Mechanism of miR-122-5p regulating the activation of PI3K-Akt-mTOR signaling pathway on the cell proliferation and apoptosis of osteosarcoma cells through targeting TP53 gene

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Abstract. – OBJECTIVE: To explore the regulatory mechanism of microRNA-122-5p (miR-122-5) targeting tumor protein p53 (TP53) gene to mediate PI3K-Akt-mTOR signaling pathway on the proliferation and apoptosis of osteosarcoma (OS) cells.

PATIENTS AND METHODS: With the collection of osteosarcoma and normal adjacent tissues, the mRNA of miR-122-5p, TP53, PTEN, PI3K, Akt, mTOR, Bim, Bax, and Bcl-2 was detected by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), followed by the detection of the protein expression by Western blot. The target relationship between miR-122-5p and TP53 gene was verified. The third generation osteosarcoma cells were divided into Blank group, miR-122-5p mimic negative control (NC) group, miR-122-5p mimic group, miR-122-5p inhibitor NC group, miR-122-5p inhibitor group, rapamycin group and miR-122-5p inhibitor + rapamycin group. Furthermore, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry were used to detect the proliferation ability, cell cycle distribution and apoptosis of each group after transfection.

RESULTS: The expression level of miR-122-5p in osteosarcoma was lower than that in normal tissues ($p < 0.05$), TP53, PTEN, Bim and Bax expression levels were decreased (all $p < 0.05$), while the expression levels of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR and Bcl-2 were highly up-regulated (all $p < 0.05$). TP53 had the lowest expression in osteosarcoma cell line U-2OS ($p < 0.05$), which was selected for subsequent cell experiments. TP53 was the target gene of miR-122-5p. Compared with Blank group, miR-122-5p mimic group had increased expression of miR-122-5p (all $p < 0.05$); besides, there were significantly increased expression of TP53, PTEN, Bim, and Bax in miR-122-5p mimic group and rapamycin group, while remarkably decreased expression of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, and Bcl-2 (all $p < 0.05$), accompanied by increased proportion of cells in G0/G1 phase, decreased cell proportion in S phase, increased cell apoptosis and inhibited cell proliferation (all $p < 0.05$). The opposite trends were found in miR-122-5p inhibitor group relative to miR-122-5p mimic group and rapamycin group (all $p < 0.05$). Meanwhile, no significant difference was found in miR-122-5p inhibitor+rapamycin group when compared with that in Blank group (all $p > 0.05$) except for significantly decreased miR-122-5p expression ($p < 0.05$).

CONCLUSIONS: Upregulation of miR-122-5p may inhibit the proliferation and promote the apoptosis of osteosarcoma cells by inhibiting the activation of PI3K-Akt-mTOR signaling pathway, which may be related to the targeted up-regulation of TP53 expression.

Key Words:
MiR-122-5p, TP53, PI3K-Akt-mTOR signaling pathway, Human osteosarcoma cells, Proliferation, Apoptosis.

Introduction

Osteosarcoma is an aggressive bone tumor with high degree of malignancy, which occurs frequently in young populations[1]. A great proportion of patients has systemic metastasis at the time of diagnosis. Amputation is the standard method to treat osteosarcoma before the 1970s, but it has a great influence on the limb function of patients, and the psychological injury is also inestimable[2,3]. With rapid development of medical technology, surgery, chemotherapy drugs, adjuvant radiothera-

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py and chemotherapy and other means have made great progress for the treatment of osteosarcoma[4]. Even so, there are still many patients with tumor recurrence and distant metastasis after surgery and chemotherapy, which significantly reduces the 5-year survival rate of osteosarcoma patients[5,6]. After that, molecular targeted therapy, immunotherapy and gene therapy gradually become the research hot-spot, providing broader choice for osteosarcoma treatment[7]. Osteosarcoma is characterized by high-grade malignant tumor at the time of diagnosis and is prone to early metastasis[8]. Distant metastasis can be explained by the abnormal expression of tumor metastasis related genes and metastasis inhibition related genes. To explore the molecular mechanism of osteosarcoma is helpful to screen molecular biomarkers and gene targeted therapeutic drugs, and to find out new approaches to improve the survival and prognosis of osteosarcoma patients.

Targeted therapy refers to such a treatment mode of selective concentration under the specific guiding mechanism through local administration, gastrointestinal administration or intravenous administration, targeting at the target organ, target tissue, target cell or specific structure within the cell. In such way, the therapeutic substance can be delivered to specific pathological tissue or organ specifically, so as to realize local treatment to alleviate or reverse pathological changes[9,10]. The main differences between tumor cells and normal cells lie in the continuous angiogenesis, unlimited proliferation, invasion and metastasis, and escape from apoptosis. Targeted therapy is an updated treatment strategy to improve the specific killing effect of drugs on tumor cells and reduce the side effects of drugs[11]. Some specific antigens or signal transduction molecules have been found expressed in osteosarcoma, which not only provide a more accurate research direction for the pathogenesis of osteosarcoma, but also a good prospect for targeted therapy[12-14].

