microRNA-625 targets Yes-associated protein 1 to suppress cell proliferation and invasion of osteosarcoma

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Abstract. Osteosarcoma (OS) is the leading cause of cancer-associated mortality in adolescents and children. MicroRNAs (miRNAs) have critical roles in cancer, particularly in the initiation and progression of OS. Therefore, OS-associated miRNAs should be identified for use as therapeutic targets for treatment of OS. This study aimed to investigate the expression pattern, potential roles and underlying mechanism of microRNA-625 (miR-625) in OS. miR-625 was markedly downregulated in OS tissues and cell lines compared with that in associated adjacent non-tumor tissues and human normal osteoblasts, respectively. The enforced expression of miR-625 using miRNA mimics significantly reduced the proliferation and invasion of OS cells. Bioinformatics analysis and luciferase reporter assays indicated that miR-625 targeted the 3'-untranslated region of Yes-associated protein 1 (YAP-1). Furthermore, upregulation of miR-625 reduced endogenous YAP1 expression at the mRNA and protein levels. The upregulated expression of YAP1 in OS tissues was inversely correlated with miR-625 expression. YAP1 restoration using a recombinant plasmid rescued the miR-625-mediated tumor-suppressive effects in OS cells. In conclusion, miR-625 attenuated the cell proliferation and invasion of OS by suppressing YAP1. Thus, miR-625 may be a potential target for OS therapy.

Introduction

Osteosarcoma (OS), an aggressive bone neoplasm, is the leading cause of cancer-associated mortality in adolescents and children (1). OS predominantly occurs in long bones, especially in the metaphyses, proximal tibia, proximal humerus and distal femur (2). Currently, treatment modalities for OS predominantly include chemotherapy, surgery and radiotherapy (3). Despite the development of multiple therapeutic strategies, the five-year survival rate and prognosis of patients with OS remain poor; the majority of patients eventually die due to local relapse or pulmonary metastases following surgical resection (4,5). The five-year survival rate is 60-80% for patients with localized lesions and 15-30% for those with metastasis (6). The molecular mechanisms underlying the progression and metastasis of OS remain poorly understood (7). These mechanisms must be elucidated to provide novel therapeutic targets or candidates for treatment of patients with OS.

MicroRNAs (miRNAs) are small, endogenous and noncoding RNA molecules, of 17-24 nucleotides (8). miRNAs post-transcriptionally modulate gene expression by base pairing to complementary sites in the 3'-untranslated region (3-UTRs) of their target mRNAs; this phenomenon leads to mRNA degradation or inhibition of translation (9). Over 1,000 miRNAs are predicted to exist in the human genome and may regulate as much as 60% of protein coding genes in humans (10). Increasing evidence has demonstrated that miRNAs have significant roles in various physiological processes, including cell proliferation, cycle, differentiation, apoptosis, angiogenesis, invasion and metastasis (11-14). Studies have reported that numerous miRNAs are aberrantly expressed in human cancers and are significantly correlated with tumorigenesis and tumor development by acting as oncogenes or tumor suppressors (15-17). Upregulated miRNAs function as oncogenes by negatively regulating tumor suppressor genes. By contrast, downregulated miRNAs act as tumor suppressors by blocking oncogenes in tumor progression (18,19). Hence, miRNAs are considered putative targets for diagnosis, prognosis and treatment of patients with tumors.

Researchers have investigated the role of miRNA-625 (miR-625) in several types of human cancer (20-22); however, miR-625 in OS has not been investigated yet, to the best of our knowledge. In the present study, the expression levels of miR-625 in OS tissues and cell lines were determined and the biological role was examined in OS cells. The molecular mechanisms underlying the functions of miR-625 in OS cells were also investigated.

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Materials and methods

Tissue samples and cell lines. Between February 2012 and April 2015, 29 paired OS tissues and associated adjacent non-tumor tissues were obtained from patients (18 males, 11 females; age range, 19-63 years; mean age, 34 years) during surgery at the Central Hospital of Enshi Autonomous Prefecture (Enshi, China). None of these patients had received chemotherapy or radiotherapy prior to surgery. All tissues were immediately snap-frozen in liquid nitrogen and then stored at -80˚C. This study was approved by the Ethics Committee of The Central Hospital of Enshi Autonomous Prefecture. Additionally, signed informed consent was provided by all patients enrolled in this study.

