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The Effect of miR-32-5p Targeting ITGAV on the Proliferation, Invasion, Migration and Apoptosis of OS Cells

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Abstract

Objective: This study aims to analyze the regulatory mechanism of miR-32-5p on the biological behavior of osteosarcoma (OS) cells and to elucidate the role and mechanism of miR-32-5p in OS. **Methods:** TargetScan, dual-luciferase reporter gene assays, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and Western blotting were employed to confirm whether ITGAV serves as the target gene of miR-32-5p. qRT-PCR was utilized to assess the impact of up-regulated miR-32-5p on ITGAV mRNA levels. Cell proliferation, invasion, and migration were evaluated using the CCK-8 assay and Transwell chamber, while the effects of miR-32-5p and ITGAV on the apoptosis of MG63 cells were analyzed through flow cytometry. **Results:** Experimental findings demonstrated that miR-32-5p specifically binds to the 3'-UTR of ITGAV, thereby regulating its expression. The miR-32-5p mimics group significantly inhibited the expression levels of both ITGAV mRNA and its protein. Concurrently, the expression of the pro-apoptotic protein BAX increased, while the levels of the anti-apoptotic protein BCL2 decreased. Furthermore, the miR-32-5p mimics group inhibited the proliferation, invasion, and migration capabilities of MG63 OS cells and promoted their apoptosis. **Conclusion:** miR-32-5p exerts an inhibitory effect on the proliferation, invasion, and migration of OS cells while promoting their apoptosis by targeting and down-regulating ITGAV expression, indicating its potential as a therapeutic agent in OS.

Keywords

Osteosarcoma, miR-32-5p, ITGAV

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1. Introduction

Osteosarcoma (OS) is a common primary malignant tumor of bone, mainly involving the metaphysis of long bones [1]. MicroRNA (miRNA) is a small molecule RNA (22nt) that is widely present in human tissues and can regulate the expression of corresponding mRNA targets in many eukaryotes by interacting with the 3' untranslated region of the target gene. UTR (base pairing) inhibits the translation and cleavage of mRNA, regulates different biological processes, exerts multiple biological functions and participates in tumor occurrence [2]-[4]. According to research reports, miR-32-5p is an anti-tumor gene in cancer, which inhibits the migration, clone formation, proliferation and invasion of HeLa cells in cervical cancer tissues and cells [5]. ITGAV is a member of the integrin α chain family, and its expression level plays an important role in inducing tumor neovasangiogenesis and promoting tumor migration and invasion [6]. Previous research reports have shown that miR-32-5p is lowly expressed in osteosarcoma tissues and cells, and miR-32-5p can target HMGB1 to inhibit the development of osteosarcoma [7] [8]. However, the role and mechanism of it in the proliferation, invasion, migration and apoptosis of osteosarcoma cells remain unclear and need further exploration.

2. Materials and Methods

2.1. Materials

Human osteosarcoma MG63 cells were obtained from Wuhan Ponsure Life Science and Technology Co., LTD. The dual-luciferase assay kit was acquired from Promega Corporation in the United States. Lipofectamine 3000 was sourced from Invitrogen Corporation in the United States. Both the negative control mimic and the miR-32-5p mimic were synthesized by Wuhan Jinkairui Bioengineering Co., LTD. The upstream and downstream primers for miR-32-5p and ITGAV were synthesized by Nanning Genis Biotechnology Co., LTD. Antibodies for ITGAV, BAX, and BCL2 were purchased from Wuhan ABclonal Biotechnology Co., LTD. The Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit was obtained from Beijing Solaibao Technology Co., LTD.

