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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, and Beclin-1 expressions in osteosarco[illegible]sue and normal control were compared. T[illegible]lationship between miR-100 and mTOR was [illegible]ified by dual luciferase assay. MiR-100, mT[illegible] and Beclin-1 levels in MG-63 c[illegible]nd MG-6[illegible] DDP cells were tested. Cell ap[illegible]s deter[illegible]mined by using flow cytome[illegible]Cell [illegible]gnancy was evaluated by colony f[illegible]ation as[illegible].

RESULTS: MiR-100 a[illegible]clin-1[illegible]ly declined, while mT[illegible]R s[illegible]c[illegible]increa[illegible]d in osteosarcoma ti[illegible]com[illegible]with that of normal tissue (p<[illegible]). MiR-10[illegible]eting significantly inhibit[illegible]OR expressi[illegible]mpared to that of unt[illegible]e[illegible].05). MiR-1[illegible] expression was do[illegible]regula[illegible]nd mTOR level was elevated i[illegible]MG-63/DDP[illegible] compared with MG-63 c[illegible](p<0.05). MG-6[illegible]R cells exhibited red[illegible]d cell [illegible]utophagy a[illegible] apoptosis, and enha[illegible]d colo[illegible]ormation induced by DDP. Mi[illegible]mimi[illegible]d/or s[illegible]all interfere mTOR (si-mTO[illegible]antly p[illegible]oted Beclin-1 expression, ce[illegible]phag[illegible]d cell apoptosis, while [illegible]ated[illegible]w[illegible]iation.

[illegible]CLUSI[illegible] MiR-100 declined, while mT[illegible] up-regu[illegible]ed in osteosarcoma tissue. Mi[illegible]-regulation enhanced cell autophagy a[illegible]nduced by cisplatin via targeted [illegible]biting of mTOR.

Keywords:
[illegible]00, mTOR, Apoptosis, Autophagy, Osteosarcoma.

Introductio[illegible]

Osteosa[illegible]coma is [illegible]mon primary malignant bone t[illegible] originated [illegible] mesenchymal tissue th[illegible]ts for more tha[illegible]0% of all malignant [illegible] tumors[1]. Reinforcement and neoadjuvant [illegible]motherapies [illegible]rkedly improve the survival [illegible]prognosis of [illegible]osarcoma. However, multiple p[illegible]s appear [illegible]tance to chemotherapy, which dec[illegible]apeutic efficacy[2]. Autophagy is the proc[illegible] autophagy-lysosome forming and [illegible]egrading denatured proteins and damaged or[illegible] which plays an important role in the re[illegible]wa[illegible] 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 chemoresistance. 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],

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progress[12], and chemo-sensitivity[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 GenePh Co. Ltd. (Shanghai, China). Si-mTOR an were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Mouse anti-mTOR β-actin antibodies were obtained from Gene Inc. (Irvine, CA, USA). Mous i-Beclin primary and horseradish pero P) labeled secondary antibodies purc d from Abcam Biotechnology (C bridge, USA). Radioimmunoprecipitation (R chased from Beyotim iotec (Shanghai, China). Apoptosis ction kit v ived from BD Biosciences lin Lakes, N A). ViaFect™ Transfe R dual lucife se reporter gene pla pGL3, a l luciferase activity detection were provided mega (Madison, MI, US Cisplatin (DDP) w purchased from Qilu macy Ltd. (Jinan, China). CCK-8 kit wa ined Dojin (Tokyo, Japan).

Clinical Sample

Total of arcoma patients who received gery bet Nov 2016 and Aug 2017 were enr in this study from The Second Affiliated H ian Medical University (Liaoning, na). There were 14 males and 11 females with n age at 18.7 ± 3.4 years old. Another 17 cases o al bone tissue obtained from the patients rece ed amputations because of severe trauma were selected as control, including 9 males and 8 females with average age at 19.6 ± 2.9 years old. No statistical significance was observed on age or gender between two groups. This st approved by Ethics Committee of Affiliated Hospital of Dalian Medi University (Liaoning, China). All of the enr patients had signed informed consent.

