MicroRNA-199a-5p inhibits cisplatin-induced drug resistance via inhibition of autophagy in osteosarcoma cells

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Abstract. Osteosarcoma (OS) is the most common cancer of the bone. Chemotherapy is commonly used for the clinical treatment of OS. However, chemoresistance to cisplatin [also known as diamminedichloridoplatinum (II) (DDP)] is a major obstacle for OS therapy, the underlying mechanism of which is not fully understood. The present study aimed to investigate the role of microRNA (miR)-199a-5p in the regulation of chemoresistance to DDP in OS cells. Reverse transcription-quantitative polymerase chain reaction demonstrated that the expression level of miR-199a-5p was significantly reduced in human OS MG63 cells. In addition, DDP treatment also upregulated the protein levels of light chain 3 (LC3)-II and Beclin1 as well as the ratio of LC3-II vs. LC3-I in MG63 cells, indicating that autophagy was activated. Restoration of miR-199a-5p expression promoted DDP-induced inhibition of MG63 cell proliferation and inhibited DDP-induced autophagy, as indicated by the reduced protein levels of LC3-II and Beclin1 and the ratio of LC3-II vs. LC3-I. Finally, luciferase reporter assay data revealed that miR-199a-5p directly targeted Beclin1 and negatively mediated Beclin1 expression at a post-transcriptional level in MG63 cells. In conclusion, our study suggests that miR-199a-5p promotes the cytotoxicity of DDP in OS cells via inhibition of autophagy. Therefore, miR-199a-5p/autophagy signaling is involved in chemoresistance and may become a potential target for the treatment of DDP-resistant OS.

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Introduction

Osteosarcoma (OS) is the most common mesenchymal sarcoma in bone, mainly arising from the metaphysis of the long bones (1). Over the past few decades, the prognosis of advanced OS has remained poor, mainly due to its resistance to radiotherapy (2). Cisplatin [also known as diamminedichloridoplatinum (II) (DDP)] is commonly used for the clinical treatment of OS (3,4). However, treatment with DDP may induce tumor chemotherapy resistance (5). Although the underlying molecular mechanism has not been fully uncovered yet, it has been demonstrated that the activation of autophagy plays a key role in DDP-induced chemotherapy resistance (6).

Autophagy is an evolutionarily conserved self-catabolic degradation process, which is responsible for the lysosomal degradation of long-lived proteins and aged or damaged organelles (7). Autophagy can generate amino acids and fatty acids, which can be reused for cell growth and proliferation (7). Therefore, autophagy is important for sustainable cell survival (8). Recently, accumulating evidences have demonstrated that autophagy is involved in DDP-induced chemotherapy resistance in multiple types of human cancer (9,10). Additionally, inhibition of autophagy could increase the chemotherapeutic sensitivity to DDP of OS (11).

MicroRNAs (miRNAs or miRs) are a class of 18-25 nucleotides-long non-coding RNAs that generally lead to messenger RNA (mRNA) degradation or inhibition of translation via directly binding to 3'-untranslated regions (UTRs) of the mRNA of their target genes (12). Through negatively mediation of their targets, miRs have been implicated in numerous cellular processes, including cell survival, proliferation, differentiation, apoptosis and autophagy (13). miR-199a-5p has been observed to be deregulated and important in a variety of human cancers, including gastric cancer, non-small cell lung cancer, melanoma and colorectal cancer (14-18). In addition, miR-199a-5p also

has an inhibitory effect on autophagy (19,20). Xu *et al* noticed that miR-199a-5p could suppress DDP-induced autophagy and chemotherapy resistance in hepatocellular carcinoma cells (20). Yi *et al* reported that overexpression of miR-199a-5p inhibited irradiation-induced autophagy and sensitized breast cancer cells to irradiation (19). However, the exact role of miR-199a-5p in OS, particularly in DDP-induced chemotherapy resistance, has not been previously studied.

The present study aimed to investigate the role of miR-199a-5p in DDP-induced chemoresistance in OS cells, as well as the underlying mechanism.

Materials and methods

Cell culture. The human OS MG63 cell line was obtained from the Cell Bank of Central South University (Changsha, China) and cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Thermo Fisher Scientific, Inc.) and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2.