2.2. Methods

2.2.1. Cell Grouping

MG63 cells were categorized into two groups: the mimic negative control group, which was transfected with the mimic negative control, and the miR-32-5p mimic group, which was transfected with the miR-32-5p mimic. The transfection procedure adhered to the instructions provided in the Lipofectamine 3000 transfection reagent manual. Following transfection, the cells were selected for subsequent experiments. After 48 hours, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted to assess the transfection efficiency and the expression levels of ITGAV mRNA. Additionally, Western blot analysis was performed to evaluate the expression of ITGAV, Bax, and Bcl-2 proteins in the cells. After the same 48-hour period, a Cell Counting Kit-8 (CCK-8) assay was utilized to assess cell proliferation, while flow cytometry was employed to evaluate apoptosis. Furthermore, the invasion and migration capabilities of the cells were analyzed using a transwell chamber. The TargetScan target gene prediction library was utilized to predict the regulatory relationship between miR-32-5p and ITGAV, which was subsequently confirmed through dual luciferase reporter assays.

2.2.2. Cell Culture and Transfection

The MG63 cell line was obtained from Wuhan Ponsure Life Science and Technology Co., Ltd. and cultured in DMEM supplemented with 10% fetal bovine serum in a cell incubator maintained at 37˚C and 5% CO_2. The MG63 cells were divided into two groups: the mimic-negative control group and the miR-32-5p mimics group. Once the cells reached 70% to 80% confluence, they were transfected according to their respective groups using mimic-negative and miR-32-5p mimics, following the instructions provided for the LipofectamineTM 3000 transfection reagent. The cells were then cultured for an additional 48 hours.

2.2.3. qRT-PCR

Total RNA was extracted from the MG63 cell line following the Trizol protocol. The RNA concentration was quantified using ultraviolet spectrophotometry. cDNA synthesis was performed with the TaqMan microRNA Reverse Transcription Kit, utilizing specific neck ring primers. Real-time PCR was conducted according to the SYBR Premix EX Taq protocol, with U6 serving as the internal reference. The expression level of miR-32-5p was semi-quantitatively analyzed based on the Ct values, employing the 2-△△Ct method for standardization. The extracted total RNA was transcribed into cDNA in accordance with the reverse transcription kit instructions. The expression level of ITGAV was assessed using GAPDH as the internal reference, with standardization performed similarly using Ct values. All primers were synthesized by Guangxi Ruisai Biotechnology Co., LTD.

2.2.4. Western Blotting

Following the aspiration and disposal of the culture medium from MG63 cells, the cells were washed with ice-cold PBS. RIPA lysis buffer containing PMSF was then added to facilitate cell lysis. The supernatant was collected via low-temperature high-speed centrifugation, and protein concentration was quantified using the BCA method. A total of 20 μg of protein was mixed with 5× loading buffer and denatured at 95˚C for 10 minutes. The protein samples were separated using 10% SDS-PAGE gel electrophoresis (5% concentrated gel, initial voltage set at 80 V) and subsequently transferred to a PVDF membrane pre-treated with methanol. After the transfer, the membrane was blocked with 5% skimmed milk powder at room temperature for 1 hour. Primary antibodies (ITGAV 1:1100, BCL-2 1:1000, BAX 1:800, GAPDH 1:10,000) were added individually and incubated overnight at 4˚C. Following washing with TBST, the corresponding horseradish peroxidase-labeled secondary antibody (goat anti-rabbit or goat anti-mouse, 1:3000) was applied and incubated at room temperature for 1 hour, followed by another TBST wash. The membrane was developed using ECL luminescent reagent, and the Xray film was exposed for 1 to 10 seconds, yielding bands after development and fixation. Finally, images were captured using a scanner, and the gray values of the target protein and the internal reference GAPDH were analyzed with Image J software.

2.2.5. CCK-8 Experiment

MG63 cells were seeded into 96-well plates at a density of 1500 cells per well. Following a 12-hour incubation period, cell proliferation was assessed using the CCK-8 kit according to the manufacturer's instructions. During the assessment, 100 μL of diluted detection solution was added to each well. After an additional incubation of 1.5 hours, the optical density of each well was measured at a wavelength of 450 nm using a microplate reader.