PI3K-Akt-mTOR signaling pathway is well-known in mediating cell survival, and its activation has been accepted as one of the main mechanisms of anti-apoptosis of tumor cells[15]. Among them, AKT regulates several downstream targets leading to cell growth, survival and cisplatin resistance[16]. Phosphatidylinositol 3-kinases (PI3K) can mediate the cell growth and cell apoptosis of osteosarcoma cell line treated by cisplatin, so as to realize the goal of attenuating the malignant progression of osteosarcoma[17]. In addition, mTOR functions remarkably in the biological behavior of mesenchymal stem cells and bone[18]. It is a member of PI3K family, which can control the translation of mRNA and protein transporter re-regulatory protein[19] to regulate cell growth, cell cycle and DNA damage repair, which is an indispensable material for cell growth and development[20]. Its absent or abnormal expression may cause extremely serious consequences to the organism itself. Changes in mTOR signaling pathway are common in many tumors, including osteosarcoma[21,22]. In osteosarcoma cell lines, rapamycin can down regulate the activity of mTOR signaling pathway in osteosarcoma cells, delay the transition from G1 phase to S phase of cell cycle and inhibit the growth of osteosarcoma cells[23]. Rapamycin, as an immunosuppressant blocking mTOR pathway, has been proved to play a wide range of anti-tumor effects in clinical drug trials of rapamycin, including osteosarcoma[24,25]. In this regard, in-depth study of this signaling pathway may provide a new insight for further understanding of the mechanism of tumor cell activity and tumor treatment.

MicroRNAs and their specific target genes have been observed to play a wide range of roles in the tumor process and has become one of the key mechanisms of targeted therapy for human tumors. MiR-122 is a kind of liver specific microRNA, which plays an important role in the growth and development of liver, pressure response, lipid metabolism, infection and replication of a variety of hepatotropic viruses and maintenance of organ morphology[26]. Up-regulation of miR-122 expression can inhibit the proliferation and invasion of several malignant bone tumor cells, suggesting that there is a certain relationship between regulation of miR-122 expression and osteosarcoma occurrence and inhibition of biological behaviors[27,28]. At present, there are still few reports about miR-122-5p mediated PI3K-Akt-mTOR signaling pathway and its targeted mRNA. Therefore, this study was carried out to explore whether miR-122-5p could regulate the proliferation and apoptosis of osteosarcoma cells by targeting specific target gene to mediate PI3K-Akt-mTOR signaling pathway.

Patients and Methods

Patients

From March 2016 to September 2018, 50 cases of osteosarcoma tissue samples were collected and confirmed by pathology. All patients were

diagnosed as classic osteosarcoma by clinical data and pathological examination, with informed consent provided. The present study has been approved by the Ethics Committee of Zhengzhou Orthopedics Hospital. Inclusion criteria: all the patients were diagnosed as classic osteosarcoma according to the definition of World Health Organization (WHO) classification of soft tissue tumors, aged between 14-52 years old, and received the operation for the first time. Exclusion criteria: patients who had diabetes mellitus, cardiovascular and cerebrovascular diseases, systemic osteopathy, multiple metastases, cachexia, etc. The 50 normal tissues samples were collected from amputees. After the collection of the specimen, it was numbered and registered, with part of which used to make paraffin section, and the other part of which stored in liquid nitrogen at - 80°C.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Detection of the mRNA Expression of MiR-122-5p, Tumor protein p53 (TP53) and Related Indexes to PI3K-Akt-mTOR Signaling Pathway

The primer sequences of miR-122-5p, TP53, PTEN, PI3K, Akt, mTOR, Bim, Bax, and Bcl-2 were designed and then synthesized by Beyotime (Shanghai, China). Following RNA extraction, the RNA was reverse transcribed into cDNA using PrimeScript RT kit, and the procedure was carried out according to the instructions: add 75 μL diethylpyrocarbonate (DEPC) H_2O and 0.5 μL Olidgo dT (1 μg/μL) into the 0.5 mL Polymerase Chain Reaction (PCR) tube, followed by mixing with shaking; add 1 g/μL RNA into the above system to mix well by shaking; make denaturation at 70°C for 5 min, then immediately put it on ice for about 5 min; add 8.5 μL DEPC H_2O, 5 μL RT buffer, 0.5 μL dNTP (2.5 mM each) and 1 μL reverse transcriptase M-MLV RTase into each tube, shake and mix; react the above reaction system at 37°C for 60 min, and at 70°C for 10 min to inactivate reverse transcriptase. The obtained DNA was stored in refrigerator at -20°C. The above reaction solution was taken for fluorescence quantitative PCR in a 20 μL system, with three replicates for each sample. By using Eva Green premixed solution of Bio-Rad company (Hercules, CA, USA) the above ingredients were added into the reaction system to shake and mix evenly, followed by the reaction according to the following conditions: 1) predenaturation for 1 min at 94°C; 2) denaturation at 94°C for 10 s; 3) annealing + extension at 58°C for 45 s; 4) with a total cycle for 40 times, and the fluorescence value was read after each cycle. The relative expression of miR-122-5p was measured with U6 (forward: 5'-CTCGCTTCGGCAGCACA-3'; reverse: 5'-AACGCTTCACGAATTTGCGT-3') as the internal reference, and that of TP53, PTEN, PI3K, Akt, mTOR, Bim, Bax and Bcl-2 with GAPDH (forward: 5'-GATTTGGTCGTATTGGGCGC-3'; reverse: 5'-GCCTTCTCCATGGTGGTGAA-3') as the internal reference.

