450 nm for each well was measured using a microplate reader (BioTek, Vermont, USA).

2.3. Annexin V/propidium iodide (PI) staining

Cell apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). After transfection, the SAOS-2 and U2OS cells were cultured for 48 h before harvesting cells. The cells were washed and then resuspended in 195 μl of binding buffer containing 5 μl Annexin V-FITC and 10 μl propidium iodide (PI). After incubation at room temperature for 20 min in darkness, the samples were analyzed using the flow cytometry (Aceabio, San Diego, CA, USA)

2.4. Cell cycle analysis

The transfected cells were cultured for 48 h and collected by centrifuging. Then the cells were fixed with ice-cold 70% ethanol at 4 ℃ overnight. After washing, the cells were stained with 25 μl propidium iodide (PI) and 10 μl RNase A in 500 μl binding buffer at 37 ℃ for 30 min in darkness, followed by flow cytometry analysis (Aceabio).

2.5. Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was used to measure miR-95-3p and HDGF expression levels in transfected SAOS-2 and U2OS cells. Briefly, total RNAs from cultured SAOS-2 and U2OS cells were extracted by the RNA simple total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The cDNA of miR-95-3p and HDGF was reversely transcribed using a TIANSeq M-MLV (Tiangen) and expression levels of miR-15a-3p and HDGF were measured by qRT-PCR system containing 2 × Taq PCR MasterMix (Tiangen) and SYBR Green (Solarbio, Beijing, China) according to the manufacturer's protocol. U6 and GAPDH were used as normalizing controls to normalize miR-95-3p and HDGF expression using the $2^{-\triangle\triangle CT}$ method, respectively.

2.6. Western blot

Protein levels were determined by western blot. Briefly, cells were lysed in RIPA buffer (Solarbio) containing 10 μl PMSF (Solarbio) and the protein concentrations were determined using BCA Protein Assay Kit (Solarbio). 10–20 μg equal amounts of protein were separated by SDS-PAGE and then transferred to the PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in TBST buffer for 1 h and then incubated overnight at 4 °C with primary antibodies: anti-PCNA (1:5000, Proteintech, Wuhan, China), anti-cyclin E (1:1000, Proteintech), anti-CDK2 (1:500, Proteintech), anti-Bcl-2 (1:2000, Proteintech), anti-Bax (1:5000, Proteintech), anti-cleaved caspase-3 (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-caspase-9 (1:1000, Cell Signaling Technology), anti-HDGF (1:2000, Proteintech) and anti-GAPDH (1:10000, Proteintech). After washing for 4 times with TBST, membranes were incubated with the HRP-conjugated (1:3000, Solarbio) at 37 °C for 1 h and the signals were visualized using ECL reagent (Solarbio). The relative level of target proteins was normalized to those of GAPDH.

2.7. Wound healing assay

Whether HDGF could rescue the miR-95-3p affecting U2OS cells migration was determined using wound healing assay. U2OS cells were co-transfected with constructed HDGF plasmid or vector and miR-95-agomir or NC. After 24 h, cells were treated with serum-free medium containing 1 μg/ml mitomycin C (Sigma, St. Louis, MO, USA) for 1 h. A scratch wound was made by a 200 μl aseptic sterile pipette tip. After washing with serum-free medium, the wound area was marked and photographed using a microscope. Then cells were cultured in serum-free medium for 24 h and then photographed.

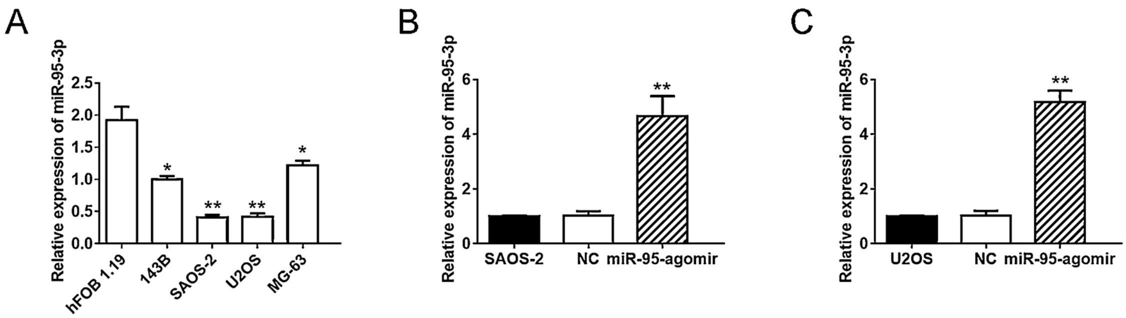

Fig. 1. Expression of miR-95-3p is downregulated in osteosarcoma cells.
(A) The miR-95-3p expression levels in 143B, SAOS-2, U2OS, MG 63 cells and normal osteoblast cell line hFOB 1.19 were determined by quantitative real-time PCR (qRT-PCR). The miR-95-agomir or negative control (NC) was transfected in SAOS-2 and U2OS cells, respectively. Expression of miR-95-3p in miR-95-agomir or NC transfected SAOS-2 (B) and U2OS (C) cells was determined by qRT-PCR. N = 3 for each experiment and the data were presented as ± standard deviation (SD). Significant differences compared to control groups were indicated as *$P < 0.05$, **$P < 0.01$.