OS cell lines (U2OS, Saos-2, and MG-63) and human normal osteoblasts (hFOB1.19) were purchased from the Institute of Cell Bank for Biological Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in 5% CO_2.

Transfection. Cells were seeded in 6-well plates and cultured to 70% confluence. Cells were transfected with miR-625 mimics, scramble miRNA negative control (NC; Guangzhou RiboBio Co., Ltd., Guangzhou, China), pCDNA3.1-YAP1 or pCDNA3.1 blank vector (Chinese Academy of Sciences, Shanghai, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's protocols. The miR-625 mimics sequence was 5'-AGGGGG AAAGUUCUAUAGUCC-3' and the miR-NC sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. After incubation at 37˚C with 5% CO_2 for 8 h, the culture medium was replaced by DMEM supplemented with 10% FBS. Then, 48 h post transfection, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine transfection efficiency. Cell Counting Kit-8 (CCK-8) and Transwell invasion assays were conducted at 24 and 48 h post transfection. Western blotting analysis was carried out at 72 h following transfection.

RNA extraction and RT-qPCR. Total RNA in tissues and cells was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The concentration of total RNA was determined by measuring absorbance at 260 nm using a NanoDrop spectrophotometer (ND-1000; Thermo Scientific Inc., Wilmington, DE, USA). For miR-625 detection, RT was performed using TaqMan MicroRNA Reverse Transcription kit, and qPCR was conducted with a TaqMan MicroRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI7900 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 was used as an internal control for miR-625. For the detection of YAP1 and GAPDH, used as the internal control, cDNA was synthesized using PrimeScript RT reagent Kit (Takara Biotechnology Co., Ltd., Dalian, China) and qPCR was performed with SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd., Dalian, China). The primers were designed as follows: miR-625, 5'-AGGGGGAAAGTTCTATAGTCC-3' (forward) and 5'-TGGTGTCGTGGAGTCG-3' (reverse); U6, 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCT TCACGAATTTGCGT-3' (reverse); YAP1, 5'-CAACTCCAA CCAGCAGCAACA-3' (forward) and 5'-GCAGCCTCTCCT TCTCCATCTG-3' (reverse); and GAPDH, 5'-CGGAGTCAA CGGATTTGGTCGTAT-3' (forward) and 5'-AGCCTTCTC CATGGTGGTGAAGAC-3' (reverse). Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (23).

CCK-8 assay. Cell proliferation was determined using the CCK-8 assay (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Cells were seeded into 96-well plates at a density of $3x10^3$ cells/well, and transfected with miR-625 mimics, NC, pCDNA3.1-YAP1 or pcDNA3.1. Cells were incubated at 37˚C in the presence of 5% CO_2 for 0, 24, 48 or 72 h. Following incubation, 10 μl CCK-8 solution was added to each well, and the cells were incubated at 37˚C for another 2 h. The absorbance was read at 450 nm using a microplate reader (Biotek Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA).

Transwell invasion assay. Cell invasion capacity was assessed using Transwell chambers (Corning Incorporated, Corning, NY, USA) precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Transfected cells suspended in FBS-free culture medium ($1x10^5$) were added into the upper chambers and the lower chambers were filled with 800 μl DMEM containing 10% FBS. After incubation for 48 h at 37˚C in the presence of 5% CO_2, the non-invasive cells on the upper chambers were carefully wiped out by a cotton-tipped swab. The cells that had invaded to the lower chambers were fixed with 90% alcohol and stained with 0.5% crystal violet. The number of cells invading through the membranes were counted in five randomly selected fields under a light microscope (x200 magnification; Olympus Corporation, Tokyo, Japan).

Bioinformatics analysis and luciferase reporter assay. To predict the putative targets of miR-625, bioinformatics analysis was performed using TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/). The pGL3-YAP1-3'-UTR wild-type (Wt) and pGL3-YAP1-3'-UTR mutant (Mut) reporter plasmids were synthesized and obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were plated in 24-well plates at a density of 60-70% confluence, and were transfected with miR-625 mimics or NC, together with pGL3-YAP1-3'-UTR Wt or pGL3-YAP1-3'-UTR Mut using Lipofectamine 2000. The firefly luciferase activity and *Renilla* luciferase activity were detected at 48 h post-transfection using Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's guidance. The firefly luciferase activity was normalized to the *Renilla* luciferase activity.