Western Blot Detection of the Protein Expression of TP53 and Related Indexes to PI3K-Akt-mTOR Signaling Pathway

In terms of total protein extraction, tissue and cell proteins were extracted from radio immunoprecipitation assay (RIPA) lysate containing protease inhibitors and phosphatase inhibitors. After centrifugation at 4°C for 15 min (12000 rpm), the protein concentration was determined by bicinchoninic acid assay (BCA) method. The 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was performed, followed by antigen antibody reaction after membrane transfer. Then, the sealing solution was added into the incubation box to immerse the polyvinylidene difluoride (PVDF) membrane after membrane transfer to seal in shaker at room temperature for 1 h. The primary antibodies were: mouse anti-p53 antibody Trp53 (1:2000, ab26), rabbit anti-PTEN antibody (1:2000, ab2199), rabbit anti-PI3K antibody (1:2000, ab32089), rabbit anti-pan-Akt antibody (1:2000, ab8805), rabbit anti-mTOR antibody (1:2000, ab109268), rabbit anti-Bim antibody (1:2000, ab32158), rabbit anti-Bax antibody (1:2000, ab32503), rabbit anti-Bcl-2 antibody (1:2000, ab32124), and GAPDH antibody (1:1000, ab9485), all of which were purchased from Abcam, Cambridge, MA, USA. According to the recommended working concentration in the instructions for the use of the selected antibody, the primary antibodies were diluted to the corresponding working concentration with the sealing solution. The PVDF membrane was further placed into the antibody solution, with the incubation box sealed by fresh-keeping film and incubated overnight on the shaking table in the cold storage. After incubation, Tris-Buffered Saline and Tween-20 (TBST) was used to rinse 4 times for 5 min each time. In the next step of incubation with the secondary antibody: according to the recommended working concentration in the instructions for the use of the selected anti-

body, the Goat anti-rabbit IgG antibody labeled with horseradish peroxidase (HRP) was diluted to the corresponding working concentration with the sealing solution. The PVDF membrane was once again placed into the antibody solution, with the incubation box sealed by fresh-keeping film and incubated overnight on the shaking table in the cold storage. After incubation, TBST was used to rinse again, 4 times for 5 min each time. After that, the membrane was immersed into the enhanced chemiluminescence (ECL) solution for further observation and photography. Relative protein expressions were tested with GAPDH as the internal reference.

Cell Culture and Grouping

Hs888T, U-2OS and MG63 cells were all purchased from Shanghai Institute of Cell Biology. Before treatment, the cells should be in good condition, i.e., normal morphology, good adherence to the wall, smooth edge and no pollution. When the cell density reached about 75%, it can be subcultured or frozen in liquid nitrogen tank. Hs888T and MG63 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM), and U-2OS cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium. All the above cells were cultured in a constant temperature incubator with humidity of 95%, 5% CO_2 at 37°C. qRT-PCR was used to detect the expression level of TP53 in each cell line, and the cell line with the lowest relative expression was selected for subsequent cell experiments. Cells were divided into Blank group, miR-122-5p mimic negative control (NC) group, miR-122-5p mimic group, miR-122-5p inhibitor NC group, miR-122-5p inhibitor group, rapamycin group and miR-122-5p inhibitor + rapamycin group.

Dual-Luciferase Reporter Gene Assay

Target gene prediction of miR-122-5p was performed using biological prediction website (microRNA.org). The Dual-Luciferase reporter gene assay was then used to verify that TP53 was the direct target of miR-122-5p. The TP53 3'-untranslated region (UTR) gene fragment was synthesized, and the TP53 wild-type group was constructed by cloning the endonuclease site SpeI and Hind III. The binding sites of miR-122-5p and target gene were predicted by target gene database, and TP53 mutant-type vector was constructed by site-directed mutagenesis method. The constructed Luciferase reporter plasmids were co-transfected, and Dual-Luciferase Reporter Assay System was applied to detect the Luciferase activity (Promega, Madison, WI, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay for the Detection of Cell Proliferation

When the number of cells in each group reached about 80%, phosphate-buffered saline (PBS) solution was used for twice washing. After trypsin digestion, cells were blown with a straw to make a single cell suspension. Cell counting was performed using FACSCalibur cell counter (Beckman Coulter, CA, USA). Cells were inoculated in 96-well plate and the volume of 200 μL in each well, with 6 replicates each. Cells were cultured in 5% CO_2 incubator at 37°C for 24-96 h, followed by the addition of 30 μL MTT solution to each well of 96-well plate in 5% CO_2 cell incubator for 4 h in the dark. With the culture terminated, the culture solution in the 96-well plate was sucked and added with 120 μL dimethyl sulfoxide (DMSO) solution to each well containing the cells of the control group and the experimental group respectively, and then the 96-well plate was placed on the shaking table to vibrate at 80 times/min to make the crystal in the well fully dissolved progressively. After that, the 96 well-plates were placed on the enzyme-linked immunosorbent assay (Bio-Rad, Hercules, CA, USA) to measure the absorbance of each well at the wavelength of 490 nm.

Flow Cytometry to Detect Cell Cycle Distribution and Cell Apoptosis in Each Group

PI single staining and double parameter flow cytometry were performed to detect cell cycle distribution and cell apoptosis in each group after transfection, respectively. For the first test, after cell treatment and collection, 70% pre-cooled ethanol was added for fixation of osteosarcoma cells at 4°C overnight; after centrifugation at 4°C (800×g), the supernatant was discarded and washed twice with PBS containing 1% fetal bovine serum. An amount of 400 μl binding buffer was used to re-suspend cells, 50 μL RNA enzyme was added for incubation at 37°C for 30 min, followed by the addition of 50 μL 50 mg/L PI for reaction in the dark. For the second test, osteosarcoma cells were inoculated into 6-well plates. After 48 h of transfection, the cells were digested with trypsin without Ethylene Diamine

Tetraacetic Acid (EDTA) and centrifuged to discard the supernatant. Cells were then re-suspended in 500 µl binding buffer with 5 µL fluoresceine isothiocyanate (FITC) and 5 µL propidium iodide (PI) added to react in the dark. Apoptosis was detected at wavelength of 488 nm.

