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MicroRNA-222 contributed to cell proliferation, invasion and migration via regulating YWHAG in osteosarcoma

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Abstract. – OBJECTIVE: The aim of this study was to investigate the role of microRNA-222 (miR-222) in osteosarcoma (OS), and to further explore the potential molecular mechanism.

PATIENTS AND METHODS: We measured the level of miR-222 in OS tissues and cell lines using quantitative Real-time polymerase chain reaction. Synthesized miR-222 mimics or inhibitors were obtained to up-regulate or down-regulate the expression of miR-222 in U2OS or Saos2 cells. Cell counting kit-8 (CCK8) and colony formation assay were employed to detect the ability of cell proliferation, and transwell assay was used to confirm the ability of cell invasion. Furthermore, luciferase assay and Western blot were applied to verify the target of miR-222 in OS.

RESULTS: The level of miR-222 in OS tumor tissue samples was significantly lower than that in normal group. Over-expression of miR-222 decreased cell proliferation and invasion in U2OS cells while knockdown of miR-222 promoted cell growth and metastasis in Saos2 cells. Furthermore, YWHAG was found to be a candidate target of miR-222 using several databases. Elevated level of miR-222 inhibited YWHAG expression while reduced miR-222 promoted YWHAG expression. Also, up-regulation of YWHAG restored the inhibiting effect of miR-222 mimics.

CONCLUSIONS: We identified for the first time that the expression level of miR-222 was reduced in OS tissues as well as in OS cell lines. miR-222 could inhibit cell proliferation and invasion via down-regulating YWHAG. These data could provide a potential target for the biological treatment of OS.

Key Words:
miR-222, Proliferation, Invasion, YWHAG, Osteosarcoma.

Introduction

Osteosarcoma (OS) is a common malignant osteogenic tumor, which is predisposed to attack the adolescence, and has a poor prognosis with a high degree of malignancy[1]. The current 5-year survival rate is only about 55% to 68%, with the vast majority of patients eventually occurring tumor metastasis[2]. Therefore, looking for new and effective prevention and control measures to improve the quality of life and survival of patients and to prevent or delay the transfer of OS become a high priority.

With the continuous innovation of experimental techniques in molecular biology and the completion of the human genome project, researchers have become more and more understanding about the RNA and its functions[3]. microRNAs are a huge family which relative to the functional RNA regulation mechanism[4]. About 50% of the known miRNAs in human are found to locate in the chromosome region closely related to the tumor, suggesting that they play important roles in the development of tumors[5]. In OS, several studies[6] have identified miRNAs participated in the development and progression of OS. For example, miR-92b could promote osteosarcoma proliferation, invasion, and migration *via* targeting RECK; miR-199a-5p could dual-target PIAS3 and p27 and the promote tumor growth in human OS; also, miR-135b and TAZ formed a feedback loop which promoted epithelial-mesenchymal transition (EMT) and tumorigenesis in OS; miR-491 acted as a tumor suppressor *via* inhibiting OS lung metastasis and chemoresistance through αB-crystallin[7-10].

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Several researches have reported miR-222 functioned as a tumor suppressor in many cancers, which belongs to a miRNA cluster miR-221/222. Liu et al[11] found that in colorectal cancer cells, a miR-221/222-mediated feedback loop maintained constitutive activation of NF-κB and STAT3. Galardi et al[12] confirmed that in human prostate carcinoma cell lines, miR-221/222 targeting p27Kip1, affected the growth potential. Wong et al[13] demonstrated in hepatocellular carcinoma, over-expression of miR-222 conferred cell metastasis through activating protein kinase B (AKT) pathway. However, the expression and function in OS have not been mentioned before.

In present study, we found that miR-222 expression level was decreased in OS samples compared to normal control using quantitative Real-time polymerase chain reaction (PCR). Gain-of-function and loss-of-function experiments were conducted to further confirm the influences of miR-222 in OS. Furthermore, we verified YWHAG as an immediate target of miR-222 in OS. This study might provide a novel target for OS biological diagnosis and prognosis prediction.

