MiR-100 up-regulation enhanced cell autophagy and apoptosis induced by cisplatin in osteosarcoma by targeting mTOR

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Abstract. – OBJECTIVE: Mammalian target of rapamycin (mTOR) can negatively regulate cell autophagy, while its expression and activity are associated with the pathogenesis of osteosarcoma. MicroRNA 100 (MiR-100) down-regulation is associated with the pathogenesis and chemo-sensitivity of osteosarcoma. Bioinformatics analysis revealed the targeted relationship between miR-100 and the 3'-UTR of mTOR. We investigate the role of miR-100 in affecting mTOR expression, osteosarcoma cell autophagy, and sensitivity to cisplatin.

PATIENTS AND METHODS: MiR-100, mTOR, and Beclin-1 expressions in osteosarcoma tissue and normal control were compared. The relationship between miR-100 and mTOR was verified by dual luciferase assay. MiR-100, mTOR, and Beclin-1 levels in MG-63 cells and MG-63/DDP cells were tested. Cell apoptosis was determined by using flow cytometry. Cell malignancy was evaluated by colony formation assay.

RESULTS: MiR-100 and Beclin-1 significantly declined, while mTOR significantly increased in osteosarcoma tissue compared with that of normal tissue (p<0.05). MiR-100 targeting significantly inhibited mTOR expression compared to that of untreated (p<0.05). MiR-100 expression was down-regulated and mTOR level was elevated in MG-63/DDP cells compared with MG-63 cells (p<0.05). MG-63/DDP cells exhibited reduced cell autophagy and apoptosis, and enhanced colony formation induced by DDP. MiR-100 mimic and/or small interfere mTOR (si-mTOR) significantly promoted Beclin-1 expression, cell autophagy, and cell apoptosis, while attenuated colony formation.

CONCLUSIONS: MiR-100 declined, while mTOR up-regulated in osteosarcoma tissue. MiR-100 up-regulation enhanced cell autophagy and apoptosis induced by cisplatin via targeted inhibiting of mTOR.

Key Words: miR-100, mTOR, Apoptosis, Autophagy, Osteosarcoma.

Introduction

Osteosarcoma is a common primary malignant bone tumor originated from mesenchymal tissue that accounts for more than 50% of all malignant bone tumors.1 Reinforcement and neoadjuvant chemotherapies markedly improve the survival and prognosis of osteosarcoma. However, multiple patients appear resistance to chemotherapy, which decreases the therapeutic efficacy.2 Autophagy is the process of autophagy-lysosome forming and degrading denatured proteins and damaged organelles, which plays an important role in the renewal of intracellular organelles, metabolic energy balance, homeostasis, and genomic stability.3 It was showed that autophagy is closely related to tumor cell survival and death during chemotherapy, suggesting that autophagy can affect the sensitivity of tumor cells to chemotherapy drugs.3,4 Phosphatidylinositol-3-kinase/protein kinase B/mammalian target of Rapamycin (PI3K/Akt/mTOR) signaling is mostly investigated pathway-regulating autophagy. mTOR is an important target effector of PI3K/Akt/mTOR signaling. It plays an inhibitory role in autophagy by suppressing the formation of ULK complex during induction and initialization phase, blocked endoplasmic reticulum membrane falling off to form autophagosome membrane.5,6 The expression and function of mTOR are closely associated with tumor occurrence, progression, and chemo-resistance. It was revealed that mTOR up-regulation plays a critical role in osteosarcoma and is correlated with prognosis and chemo-resistance.7,8 MicroRNA is a type of endogenous small non-coding RNA at 22-25 nucleotides. It can degrade or inhibit target mRNA translation to regulate target gene expression through complementary binding with the 3'-UTR.9,10 It was demonstrated that miR-100 is associated with osteosarcoma pathogenesis.11
progress\textsuperscript{12}, and chemo-sensitivity\textsuperscript{13}, suggesting that miR-100 may play a tumor suppressor gene role in osteosarcoma. Microrna.org online prediction revealed the binding site between miR-100 and the 3'-UTR of mTOR mRNA. This study intends to investigate the role of miR-100 in affecting mTOR expression, osteosarcoma cell autophagy, and sensitivity to cisplatin.