Western blotting. Tissues and cells were solubilized in cold RIPA Lysis and Extraction Buffer (Beyotime Institute of Biotechnology, Haimen, China). A BCA Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China) was used to determine protein concentration. Equivalent protein (20 μg)

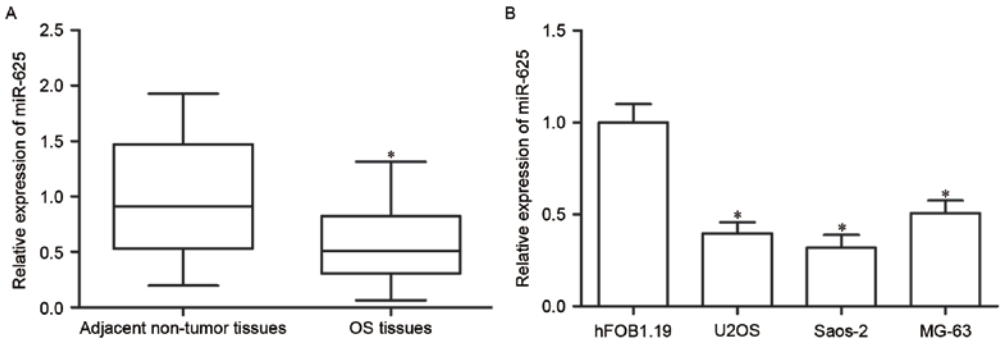

Figure 1. Expression of miR-625 in OS tissues and cell lines. (A) RT-qPCR was used to determine the relative expression of miR-625 in 29 paired OS tissues and associated adjacent non-tumor tissues. *P<0.05 vs. adjacent non-tumor tissues. Each assay was repeated three times. (B) miR-625 expression in OS cell lines (U2OS, Saos-2, and MG-63) and human normal osteoblasts (hFOB1.19) were determined by using RT-qPCR. *P<0.05 vs. hFOB1.19. OS, osteosarcoma; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. Each assay was repeated three times.

were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), which were then blocked with Tris-buffered saline containing 0.05% Tween-20 (TBST; Beyotime Institute of Biotechnology) containing 5% skimmed milk at room temperature for 2 h. The membranes were incubated with following primary antibodies at 4˚C overnight: Mouse anti-human monoclonal Yes associated protein 1 (YAP1; cat. no. ab56701; 1:1,000; Abcam, Cambridge, UK) and mouse anti-human monoclonal GAPDH (cat. no. sc-69778; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following washing three times in TBST, the membranes were incubated for 2 h at room temperature with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (cat. no. sc-2005; 1:3,000; Santa Cruz Biotechnology, Inc.). An ECL kit (Pierce; Thermo Fisher Scientific, Inc.) was used visualization of the protein bands. Relative expression of YAP1 protein was analyzed using Image Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. Two-tailed Student's t test or one-way analysis of variance was used to compare difference between groups by using SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). The SNK test was used as a post hoc test following analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR-625 in OS tissues and cell lines. To explore the roles of miR-625 in OS formation and progression, the expression levels of miR-625 in OS tissues and associated adjacent non-tumor tissues were determined. The expression of miR-625 was decreased in OS tissues compared with those in the adjacent non-tumor tissues (P<0.05; Fig. 1A). As such, the expression levels of miR-625 in three common OS cell lines (U2OS, Saos-2 and MG-63) and human normal osteoblasts (hFOB1.19) were then measured. Results revealed that miR-625 was downregulated in the three OS cell lines compared with that in hFOB1.19 (P<0.05; Fig. 1B). Thus, miR-625 may have an important role in OS.