2.8. *Lentivirus infection*

U2OS cells were seeded in 6-well plates in fresh complete medium and the lentivirus containing a pre-miR-95 or negative control (NC) was added to cells, respectively. After incubation 24 h, the cells were taken photos under a fluorescence microscope (Olympus, TKY, Japan). The infected-cells were selected by 2 μg/ml puromycin and the medium was changed every 2 days for 5 days till several puromycin-resistant single cells appeared. Then the cells were cultured in complete medium without puromycin for 10 days to form puromycin-resistant colonies. The healthy cell colonies were passaged and cultured in a humidified incubator at 37 °C with 5% CO_2 for subsequent studies.

2.9. *Tumor formation assay*

BALB/c nude mice (HFK Bioscience, Beijing, China) at the age of 6–8 weeks weighing 18–20 g were housed under pathogen-free, air-controlled conditions. The mice were randomly divided into three groups and each group consisted of 6 mice. The 1×10^7 osteosarcoma tumor cells stably expressing pre-miR-95 (U2OS), NC or U2OS only in 200 μl PBS were injected subcutaneously into the right flank of the mice. Tumor volume was measured every 5 days. After 27 days, all mice were sacrificed and tumor weights and volumes were measured. The study was approved by the legislation and ethical boards of Honghui Hospital. All protocols were conformed with the Guideline for the care and use of laboratory animals.

2.10. *Hematoxylin-Eosin (HE) staining*

The osteosarcoma tissue sections were made and stained with hematoxylin (Solarbio) and eosin (Sangon). Briefly, the sections were deparaffinized in xylene (Sinopharm, Shanghai, China) and successively rehydrated in ethanol (Sinopharm). The sections were stained in hematoxylin for 5 min and eosin for 3 min. After dehydration, the slides were mounted with coverslip and photographed under the microscope with a 200 × magnification (Olympus).

2.11. *Immunohistochemical analysis*

Immunohistochemical staining was performed on paraffin-embedded osteosarcoma tissue sections to determine the expression of proliferating cell nuclear antigen (PCNA). Briefly, the sections were incubated with PCNA antibody (1:50, Proteintech) at 4 ℃ overnight and detected by the HRP-conjugated secondary antibody (1:500, Thermo Fisher Scientific, Waltham, MA, USA) after incubation at 37 ℃ for 60 min. Sections were incubated with peroxidase substrate DAB (Solarbio) for color development and counterstained with hematoxylin for 3 min. The slides were observed and photographed under the microscope with a 400 × magnification (Olympus).

2.12. *TUNEL assay*

In situ Cell Death Detection Kit (Roche, Basel, Switzerland) was used for detection of apoptotic cells of osteosarcoma tissue. The deparaffinized sections were incubated with TUNEL reaction mixture for 60 min at 37 ℃ in darkness. After washing, the sections were incubated with converter-POD at 37 °C for 30 min and followed by incubation in DAB solution. The slides were counterstained with haematoxylin for 3 min and photographed using the microscope with a 400 × magnification (Olympus).

2.13. *Luciferase reporter assay*

Partial DNA sequences of HDGF 3′-UTR containing wild type or mutant binding sites for miR-95-3p were amplified by PCR and then cloned into pmir-GLO vector (Promega, Madison, WI, USA). The recombinant plasmids pGL3-HDGF-wild type and pGL3-HDGF-mutant were respectively co-transfected with miR-95-agomir or NC into 293 T cells using Lipofectamine 2000. After 48 h, the luciferase activities were measured using the Dual Luciferase Reporter Gene Assay Kit (KeyGen Biotech) according to the manufacturer's instructions.

2.14. *Statistical analysis*

All analyses were performed using GraphPad Prism software version 6.00 (San Diego, CA, USA). Data were expressed as the mean ± standard deviation (SD). Differences were evaluated using the one-way and two-way ANOVA. Significant differences were accepted at a $P < 0.05$.

3. Results

3.1. *Expression of miR-95-3p in osteosarcoma cells*

We chose 4 osteosarcoma cell lines and a normal osteoblast cell line hFOB 1.19 to detect the expression of miR-95-3p by RT-qPCR, the results in Fig. 1A showed that expressions of miR-95-3p were remarkably decreased in osteosarcoma cell lines compared to normal osteoblast cell, especially in SAOS-2 and U2OS cell lines. Thus SAOS-2 and U2OS cells were used for further study. To investigate the role of miR-95-3p in the osteosarcoma progression, we transfected miR-95-agomir or NC to SAOS-2 and U2OS cells. The expression of miR-95-3p was significantly increased in miR-95-agomir transfected cells compared to NC transfected cells (Fig. 1B and C).