Cell treatment. MG63 cells were treated with DDP (10 mM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 6 h, and the following assays were conducted.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. RT-qPCR assay was used to determine the miR expression. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. All-in-One™ miRNA Reverse Transcription kit (GeneCopoeia, Inc., Rockville, MD, USA) was used to convert RNA into cDNA according to the manufacturer's protocol. qPCR was then performed using mirVana™ qRT-PCR miRNA Detection kit (Thermo Fisher Scientific, Inc.) on an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR primers were provided by Yearthbio (Changsa, China). The conditions were 95˚C for 10 min, and 40 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 60 sec. U6 was used as an internal reference. The relative expression was analyzed by the $2^{-\Delta\Delta Cq}$ method (21).

Transfection. MG63 cells were cultured to 70% confluence and resuspended in serum-free DMEM. Serum-free DMEM was also used to dilute Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), miR-199a-5p mimic, miR-199a-5p inhibitor and scramble miR mimic [which served as miR-negative control (NC)] . The diluted Lipofectamine 2000 was then added to the above diluted oligonucleotides, which were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China), and then incubated at room temperature for 20 min prior to addition of the mixture to the cell suspension. Upon incubation at 37˚C in the presence of 5% CO_2 for 6 h, the medium was replaced by DMEM supplemented with 10% FBS. After transfection for 48 h, the following assays were performed.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MG63 cells (100,000) were plated in a 96-well plate and incubated for 48 h at 37˚C with 5% CO_2. MTT (5 mg/ml; Thermo Fisher Scientific, Inc.) was added to each well and incubated for 4 h at 37˚C with 5% CO_2. The supernatant was removed, and 100 μl dimethylsulfoxide (Thermo Fisher Scientific, Inc.) was added to dissolve the precipitate. The absorbance was detected at 492 nm using the ELX-800™ Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Western blotting. MG63 cells were solubilized in cold radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) to extract the proteins, which were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Pierce Biotechnology, Inc., Rockford, USA), and transferred onto a polyvinylidene difluoride (PVDF) membrane (Pierce Biotechnology, Inc.). The PVDF membrane was incubated with rabbit anti-Beclin1 monoclonal antibody (1:50; ab55878; Abcam, Cambridge, MA, USA), rabbit anti-light chain 3 (LC3)-II polyclonal antibody (1:50; ab48394; Abcam), rabbit anti-LC3-I polyclonal primary antibody (1:50; ab62721; Abcam) and rabbit anti-GAPDH polyclonal primary antibody (1:50; ab9484; Abcam) at 4˚C overnight. Upon being washed three times with PBS containing Tween 20, the PVDF membrane was then incubated with mouse anti-rabbit secondary antibody (1:20,000; ab99702; Abcam) at room temperature for 40 min. Chemiluminescent detection was conducted using the ECL Western Blotting Substrate kit (Pierce Biotechnology, Inc.). Protein expression was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA), and represented as the density ratio vs. GAPDH.

Dual luciferase reporter assay. Bioinformatics analysis was conducted to predict the putative target genes of miR-199a-5p using TargetScan 3.1 online software (http://www.targetscan.org/). The predicted seed sequence of miR-199a-5p in the Beclin1 3'-UTR as well as the mutant seed sequence in the Beclin1 3'-UTR were cloned downstream of the luciferase gene in the pGL3 Luciferase Reporter Vector (Promega Corporation, Madison, WI, USA), generating vectors containing wild type Beclin1 3'-UTR (Luc-Beclin1 vector) or mutant Beclin1 3'-UTR (Luc-mutant Beclin1 vector). MG63 cells were co-transfected with miR-199a-5p mimics and Luc-Beclin1 or Luc-mutant Beclin1 vector. Luciferase activity was determined after transfection for 24 h using the ELX-800™ Absorbance Microplate Reader.