Transwell Assay to Detect Cell Invasion

After the culture of cells with bovine serum overnight, the logarithmic growth cells were collected, and the cells were digested by trypsin. With centrifugation at 3,000 rpm for 5 min and three times of PBS washing, cells were re-suspended with bovine serum medium. Using a pipette, 100 µl cell suspension was added into each well to the upper chamber, with the chamber placed into the culture plate; and 600 µl Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% newborn bovine serum was added into the lower chamber. Each group was provided with 3 replicates. After incubation in an incubator at 37°C for 24 h, the transwell chamber was taken out, washed with a small amount of PBS for three times, and cells in the PVPF membrane near the upper chamber was wiped gently with a cotton swab. After another washing with PBS twice, cells were fixed with 95% alcohol at 37°C for 30 min, and stained with crystal violet for 10 min. Cells passed were observed under microscope.

Statistical Analysis

All data were processed by SPSS 21.0 statistical software (SPSS, IBM, Armonk, NY, USA). All the measurement data were expressed in the form of mean ± standard deviation. The tool used in the analysis of data were *t*-test for the comparison between the two groups, and one-way analysis of variance (ANOVA) for the comparison among groups, respectively, followed by the use of Bonferroni's post-hoc test for group comparison. $p < 0.05$ presented that the difference was statistically significant.

Results

Expression of MiR-122-5p as well as mRNA and Proteins Expression of TP53, PTEN, PI3K, Akt, mTOR, Bim, Bax, and Bcl-2 Detected by RT-PCR and Western Blot

According to the results of qRT-PCR (Figure 1A), compared with normal tissues, the expression of miR-122-5p and TP53, PTEN, BIM and

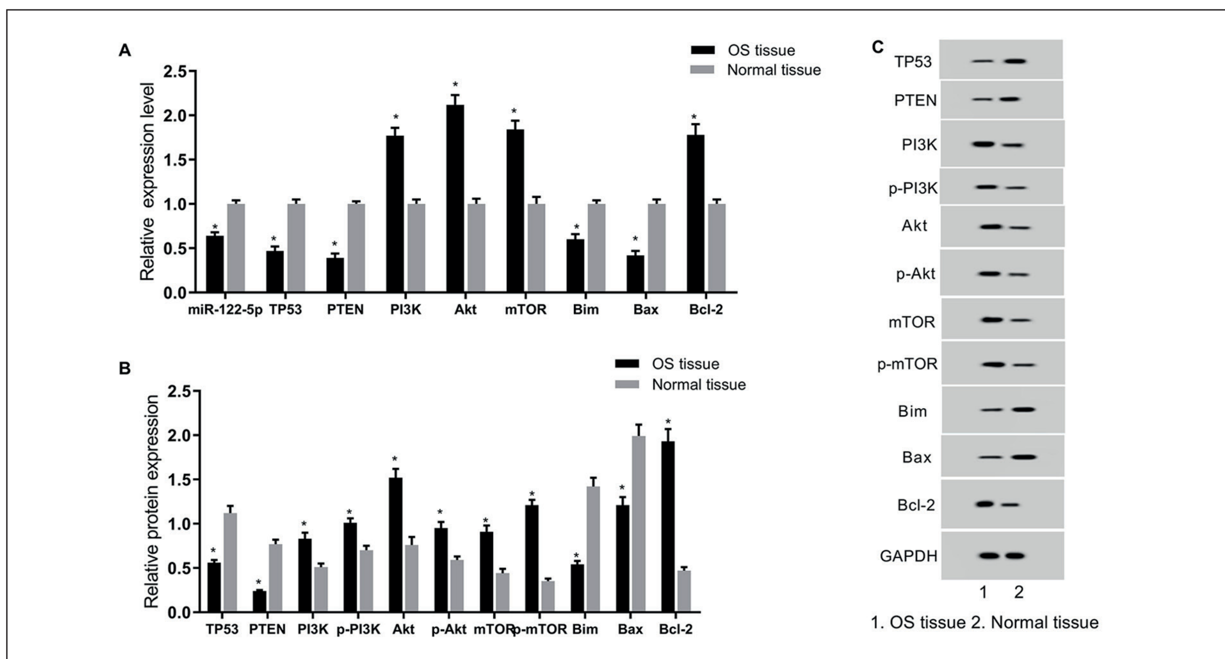

Figure 1. Expression of miR-122-5p as well as mRNA and proteins expression of TP53, PTEN, PI3K, Akt, mTOR, Bim, Bax, and Bcl-2 detected by qRT-PCR and Western blot. Note: **A**, Detection of mRNA expression in osteosarcoma and normal tissues by qRT-PCR; **B**, Detection of protein expression in osteosarcoma and normal tissues by Western blot; **C**, Electrophoretic bands of each protein detected by Western blot; *Compared with the Normal group, $p < 0.05$; OS, Osteosarcoma.

Bax decreased markedly in osteosarcoma (all $p < 0.05$), while that of PI3K, Akt, mTOR and Bcl-2 were significantly increased (all $p < 0.05$). Similar trends were indicated by Western blot (Figure 1B) to detect TP53, pathway related indexes (PTEN, PI3K, Akt, mTOR) and apoptosis related factors (Bim, Bax, and Bcl-2) (all $p < 0.05$).

Screening of Cell Line with Low Expression of TP53 to Determine Suitable experimental cells

The expression of TP53 in three human osteosarcoma cell lines (Hs888T, U-2OS, MG63) was detected by qRT-PCR, and the cell line with the lowest expression of TP53 was selected. The results showed that TP53 was expressed in different degrees in human osteosarcoma cell lines (Figure 2), showing the lowest expression level in U-2OS ($p < 0.05$). Hence, U-2OS was selected as the suitable cell line for subsequent cell experiment.