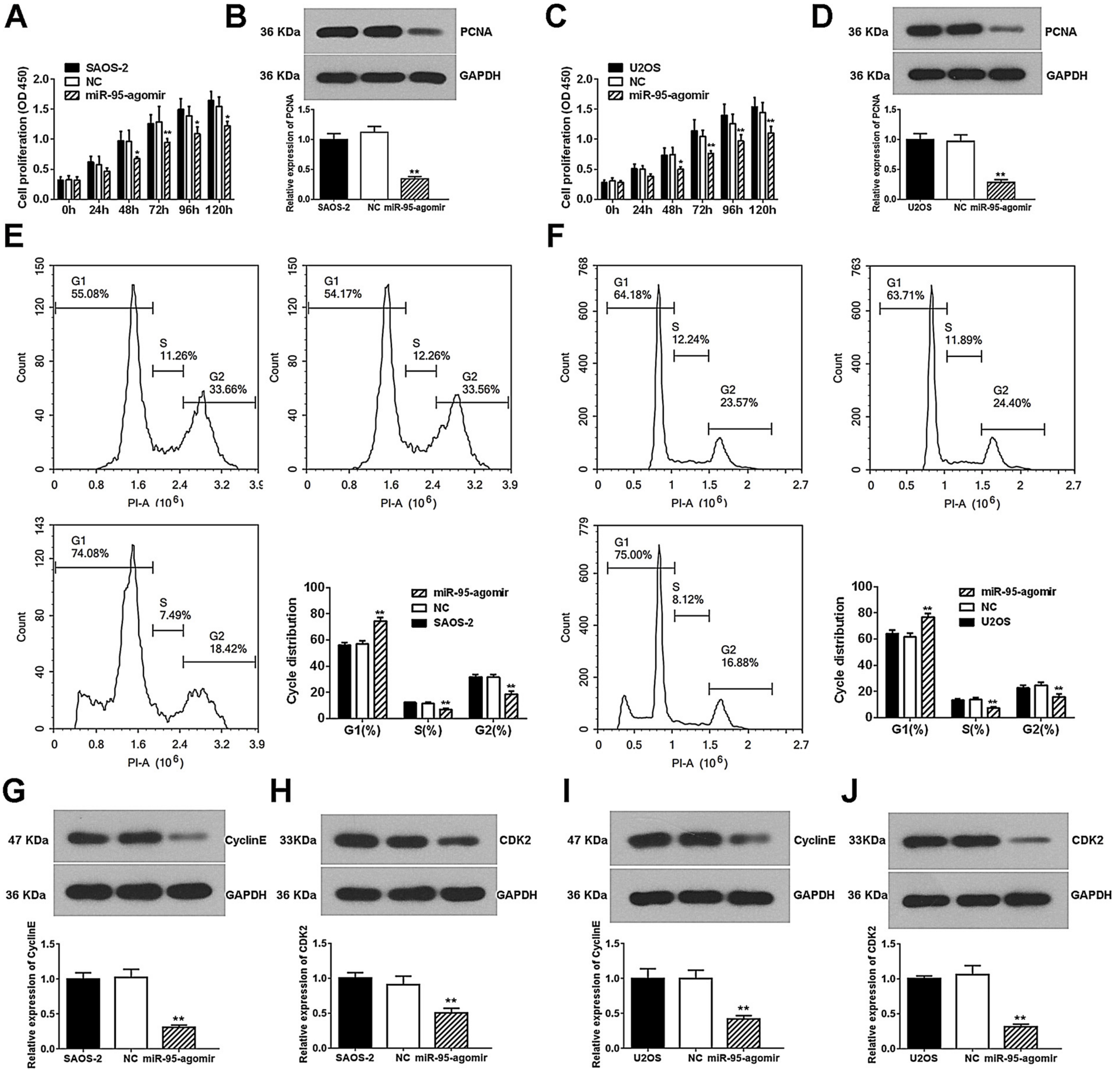

Fig. 2. Upregulating of miR-95-3p inhibits proliferation of osteosarcoma cells.
SAOS-2 and U2OS were transfected with miR-95-agomir or negative control (NC). Transfected SAOS-2 (A) and U2OS (C) cells were subject to the CCK-8 assay to evaluate degree of cell proliferation was determined at 450 nm. The PCNA protein levels in SAOS-2 (B) and in U2OS (D) cells were detected by western blot. Cell cycle assay of SAOS-2 (E) and U2OS (F) cells was detected by flow cytometry. The protein levels of cyclin E and CDK2 in SAOS-2 (G, H) and in U2OS (I, J) cells were determined by western blot. N = 3 for each experiment and the data were presented as mean ± standard deviation (SD). Significant differences compared to control groups were indicated as $*P < 0.05$, $**P < 0.01$. PCNA, proliferating cell nuclear antigen. CKD2, Cyclin-dependent kinase 2.

3.2. Upregulating of miR-95-3p inhibits proliferation of osteosarcoma cells

The CCK-8 assay was used to assess the effect of miR-95-3p on osteosarcoma cell proliferation. According to the results in Fig. 2A and C, compared to NC, upregulation of miR-95-3p significantly inhibited the proliferation of SAOS-2 and U2OS cells. As shown in Fig. 2B and D, the expression levels of PCNA were remarkably decreased in miR-95-agomir groups both in SAOS-2 and U2OS cells. Compared to NC, cell cycle analysis results showed miR-95-3p induced cells accumulating in the G1 phase of cell cycle progression from 54.17% to 74.08% in SAOS-2 cells and from 63.71% to 75.00% in U2OS cells and reduced the cell percentages in S phase from 12.26% to 7.49% in SAOS-2 cells and from 11.89% to 8.12% in U2OS cells. The cell percentages in G2 phase were also decreased from 33.56% to 18.42% in SAOS-2 cells and from 24.40% to 16.88% in U2OS cells (Fig. 2E and F). Meanwhile, the expression levels of cyclin E and cyclin-dependent kinase 2 (CDK2) were remarkably decreased both in SAOS-2 and U2OS cells (Fig. 2G–J). The results indicated that upregulation of miR-95-3p inhibits the osteosarcoma cell proliferation.

3.3. Upregulating of miR-95 induces apoptosis of osteosarcoma cells

For apoptosis detection, miR-95-agomir transfected SAOS-2 and U2OS cells had a significant increase in numbers of apoptosis cells compared to NC group (Fig. 3A and B). In Fig. 3C–J, the expression levels of cleaved caspase-3, cleaved caspase-9 and Bax were markedly increased, whereas Bcl-2 was remarkably decreased in miR-95-agomir group compared to NC both in SAOS-2 and U2OS cells. The data showed that upregulation of miR-95-3p induces apoptosis of osteosarcoma cells.