Statistical analysis. All data were represented as the mean of at least triplicate samples ± standard deviation. Statistical analysis of differences was performed by one-way analysis of variance using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

DDP treatment leads to decreased expression of miR-199a-5p and activation of autophagy in OS MG63 cells. MG63 cells were treated with DDP (30 μg/ml). After treatment for 6 h, the expression level of miR-199a-5p was significantly reduced

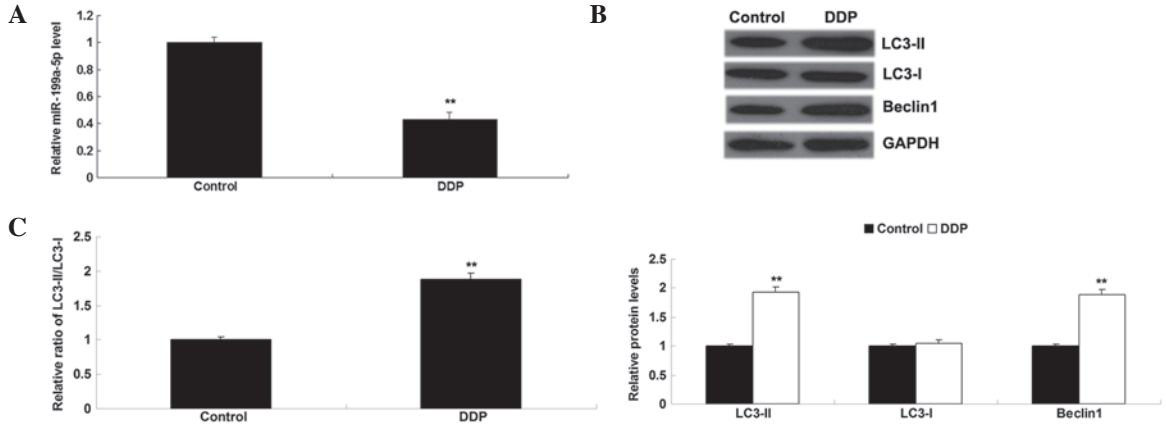

Figure 1. (A) Reverse transcription-quantitative polymerase chain reaction assay was conducted to determine the relative level of miR-199a-5p in MG63 cells treated with DDP for 6 h. (B) Western blot assay was conducted to examine the protein levels in each group. (C) The ratio of LC3-II vs. LC3-I in each group was determined. Non-treated MG63 cells were used as control. **P<0.01 vs. control. miR, microRNA; LC3, light chain 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DDP, diamminedichloridoplatinum (II).

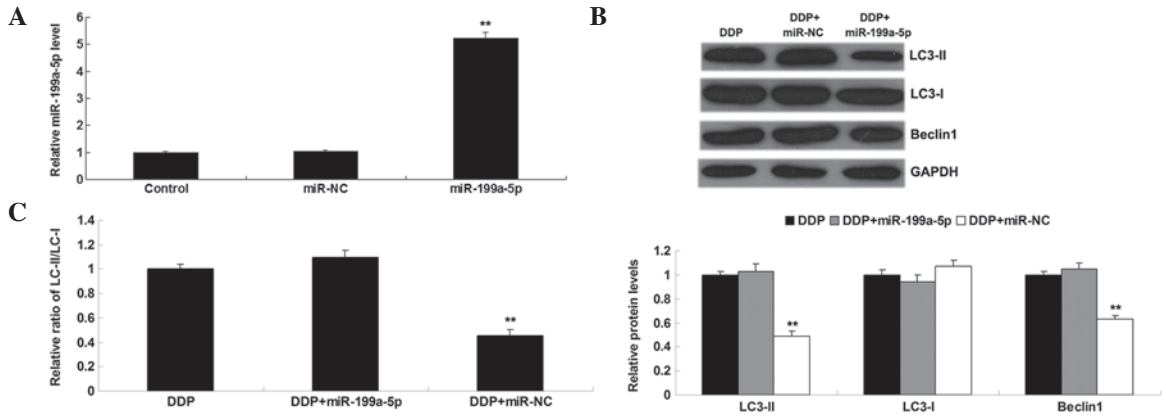

Figure 2. (A) Reverse transcription-quantitative polymerase chain reaction assay was conducted to determine the relative level of miR-199a-5p in MG63 cells transfected with miR-199a-5p mimic or miR-NC. Non-treated MG63 cells were used as control. **P<0.01 vs. control. (B) Western blot assay was conducted to examine the protein levels in DDP-treated MG63 cells transfected with miR-199a-5p mimic or miR-NC. **P<0.01 vs. DDP. (C) The ratio of LC3-II vs. LC3-I in each group was determined. **P<0.01 vs. DDP. miR, microRNA; LC3, light chain 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DDP, diamminedichloridoplatinum (II); NC, negative control.

in MG63 cells treated with DDP, compared with the control group (Fig. 1A). Next, the autophagy-related protein levels were determined in MG63 cells treated with DDP. Western blotting revealed that treatment with DDP upregulated the protein levels of LC3-II and Beclin1 as well as the ratio of LC3-II vs. LC3-I in MG63 cells (Fig. 1B and C).