Restoration of miR-625 expression inhibits cell proliferation and invasion in OS. U2OS and Saos-2 cells expressed significantly reduced miR-625 levels compared with normal human osteoblasts; therefore, these cell lines were transfected with miR-625 mimics to increase the expression of miR-625 (P<0.05; Fig. 2A and B). The functional effect of miR-625 on the proliferation of U2OS and Saos-2 cells was investigated using a CCK-8 assay. As presented in Fig. 2C and D, treatment with miR-625 mimics reduced the proliferation of U2OS and Saos-2 cells compared with cells transfected with an NC miRNA mimic (P<0.05). Transwell invasion assay was then performed to evaluate the effects of miR-625 overexpression on the invasion capacity of U2OS and Saos-2 cells. The results revealed that miR-625 upregulation reduced the number of invasive U2OS and Saos-2 cells compared with that in the NC groups (P<0.05; Fig. 2E). Therefore, miR-625 may suppress OS growth and metastasis.

miR-625 directly targets YAP1 in OS. To further explore the molecular mechanism underlying the tumor-suppressive roles of miR-625 in OS, the putative target genes of miR-625 were predicted using bioinformatics analysis. YAP1 was identified as a potential target of miR-625 (Fig. 3A) and is involved in the initiation and development of various cancer types (24). To confirm this hypothesis, a luciferase reporter assay was performed in U2OS and Saos-2 cells. The cells were then transfected with miR-625 mimics or NC, along with luciferase reporter plasmid carrying the wild type or the mutant 3'-UTR of YAP1. As presented in Fig. 3B and C, miR-625 overexpression significantly decreased the luciferase activity of the vector carrying the wild type 3'-UTR of YAP1 (P<0.05); however, the luciferase activity did not differ between cells co-transfected with vectors carrying the mutant 3'-UTR of YAP1 and miR-625 mimics.

RT-qPCR and western blot analyses were conducted to determine the mRNA and protein expression levels of YAP1 in U2OS and Saos-2 cells transfected with miR-625 mimic or NC. The mRNA and protein expression levels of YAP1 were suppressed in U2OS and Saos-2 cells following transfection with the miR-625 mimic (P<0.05; Fig. 3D and E). The mRNA and protein expression levels of YAP1 were then measured in OS tissues and associated adjacent non-tumor tissues. YAP1 was upregulated at both mRNA and protein level in OS tissues compared with that in the adjacent non-tumor tissues (P<0.05;