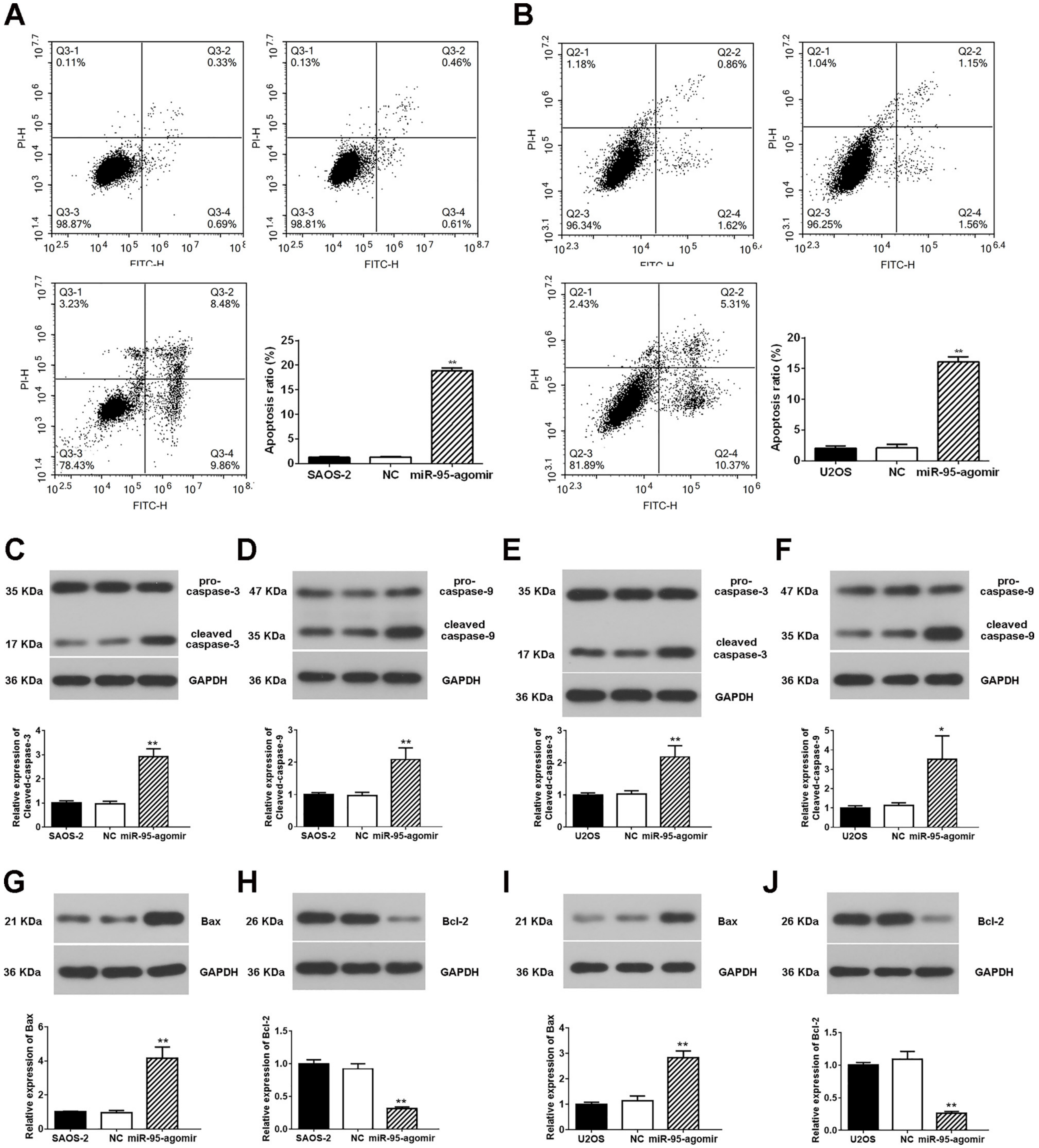

Fig. 3. Upregulating of miR-95-3p induces apoptosis of osteosarcoma cells.
SAOS-2 and U2OS were transfected with miR-95-agomir or negative control (NC). Cell apoptosis of SAOS-2 (A) and U2OS (B) cells after Annexin V/PI staining was detected by flow cytometry. The protein levels of pro-caspase-3, cleaved caspase-3, pro-caspase-9, cleaved caspase-9, Bax and Bcl-2 in SAOS-2 cells (C, D, G, H) and in U2OS cells (E, F, I, J) cells were determined by western blot. N = 3 for each experiment and the data were presented as mean ± standard deviation (SD). Significant differences compared to control groups were indicated as $^{*}P < 0.05$, $^{**}P < 0.01$.

3.4. MiR-95-3p regulates proliferation and apoptosis of osteosarcoma cells through targeting HDGF

The protein and mRNA levels of HDGF in miR-95-agomir transfected SAOS-2 and U2OS cells were detected. As shown in Fig. 4A–D, compared to NC transfected cells, the expression level of HDGF was remarkably reduced in miR-95-agomir transfected cells. To confirm the relationship between miR-95-3p and HDGF, in Fig. 4E, we cloned the wild-type HDGF 3′-UTR or mutant HDGF 3′-UTR sequences into the pGL3 luciferase reporter vector, respectively. As shown in Fig. 4F, miR-95-agomir decreased the luciferase activity in the HDGF-wild type group in U2OS cells compared to NC group but HDGF-mutant didn't show any changes. In Fig. 4G and H, when HDGF siRNA was transfected into U2OS cells, cell proliferation was markedly decreased and the proportion of cells in G1 phase was increased from 65.59% to 75.05%, whereas the proportion of cells in S and G2 phases were reduced from