Enforced expression of miR-199a-5p suppresses DDP-induced autophagy in MG63 cells. The role of miR-199a-5p in DDP-induced autophagy in OS cells was further studied. MG63 cells were transfected with miR-199a-5p mimic or miR-NC prior to DDP treatment. Upon transfection, RT-qPCR was conducted to determine the miR-199-5p levels in MG63 cells, and it was observed that transfection with miR-199a-5p mimic led to a significant increase in miR-199a-5p levels, when compared with the control group (Fig. 2A). Next, it was further studied whether enforced expression of miR-199a-5p could suppress DDP-induced autophagy in MG63 cells. MG63 cells were treated with DDP for 6 h, and the levels of autophagy-related proteins were examined by western blot assay. As shown in Fig. 2B and C, the protein levels of LC3-II and Beclin1 as well as the ratio of LC3-II vs. LC3-I were lower in DDP-treated MG63 cells overexpressing miR-199a-5p compared with those in the control group. These findings indicate that

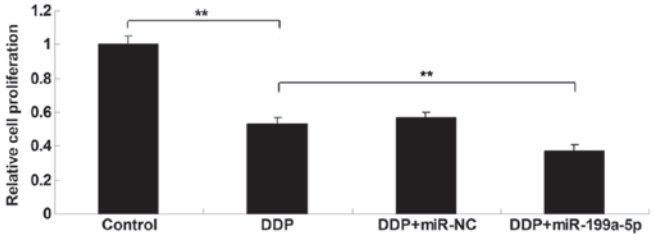

Figure 3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was conducted to determine cell proliferation in DDP-treated MG63 cells with or without transfection with miR-199a-5p or miR-NC. Non-transfected MG63 cells without DDP treatment were used as control. **P<0.01 vs. control. miR, microRNA; DDP, diamminedichloridoplatinum (II); NC, negative control.

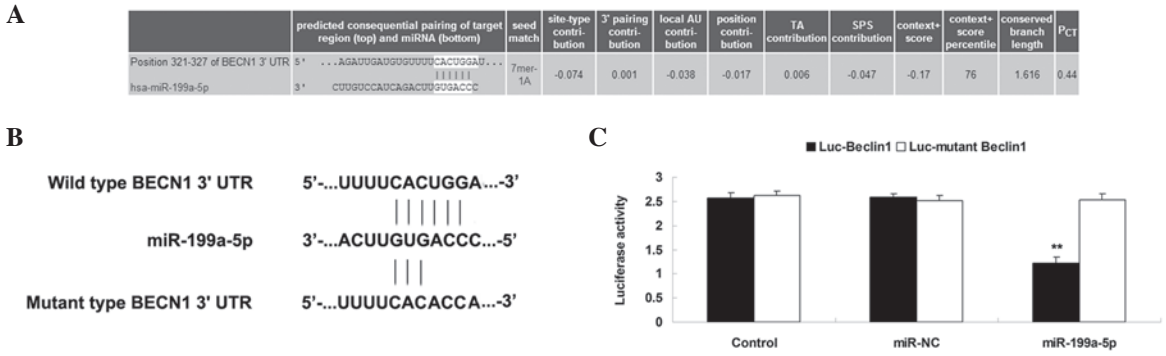

Figure 4. (A) TargetScan data indicated that Beclin1 was a target of miR-199a-5p. (B) The wild type and mutant type of Beclin1 3'-UTR containing the putative miR-199a-5p binding sequences are indicated. (C) MG63 cells were co-transfected with miR-199a-5p mimics or miR-NC and Luc-Beclin1 or Luc-mutant Beclin1 vector. Luciferase reporter assay was conducted to determine the luciferase activity in each group. MG63 cells transfected with only Luc-Beclin1 or Luc-mutant Beclin1 vector were used as control. **P<0.01 vs. control. miR/miRNA, microRNA; UTR, untranslated region; BECN1, Beclin1; Luc, luciferase; NC, negative control; hsa, *Homo sapiens*.

enforced miR-199a-5p expression suppresses DDP-induced autophagy in OS MG63 cells.