Patients and Methods

Tissue Samples of OS and Normal Control Group

A total of 21 cases of osteosarcoma tissue specimens surgically removed from Department of Orthopedic in our hospital from 2010 to 2016 were collected. There were 13 males and 8 females, ranged from 6 to 35 years, with an average of 20.42 years. 21 cases of normal bone tissue samples collected from normal bone tissue specimens after the hip joint replacement and the sex, age, and tumor group, had no significant difference. Specimens were cut and then placed in liquid nitrogen at -80°C for preservation. This study was approved by the Ethics Committee of the Third People's Hospital of Yancheng. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Culture

Human OS-derived cell line Saos2, 143B, HOS, MG63, U2OS and normal osteoblast line NHost cells were purchased from Shanghai Keilton Biological Co., Ltd. (Shanghai, China). All these cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI)-1640 medium containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and maintained at 37°C in humid air containing 5% CO_2. The cells were passaged when the cell confluence reached 90%, and kept in logarithmic growth state. We took cells under logarithmic growth phase to conduct the experiments.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA of tissue samples and established cell lines were lysed using RIPA reagent (Invitrogen, Carlsbad, CA, USA). ATaqMan microRNA Reverse Transcription kit (Biosystems, Medford, MA, USA) was employed to reverse the RNA to complementary Deoxyribose Nucleic Acid (cDNA), and the expression level of miR-222 was detected using a SYBR Green PCR master mix Kit (TaKaRa, Dalian, China). U6 was applied as an internal control and the relative expression level was measured by $2^{-\Delta\Delta CT}$ method. Each experiment was repeated at least three times.

Cell Transfection

miR-222 mimcs, inhibitors, negative control (NC), inhibitors negative control (INC) and pcDNA for over-expressing YWHAG (YWHAG) were synthesized by GenePharma (Shanghai, China). Cells were transfected with these synthetics using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The efficiency of transfection was confirmed using qRT-PCR.

Cell Counting Kit-8 (CCK8) Assay

The Cell Counting Kit (Dojindo, Kumamoto, Japan) was employed to measure the ability of cell proliferation. Cells after treatment were seeded in 96-well plates and cultured in 100 μL normal medium at a density of 3000 cells per well. At 0 h, 24 h, 48 h, and 72 h after culture, 10 μLCCK8 reagents were added into the well and the absorbance was measured at 450 nm. Each experiment was replicated for at least three times.

Colony Formation Assay

A total of 400 cells were planted in each 3.5 cm plate after transfection, and maintained in normal RPMI-1640 medium for two weeks. Then, the colony containing more than 50 cells was calculated. We repeated the assay for three times.

Transwell Assay

To detect the cell invasion and migration ability, we used 8-μm transwell inserts (Millipore, Billeri-

ca, MA, USA) to realize the experiments. For invasion, the upper surface of the membrane was covered with Matrigel (BD Science, Franklin Lakes, NJ, USA). A total of $4x10^4$ treated cells in FBS-free medium were seeded into the top chamber of the insert, and 500 μL RPMI-1640 medium containing 10% fetal bovine serum (FBS) were added into the lower chamber. After 48 h incubation, cells that failed to pass through the membrane were removed. The membrane containing cells on its lower surface was fixed with pre-cooling methanol and stained with 0.5% crystal violet. Next, the cells stained were calculated after pictures taken using a microscope in five random visions. For migration, the upper surface of the membrane was covered with nothing. The other steps were the same as the invasion one.

Dual-Luciferase Assay

Dual-Luciferase assay (Promega, Madison, WI, USA) was recruited to measure the luciferase activity. The YWHAG3'-UTR cDNA fragments carrying with wild type or mutant miR-222 binding site after being amplified were cloned into the pGL3 luciferase vector (Promega, Madison, WI, USA), relatively. Cells were transfected with miR-222 mimics and the conducted PGL3 vector. The cells were maintained for 24 h, and then, the activity of luciferase was determined using a luminometer (Promega, Madison, WI, USA) and measured. The experiment was repeated for three times.

Protein Extraction and Western-Blot

Protein of established cell lines was isolated using RIPA reagent from Beyotime, Shanghai, China. The protein concentration was detected using bicinchoninic acid (BCA) quantitative detection reagent kit (Beyotime, Shanghai, China) based on kit instructions. Proteins were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of primary antibody at 4°C overnight. After washing with Tris buffered saline-Tween 20 (TBST-20) buffer for three times, the membranes were incubated with secondary antibody carrying horseradish peroxide (HRP) for 1 h at room temperature. We next detected the membranes using enhanced chemiluminescence (ECL, Millipore, Billerica, MA, USA) following the instructions. All these antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical Analysis

Statistical product and service solutions 17.0 software (SPSS Inc., Chicago, IL, USA) were applied to analyze the data. The measurement data were shown as mean ± SD. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). p-values < 0.05 were considered having significant difference.