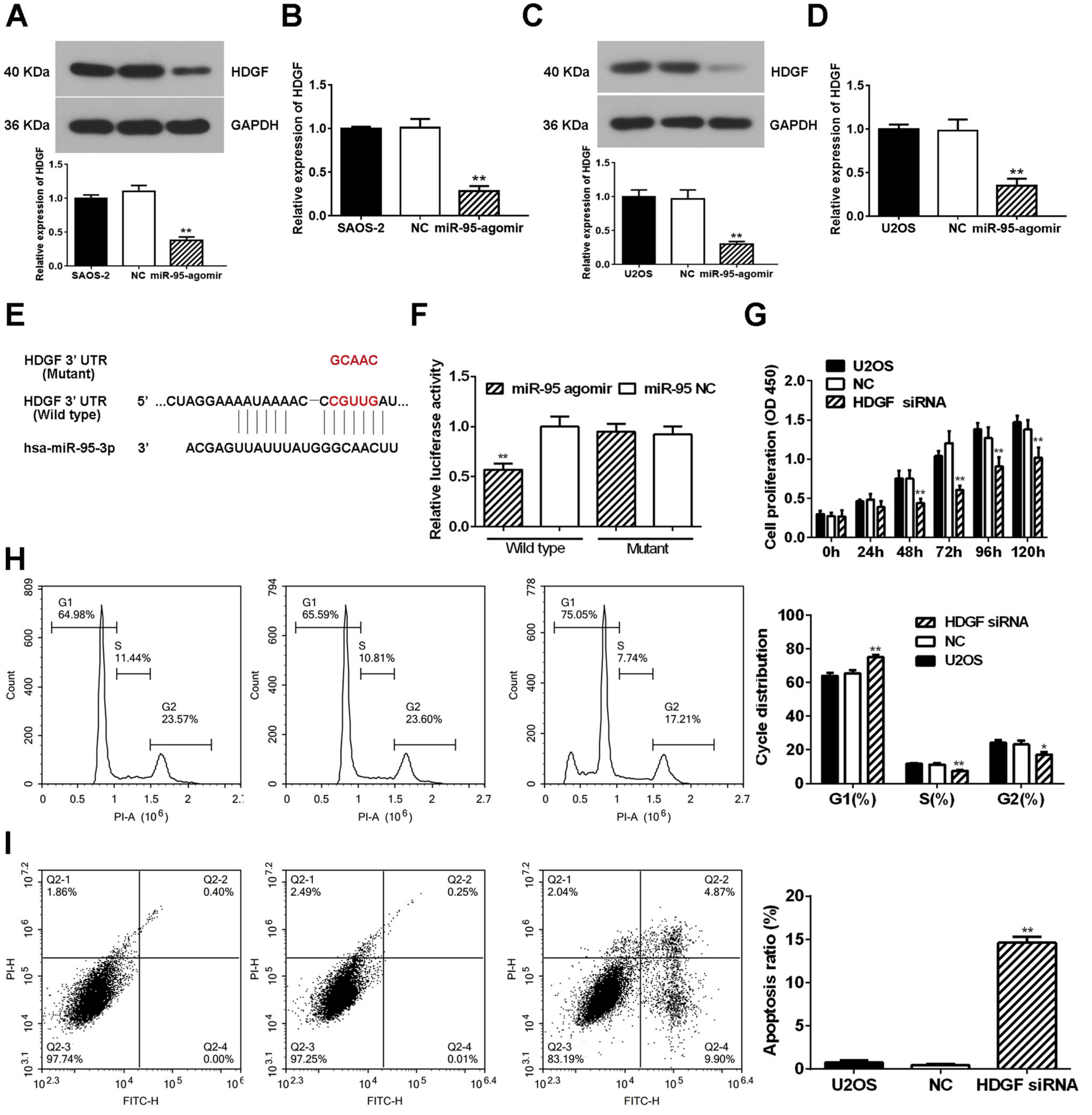

Fig. 4. MiR-95-3p regulates proliferation and apoptosis of osteosarcoma cells through targeting HDGF.
The protein and mRNA level of HDGF in miR-95-agomir or negative control (NC) transfected SAOS-2 (A, B) and U2OS (C, D) cells were detected. (E) Schematic representation of HDGF 3′-UTR showed a putative miR-95-3p binding site. The seed location for wild type 3′-UTR and mutant 3′-UTR of HDGF was indicated with red. The miR-95-agomir or NC was co-transfected with pGL3-HDGF-wild type or pGL3-HDGF-mutant into U2OS cells for 48 h. (F) The luciferase activity of HDGF-wild type and HDGF-mutant in U2OS cell following transfection with miR-95-agomir or NC was measured. HDGF siRNA or negative control (NC) were transfected into U2OS cells. (G) CCK-8 assay was used to evaluate degree of cell proliferation of HDGF knockdown and control cells. Cell cycle assay (H) and cell apoptosis (I) of HDGF knockdown and control cells were detected by flow cytometry. N = 3 for each experiment and the data were presented as mean ± standard deviation (SD). Significant differences compared to control groups were indicated as $*P < 0.05$, $**P < 0.01$. HDGF, hepatoma-derived growth factor.

10.81% to 7.74% and from 23.60% to 17.21%, respectively. Moreover, the results from Fig. 4I showed that knockdown of HDGF significantly induced the apoptosis of U2OS cells. Taken together, we demonstrated that HDGF is the potential target of miR-95-3p.