**Patients and Methods**

**Main Reagents and Materials**

Human osteosarcoma cell MG-63 was obtained from Shandong University. (Shandong, China). Dulbecco’s Modified Eagle Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). SPLIT RNA Extraction Kit was purchased from Lexogen (Vienna, Austria). Quantitect SYBR Green Real Time-PCR (RT-PCR) Kit was derived from Qiagen (Hilden, Germany). PCR primers were synthesized by Generay (Shanghai, China). MiR-100 mimic, miR-100 inhibitor, and miR-NC were provided by Shanghai GenePharma Co. Ltd. (Shanghai, China). Si-mTOR and si-NC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-mTOR and β-actin antibodies were obtained from GeneTex Inc. (Irvine, CA, USA). Mouse anti-Becline-1 primary and horseradish peroxidase (HRP) labeled secondary antibodies were purchased from Abcam Biotechnology (Cambridge, MA, USA). Radiomunoprecipitation assay (RIPA) was purchased from Beyotime Biotechnology (Shanghai, China). Apoptosis detection kit was derived from BD Biosciences (Franklin Lakes, NJ, USA). ViaFect™ Transfection Reagent was applied to co-transfect 1 μg pGL3-mTOR-wt or pGL3-mTOR-mut with 50 nm/l miR-100 mimic or miR-100 inhibitor to HEK293T cells. Dual luciferase activity was tested after 48 h.

**Clinical Sample**

A total of 25 osteosarcoma patients who received surgery between Nov 2016 and Aug 2017 were enrolled in this study from The Second Affiliated Hospital of Dalian Medical University (Liaoning, China). There were 14 males and 11 females with average age at 19.6 ± 2.9 years old. No statistical significance was observed on age or gender between two groups. This study was approved by Ethics Committee of The Second Affiliated Hospital of Dalian Medical University (Liaoning, China). All of the enrolled patients had signed informed consent.

**MG-63/DDP Cell Model Establishment and Resistance Index (RI) Calculation**

MG-63 cells were cultured in Dulbecco’s modified eagle medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% streptomycin and maintained at 37°C and 5% CO\textsubscript{2}. The cells were treated by 0.1 mg/l DDP for 24 h when the fusion reached 60-70%. After cell recovers by passage, DDP concentration was gradually increased to 0.25, 0.5, 1.0, and 2.0 mg/l. Finally, the cells kept stable in 2.0 mg/l DDP were identified as MG-63/DDP. MG-63 and MG-63/DDP cells were treated by DDP at 0, 0.025, 0.25, 0.5, 1.0, 2.5, 5, and 10, and 20 mg/l for 48 h. Cell viability was determined by CCK-8 kit at 450 nm. Inhibitory rate = (1-A\textsubscript{450} at experimental group)/A\textsubscript{450} at control × 100%. IC\textsubscript{50} was calculated by Excel software. RI = IC\textsubscript{50} at MG-63/DDP/IC\textsubscript{50} at MG-63.

**Dual Luciferase Assay**

The full-length fragment of mTOR 3'-UTR was connected to pGL3 luciferase reporter vector to form pGL3-mTOR-wt. The mutation of mTOR 3'-UTR was used to construct pGL3-mTOR-mut. ViaFect™ Transfection Reagent was applied to co-transfect 1 μg pGL3-mTOR-wt or pGL3-mTOR-mut with 50 nm/l miR-100 mimic or miR-100 inhibitor to HEK293T cells. Dual luciferase activity was tested after 48 h.

**MG-63/DDP Cell Transfection**

MG-63/DDP cells were divided into five groups, including miR-NC group, miR-100 mimic group, si-NC group, si-mTOR group, and miR-100 mimic + si-mTOR group. The cells were treated by 0.4 mg/l DDP for 24 h.

**qRT-PCR**

Total RNA was extracted using SPLIT RNA Extraction Kit and detected using QuantiTect SYBR Green RT-PCR Kit for one-step qRT-PCR. The reaction system contained 10.0 μl 2× QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μl primer at 0.5 μm/l, 2 μg Template RNA, 0.5 μl QuantiTect RT Mix, and ddH\textsubscript{2}O. The primer sequences were as follows, miR-100F: 5'-ACACTCCAGCTGG-
GAACCGTGATCCGAAC-3′, miR-100 P: 5′-TGGTGTCGTGGAGTCG-3′; U6 P: 5′-ATTG-GAACGATACAGAAGATT-3′, U6 R: 5′-GGA-ACGCTTACGGAATTG-3′; mTOR P: 5′-GCA-GATTGGCCAACATCTTCGG-3′, mTOR R: 5′-CAGCGGTAAAAGTGTCCCCTG-3′; Beclin-1 P: 5′-GGTGTCTCTCGAGATTCATC-3′, Beclin-1 R: 5′-GGGCATTACAGAGAAGATT-3′, β-actin P: 5′-TGTCACGCACGATTTCC-3′.

**Western Blot**

Total protein was extracted by RIPA for quantification. A total of 40 μg protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was incubated in primary antibody at 4°C for 12 h (mTOR, Beclin-1, and β-actin at 1:200, 1:100, and 1:500, respectively). Then, the membrane was incubated in secondary antibody (1:8000) for 60 min after washed by PBST for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

**Flow Cytometry**

Cells were collected and incubated in 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) avoiding light. The cell apoptosis was tested on flow cytometry.