Results

miR-222 was Reduced in OS Tissue and Cell Lines

To examine the expression level of miR-222, we measured the expression of miR-222 in 21 OS patients' tissue samples compared with 21 normal control tissue samples. miR-222 significantly reduced in the tumor group compared with the normal group (Figure 1A). Also, we analyzed the expression level of miR-222 in five OS-derived cell lines compared with normal osteoblast lineage NHost and found miR-222 was down-regulated in all the five OS cell lines (Figure 1B). Next to further study, the function of miR-222 in OS, we chose U2OS and Saos2 cells to over-express or knockdown miR-222 level using miR-222 mimics or inhibitors, relatively (Figure 1C-D). These results indicated miR-222 could act as a tumor-inhibiting factor in OS.

miR-222 Affected the Proliferation of OS Cells

CCK8 assay was recruited to measure the cell proliferation. We found miR-222 over-expression significantly reduced U2OS cell growth compared with the negative group (Figure 2A), while miR-222 knockdown increased Saos2 cell proliferation compared with INC group (Figure 2B). Also, we confirmed the results with colony formation assay, and U2OS cells formed fewer colonies after treatment with miR-222 mimics. On the contrary, Saos2 cells formed more colonies after miR-222 inhibitors treatment when comparing to relative control group (Figure 2C-D). All these data suggested miR-222 could inhibit cell proliferation of OS.

Ectopic Expression of miR-222 Influenced Cell Invasion and Migration in OS Cells

As tumor metastasis is the major lethal factor of OS, we next evaluate the influence of miR-222 in cell metastasis. The ability of cell invasion was significantly inhibited in miR-222 mimics treated