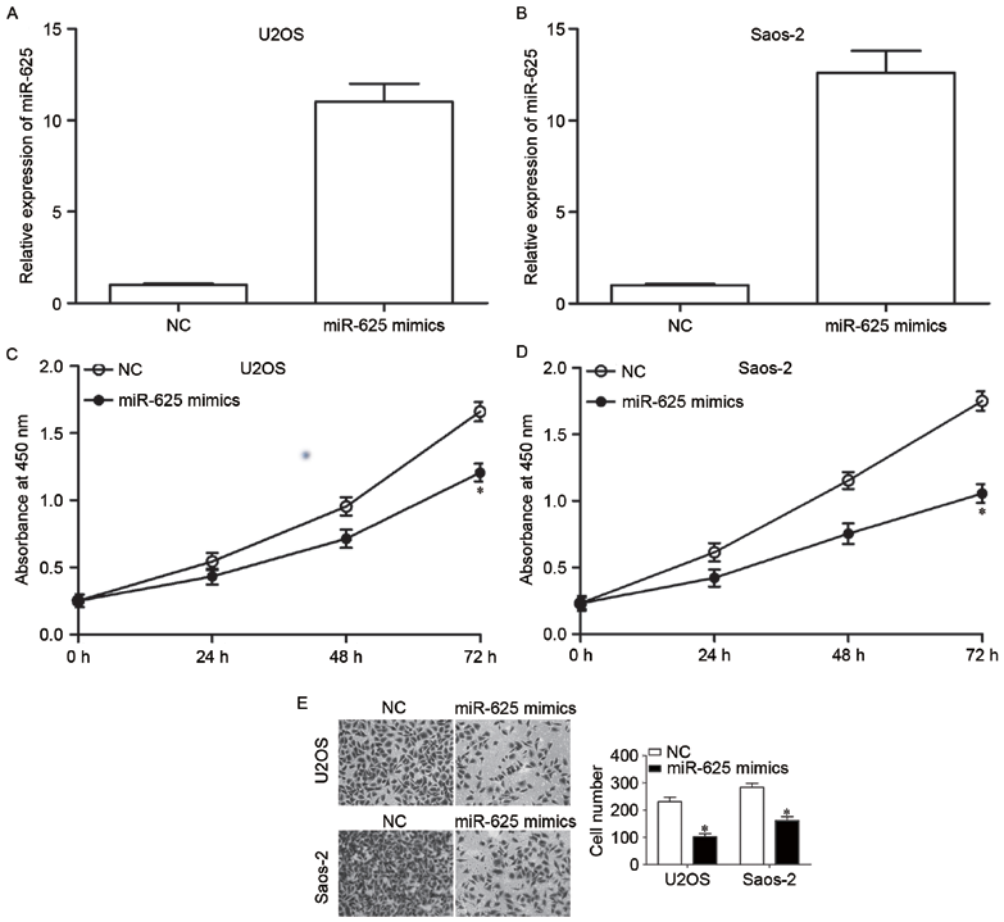

Figure 2. Effects of miR-625 overexpression on proliferation and invasion of U2OS and Saos-2 cells. Relative miR-625 expression in (A) U2OS and (B) Saos-2 cells following transfection with miR-625 mimics or NC was detected using reverse transcription-quantitative polymerase chain reaction. Each assay was repeated three times CCK-8 assays using (C) U2OS and (D) Saos-2 cells were performed to evaluate the inhibitory effects of miR-625 on cell proliferation. Each assay was repeated three times (E) Transwell invasion assays of U2OS and Saos-2 cells were conducted to assess the effect of miR-625 on cell invasion ability. *P<0.05 vs. NC. miR, microRNA; NC, negative control microRNA. Each assay was repeated three times.

Fig. 3F and G). Spearman's correlation analysis further indicated that YAP1 mRNA expression was inversely correlated with miR-625 expression in OS tissues (r=-0.7665, P<0.001; Fig. 3H). Collectively, these results suggested that YAP1 was directly and negatively regulated by miR-625 in OS.

Upregulation of YAP1 rescues the inhibitory effects of miR-625 on cell proliferation and invasion. To further determine whether YAP1 mediates the inhibitory effects of miR-625 on OS proliferation and invasion, rescue experiments were performed. U2OS and Saos-2 cells were transfected with pcDNA3.1-YAP1 or pcDNA3.1. Following transfection, YAP1 protein expression was upregulated in pcDNA3.1-YAP1-transfected U2OS and Saos-2 cells (P<0.05; Fig. 4A). The rescue experiments demonstrated that the enforced YAP1 expression rescued the impaired cell proliferation and invasion induced by miR-625 mimics (P<0.05; Fig. 4B-D); thus, YAP1, at least partially, mediated the functional roles of miR-625 in OS.

Discussion

Accumulated evidence supports the critical roles of miRNAs in cancer initiation and progression (25-27). Therefore, OS-associated miRNAs must be identified for use as therapeutic targets for treatment of OS. In the present study, the expression and functions of miR-625 in OS were investigated. The expression of miR-625 was low in OS tissues and cell lines compared with adjacent normal tissue and a normal osteoblast cell line, respectively. Functional experiments demonstrated that the ectopic expression of miR-625 suppressed OS cell proliferation and invasion *in vitro*. Mechanistically, YAP1 was identified as the direct target of miR-625 in OS. These results suggested that miR-625 may act as a tumor suppressor in the tumorigenesis and development of OS.

The expression of miR-625 is reduced in several types of human cancer. For example, miR-625 was reported to be downregulated in colorectal cancer tissues and cell lines (20-22). Low expression of miR-625 was previously demonstrated to be strongly correlated with lymph node metastasis, liver metastasis, poor overall survival and an unfavorable prognosis for patients with colorectal cancer (20). In gastric cancer, miR-625 was expressed at low levels in gastric cancer and inversely associated with lymph node metastasis (21). In hepatocellular carcinoma, miR-625 was reduced in tissues. The low expression level of miR-625 was closely correlated with lymph node and distant metastases, the presence of portal venous invasion, TNM staging and unfavorable overall survival (22). In esophageal squamous cell carcinoma (ESCC), the expression of miR-625