**Colony Formation Assay**

The cells were seeded in 10 cm dish at 100/dish. After cultured for 14-21 weeks, the cells were fixed by paraformaldehyde and stained by Giemsa. Next, the cells were observed under the microscope to record the clone number. Cloning efficiency = clone number/seed number×100%.

**Statistical Analysis**

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation (SD). The Student’s t-test was used to compare the differences between two groups. The Tukey’s post-hoc test was used to validate the ANOVA for comparing measurement data among groups. p<0.05 was considered as statistical significance.

**Results**

**MiR-100 and Autophagy Reduced, while mTOR Elevated in Osteosarcoma Tissue**

Quantitative RT-PCR (qRT-PCR) showed that miR-100 expression significantly decreased (Figure 1A), while mTOR mRNA significantly elevated (Figure 1B) in osteosarcoma tissue compared with normal control. Western blot revealed that Beclin-1 protein was lower, whereas mTOR protein was higher in osteosarcoma tissue than the normal tissue (Figure 1C).

**MiR-100 Targeted Inhibited mTOR Expression**

Bioinformatics analysis demonstrated the complementary binding site between miR-100 and the 3'-UTR of mTOR mRNA (Figure 2A). Dual luciferase assay showed that miR-100 mimic significantly declined relative luciferase activity, while miR-100 inhibitor significantly enhanced luciferase activity in HEK293T cells (Figure 2B), indicating the regulatory relationship between miR-100 and mTOR mRNA.

**Figure 1.** MiR-100 declined, while mTOR upregulated in osteosarcoma tissue. (A) qRT-PCR detection of miR-100 expression. (B) qRT-PCR detection of mTOR mRNA expression. (C) Western Blot detection of protein expression.
MiR-100 Downregulation and mTOR Enhancement Were Related to Autophagy Reduction in MG-63/DDP Cells

IC₅₀ was 0.87 mg/L in MG-63 cells, while it was 15.26 mg/L in MG-63/DDP cells. RI was 17.54, suggesting the successful establishment of DDP resistant MG-63 cells. Flow cytometry exhibited that MG-63/DDP cell apoptosis was markedly lower than that of MG-63 cells after treated by 0.87 mg/L DDP (Figure 3A). In addition, MG-63/DDP cells showed stronger malignancy potential than MG-63 cells treated by 0.87 mg/l DDP (Figure 3B). qRT-PCR demonstrated that miR-100 and Beclin-1 mRNA significantly decreased, while mTOR mRNA markedly elevated in MG-63/DDP cells compared with MG-63 cells (Figure 3C). Western blot revealed the similar result with PCR (Figure 3D). It indicated that DDP markedly induced MG-63 cell autophagy, while MG-63/DDP cells showed obviously lower autophagy sensitivity induced by DDP. MiR-100 reduction and mTOR upregulation may play critical roles in attenuating osteosarcoma cell autophagy.

Figure 2. MiR-100 targeted inhibited mTOR expression. (A) The binding site between miR-100 the 3'-UTR of mTOR mRNA. (B) Dual luciferase assay. *p<0.05, compared with miR-NC.

Figure 3. MiR-100 downregulation and mTOR enhancement were related to autophagy reduction in MG-63/DDP cells. (A) Flow cytometry detection of cell apoptosis. (B) Colony formation assay detection of cell malignancy. (C) qRT-PCR detection of gene expressions. (D) Western blot detection of protein expression. *p<0.05, compared with MG-63 cells.
MiR-100 promotes osteosarcoma autophagy

**MiR-100 Suppressed mTOR Expression and Facilitated Cell Autophagy and Apoptosis Induced By DDP**

MiR-100 mimic and/or si-mTOR significantly reduced mTOR expression (Figure 4A-B), promoted Beclin-1 expression (Figure 4A-B), attenuated colony formation (Figure 4C), and enhanced cell apoptosis (Figure 4D).

**Discussion**

mTOR is an important effector of PI3K/Akt/mTOR signaling that can regulate cell autophagy. A variety of factors participate in triggering cell autophagy. The activation of ULK complex composed by Atg13, FIP200, and ULK1/2 plays a key role in inducing and triggering autophagy. mTOR can block the formation of ULK-A

- **Figure 4.** MiR-100 suppressed mTOR expression and facilitated cell autophagy and apoptosis induced by DDP. (A) qRT-PCR detection of gene expression. (B) Western blot detection of protein expression. (C) Colony formation assay. (D) Flow cytometry detection of cell apoptosis. *p* < 0.05, compared with miR-NC. *p* < 0.05, compared with miR-NC. *p* < 0.05 compared with miR-NC. *p* < 0.05 compared with si-NC.