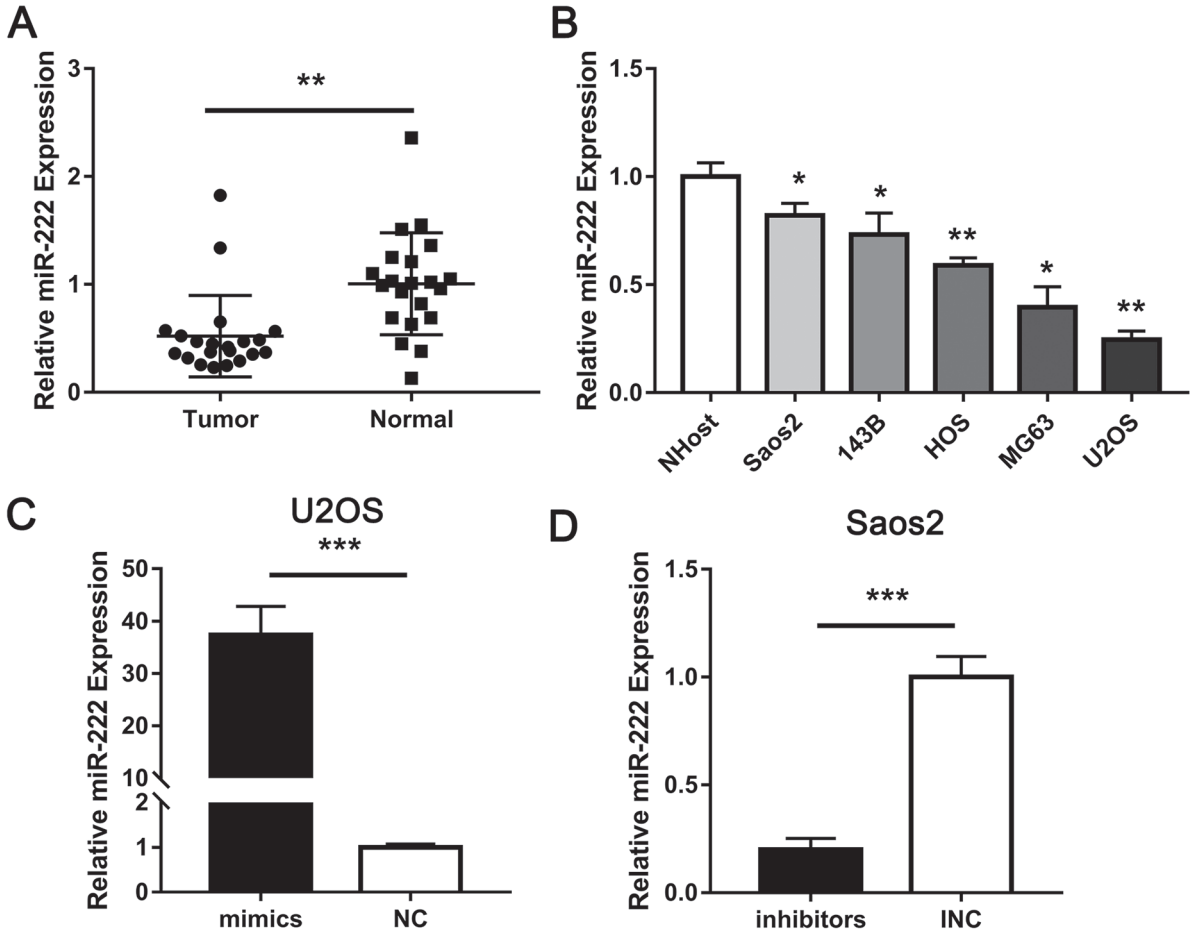

Figure 1. miR-222 was down-regulated in OS tissues and cell lines. ***A,*** Analysis of the expression level of miR-222 in 21 OS samples and 21 normal tissues. ***B,*** Analysis of miR-222 expression level in OS cell lines (Saos2, 143B, HOS, MG63, U2OS) and normal osteoblast line NHost cells. ***C,*** Expression of miR-222 in miR-222 mimics treated U2OS cells. ***D,*** expression of miR-222 in miR-222 inhibitors treated Saos2 cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

U2OS cells but increased in miR-222 inhibitors treated Saos2 cells (Figure 3A). Also, we detected cell migration ability using transwell assay. The over-expression of miR-222 significantly reduced migrated cell numbers of U2OS cells while knockdown of miR-222 improved migrated cell numbers of Saos2 cells (Figure 3B). These results indicated that miR-222 could inhibit cell metastasis of OS.

YWHAG Was Direct Target of miR-222 in OS

Furthermore, to investigate the underlying mechanism of miR-222 in OS, we next searched several database including TargetScan (http://www.targetscan.org/), PicTar (http://www.pictar.org/), and miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), and found YWHAG as a potential target of miR-222 (Figure 4A). To verify this, we conducted luciferase assay using pGL3 luciferase vector containing mutant or wild type binding site of YWHAG's 3'-UTR and found the luciferase activity of wild type group was significantly lower than that of the mutant group (Figure 4B). Next, we confirmed the protein expression level of YWHAG in established cell lines. The expression of YWHAG was reduced in miR-222 mimics treated U2OS cells but increased in miR-222 treated Saos2 cells (Figure 4C-E). These results suggested YWHAG function as a target of miR-222 in OS.

Over-Expression of YWHAG Rescued the Effects of miR-222

To further verify our result of YWHAG as a target of miR-222, we over-expressed YHWAG level in miR-222 mimics treated U2OS cells. The expression of YHWAG was confirmed using Western-blot (Figure 5A-B). The inhibiting functions of miR-222 mimics on cell proliferation were significantly rescued by over-expression of YWHAG and the influence of miR-222 mimics on cell invasion was significantly restored by up-regulation of YWHAG (Figure 5C-E). These data confirmed miR-222 effected OS cell function via regulating YWHAG.

Discussion

In recent years, miRNAs have received more and more attention as a research focus on tumorigenesis and progression. Many miRNAs have been found to exert a role in promoting or inhibiting tumors by acting selectively on the target genes[3,4]. miR-30c can suppress esophageal squamous cell carcinoma progression via targeting SNAI1[14]. miR-106b-5p can promote cell growth and inhibit cell apoptosis in non-small cell lung cancer though regulating BTG3[15]. miR-409-3p has been reported to enhance sensitivity of ovarian cancer cells to cisplatin through block-