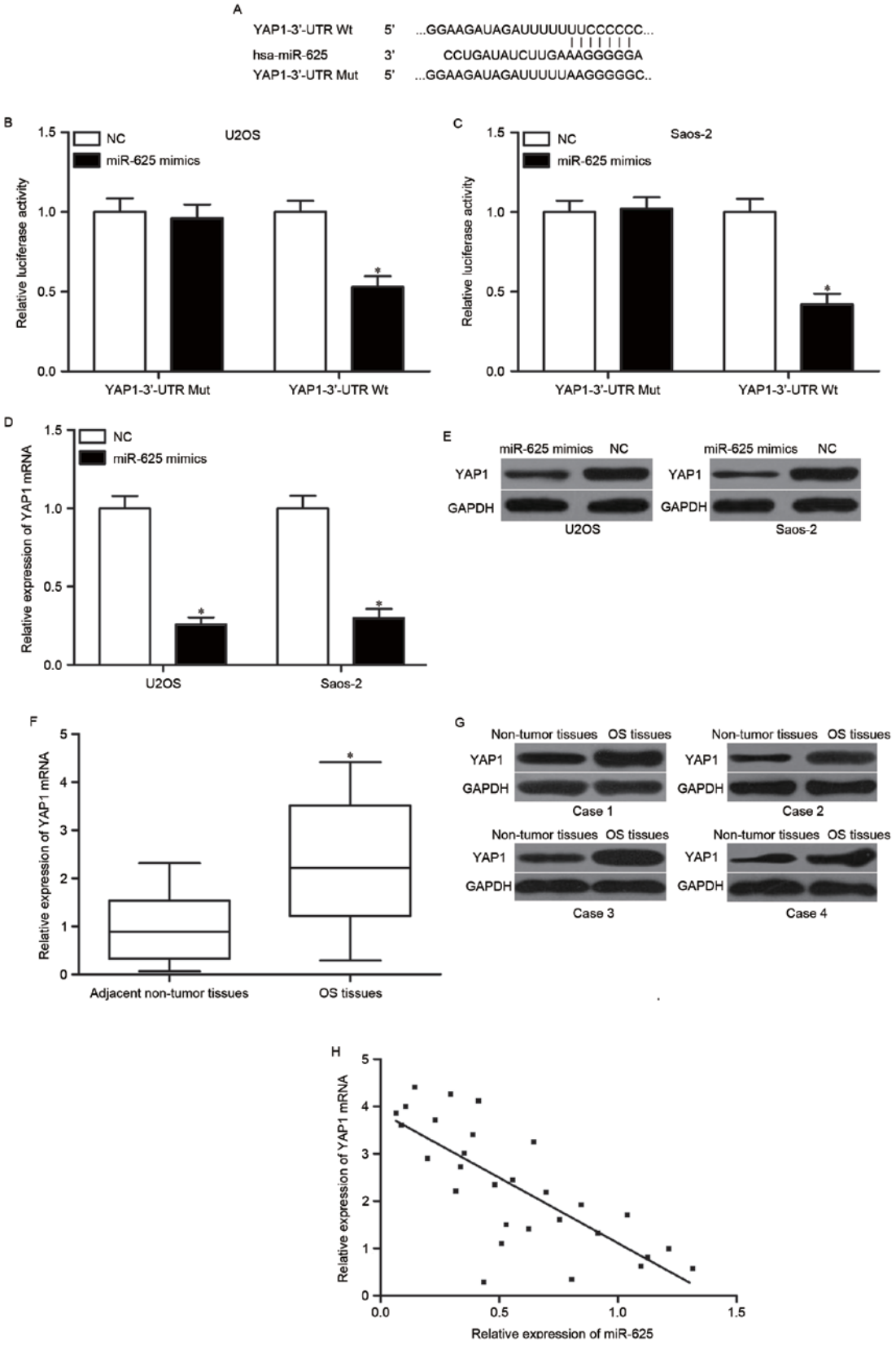

Figure 3. YAP1 is a direct target of miR-625 in OS. (A) Schematic representation of miR-625 putative binding sites in the 3'-UTR of YAP1 mRNA. (B) U2OS and (C) Saos-2 cells were co-transfected with pGL3-YAP1-3'-UTR Wt or pGL3-YAP1-3'-UTR Mut, and miR-625 mimics or NC. Luciferase reporter assay was performed to detect the luciferase activity in each group. Each assay was repeated three times. (D) RT-qPCR and (E) western blotting were used to analyze YAP1 mRNA and protein expression in U2OS and Saos-2 cells following transfection with miR-625 mimics or NC. *P<0.05 vs. NC. Each assay was repeated three times (F) YAP1 mRNA level in 29 paired OS tissues and associated adjacent non-tumor tissues was examined using RT-qPCR. *P<0.05 vs. adjacent non-tumor tissues. Each assay was repeated three times (G) Western blotting was performed to measure YAP1 protein expression in OS tissues compared to adjacent non-tumor tissues. Each assay was repeated three times (H) A negative correlation between the miR-625 expression and YAP1 mRNA expression was observed in OS tissues, r=-0.7665, P<0.001. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; YAP1, Yes-associated protein 1; UTR, untranslated region; Wt, wild-type; miR, microRNA; Mut, mutant; NC, negative control microRNA; OS, osteosarcoma.

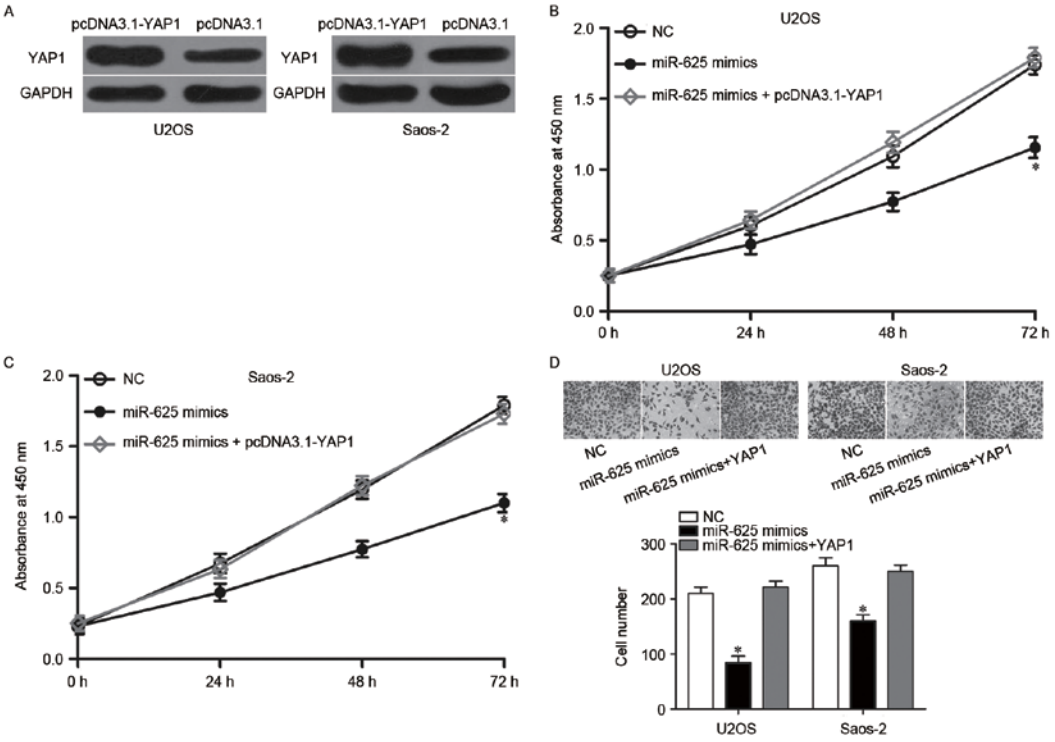

Figure 4. Upregulation of YAP1 rescued the antitumor effects of miR-625 on proliferation and invasion in U2OS and Saos-2 cells. (A) U2OS and Saos-2 cells transfected with pcDNA3.1-YAP1 or pcDNA3.1 were subjected to western blotting for YAP1 protein expression. Each assay was repeated three times Enforced expression of YAP1 partially rescued the suppressive roles of miR-625 on (B) U2OS cell proliferation and (C) Saos-2 cell proliferation and (D) U2OS and Saos-2 cell invasion. *P<0.05 vs. NC and miR-625 mimics + pcDNA3.1-YAP1. YAP1, Yes-associated protein 1; NC, negative control microRNA; miR, microRNA. Each assay was repeated three times.