2.2.6. Cell Invasion and Migration Were Determined by Transwell Chamber

The cells were categorized into the miR-32-5p-NC group and the miR-32-5p mimics group. Following a 48-hour transfection period, the cells were digested with trypsin, resuspended in serum-free medium, and the concentration was adjusted to 5×10^4 cells/mL (*i.e.*, 10^4 cells/200 μL). For the migration assay, 200 μL of the cell suspension was directly added to the upper chamber of the Transwell. In the invasion assay, 50 μL of Matrigel matrix gel was pre-coated on the chamber membrane (liquefied overnight at 4˚C and cured for 30 minutes at 37˚C), followed by the addition of an equal volume of cell suspension. Subsequently, 600 μL of complete medium containing serum was added to the lower chamber, and the setup was incubated in a 37˚C, 5% CO_2 incubator for 24 hours. After incubation, the small chamber was removed, the liquid in the upper chamber was discarded, and non-migrating/invasive cells on the inner side of the membrane were gently wiped off with a cotton swab. The membrane was washed with PBS, fixed with 4% paraformaldehyde for 30 minutes, stained with crystal violet for 15 minutes, washed again, and then placed on a slide. Using an inverted microscope at 200× magnification, a random field of view was selected for photography, and the number of transmembrane cells was counted to assess the migration and invasion capabilities of the cells in each group.

2.2.7. Flow Cytometry

MG63 cells in the logarithmic growth phase were harvested. Following digestion with 0.01% trypsin (excluding EDTA), the cells were washed and resuspended three times in PBS solution. Subsequently, they were centrifuged at 800× g for 10 minutes, and the supernatant was discarded. The cells were then resuspended in binding buffer, adjusting the concentration to 1×10^6 cells/mL. A volume of 100 μL of the cell suspension was transferred into a 5 mL flow tube, and 5 μL of Annexin V-FITC and PI staining solution was added according to the reagent instructions. The mixture was gently mixed and incubated in the dark for 15 minutes. After the incubation, 400 μL of binding buffer was added, and flow cytometry was performed immediately to assess cell apoptosis.

2.2.8. Dual-Luciferase Reporter Gene Assay

Using TargetScan (http://www.Targetscan.org), online predictions indicated that miR-32-5p possesses a potential binding site within the 3'-UTR of the ITGAV gene. Accordingly, we constructed wild-type (pmirGLO-WT) and mutant (pmirGLO-MUT) dual-luciferase reporter gene vectors containing this site. MG63 cells were seeded in 24-well plates, and upon reaching a density of 50% to 60%, pmirGLO-WT or pmirGLO-MUT were co-transfected with miR-32-5p mimics or negative control mimics (NC), respectively. Twenty-four hours post-transfection, the cells were lysed using the dual-luciferase reporter assay system (Promega, E1910), and fluorescence values were measured. The luciferase activity of Renilla served as an internal reference for calculating the relative luciferase activity of each group.

2.2.9. Statistical Processing

A minimum of three independent biological replicates (n ≥ 3) were conducted for all quantitative experiments in this study. Each biological replicate corresponds to a complete experimental process utilizing independently prepared cell samples collected at different times. Data analysis was performed using GraphPad Prism 10.6 statistical software, and results are presented as mean ± standard deviation (±s). A t-test was employed to compare data between the two groups.

3. Results

3.1. Changes in miR-32-5p Expression Level Mediated by miR-32-5p Mimics Transfection

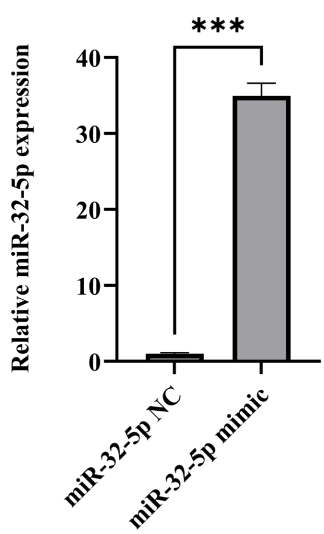

Figure 1. Expression of miR-32-5p in MG63 cells after transfection.