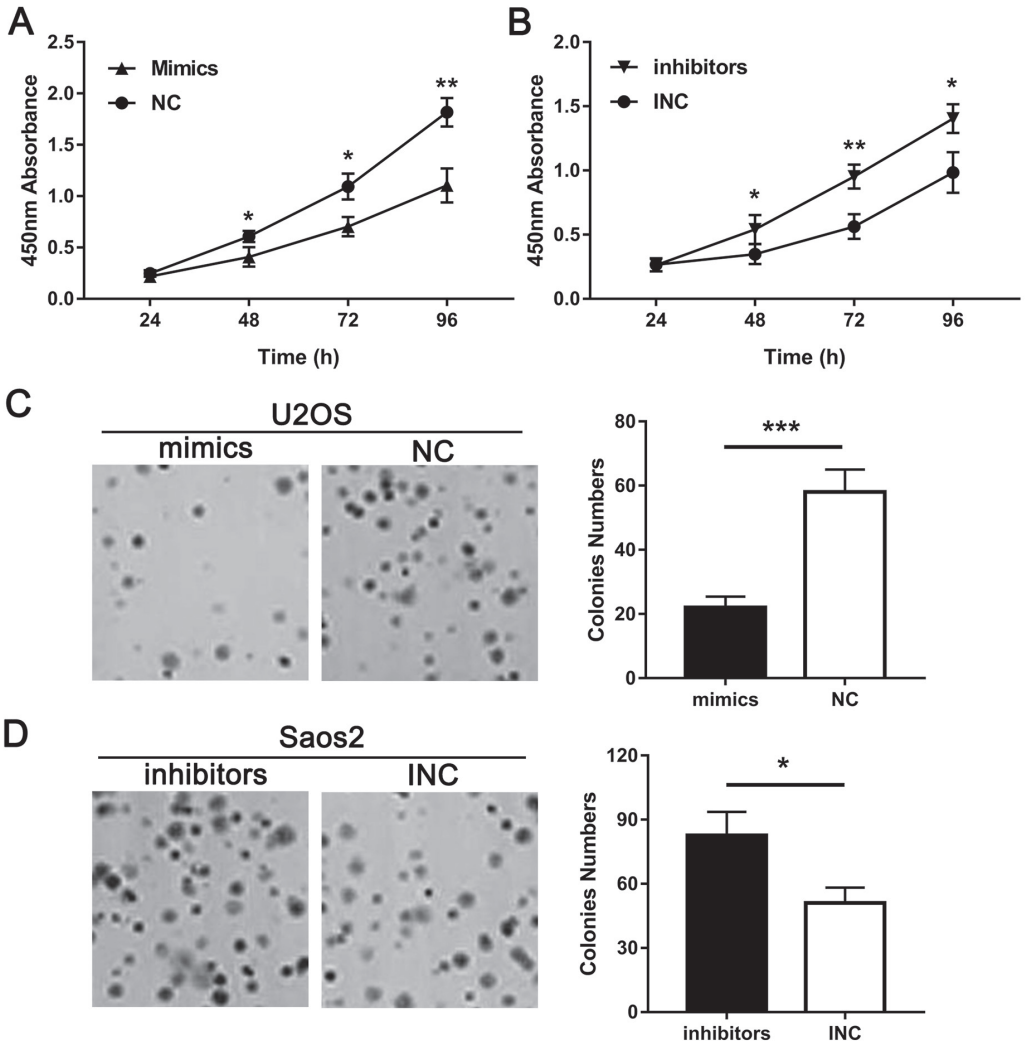

Figure 2. miR-222 effected the proliferation and invasion of OS cells. ***A-B,*** CCK8 assay was performed to determine proliferation of U2OS (***A,***) or Saos2 (***B,***) cells treating with miR-222 mimics or inhibitors compared to each negative control. ***C, D,*** Colony formation assay was performed to determine the growth of U2OS (***C,***) or Saos2 ***E,*** cells transfected with mimics or inhibitors, respectively. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