was decreased in tumor tissues and was significantly associated with lymph node and distant metastases, tumor differentiation and TNM staging. In patients with ESCC, low miR-625 expression levels were associated with short overall survival. Multivariate Cox regression analysis indicated miR-625 may be an independent prognostic factor for overall patient survival in ESCC (28). Thus, miR-625 downregulation may be a prognostic factor for these cancer types.

Previous studies validated that miR-625 is a tumor suppressor in numerous human cancers (20-22). In colorectal cancer, miR-625 re-expression suppressed tumor cell metastasis *in vitro* and *in vivo* (20). Wang *et al* (21) revealed that miR-625 expression level restoration inhibited the invasive and metastatic abilities of gastric cancer cells *in vitro* and *in vivo* by directly targeting integrin linked kinase. Zhou *et al* (22) reported that miR-625 upregulation impeded cell motility in hepatocellular carcinoma *in vitro* and *in vivo* through negative regulation of insulin like growth factor 2 mRNA binding protein 1. Wang *et al* (29) demonstrated that the resumption expression of miR-625 attenuated cell proliferation and invasion in esophageal cancer by blocking SRY-box 2. Zhou *et al* (30) revealed that enforced expression of miR-625 targeted high mobility group AT-hook 2 to repress the proliferation and invasion of breast cancer cells. These findings suggest that miR-625 is a potential anticancer agent.

It is well established that miRNAs exert biological activities through negative regulation of their target mRNAs (31). To investigate the mechanisms by which miR-625 suppressed OS cell proliferation and invasion, bioinformatics analysis was used to search for potential targets of miR-625. The analysis indicated that YAP1 mRNA contained a miR-625 seed match at position 1,896-1,902 of the YAP1 3'-UTR. Furthermore, a luciferase reporter assay demonstrated that upregulation of miR-625 decreased the luciferase activity in OS cells transfected with luciferase reporter plasmid carrying the wild type 3'-UTR of YAP1, but not affect the luciferase activity of plasmid carrying the mutant 3'-UTR of YAP1, suggesting that miR-625 directly targeted the 3'-UTR of YAP1. In addition, miR-625 overexpression repressed YAP1 mRNA and protein expression in OS cells. Furthermore, YAP1 was upregulated in OS tissues and inversely correlated with miR-625 expression. Additionally, enforced expression of YAP1 rescued the impaired cell proliferation and invasion induced by miR-625 in OS, which demonstrated that YAP1 is a direct functional target of miR-625 in OS cells.

YAP1, which is located on chromosome 11q22.1, is abnormally expressed in various tumor types, including glioma (32), colorectal cancer (33), gastric cancer (34) and bladder cancer (35). As a member of the Hippo pathway, YAP1 has important roles in regulating cell proliferation, invasion, epithelial mesenchymal transition, metastasis, differentiation and survival (24). In OS, YAP1 was previously reported to be upregulated in tumor tissues, and significantly correlated with gender and Enneking staging. Additionally, downregulating YAP1 suppressed OS cell proliferation and invasion *in vitro* (36). Yang *et al* (37) reported that YAP1 knockdown inhibited cell proliferation, colony formation, cell cycle progression *in vitro* and the growth of OS xenograft tumors *in vivo*. In the present study, it was demonstrated that miR-625 targeted YAP1 to inhibit OS cell proliferation and invasion. Thus, the miR-625/YAP1 axis is a potential target for inhibiting the rapid growth and metastasis of OS.

In conclusion, the results of the current study demonstrate that miR-625 was downregulated in OS tissues and cell lines. Ectopic expression of miR-625 attenuated the proliferation and invasion of OS cells by directly targeting YAP1. The abnormal miR-625 expression may potentially be used as a therapeutic target for treatment of patients with OS.

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