MG63 osteosarcoma cells were transfected with miR-32-5p mimics and miR-NC, respectively, and the changes in miR-32-5p expression levels in MG63 cells were assessed using real-time fluorescence quantitative PCR. The results indicated that the expression level of miR-32-5p in the miR-32-5p mimics treatment group was significantly elevated compared to the miR-NC group. The relative expression levels of miR-32-5p in the two groups were 34.94 ± 1.68 and 1.007 ± 0.144, respectively ($t = 34.84$, $p < 0.0001$). These findings demonstrate that the miR-32-5p mimics overexpression cell model has been successfully established, providing a basis for subsequent investigations into the malignant phenotypes of osteosarcoma (Figure 1).

3.2. qRT-PCR Was Used to Detect the Effect of miR-32-5p Overexpression on the mRNA Expression of ITGAV in Osteosarcoma Cells

MG63 osteosarcoma cells were transfected with miR-32-5p mimics or miR-NC, and the mRNA expression level of ITGAV in these cells was assessed using Real-Time PCR. The results indicated that miR-32-5p mimics significantly inhibited the expression of ITGAV in MG63 cells. Specifically, the mRNA expression levels of ITGAV in the miR-32-5p mimics and miR-NC groups were 0.25 ± 0.006 and 1.02 ± 0.26, respectively ($t = 4.742$, $p = 0.009$). This difference was statistically significant (Figure 2).

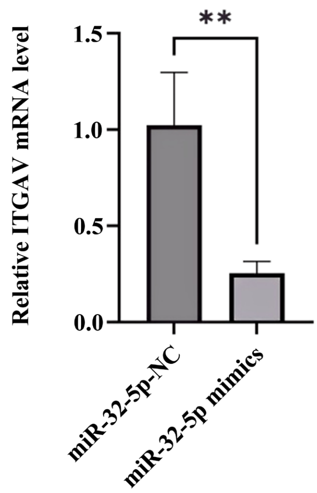

Figure 2. The effect of miR-32-5p on ITGAV mRNA expression in MG63 cells.

3.3. Western Blot Was Used to Detect the Effect of miR-32-5p Overexpression on the Expression of ITGAV and Apoptosis-Related Proteins in Osteosarcoma Cells

MG63 osteosarcoma cells were transfected with miR-32-5p mimics and miR-NC, respectively. Western blot analysis was conducted to assess the impact of miR-32-5p mimics on the expression of ITGAV and apoptosis-related proteins BCL2 and BAX. The results indicated that, compared to the miR-NC group, the levels of ITGAV and the anti-apoptotic protein BCL2 in the miR-32-5p mimics group were reduced, while the expression of the pro-apoptotic protein BAX significantly increased. These findings suggest that miR-32-5p inhibits the expression of ITGAV protein and simultaneously modulates the levels of various apoptosis-related proteins, thereby promoting apoptosis in osteosarcoma cells (Figure 3).

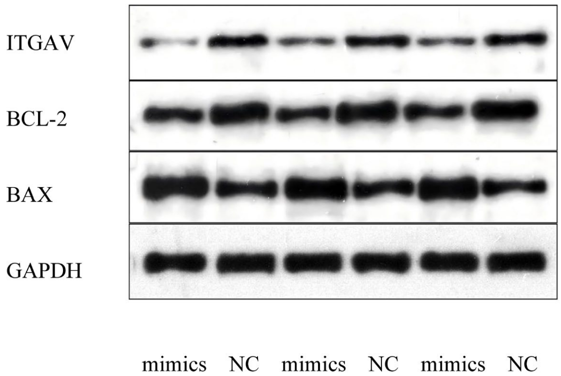

Figure 3. The effect of miR-32-5p on ITGAV and apoptosis-related protein expression of MG63 cells.