Enforced expression of miR-199a-5p enhances the inhibitory effect of DDP on MG63 cell proliferation. Next, the role of miR-199a-5p in mediating the proliferation of MG63 cells treated with DDP was investigated. MTT assay revealed that enforced expression of miR-199a-5p significantly suppressed the proliferation of DDP-treated MG63 cells compared with the control group, indicating that miR-199a-5p enhances the inhibitory effect of DDP on MG63 cell proliferation (Fig. 3).

Beclin1 is a direct target of miR-199a-5p. Bioinformatics prediction was further conducted to analyze the putative target genes of miR-199a-5p. As shown in Fig. 4A, Beclin1 was a putative target of miR-199a-5p. Next, the predicted seed sequence of miR-199a-5p in the Beclin1 3'-UTR as well as a mutant seed sequence in the Beclin1 3'-UTR were cloned downstream of the luciferase gene driven by the cytomegalovirus promoter, generating Luc-Beclin1 vector or Luc-mutant Beclin1 vector, respectively (Fig. 4B). MG63 cells were co-transfected with miR-199a-5p mimics and Luc-Beclin1 or Luc-mutant Beclin1 vector. Luciferase reporter assay demonstrated that the luciferase activity was significantly reduced in MG63 cells co-transfected with Luc-Beclin1 vector and miR-199a-5p mimics, compared with the control group. However, the luciferase activity exhibited no difference in MG63 cells co-transfected with Luc-mutant Beclin1 vector and miR-199a-5p mimics, when compared with that in the control group (Fig. 4C). These data indicate that miR-199a-5p can directly bind to the seed sequences in the Beclin1 3'-UTR in MG63 cells. Therefore, Beclin1 was identified as a target of miR-199a-5p in MG63 cells.

miR-199a-5p negatively mediates the protein expression of Beclin1 in MG63 cells. The effect of miR-199a-5p on the expression of Beclin1 in MG63 cells was further investigated. MG63 cells were transfected with miR-199a-5p mimic or inhibitor. Upon transfection, RT-qPCR was performed to determine the miR-199a-5p level. As shown in Fig. 5A, transfection with miR-199a-5p mimic enhanced the miR-199a-5p level, while transfection with miR-199a-5p inhibitor decreased the miR-199a-5p level, compared with the control group. Subsequently, western blot assay was conducted to examine the Beclin1 protein level. As shown in Fig. 5B, overexpression of miR-199a-5p suppressed the Beclin1 protein level, while knockdown of miR-199a-5p enhanced the Beclin1 protein level, indicating that miR-199a-5p negatively mediates the protein expression of Beclin1 in MG63 cells.

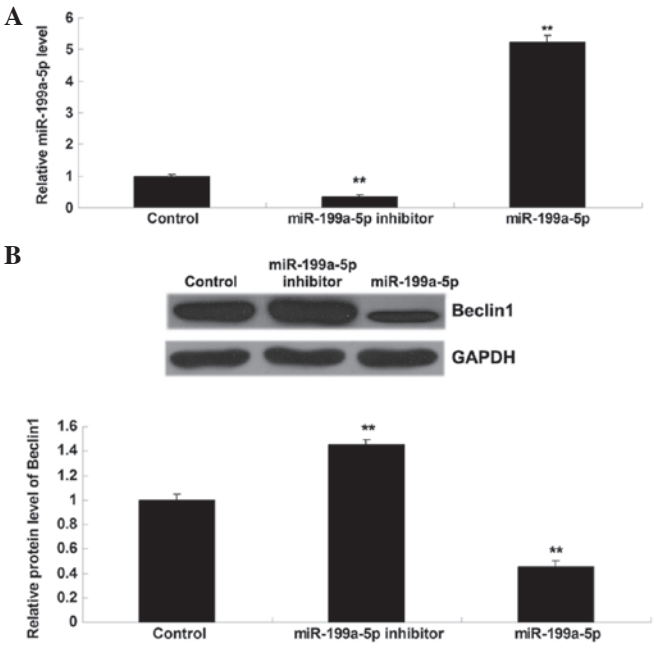

Figure 5. (A) Reverse transcription-quantitative polymerase chain reaction assay was conducted to determine the relative level of miR-199a-5p in MG63 cells transfected with miR-199a-5p mimic or miR-199a-5p inhibitor. (B) Western blot assay was conducted to examine the protein level of Beclin1 in each group. Non-treated MG63 cells were used as control. **P<0.01 vs. control. miR, microRNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Discussion