3.5. Upregulating of miR-95-3p inhibits growth of osteosarcoma through regulating HDGF

After lentivirus infection, the stable pre-miR-95 or NC transfected U2OS cells were achieved (Fig. 5A). The miR-95-3p expression was upregulated in stable pre-miR-95 transfected U2OS cells and in lenti-pre-miR-95 xenografted tumor tissue than that in NC cells and lenti-vector xenografted tumor tissue, respectively (Fig. 5B). In addition, the tumor growth (volume and weight) in pre-miR-95 injected mice were significantly reduced about 4 times compared to NC injected mice (Fig. 5C, E and F). Moreover, miR-95-3p clearly inhibited the expression of PCNA showing in immunohistochemistry staining and induced the apoptosis of xenografted tumor cells in HE and TUNEL staining

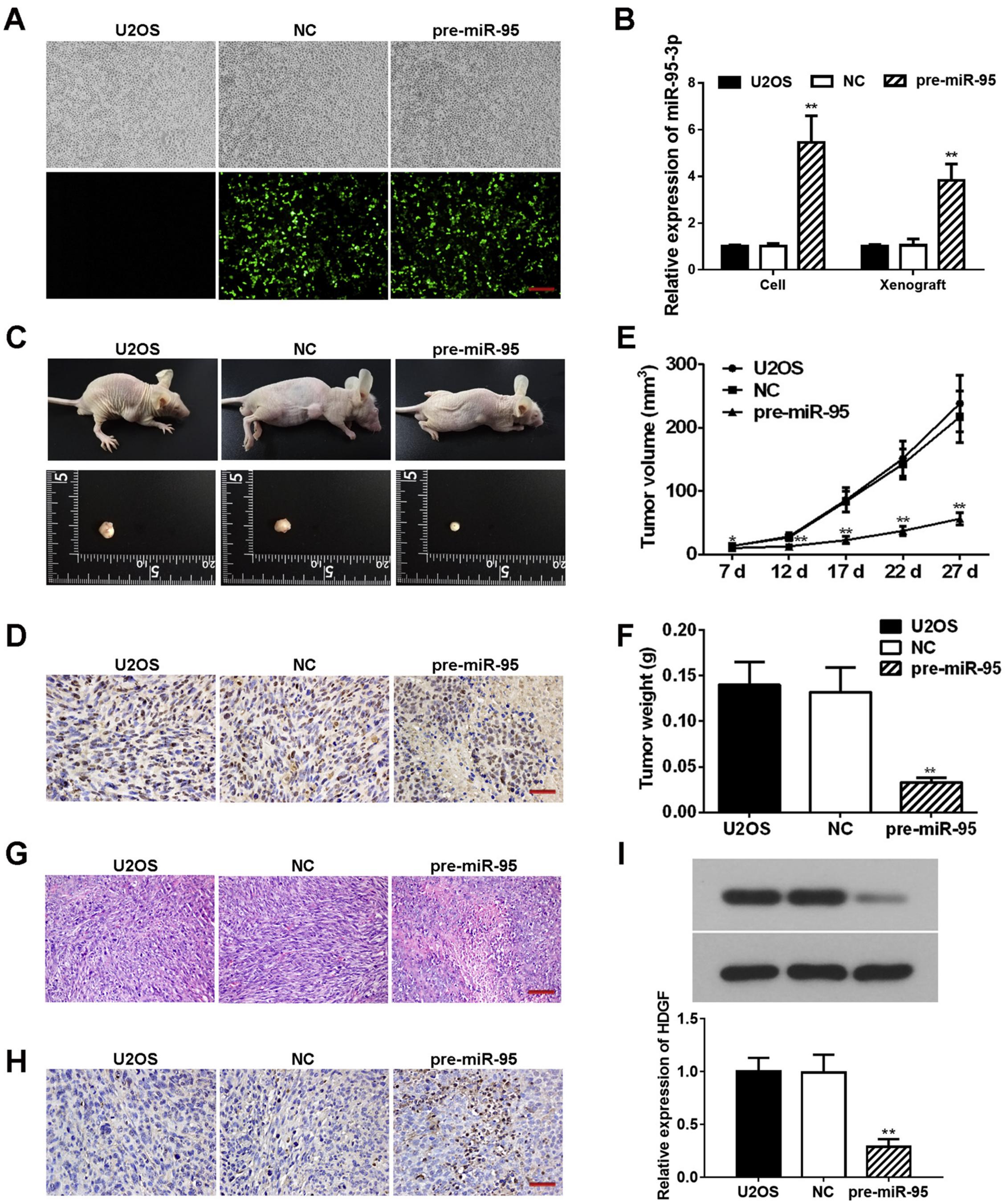

Fig. 5. Upregulating of miR-95-3p inhibits growth of osteosarcoma.

Lentivirus expressing pre-miR-95 or negative control (NC) stably infected U2OS cells were subcutaneously injected into nude mice. (A) Transfection efficiency of pre-miR-95 or NC was detected after 24 h infection by fluorescent microscopy; Scale bar = 200 μm. (B) The expression of miR-95-3p in U2OS cells and the tumor tissues from pre-miR-95 and NC group mice was detected by quantitative real-time PCR (qRT-PCR). (C) Photograph showing excised tumors in representative mice in each group mice on experimental day 27. (D) Immunohistochemical staining of PCNA in tumor tissues from pre-miR-95 and NC group mice; Scale bar = 50 μm. (E) Growth curves for tumor volume for the pre-miR-95 and NC group mice at the indicated times. (F) Tumor weight of the pre-miR-95 and NC group mice. The tumor sections from the pre-miR-95 and the NC group mice were stained with (G) HE-haematoxylin and eosin (Scale bar = 100 μm) and (H) TUNEL (Scale bar = 50 μm). (I) The mRNA of HDGF in the tumor tissues from pre-miR-95 and NC group was determined by western blot. N = 3 for U2OS cells and N = 6 for tumor tissues. Data were shown as mean ± standard deviation (SD). Significant differences compared to control groups were indicated as $^*P < 0.05$ and $^{**}P < 0.01$. HDGF, hepatoma-derived growth factor.