3.4. The Effect of miR-32-5p Overexpression on the Proliferation Ability of Osteosarcoma Cells Was Detected by the CCK-8 Method

MG63 osteosarcoma cells were transfected with miR-32-5p mimics and miR-NC, respectively. The proliferation capacity of MG63 cells was assessed using the CCK-8 method at 12, 24, 48, and 72 hours post-transfection. The results indicated that, compared to themiR-NC group, the proliferation of the miR-32-5p mimics treatment group was significantly inhibited (figure). At 12 hours post-transfection, the proliferation rates for the miR-NC group and the miR-32-5p mimics group were (100 ± 4.14)% and (76.47 ± 3.77)%, respectively ($t = 3.933$, $p = 0.0017$). After 24 hours, the proliferation rates for the miR-NC group and the miR-32-5p mimics group were (151.2 ± 4.47)% and (91.60 ± 1.75)%, respectively ($t = 9.965$, $p < 0.0001$). At 48 hours, the proliferation rates for the miR-NC group and the miR-32-5p mimics group were (196.9 ± 7.20)% and (120.6 ± 6.99)%, respectively ($t = 12.75$, $p < 0.0001$). Finally, at 72 hours, the proliferation rates for the miR-NC group and the miR-32-5p mimics group were (294.8 ± 13.97)% and (193.4 ± 13.87)%, respectively ($t = 16.94$, $p < 0.0001$). These findings suggest that elevated expression of miR-32-5p significantly inhibits the proliferation of MG63 osteosarcoma cells (**Figure 4**).

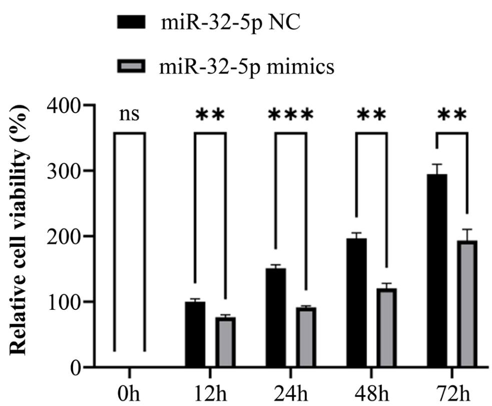

Figure 4. The effect of miR-32-5p on proliferation of MG63 cells.

3.5. The Transwell Assay Was Used to Detect the Effect of miR-32-5p Overexpression on the Invasion Ability of Cells

MG63 osteosarcoma cells were transfected with miR-32-5p mimics and miR-NC, respectively, to assess the impact of miR-32-5p on cell invasion capability using a Transwell assay. The results indicated that the invasion ability of cells in the miR-32-5p mimics group was significantly reduced compared to the miR-NC group. The number of cells that traversed the Transwell chamber was quantified, revealing cell counts of 147 ± 20.01 for the miR-NC group and 46.89 ± 15.46 for the miR-32-5p mimics group ($t = 12.23$, $p < 0.001$). These findings suggest that elevated expression of miR-32-5p markedly inhibits the invasion ability of MG63 osteosarcoma cells (Figure 5).

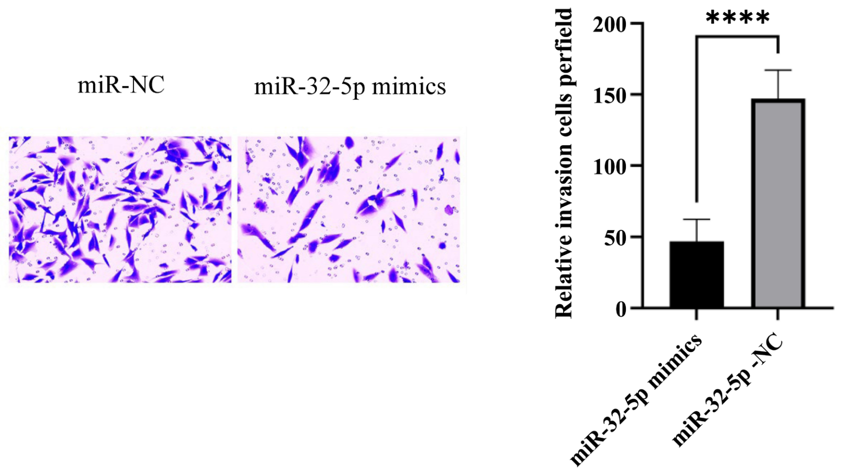

Figure 5. Effect of miR-32-5p on the invasive ability of MG63 by Transwell (crystal violet staining × 200).