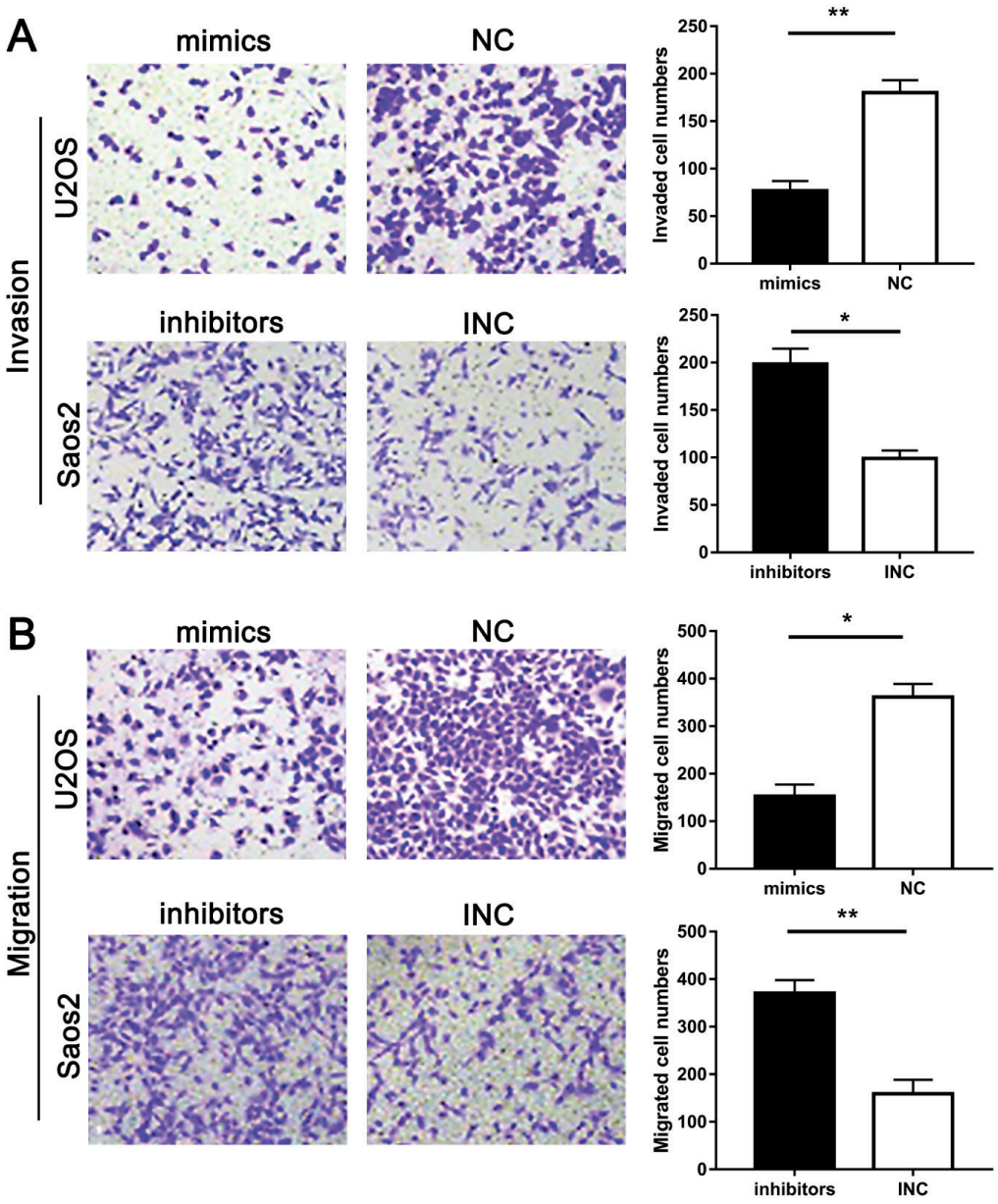

Figure 3. miR-222 effected the invasion and migration of OS cells. ***A,*** Transwell invasion assay was used to detect the invasion ability of miR-222 mimics treated U2OS cells or miR-222 inhibitors treated Saos2 cells. ***B,*** Transwell invasion assay was used to detect the migration ability of miR-222 mimics treated U2OS cells or miR-222 inhibitors treated Saos2 cells. Data are presented as the mean ± SD of three independent experiments. * $p<0.05$, ** $p<0.01$.