MG-63/DDP Cell Mod stablishm and Resistance Inde RI) Ca ulation

MG-63 cells were ured i lbecco's modified eagle mediu EM medium taining 10% fetal b se BS) an strep-tomycin and m ained at 3 d CO_2. The cells were t y 0.1 mg/l E 24 h when the fusion h 70%. After l recovers by passage, DDP conc on was gradually increased t 0.25, 0.5, 1.0, 0 mg/l. Finally, the cel table in 2.0 mg DDP were identified a G-63/DDP. MG-63 and MG-63/DDP cells e treated by at 0, 0.025, 0.25, 0.5, 1.0, 2.5, and 20 mg r 48 h. Cell viability was detected by CC kit at 450 nm. Inhibitory rate = (A imental group)/A450 at control × 100%. IC_{50} was calculated by Excel software. RI = IC_{50} at MG-63/DDP/IC_{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_2O. The primer sequences were as follows. miR-100P_F: 5'-ACACTCCAGCTGG-

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GAACCCGTAGATCCGAAC-3′, miR-100P$_R$: 5′- TGGTGTCGTGGAGTCG-3′; U6P$_F$: 5′-ATTGGAACGATACAGAGAAGATT-3′, U6P$_R$:5′-GGAACGCTTCACGAATTTG-3′; mTORP$_F$: 5′-GCAGATTTGCCAACTATCTTCGG-3′, mTORP$_R$: 5′-CAGCGGTAAAAGTGTCCCCTG-3′; Beclin-1P$_F$: 5′-GGTGTCTCTCGCAGATTCATC-3′, Beclin-1P$_R$: 5′-TCAGTCTTCGGCTGAGGTTCT-3′;β-actinP$_F$:5′-GAACCCTAAGGCCAAC-3′, β-actinP$_R$: 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 [illegible] avoiding light. The cell apoptosis was tested by flow cytometry.

Colony Formation Assa

The cells were seeded [illegible]0 cm [illegible]100/dish. After cultured for [illegible] were fixed by paraf[illegible]ide[illegible] stained by Giemsa. Next, the [illegible]s were obs[illegible] under the microscope to r[illegible]e clone nu[illegible]. Cloning efficiency = cl[illegible]nu[illegible]eed number 100%.

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, Il[illegible]). The measurement data were depict[illegible] ± standard deviation (SD). The S[illegible]nt's t-test was used to compare the diff[illegible]s between two groups. The Tukey's post-hoc [illegible]as used to validate the ANOVA fo[illegible]ompari[illegible]easurement data among grou[illegible] $p<0.05$ wa[illegible]dered as statistical sig[illegible]ance.

Result[illegible]

MiR-100 [illegible]phagy Reduced, while mTOR E[illegible]vated [illegible]teosarcoma Tissue

Qu[illegible]tative RT-PC[illegible]T-PCR) showed that mi[illegible]xpression signi[illegible]antly decreased (Fi[illegible]1A), while mTOR mRNA significantly ele[illegible]d (Figure 1B[illegible] osteosarcoma tissue compa[illegible]with norma[illegible]ntrol. Western blot revealed t[illegible]eclin-1 pr[illegible]n was lower, whereas mTOR prot[illegible]r in osteosarcoma tissue than the norm[illegible]ue (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.

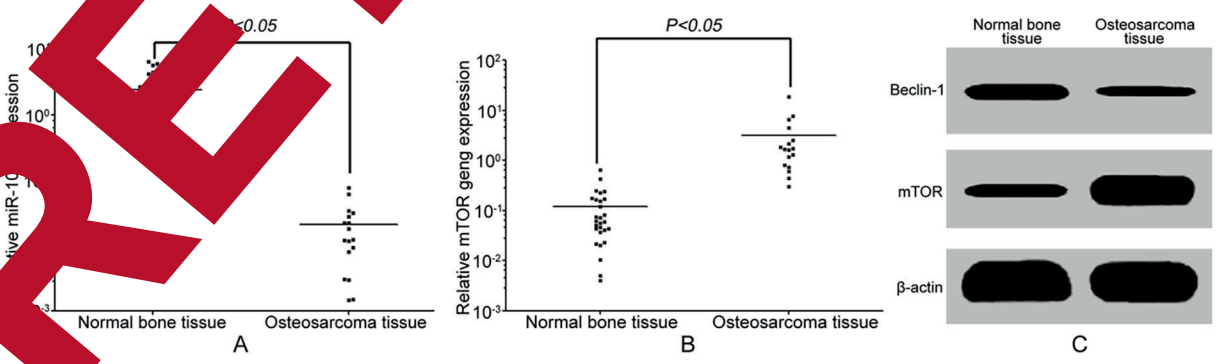

Figure 1. MiR-100 declined, while mTOR upregulated in osteosarcoma tissue. ***(A)*** qRT-PCR detection of miR-100 expression. ***(B)*** qRT-PCR detection of mTOR mRNA expression. ***(C)*** Western Blot detection of protein expression.