3.6. The Transwell Assay Was Used to Detect the Effect of miR-32-5p Overexpression on the Migration Ability of Cells

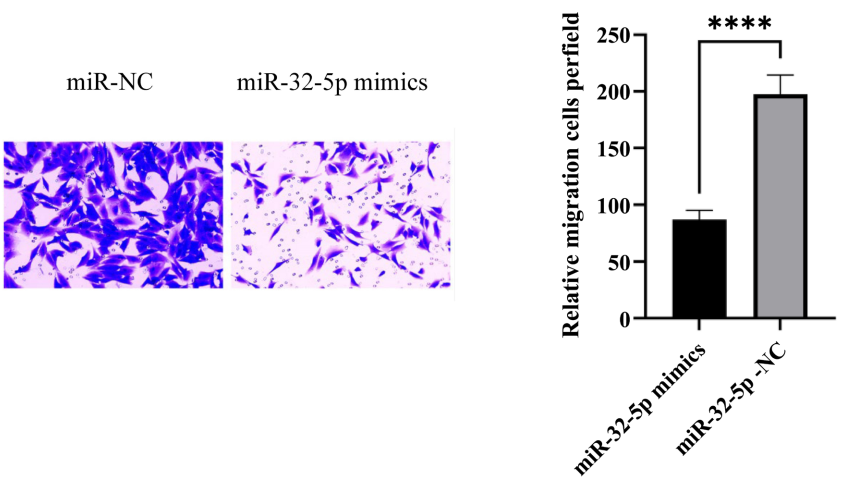

Figure 6. Effect of miR-32-5p on the migratory ability of MG63 by Transwell (crystal violet staining × 200).

MG63 osteosarcoma cells were transfected with miR-32-5p mimics and miR-NC, respectively, to evaluate the effect of miR-32-5p on cell migration ability using a Transwell assay. The results demonstrated that the migration capacity of cells in the miR-32-5pmimics group was significantly lower than that in the miR-NC group. The number of cells that passed through the Transwell chamber was quantified, revealing cell counts of 197.56 ± 16.90 for the miR-NC group and 86.89 ± 8.13 for the miR-32-5p mimics group ($t = 18.86$, $p < 0.001$). These findings indicate that elevated expression of miR-32-5p can significantly inhibit the migration of MG63 osteosarcoma cells (**Figure 6**).

3.7. Flow Cytometry Was Used to Detect the Effect of miR-32-5p Overexpression on Apoptosis

MG63 osteosarcoma cells were transfected with miR-32-5p mimics and miR-NC, respectively, and the alterations in cell apoptosis levels were assessed using flow cytometry. The results revealed that the apoptotic ratios for the miR-NC group and the miR-32-5p mimics group were (1.91 ± 1.59)% and (19.27 ± 1.31)%, respectively ($t = 14.59$, $p < 0.001$). These findings indicate that the elevated expression of miR-32-5p significantly enhanced the apoptosis levels of MG63 osteosarcoma cells (**Figure 7**).

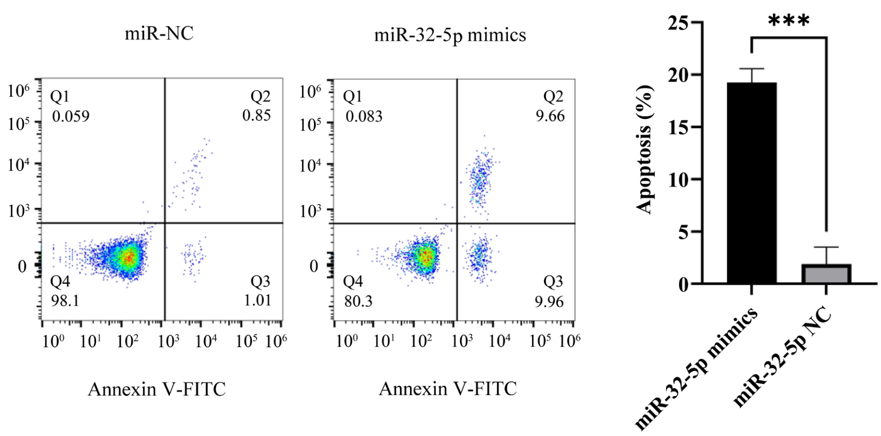

Figure 7. Effect of miR-32-5p on apoptosis in MG63 by flow cytometry.