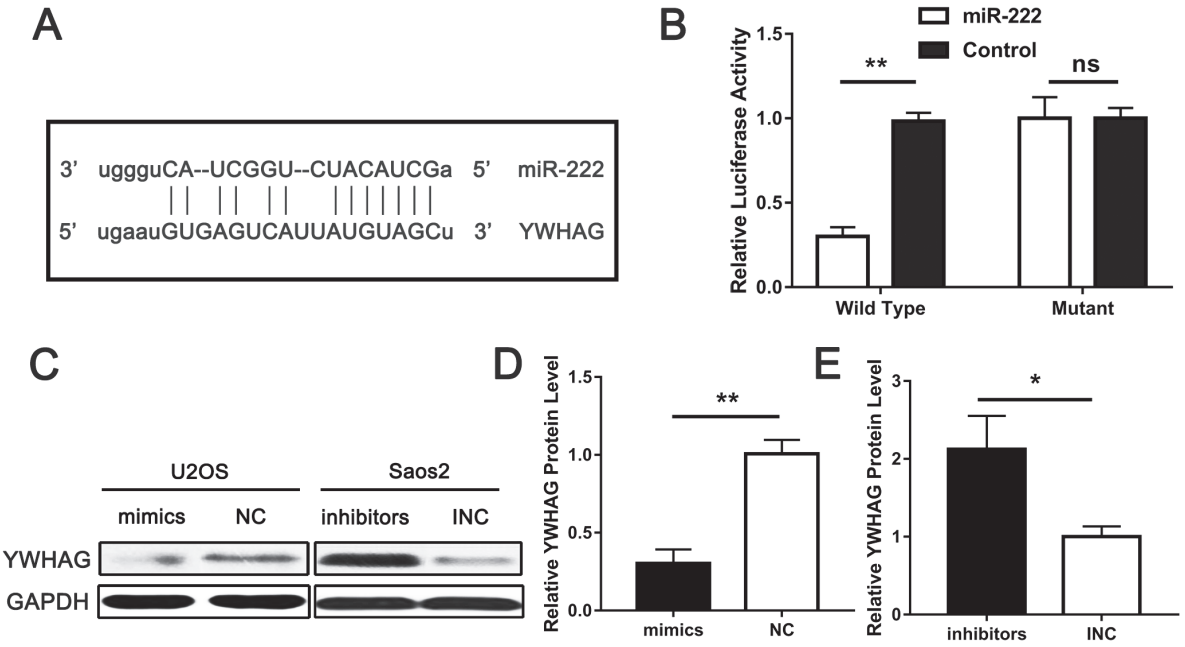

Figure 4. YWHAG was a direct target of miR-222. ***A,*** The predicted binding sites of miR-222 in the 3'-UTR of YWHAG. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. ***C,*** Levels of YWHAG and GAPDH protein measured by Western-blot in miR-222 over-expression U2OS cells and miR-222 knockdown Saos2 cells. ***D-E,*** the relative protein level of YWHAG and GAPDH. Data are presented as the mean ± SD of three independent experiments. * $p<0.05$, ** $p<0.01$, ns: non-sense.

ing the autophagy via Fip200[16]. What's more, miR-Let-7a is demonstrated to mediate prostate cancer PC-3 cell invasion, migration by inducing EMT via CCR7/MAPK pathway[17]. The ectopic expressions of miRNAs have been verified to participate in the regulation of OS tumorigenesis and progression. miR-216a and miR-494 have been reported to inhibit cell proliferation, invasion and metastasis via regulating CDK14 and CDK6, respectively, while miR-19 promotes osteosarcoma progression through targeting SOCS6[18-20]. Also, miR-17-92 cluster can encode miR-20a and increase the metastatic ability of OS cells by repressing expression Fas[21]. In addition, miR-26a was studied to reduce tumor growth and decrease a stem cell-like phenotype in OS by repressing Jagged1 expression[22]. Our study aim to study the role of miR-222 played in OS and to explore the underlying mechanism. We identified miR-222 was decreased in OS tumor tissues as well as in OS cell lines compared with normal group relatively, which indicated miR-222 as tumor suppressor in OS. Next, function experiments were obtained to explain the effect of miR-222 in OS with using established cell lines. Over-expression of miR-222 significantly reduced cell abilities of growth and metastasisinU2OS cells, while down-regulation of miR-222 promoted these abilities of Saos2 cells. These data suggested miR-222 could inhibit OS progression, which emphasized the results that miR-222 as an inhibiting factor in hepatocellular carcinoma[13].

Furthermore, this work next detected the specific mechanism of miR-222 in OS via bioinformatics analysis and luciferase reporter gene assay. YWHAG was found to be a potential target of miR-222. YWHAG as a member of 14-3-3 protein family, has been reported as a tumor promoter in several types of tumor[23]. It could accelerate cell proliferation and metastasis in different cancers including glioblastoma, non-small cell lung cancer and hematologic neoplasms[24-26]. Its oncogenic function can be regulated by several miRNAs, such as in breast cancer, miR-181b-3p promotes EMT via directly targeting YWHAG; in glioblastoma miR-217 promoted the proliferation and invasion by YWHAG[24,27]. In OS, YWHAG has been reported to promote in OS cell proliferation and migration and function as a linker protein