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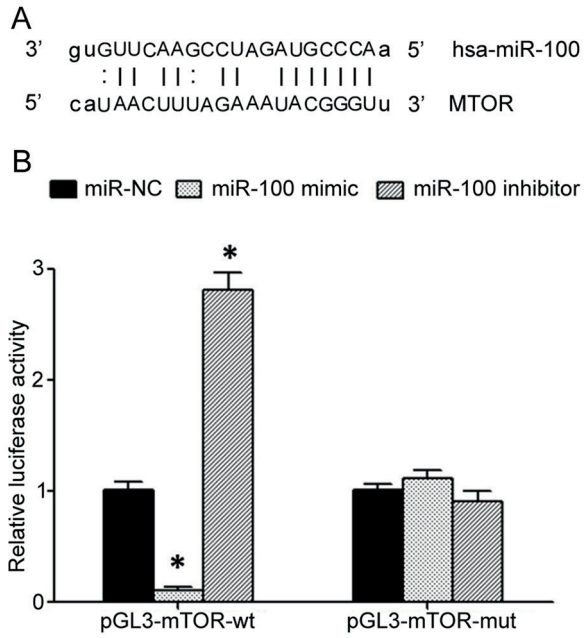

Figure 2. MiR-100 targeted inhibited mTOR expression. ***(A)*** The binding site between miR-100 the 3'-UTR of mTOR mRNA. ***(B)*** Dual luciferase assay. $^{*}p<0.05$, compared with miR-NC.

MiR-100 Downregulation and mTOR Enhancement Were Related to Autophagy Reduction in MG-63/DDP Cells

IC_{50} was 0.87 mg/L in MG-63 ce... was 15.26 mg/L in MG-63/DDP ...s. RI was 17.54, suggesting the successful ...lishment of DDP resistant MG-63 cells. Flow c... ...try exhibited that MG-63/DDP cell ...ptosis ...arkedly lower than that of MG... ...ells after t... 0.87 mg/L DDP (Figure ...). In addition, M... DDP cells showed st... ...er mal... ...ncy potential than MG-63 cells tre... ...mg/l D... (Figure 3B). qRT-P... de... ...ted th... ...R-100 and Beclin-1 ...RNA sig... ...th... ...ecreased, while mTO... ...A markedly ...ed in MG-63/DDP c... ...d with MG-... cells (Figure 3C). Weste... blot r... ...d the similar result with PCR (Fi... ...re 3D). It i... ...ed that DDP markedly ...d MG-63 cell... ...ophagy, while MG-... ...DP cells showed obviously lower autophagy ...sitivity indu... ...by DDP. MiR-100 reduction ...mTOR upreg... ...tion may play critical roles in a... ...ting oste... ...coma cell autophagy.