Targeting Relationship Verification Between MiR-122-5p and TP53

Through the analysis of online analysis software, there was a specific binding region between TP53 gene and miR-122-5p sequence. TP53 was the target gene of miR-122-5p (Figure 3A). Furthermore, Dual-Luciferase reporter gene assay (Figure 3B) showed that compared with the NC group, the luciferase signal increased in the miR-122-5p mimic group ($p < 0.05$); however, there was no significant difference in mutant-type group compared with the NC group ($p > 0.05$), suggesting that miR-122-5p can specifically bind to TP53 gene.

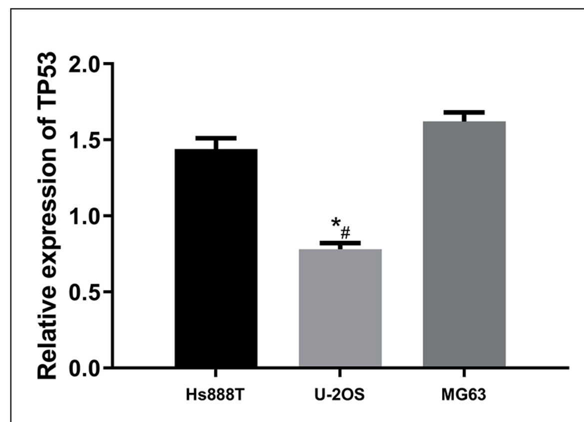

Figure 2. Expression of U-2OS in osteosarcoma cell lines. Note: *Compared with Hs888T cell line, $p < 0.05$; #Compared with MG63 cell line, $p < 0.05$.

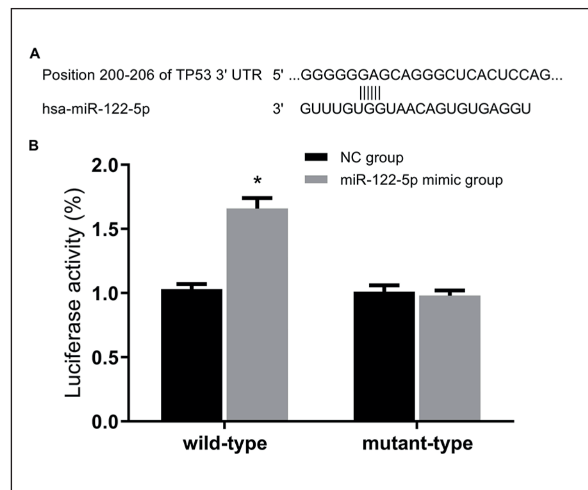

Figure 3. Targeting relationship verification between miR-122-5p and TP53. Note: **A**, Predicted binding sites of rmiR-122-5p on TP533'UTR; **B**, Luciferase activity detection by Dual-Luciferase reporter gene assay; *compared with Blank group, $p < 0.05$.

Detection of mRNA Levels of miR-122-5p, TP53, PTEN, PI3K, Akt, mTOR, Bim, Bax, and Bcl-2 after Cell Transfection as well as Corresponding Protein Expression Detection

qRT-PCR (Figure 4A) showed no significant difference in the comparison among Blank group, miR-122-5p mimic NC group and miR-122-5p inhibitor NC group ($p > 0.05$); compared with Blank group, miR-122-5p mimic group showed increased expression of miR-122-5p ($p < 0.05$), and there was no significant difference in the mRNA expression of miR-122-5p in rapamycin group ($p > 0.05$); miR-122-5p inhibitor group had significant decreased expression of miR-122-5p ($p < 0.05$); in addition, there was remarkably decreased expression of miR-122-5p in miR-122-5p inhibitor + rapamycin group ($p < 0.05$).

Furthermore, as revealed by qRT-PCR (Figure 4A) and Western blot (Figure 4B-C), there was no significant difference in the comparison Blank group, miR-122-5p mimic NC group and miR-122-5p inhibitor NC group ($p > 0.05$). In addition, compared with Blank group, miR-122-5p mimic group and rapamycin group showed significantly increased mRNA and protein expression of TP53, PTEN, Bim and Bax in U-2OS cells (all $p < 0.05$). While the mRNA and protein expression levels of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR and Bcl-2 mRNA were evi-