3.8. Dual-Luciferase Activity Was Used to Detect the Targeted Regulatory Effect of miR-32-5p on ITGAV

To verify the downstream molecules targeted by miR-32-5p in MG63 osteosarcoma cells, bioinformatics software was employed for online predictive analysis, which identified ITGAV as a potential downstream target of miR-32-5p in MG63 cells. Analysis using the TargetScan database indicated that the 3'-UTR sequence of ITGAV was partially complementary to miR-32-5p, with seed sequences of at least 8-mer (**Figure 8(A)**). Based on these predictions, wild-type (WT) and mutant (MUT) plasmids containing the ITGAV 3'-UTR were co-transfected into MG63 cells with either miR-NC or miR-32-5p mimics. The luciferase activities of these four groups were subsequently measured. The results demonstrated that co-transfection of miR-32-5p mimics with the wild-type plasmid containing the ITGAV 3'-UTR significantly inhibited luciferase activity (**Figure 8(B)**). The luciferase activities for miR-NC and miR-32-5p mimics co-transfected with the wild-type ITGAV 3'-UTR plasmid were (1.00 ± 0.03) and (0.33 ± 0.02), respectively ($t = 37.181$, $p < 0.001$). These findings indicate that miR-32-5p can inhibit ITGAV expression by targeting its 3'-UTR, suggesting that ITGAV is a direct target of miR-32-5p.

(A) Position 3176-3183 of ITGAV 3' UTR 5' ...UGUCAUUGUUCUCAAGUGCAAUA...

hsa-miR-32-5p 3' ACGUUGAAUCAUUACACGUUAU

(B)

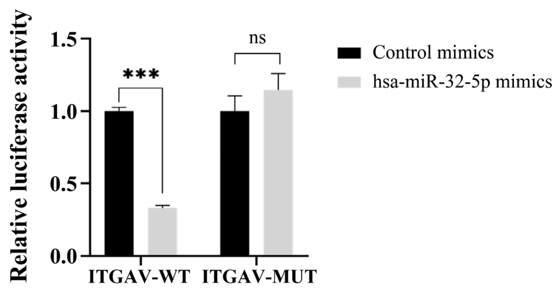

Figure 8. Targeted regulation of ITGAV by miR-32-5p through dual luciferase reporter assay. (A): The binding site of ITGAV and miR-32-5p. (B): Detection of luciferase activity.

4. Discussion

Osteosarcoma (OS) is the most prevalent primary malignant tumor. Despite advancements in treatment modalities, including radiotherapy, adjuvant chemotherapy, gene therapy, and extensive tumor resection, the 5-year survival rate for metastatic OS remains unsatisfactory [9]. Thus, the development of new strategies is crucial for the clinical targeting and immunotherapy of osteosarcoma patients. miRNA plays a significant role in the pathogenesis of various tumors by regulating the expression of multiple target genes and influencing several biological processes, including cell proliferation, invasion, and apoptosis [10] [11]. This study aims to clarify the role of miR-32-5p in regulating the malignant phenotype of osteosarcoma and the related molecular mechanisms.