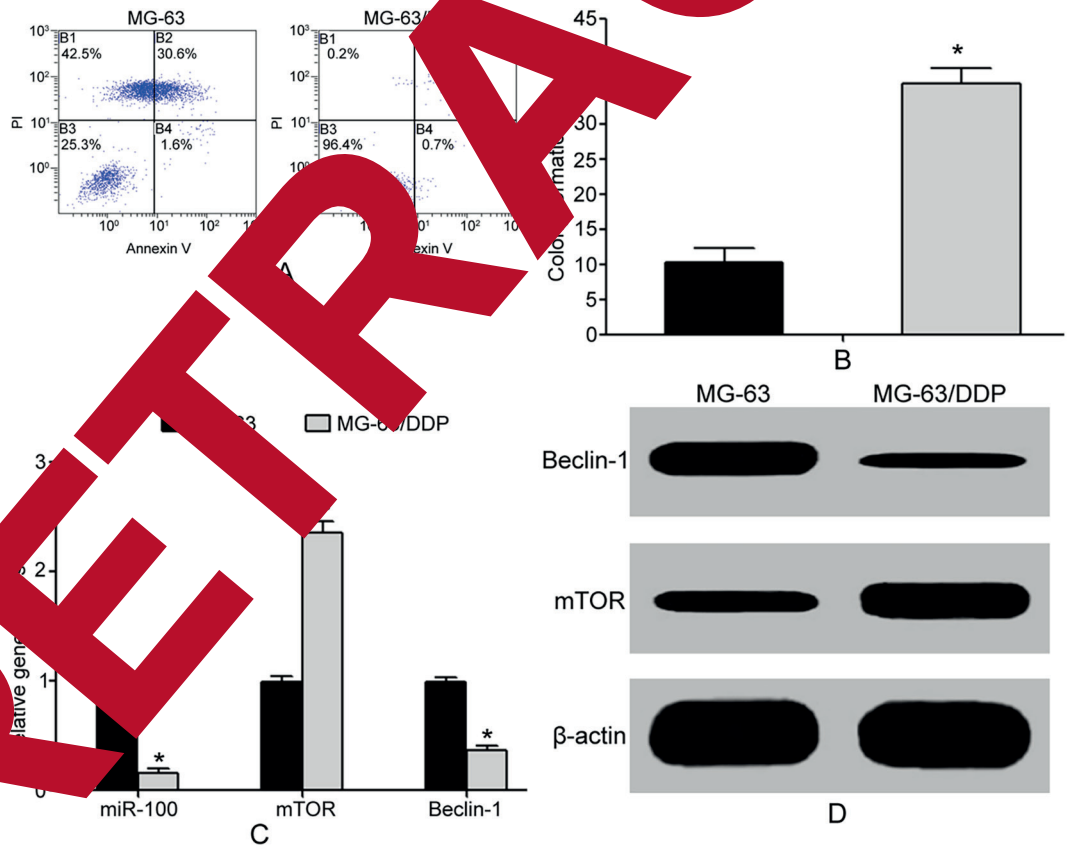

Figure 3. MiR-100 downregulation and mTOR enhancement were related to autophagy reduction in MG-63/DDP cells. ***(A)*** Flow cytometry detection of cell apoptosis. ***(B)*** Colony formation assay detection of cell malignancy. ***(C)*** qRT-PCR detection of gene expressions. ***(D)*** Western blot detection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

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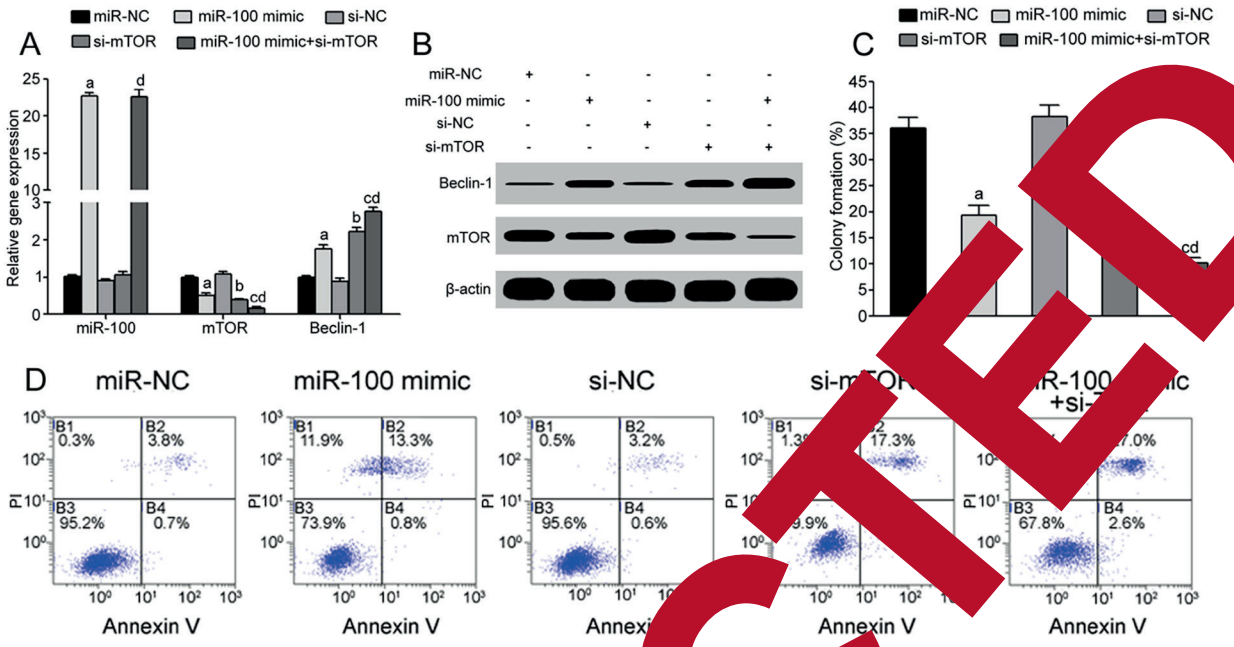

Figure 4. MiR-100 suppressed mTOR expression and facilitated [illegible]utophagy and [illegible]osis induced by DDP. ***(A)*** qRT-PCR detection of gene expression. ***(B)*** Western blot detection of prote[illegible]pression. ***(C)*** C[illegible]y formation assay. ***(D)*** Flow cytometry detection of cell apoptosis. [a]$p<0.05$, compared with miR-NC; [illegible]05, compar[illegible]th miR-NC. [c]$p<0.05$ compared with miR-NC. [d]$p<0.05$ compared with si-NC.