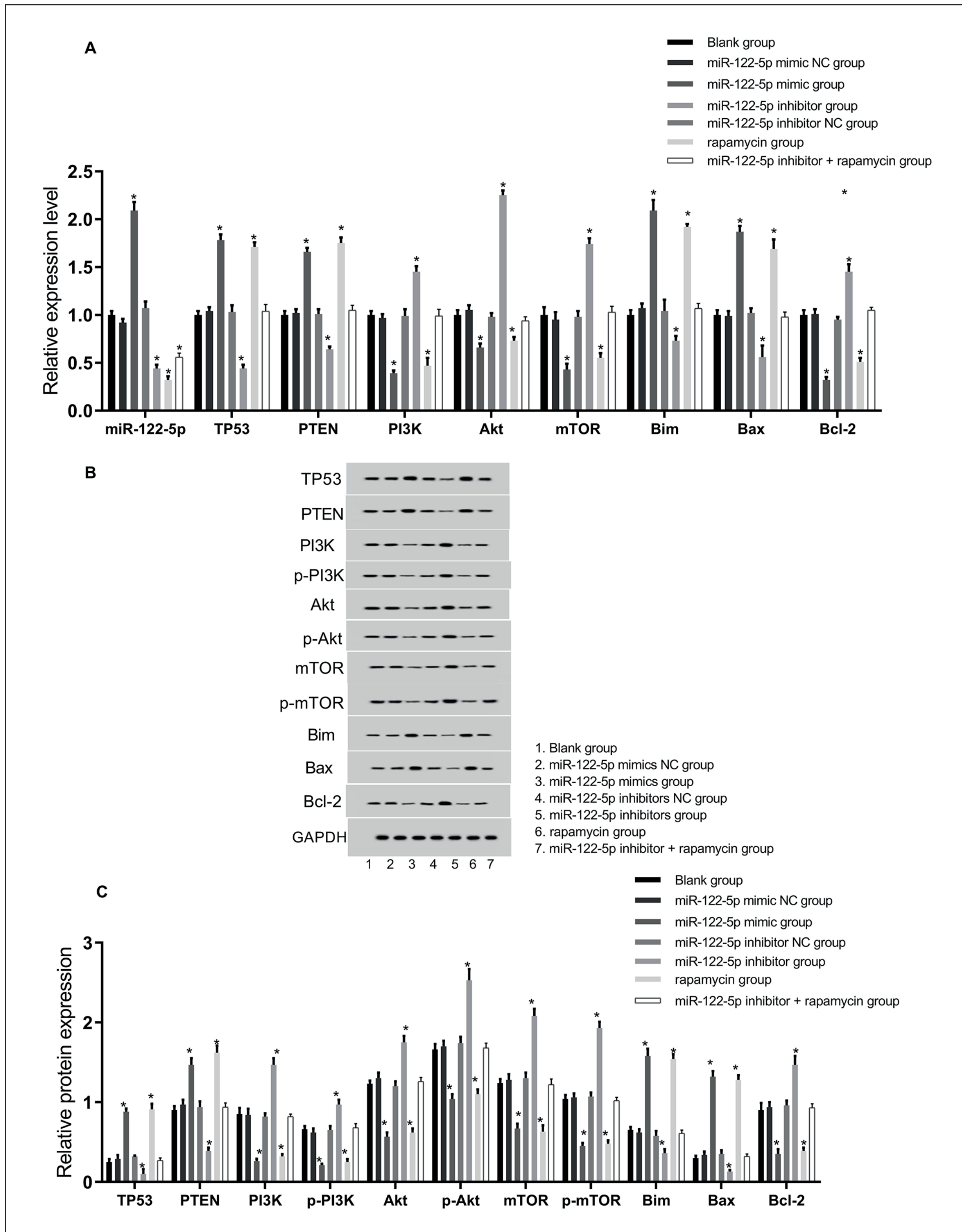

Figure 4. The detection of mRNA and protein expressions of miR-122-5p, TP53, PTEN, PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, Bim, Bax, and Bcl-2 in each group after cell transfection by qRT-PCR and Western blot respectively. Note: **A**, Histogram of the mRNA expression levels; **B**, Electrophoretic bands of each protein detected by Western blot; C. Histogram of the protein expression levels; *compared with Blank group, $p < 0.05$.

dently decreased (all $p < 0.05$). Furthermore, miR-122-5p inhibitor group had significant decreased mRNA and protein expression of TP53, PTEN, Bim and Bax (all $p < 0.05$), with evidently increased mRNA and protein expression of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR and Bcl-2 (all $p < 0.05$). In addition, there was no statistical difference in the comparison of the mRNA and protein expression levels of TP53, PTEN, Bim, Bax, PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR and Bcl-2 in miR-122-5p inhibitor + rapamycin group (all $p > 0.05$).

Detection and Comparison of Cell Proliferation and Invasiveness in Each Group after Transfection

According to the results of MTT assay (Figure 5A) and transwell invasive assay (Figure 5B), there was no statistical difference in the comparison of cell proliferation and invasion ability among Blank group, miR-122-5p mimic NC group and miR-122-5p inhibitor NC group ($p > 0.05$). Meanwhile, compared with Blank group, miR-122-5p mimic group and rapamycin group showed significantly decreased cell proliferation at 48h, 72h and 96h and invasion (all $p < 0.05$). While there was significant increase in cell proliferation at 48h, 72h and 96h and invasion in miR-122-5p inhibitor group ($p < 0.05$). Besides, no significant difference was found when compared miR-122-5p inhibitor +rapamycin group with that of the Blank group and NC group ($p > 0.05$).

Detection and Comparison of Cell Cycle and Apoptosis Rate after Transfection in Each Group

On the basis of the findings of PI single staining results (Figure 6A) and flow cytometry (Figure 6B), there was no statistical difference in the comparison of cell cycle distribution and apoptosis rate among Blank group, miR-122-5p mimic NC group and miR-122-5p inhibitor NC group ($p > 0.05$). Compared with Blank group, miR-122-5p mimic group and rapamycin group showed significantly increased cells in phase G0/G1, decreased cells in phase S, and significantly increased cell apoptosis (all $p < 0.05$). Moreover, miR-122-5p inhibitor group had significantly decreased cells in phase G0/G1 and increased cells in phase S, as well as significantly decreased cell apoptosis (all $p < 0.05$). There was no significant difference in the comparison of cell cycle distribution and cell apoptosis of miR-122-5p inhibitor+ rapamycin group with Blank group and NC group ($p > 0.05$).

Discussion

mTOR inhibitor has been reported to have the ability to control the occurrence and development of osteosarcoma[29,30]. Meanwhile, PI3K can mediate cell survival, growth and proliferation through Akt signaling pathway. Rapamycin, an inhibitor of mTOR, can inhibit the growth and activity of tumor cells by blocking the activity of mTOR[31]. Significantly at present, many molecular