miRs have been identified to play key roles in the regulation of tumorigenesis and chemotherapy resistance via directly

inhibition of their targets expression (22). In the present study, it was observed that treatment with DDP not only led to a significant decrease in miR-199a-5p levels but also induced an activation of autophagy in OS MG63 cells. Enforced expression of miR-199a-5p inhibited DDP-induced autophagy and enhanced DDP-induced inhibition of MG63 cell proliferation. In addition, it was demonstrated that Beclin1, a key autophagy inducer, was a direct target of miR-199a-5p, which suggested that the suppressive effect of miR-199a-5p on DDP-induced autophagy in MG63 cells may occur through the mediation of Beclin1 expression.

Autophagy is an evolutionarily conserved function, responsible for the reuse of degraded components to sustain metabolic homeostasis and the prevention of the toxic accumulation of damaged components (23). Recently, aberrant activation of autophagy has been detected in various cancers under administration of chemotherapy drugs such as DDP, and accumulating evidences have demonstrated that chemotherapy drug-induced autophagy often causes chemotherapy resistance of tumor cells (24). In addition, inhibition of chemotherapy-induced autophagy has been observed to be beneficial for promoting the efficiency of chemotherapy drug treatment in human cancers, including OS (25). DDP is one of the most commonly used chemotherapy drugs for the treatment of OS (25). In the present study, it was observed that treatment with DDP induced the activation of autophagy in OS MG63 cells by upregulating the protein levels of Beclin1 and LC3-II. Notably, DDP treatment also led to a significant decrease in the expression levels of miR-199a-5p, which has also been implicated in autophagy in breast cancer and hepatocellular carcinoma (19,20).

Deregulation of miR-199a-5p has been implicated in multiple types of human cancer (14,17,18). For instance, miR-199a-5p was observed to be downregulated in triple-negative breast cancer, and its levels in plasma were notably restored upon surgical resection, suggesting that it may be involved in the development of breast cancer (17). Hu *et al* reported that miR-199a-5p was frequently downregulated in colorectal cancer, which led to the upregulation of discoidin domain receptor 1 and the activation of epithelial-to-mesenchymal transition-related signaling (16). In addition, the expression level of miR-199a-5p was significantly increased in gastric cancer tissues compared with paired normal tissues, and higher miR-199a-5p level was associated with increased lymph node metastasis and higher tumor-node-metastasis stage, suggesting that miR-199a-5p may act as an oncogene in gastric cancer (26). Recently, miR-199a-5p has been reported to exhibit inhibitory effects on autophagy (27). For instance, Lee *et al* observed that protoporphyrin IX, a photocatalyzer, could increase the expression of miR-199a-5p, which further inhibited E2F transcription factor 3 and sensitized mesenchymal tumor cells to chemotherapy drugs (28). In the present study, it was observed that miR-199a-5p inhibited DDP-induced autophagy. Xu *et al* reported similar findings in hepatocellular carcinoma, where DDP treatment caused downregulation of miR-199a-5p and increased drug resistance by activating autophagy (20).

Finally, the present study demonstrated that Beclin1, a key inducer of autophagy (29), was a direct target of miR-199a-5p, and that the protein expression of Beclin1 was negatively regulated by miR-199a-5p in OS cells. These data were consistent with those of a recent study (19). Yi *et al* also identified Beclin1 as a direct target of miR-199a-5p, and miR-199a-5p negatively mediated Beclin1 expression in breast cancer cells (19). Therefore, this regulatory mechanism appears to participate in different cancers.

In summary, our study demonstrated that treatment with DDP inhibited the expression of miR-199a-5p and induced the activation of autophagy in OS cells. Enforced expression of miR-199a-5p inhibited DDP-induced autophagy and enhanced the cytotoxicity of DDP in OS cells. Therefore, we suggest that miR-199a-5p may become a potential candidate for the treatment of DDP-resistant cancers.

Acknowledgements

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