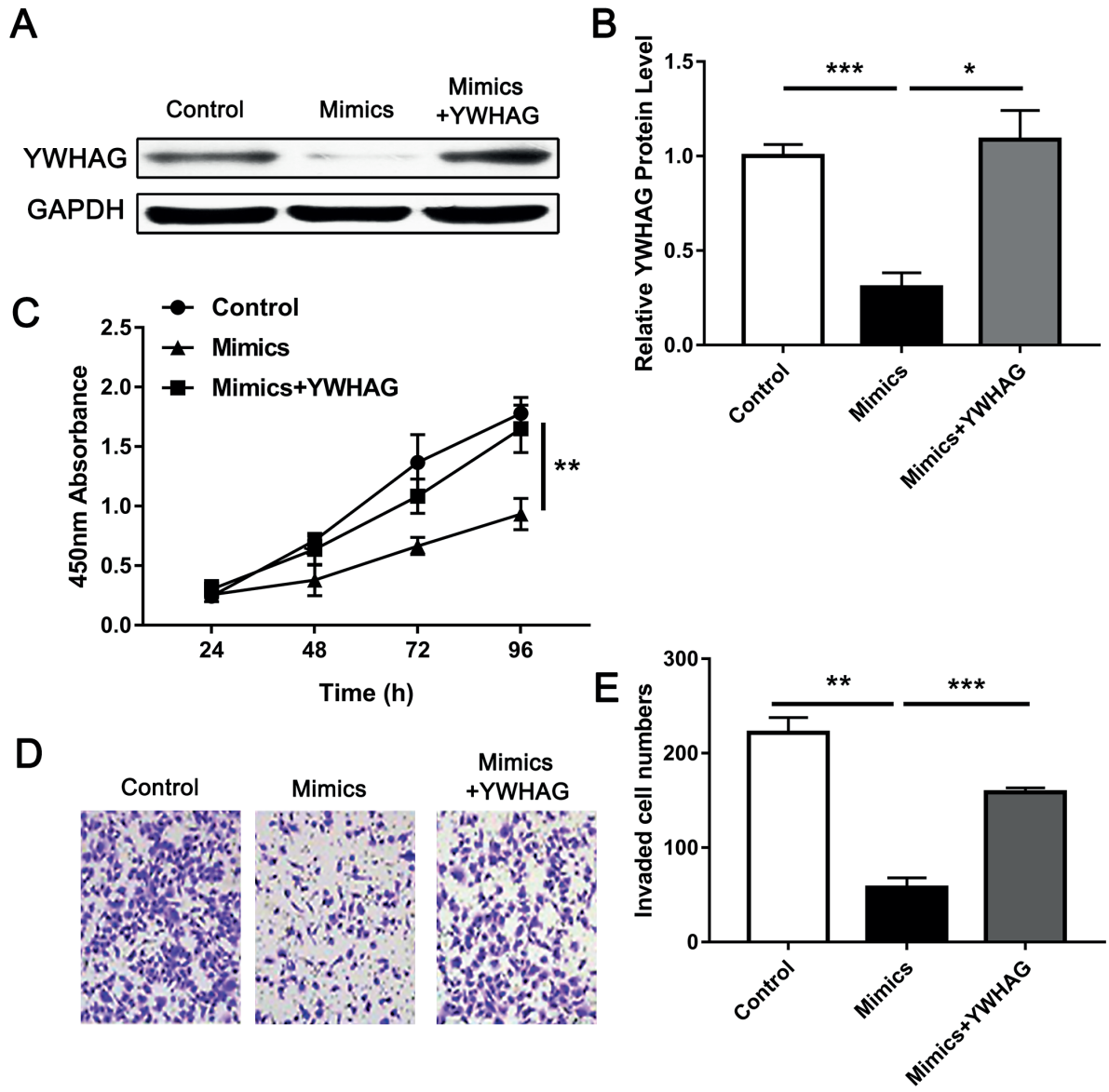

Figure 5. YWHAG rescued the effects of miR-222 mimics in U2OS cells. ***A,*** Western-blot analyses of YWHAG. GAPDH was used as an internal control. ***B,*** Relative protein band densities of YWHAG. ***C,*** Analysis of the cell proliferation ability by CCK8 assay in control, mimics, or mimics+YWHAG treated U2OS cells; ***D, E,*** Cell invasion ability was measured by transwell assay; Data are represented as the mean ± SD of three replicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

between pERK1/2 inhibition and BIM up-regulation in OS progression regulation[28-31]. In accordance with these reports, we verified YWHAG was repressed by miR-222 over-expression but increased by miR-222 knockdown. To further certify these results, we up-regulated YWHAG in miR-222 mimics treated cells and found the inhibiting influence of over-expressing miR-222 in U2OS cell on cell proliferation and invasion were significantly restored by YWHAG over-expression. Taken together, we demonstrated miR-222 inhibited OS cell proliferation and invasion via repressing YWHAG.

Conclusions

We showed that miR-222 played a tumor-suppressing role in OS through regulating YWHAG, which might function as a useful biological target for OS therapy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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