(Fig. 5D, G and H). The western blot result showed the protein level of HDGF in lenti-pre-miR-95 xenografted tumor was decreased compared to lenti-vector xenografted tumor after 27 days growth in nude mice (Fig. 5I).

In order to confirm that miR-95-3p inhibits the growth of osteosarcoma by targeting HDGF, we constructed HDGF/pcDNA3.1 plasmid to overexpress HDGF in U2OS cells (Fig. 6A). Furthermore, we co-transfected with constructed HDGF/ pcDNA3.1 plasmid or pcDNA3.1 vector and miR-95-agomir or NC agomir into U2OS cells as following groups: NC agomir + pcDNA3.1, miR-95-agomir + pcDNA3.1, miR-95-agomir + HDGF/pcDNA3.1. As the results shown in Fig. 6B and C, the miR-95-3p expression was negatively correlated to HDGF expression.

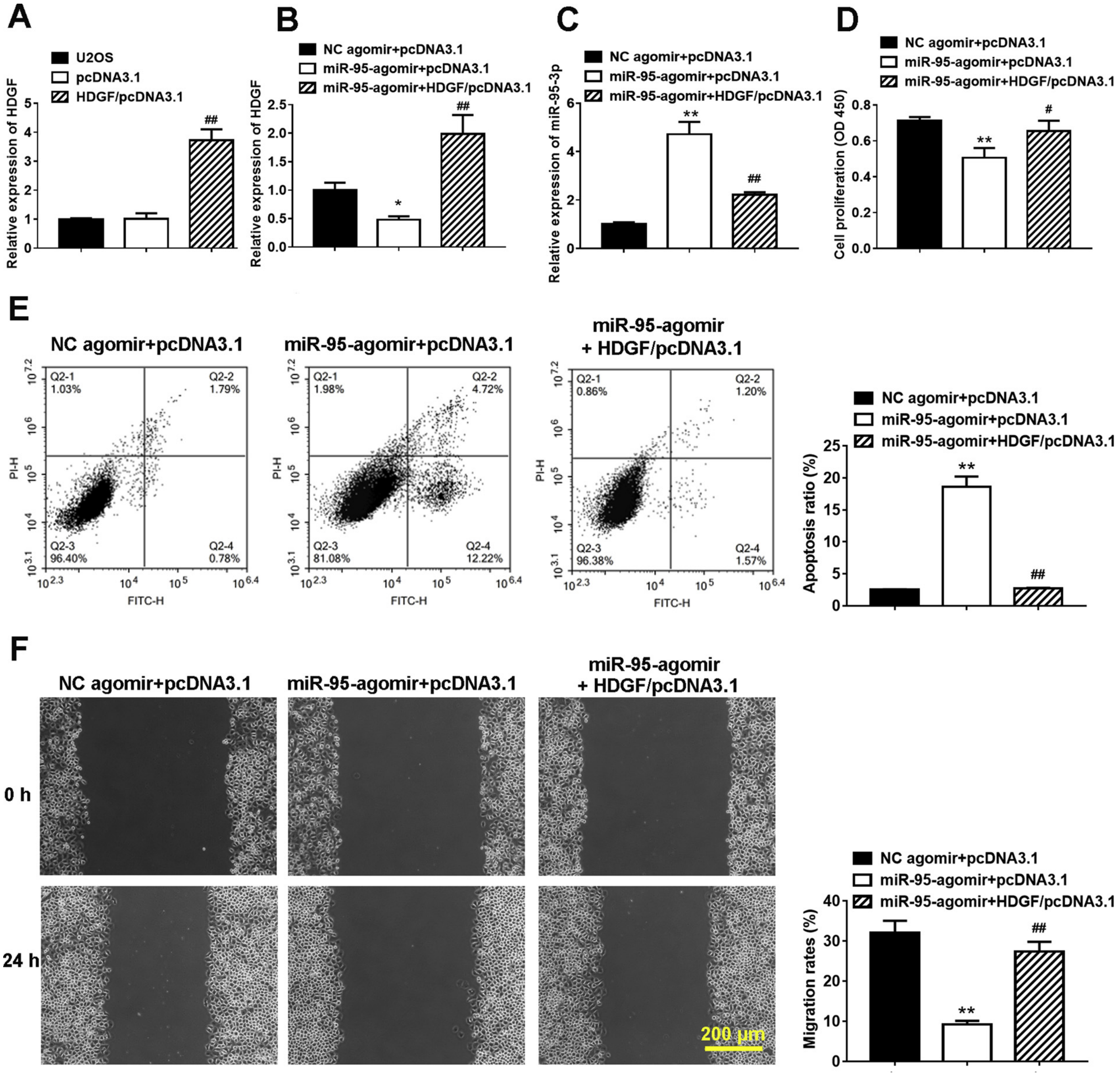

Fig. 6. Overexpression of HDGF attenuates the miR-95-3p-induced changes in osteosarcoma cells.