MiR-32 acts as a tumor suppressor in many tumors such as lung cancer, pancreatic cancer and clear cell renal cell carcinoma, and can inhibit tumor development [12]-[14]. The reduced expression of miR-32-5p in osteosarcoma tissues and cells indicates that its abnormal expression may be linked to osteosarcoma (OS) [15]. The upregulation of miR-32-5p expression can inhibit the proliferation, invasion, and migration of osteosarcoma cells via the miR-32-5p/HMGB1 axis, promote apoptosis, and impede the progression of osteosarcoma [7]. In this study, the expression of miR-32-5p in MG63 osteosarcoma cells transfected with miR-32-5p mimics was significantly elevated, demonstrating effective transfection efficiency. Research has shown that the activity and proliferative capacity of MG63 osteosarcoma cells transfected with miR-32-5p mimics are markedly lower than those of control cells, indicating that miR-32-5p effectively inhibits the proliferation of MG63 osteosarcoma cells. Enhanced invasion and migration capabilities are defining characteristics of malignant tumors. In osteosarcoma, this trait is evident in a pronounced propensity for distant metastasis, with lung metastasis being the most prevalent [16].

This study demonstrated that the upregulation of miR-32-5p expression significantly inhibited the invasion and migration abilities of MG63 cells, indicating its role in suppressing distal metastasis. Furthermore, to assess the impact of miR-32-5p on other malignant phenotypes of osteosarcoma cells, the study examined its effect on apoptosis levels in MG63 cells. The results revealed that elevated miR-32-5p expression promoted cell apoptosis, which was associated with an increase in the pro-apoptotic protein BAX and a decrease in the anti-apoptotic protein BCL2. These findings confirm the inhibitory effect of miR-32-5p on the malignant phenotype of osteosarcoma cells.

To further investigate the molecular mechanism by which miR-32-5p inhibits osteosarcoma, it is essential to consider the role of integrin subunit alpha V (ITGAV). Research indicates that ITGAV, a member of the integrin family, is significantly involved in the pathological processes of various cancers. Notably, ITGAV is aberrantly overexpressed in osteosarcoma (OS) tumor cells and in metastatic OS, thereby promoting tumor progression. This suggests that ITGAV serves as a pivotal molecule within the signaling network that mediates tumor initiation and advancement, playing a crucial role in the signaling pathways that drive tumor development [17]. The study also assessed the expression level of ITGAV. It was observed that the upregulation of miR-32-5p expression led to a reduction in the protein expression level of ITGAV in MG63 osteosarcoma cells.

To investigate the impact of miR-32-5p on the malignant phenotype of osteosarcoma through its interaction with ITGAV, a bioinformatics analysis was performed using the TargetScan online prediction software. The analysis revealed that the 3'-UTR sequence of ITGAV is partially complementary to miR-32-5p. This finding was subsequently validated through a dual-luciferase activity assay. Research indicates that miR-32-5p inhibits ITGAV expression by targeting its 3'-UTR, thereby confirming that miR-32-5p directly regulates ITGAV expression in a positive manner.

This study confirmed that miR-32-5p exerts an anti-tumor effect in osteosarcoma. Its overexpression significantly inhibits the proliferation, invasion, and migration of MG63 cells while promoting apoptosis. Mechanistically, we established that ITGAV is a direct target of miR-32-5p, with its expression being negatively regulated by miR-32-5p. This finding suggests that this regulatory axis may play a role in mediating the aforementioned biological effects.

This study has several limitations. First, the experiments were conducted using only the MG63 cell line, whereas osteosarcoma is characterized by significant heterogeneity. Future research should validate the findings using additional cell lines, such as U2-OS and Saos-2, to enhance the generalizability of the conclusions. Second, the current results primarily indicate correlation, lacking direct causal evidence. Subsequent rescue experiments, such as restoring ITGAV expression in cells overexpressing miR-32-5p to observe potential phenotypic reversal, will help ascertain whether the observed phenotypic changes are specifically mediated by the down-regulation of ITGAV, thereby ruling out the influence of other targets.

This study enhances the understanding of miR-32-5p's role and offers new potential targets and a theoretical foundation for the targeted therapy of osteosarcoma. Further validation in various cell lines and in vivo models is necessary, and the interactions between this pathway and other signaling networks should be investigated to facilitate its clinical application.

Data Availability

The datasets generated or analyzed in the current study will be available upon reasonable request.

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Author Contributions

W F and W L contributed to the study design and data acquisition, drafted the manuscript, and were the co-first authors. T S, W T and P L contributed to contributed experimental data. C L were considered the co-correspondence author. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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