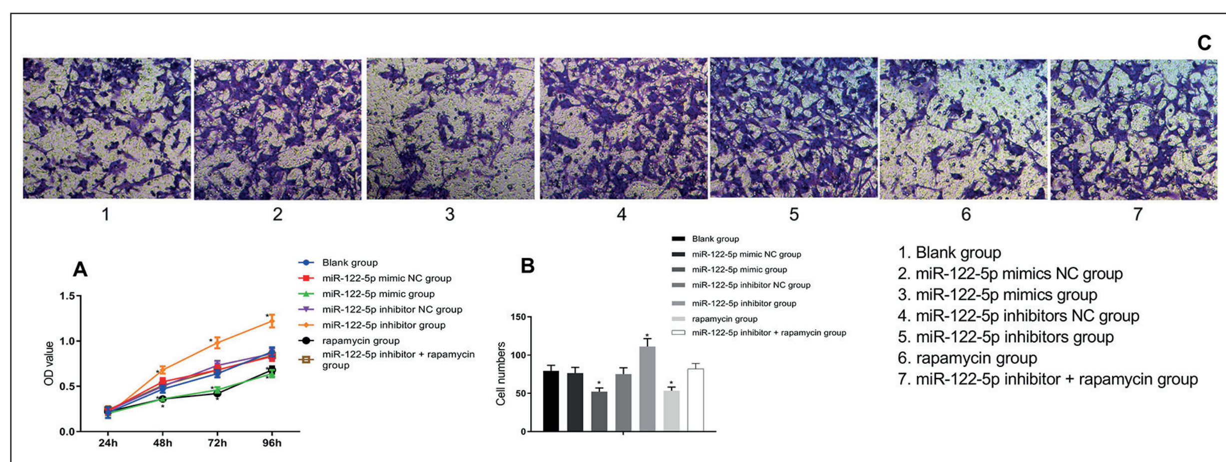

Figure 5. Changes of cell proliferation invasion in each group after cell transfection detected by MTT assay and transwell assay respectively. Note: **A**, Changes of proliferative ability of cells in each group after cell transfection detected by MTT assay; **B**, Statistical analysis of the number of cells transferred from transwell invading the upper chamber to the lower chamber; C. Microscopic observation of transwell assay (×200); *compared with Blank group, $p < 0.05$.

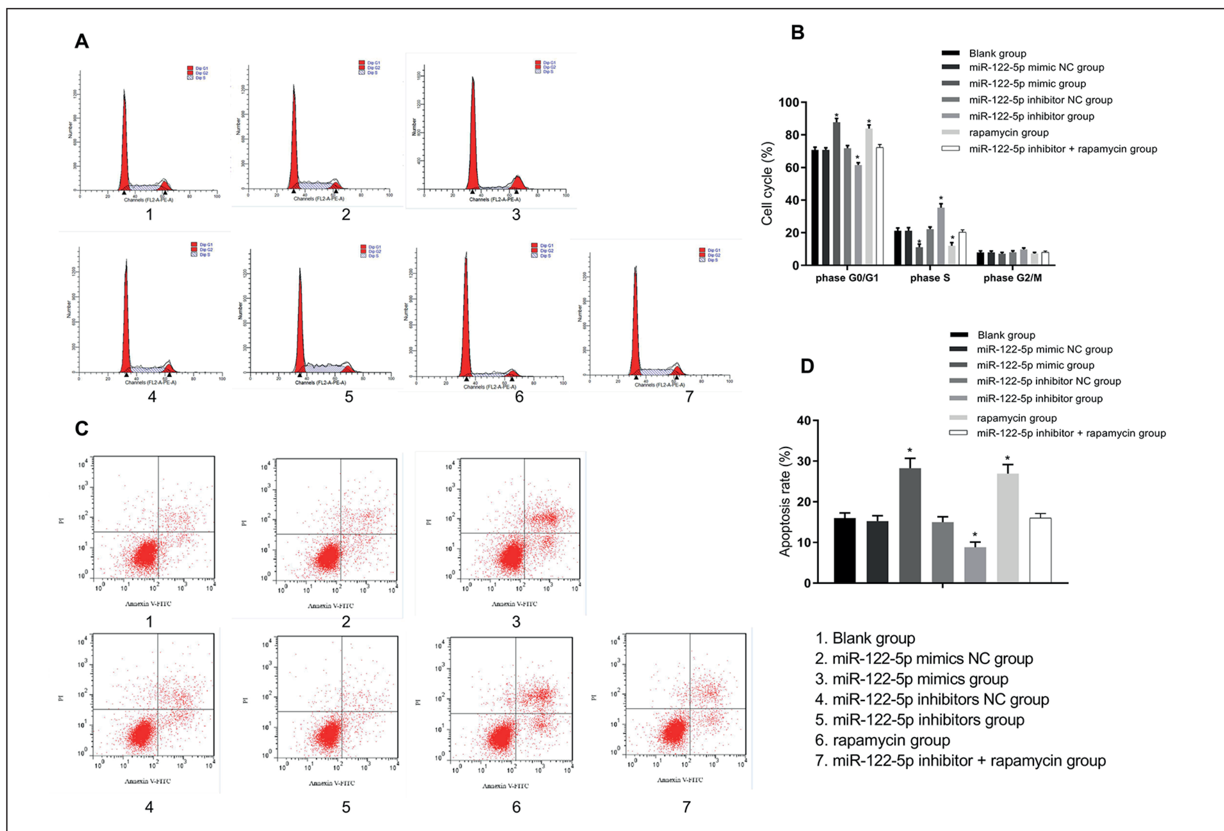

Figure 6. Comparison of cell cycle and apoptosis rate after transfection in each group. Note: **A**, Cell cycle of each group; **B**, Cycle cycle statistical analysis of each group; **C**, Cell apoptosis of each group; **D**, Cell apoptosis statistical analysis of each group;*compared with Blank group, $p < 0.05$.

pathways related to targeted therapy using molecular marker involved in tumor development are being studied, which can be used to predict the possibility of tumor metastasis and the response to treatment. These pathways may be potential targets for the treatment of osteosarcoma.