(A) HDGF/pcDNA3.1 plasmid for HDGF overexpression was constructed and transfected in U2OS cells and the expression of HDGF detected by quantitative real-time PCR (qRT-PCR). The constructed HDGF plasmid or pcDNA3.1 vector was co-transfected with miR-95-agomir or NC agomir (negative control) into U2OS cells. Expression levels of HDGF (B) and miR-95-3p (C) were detected after 48 h transfection. The proliferation (D) and apoptosis (E) of transfected U2OS cells were determined after 48 h transfection. The migration (F) of transfected U2OS cells were determined after 24 h transfection. N = 3 and data were shown as mean ± standard deviation (SD). Significant differences compared to NC agomir + pcDNA 3.1 group were indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$ and significant differences compared to pcDNA 3.1 or miR-95-agomir + pcDNA 3.1 group were indicated as $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$. HDGF, hepatoma-derived growth factor.

Fig.6D–F showed overexpression of HDGF attenuated the decrease of cell activity and migration and increase of apoptosis regulated by miR-95-3p in U2OS cells. The results from *in vivo* and *in vitro* indicated that upregulating of miR-95-3p inhibits osteosarcoma tumor growth through regulating HDGF expression.

4. Discussion

Osteosarcoma is the most common bone malignancy in adolescents and children [1]. Many studies were reported to explore the molecular mechanisms for the development and progression of osteosarcoma. In this study, we tried to investigate the effects of miR-95-3p on osteosarcoma and the potential regulatory mechanism. Much work has been carried out on regulation of osteosarcoma malignancies by miRNAs. Except for the miRNAs we mentioned above, many other miRNAs such as miR-664, miR-194 and miR-423-5p are also able to modulate osteosarcoma cell proliferation, migration, invasion and apoptosis through their target genes FOXO 4, CDH2 and stathmin1, respectively [10,24,25]. Additionally, miRNAs could be regulated by long non-coding RNAs (lncRNAs) to modulate cell proliferation, apoptosis, and migration of osteosarcoma cells [26,27].

The roles of miR-95-3p in regulating proliferation, apoptosis, migration and invasion of carcinoma have determined in multiple cancer types including colorectal carcinoma, non-small cell lung cancer, hepatocellular carcinoma, gastric cancer, lung adenocarcinoma [15,16,18,28,29]. In osteosarcoma, study found that the levels of miR-95-3p in serum of osteosarcoma patients are lower than the healthy controls, and the expression of miR-95-3p is positively correlated with the survival rate of patients with osteosarcoma [19]. However, the effects of miR-95-3p on osteosarcoma cell and the regulatory mechanisms need to be uncovered.

In this study, we demonstrated that miR-95-3p expression was significantly decreased in osteosarcoma cell lines compared to control cells, especially in SAOS-2 and U2OS cell lines. We determined that upregulation of miR-95-3p reduced cell proliferation and arrested the cell cycle at G1 phase by significantly decreasing the protein expression levels of cyclin E and CDK2 both in SAOS-2 and U2OS cells. In addition, upregulation of miR-95-3p also induced mitochondrial and caspases mediated apoptosis in osteosarcoma cells by reducing the expression of Bcl-2 and elevating the expression levels of Bax, cleaved caspase-3 and cleaved caspase-9. *In vivo*, the xenografted osteosarcoma tumor volume in miR-95-3p overexpressing group was about 4 times fold less than NC group. These findings were consistent with clinical outcome reports and it indicated that miR-95-3p has a potential role to suppress osteosarcoma growth.

In order to understand the mechanism how miR-95-3p regulates osteosarcoma development, bioinformatics prediction was performed and the result showed HDGF might be target gene for miR-95-3p. It has been reported that HDGF is highly expressed in osteosarcoma tissues and cells, and overexpression of HDGF significantly promotes cell proliferation and tumor growth of osteosarcoma [23]. In our results, the mRNA and protein levels of HDGF were remarkably reduced in miR-95-3p overexpressing cells and luciferase reporter assay confirmed the correlation of miR-95-3p and HDGF. Next, we determined knockdown of HDGF clearly inhibited cell proliferation and enhanced apoptosis of U2OS cells. In addition, the HDGF expression was dramatically decrease in miR-95-3p overexpressed xenografted tumors *in vivo*. Mechanically, overexpression of HDGF attenuated the effects of miR-95-3p on inhibiting proliferation and migration and inducing apoptosis of U2OS cells. Obviously, the results suggested that miR-95-3p may play its roles in osteosarcoma through directly targeting HDGF.

HDGF plays the important roles in tumor development through multiple regulating pathways. Studies found that knockdown of HDGF accumulates cell in G1 phase and decreases the percentage of cells in S phase of cell-cycle progression in Ewing's sarcoma cell [30]. Additionally, HDGF can activate MAPK/ERK and/or PI3K/Akt by binding a receptor on the cell surface to promote cellular proliferation, and HDGF also stimulates tumor cell DNA synthesis and proliferation by DNA binding following nuclear translocation [20,31]. Moreover, HDGF interacts with β-catenin as a positive feedback loop to regulate cell proliferation, apoptosis, migration and invasion of colorectal cancer cells [32]. Therefore, our results were consistent with previous reports and it suggested that upregulation of miR-95-3p could negatively regulate HDGF expression, leading to inactivate signaling pathways such as the MAPK/ERK and PI3K/Akt to inhibit the proliferation, migration and induce apoptosis of osteosarcoma cells.

In conclusion, our results indicated that miR-95-3p is significantly downregulated in osteosarcoma cell lines. We also demonstrated that upregulation of miR-95-3p inhibits osteosarcoma cell proliferation and migration and reduces cell apoptosis, and suppress miR-95-3p overexpressed xenografted tumor growth through targeting HDGF. Thus, our study revealed miR-95-3p might be a biomarker and tumor regulator in osteosarcoma and miR-95-3p/HDGF axis may be a potential target for the treatment of osteosarcoma.

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