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MiR-100 Suppressed mTOR Expressi[illegible] and Facilitated Cell Autophagy and Apoptosis Induced By DDP

MiR-100 mimic and/or si-mT[illegible]ignificant[illegible] reduced mTOR expression (Fi[illegible] promoted Beclin-1 expression (Fig[illegible]4A-B)[illegible]enuated colony formation (Figure[illegible] and e[illegible]cell apoptosis (Figure 4D).

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mTOR i[illegible]an importa[illegible]factor of PI3K/Akt/mTOR si[illegible]ng that can [illegible]te cell autophagy[14]. A[illegible]iety of factors partic[illegible]ate in triggering cell [illegible]phagy[illegible] activation of ULK complex co[illegible]d by[illegible]13, FI[illegible]200, and ULK1/2 plays a key [illegible]nducin[illegible]d triggering autopha-[illegible] mT[illegible]n blo[illegible]he formation of ULK-A-[illegible]IP200[illegible]omplex by phosphorylating Atg[illegible] thus to[illegible]rain autophagosome formation[illegible]eclin-1 is the homologous gene of Atg6 in [illegible]promotes autophagosome membrane[illegible]ormation and guides other autophagy proteins [illegible]lization. Moreover, it plays a crucial role in ti[illegible]nation of autophagy starting vesicle, thus reflecting the level of autophagy[17]. Several researches[7,8] showed that mTOR enhancement plays an [illegible]t role in the pathogenesis of osteosarcoma a[illegible]s closely associated with prognosis and chemoresistance. It was revealed that miR-100 down-regulation is related to the pathogenesis[11], progress[12], and chemo-sensitivity[13] of osteosarcoma, suggesting that miR-100 may play a tumor suppressor 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.

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[11] reported that miR-100 decreased in the tumor tissue of osteosarcoma. Huang et al[18] discovered that miR-100 significantly declined in osteosarcoma compared with adjacent tissue. Liu et al[13] 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[11], Huang et al[18], and Liu et al[13]. Zhou et al[8] 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 al[8] findings. Zhang et al[19] 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 al[19]. 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 suppressed[4]. Ning et al[20] 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 al[ ] and Ning et al[20] results. Further analysis revea[ ] miR-100 mimic and/or si-mTOR markedly [ ]u-ced mTOR expression, attenuated colony fo[ ] tion, and enhanced cell apoptosis and autopha[ ] induced by DDP. Bi et al[11] exhi[ ] that mi[ ] 100 overexpression inhibited [ ]oma cel[ ] proliferation *in vitro* and t[ ]rigene[ ] *in vivo* through targeted suppres[ ]FGFR3[ ] whereas miR-100 inhibitio[ ]en[ ] te results. Huang et a[ ]howe[ ]iR-100 ele-vation significantl[ ]enuated o[ ]coma cell line Saos-2 and [ ]proliferation *vitro*. Liu et al[13] demon[ ]ed [ ]miR-100 up-regulation inhibited o[ ]teosarcoma [ ]U2OS and MG-63 proliferat[ ]motility, and [ ]ion, and enhanced se[ ]ity to cisplatin via targeting IGFIR. In th[ ]tudy, [ ]100 up-regulation markedly att[ ]d os[ ]arcoma malignancy and apoptosis re[ ] chem[ ]rapy, which was in ac-[ ]dance[ ]Bi et[ ] Huang et al[18], and Liu et [ ]e et al[ ]ed that miR-100 expression and [ ]ophagy [ ]e correlated with liver cancer pat[ ]esis. MiR-100 over-expression signifi-[ ]y [ ]d liver cancer cell autophagy and [ ]optosis, and suppressed its proliferation and [ ]rigenesis *in vivo*. This work revealed that [ ]0 down-regulation plays a role in restraining [ ]utophagy, which was similar with Ge et al[21]. Xie et al[22] 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 [ ]ted that inhibition of mTOR marked[ ] tumor cell autophagy and apopto[ ]nduced by chemotherapy, and reduced c[ ]-resistance. This study suggested that miR-1[ ]pression plays a role in elevating mT[ ]R expre[ ] inhibiting cell autophagy an[ ]optosis ind[ ] cisplatin, and enhancin[ ]platin [ ]istance.

Conclusions

We showe[ ]miR-100 red[ ]hile mTOR elevated i[ ]ec[ ]ma tissue. M[ ]-100 upregulation enh[ ]ced ce[ ]phagy and apoptosis induced b[ ]isplatin by [ ]ing mTOR.

Conflict of Inte[ ]

Authors declare[ ] they have no conflict of interest.

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