The molecular mechanism of osteosarcoma is characterized by its complex karyotype as well as changes in various genes and related signaling pathways[32]. It has a wide range of unstable genomes and complex interacting protein and signal pathways. The change of oncogene and tumor suppressor gene is always the focus of research[33-35]. Therefore, it is an effective way to detect the changes of oncogenes and tumor suppressor genes or their products. TP53 has been reported to be involved in the pathogenesis of osteosarcoma[36]. Inactivation or loss of TP53 may stimulate cell resistance to DNA damage and loss of control of cell growth, leading to extensive proliferation, transformation and avoidance of the toxic effects of DNA damage agents[37]. On the basis of the analysis of online analysis software, it was discovered that there was a specific binding region between TP53 gene and miR-122-5p sequence. TP53 was identified to be the target gene of miR-122-5p in our study, which promoted our subsequent cell experiments. Significantly, it has also revealed that microRNA is a kind of small non-coding RNA with 22 nucleotides. It is quite important to regulate several pathophysiological processes, and it has been found that microRNA is involved in tumorigenesis, tumor progression and metastasis. It has been reported that the expression of miR-144 was decreased significantly, while the levels of Rock1 and Rock2 increased in osteosarcoma cell line and osteosarcoma tissue[38]. Further study on the inhibition of cell proliferation, migration, invasion and metastasis *in vivo* and *in vitro* caused by the recovered expression of miR-144 suggested that miR-144 might be an effective tumor inhibitor by down regulating the expression of Rock1 and Rock1 in osteosarcoma, suggesting that the re-upregulation of the ex-

pression may provide an available access to the treatment of osteosarcoma. Similarly, miR-326 might be a potential predictor of cell growth and apoptosis by targeting Bcl-2 in osteosarcoma, suggesting that potential microRNA can be used as diagnostic and therapeutic target for osteosarcoma therapy[39]. Our study was thus carried out on the basis of previous studies and in accordance with the biological screening of target gene of miR-122-5p, as well as osteosarcoma tissue detection *in vivo* and cell transfection *in vitro*.

In accordance with the results of our tissue detection and cell experiment, it was found that the expression level of miR-122-5p in osteosarcoma was significantly lower than that in normal tissues, TP53, PTEN, Bim and Bax expression levels were significantly decreased, but with a highly upregulated expression levels of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR and Bcl-2. The above in vivo detection results provide us a hypothesis that increased expression level of miR-122-5p may target the expression of TP53 positively to suppress pathway activation, thus participating in the suppressing of osteosarcoma progression. In the next step, our research focused on the performance of *in vitro* verification. First, to select the suitable cell line, the expression of TP53 was detected by PCR, which had the lowest expression in osteosarcoma cell line U-2OS that was thus used for subsequent cell experiments. With the observation of the targeting relationship between TP53 and miR-122-5p, our study further emphasized on the detection of miR-122-5p, TP53 and PI3K-Akt-mTOR signaling pathway related indexes, as well as cell biological characteristics of osteosarcoma cells. Consequently, upregulation of miR-122-5p significantly increased the expression of miR-122-5p, and its upregulation and suppression of the pathway activation by using rapamycin increased the expression of TP53, PTEN, Bim, and Bax, while decreased the expression of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, and Bcl-2, accompanied by significantly cell arrest in G0/G1 phase, increased cell apoptosis and markedly inhibited cell proliferation. The opposite trends were found when downregulating miR-122-5p. The above results revealed that upregulation of miR-122-5p or inhibition of the activation of the studied pathway exhibited significant positive effect on suppressing cell proliferation and cell cycle while promoting cell apoptosis, which might be related to the targeted upregulation of TP53. On the other hand, suppression of miR-122-5p showed a negative role, promoting the activation of the proposed pathway and inhibition of TP53 gene expression. Collectively, our study provides an insight that in the development of osteosarcoma, both miR-122-5p and TP53 may play the role of tumor suppression, associated with the inhibition of tumor-promoting PI3K-Akt-mTOR signaling pathway, so as to have a synergetic enhanced tumor-suppressing role in the progression of osteosarcoma. Meanwhile and importantly, inhibition of miR-122-5p inhibitor associated with the inhibition of the proposed pathway inhibited no significant difference compared with the control, suggesting that the positive role of inhibiting the activation of this pathway was reversed by the inhibited expression ofmiR-122-5p, which in turn supports our speculation that upregulation of miR-122-5p may exert a beneficial role in preventing osteosarcoma.

The starting point and significance of this study lie in that a deeper understanding of genetics, molecular basis and tumorigenesis, in addition to identifying biomarkers associated with adverse outcomes and adverse reactions to conventional therapies, can also pave the way for the discovery of potential therapeutic agents and the development of new molecular drugs. In addition, the future challenge is to identify and utilize the screened molecular therapeutic targets for multi-target combined therapy or customized personalized therapy or to improve drug delivery. The proposed updated approach can help us explore the full potential of targeted therapy in the diagnosis and treatment of human malignancies.

Conclusions

To sum up, our study for the first time clarifies that upregulation of miR-122-5p may inhibit the proliferation and promote the apoptosis of osteosarcoma cells by inhibiting the activation of PI3K-Akt-mTOR signaling pathway, which may be related to target the up-regulation of TP53 expression. Significantly, this study for the first time explores the role of miR-122-5p targeting TP53 and mediating PI3K-Akt-mTOR signaling pathway in the development of osteosarcoma. We carried out a clinical experiment and cell tests on the basis of potential biological prediction with a comprehensive grouping to verify our hypothesis reasonably. Our research discoveries

the effect and molecular mechanism of the above axis for osteosarcoma, providing new targets and pathway for this tumor with vital theoretical significance and application value. This study lays a theoretical foundation for further understanding osteosarcoma cells and finding new molecules for targeted therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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