MiR-100 is an important role in the pathogenesis of osteosarcoma and is closely associated with prognosis and chemoresistance. It was revealed that miR-100 down-regulation is related to the pathogenesis, progress, and chemosensitivity of osteosarcoma, suggesting that miR-100 may play a tumor suppressor role in osteosarcoma. Miicrorna.org online prediction revealed the binding site between miR-100 and the 3'-UTR of mTOR mRNA. This study intends to investigate the role of miR-100 in affecting mTOR expression, osteosarcoma cell autophagy, and sensitivity to cisplatin.

Our results exhibited that miR-100 expression significantly decreased, while mTOR mRNA significantly elevated in osteosarcoma tissue compared with normal control. Western blot revealed that Beclin-1 protein was lower, whereas mTOR protein was higher in osteosarcoma tissue than the normal tissue. Bi et al. reported that miR-100 decreased in the tumor tissue of osteosarcoma. Huang et al. discovered that miR-100 significantly declined in osteosarcoma compared with adjacent tissue. Liu et al. also demonstrated miR-100 markedly reduced in tumor tissue. In this study, miR-100 expression was significantly lower in osteosarcoma tissue than the normal bone tissue, which was in accordance with Bi et al., Huang et al., and Liu et al. Zhou et al. revealed that
mTOR positive rate significantly increased in osteosarcoma tissue and was closely related to clinical staging, metastasis, survival, and prognosis. This study observed mTOR abnormal expression in osteosarcoma tissue, which was similar with Zhou et al8 findings. Zhang et al19 showed that Beclin-1 level and autophagy markedly declined in osteosarcoma tissue compared with normal bone. This study showed Beclin-1 downregulation in tumor tissue, which was in accordance with Zhang et al19. MiR-100 significantly decreased, while mTOR mRNA significantly elevated in MG-63/DDP cells compared with MG-63 cells. It demonstrated that miR-100 down-regulation may play a role in elevating mTOR and inhibiting cell autophagy and apoptosis; also, mTOR was significantly enhanced in sorafenib resistant liver cancer cell line, whereas cell autophagy and apoptosis were suppressed4. Ning et al20 demonstrated that miR-100 down-regulation may play a role in elevating mTOR and inhibiting cell autophagy and apoptosis; also, mTOR was significantly enhanced in sorafenib resistant liver cancer cell line, whereas cell autophagy and apoptosis were suppressed4. Ning et al20 showed that PTEN depletion induced PI3K/Akt/mTOR activation and autophagy inhibition significantly enhanced breast cancer cell resistance to trastuzumab. In this study, mTOR level markedly up-regulated, while autophagy was attenuated in drug-resistant cell line, which was similar with He et al4 and Ning et al20 results. Further analysis revealed that miR-100 mimic and/or si-mTOR markedly reduced mTOR expression, attenuated colony formation, and enhanced cell apoptosis and autophagy induced by DDP. Bi et al11 exhibited that miR-100 overexpression inhibited osteosarcoma cell proliferation in vitro and tumorigenesis in vivo through targeted suppressing FGFR3 expression, whereas miR-100 inhibition presented the opposite results. Huang et al18 showed that miR-100 elevation significantly attenuated osteosarcoma cell line Saos-2 and MG63 proliferation in vitro. Liu et al23 demonstrated that miR-100 up-regulation inhibited osteosarcoma cell U2OS and MG-63 proliferation, motility, and migration, and enhanced sensitivity to cisplatin via targeting IGFIR. In this study, miR-100 up-regulation markedly attenuated osteosarcoma malignancy and apoptosis resistance to chemotherapy, which was in accordance with Bi et al11, Huang et al18, and Liu et al23. Ge et al24 presented that miR-100 expression and autophagy were correlated with liver cancer pathogenesis. MiR-100 over-expression significantly promoted liver cancer cell autophagy and apoptosis, and suppressed its proliferation and tumorigenesis in vivo. This work revealed that miR-100 down-regulation plays a role in restraining autophagy, which was similar with Ge et al24. Xie et al25 showed that Beclin-1 expression, autophagy, and chemo-sensitivity to cisplatin were enhanced, while proliferation was suppressed in MG-63 cells treated by cisplatin. He et al4 reported that inhibition of mTOR markedly facilitated tumor cell autophagy and apoptosis induced by chemotherapy, and reduced chemo-resistance. This study suggested that miR-100 suppression plays a role in elevating mTOR expression, inhibiting cell autophagy and apoptosis induced by cisplatin, and enhancing cisplatin resistance.

**Conclusions**

We showed that miR-100 reduced, while mTOR elevated in osteosarcoma tissue. MiR-100 upregulation enhanced cell autophagy and apoptosis induced by cisplatin by inhibiting mTOR.

**Conflict of Interest**

The Authors declare that they have no conflict of interest.

**References**


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