miR-133b induces chemoresistance of osteosarcoma cells to cisplatin treatment by promoting cell death, migration and invasion

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Abstract. As an important chemotherapeutic agent for the treatment of osteosarcoma, the effectiveness of cisplatin is considered to be due to its unique properties, which allow it to penetrate the cell membrane and form various DNA-platinum adducts, resulting in genetic alterations or DNA damage. However, chemoresistance to cisplatin remains a major challenge for its use and chemotherapeutic effects. In the present study, an isogenic model of a cisplatin resistant osteosarcoma cell line, MG63-DDP, was generated from the original MG63 cell line. The expression level of microRNA (miR)-133b in the MG63-DDP cisplatin-resistant osteosarcoma cell line was analyzed by reverse transcription-quantitative polymerase chain reaction (PCR). Cisplatin-DNA adduct formation, cell death (carboxyfluorescein succinimidyl ester/propidium iodide staining) and clonogenic survival assays (crystal violet staining) were performed, comparing various cell types. The effect of miR-133b on migration (scratch wound assay) and invasion (Transwell assay) was also evaluated. Characterization studies have previously revealed an increased level of miR-133b in MG63-DDP cells compared with normal MG63 cells. Upregulation of miR-133b was associated with the accumulation of cisplatin-DNA adducts and an increase in cisplatin-induced cell death. Furthermore, increased miR-133b expression levels enhanced the migration and invasion of MG63 cells under cisplatin stress. Concordantly, in MG63-DDP cells the neutralization of miR-133b demonstrated opposite effects, as compared with the upregulation of miR-133b. To the best of our knowledge, the present study demonstrated for the first time that cisplatin-resistant MG63 cells exhibit an increased level of miR-133b expression. The endogenous expression level of miR-133b is sufficient for inducing cisplatin resistance, which suggests that miR-133b may be a biomarker for cisplatin resistance in osteosarcoma.

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Introduction

Human osteosarcoma is a leading cause of tumor-associated mortality in children and young adults; currently there is an ~70% five-year survival rate following treatment with combination chemotherapy (1,2). However, a significant proportion of patients with osteosarcoma exhibit a poor response to chemotherapy, in particular to cisplatin treatment, and suffer a high risk of local relapse or metastasis following intensive combination chemotherapy (3).

MicroRNAs (miRNAs) are small non-coding RNAs that are 19-21 nucleotides long and are highly conserved (4). miRNAs serve regulatory roles by modulating gene expression at the posttranscriptional level by binding the 5'-untranslated region (5'-UTR), coding sequences and 3'-UTR of target mRNA (5). miRNAs directly regulate >60% of human protein coding genes or non-coding genes, indicating their crucial roles in a wide range of biological processes, including embryogenesis, development, differentiation and apoptosis (6). Previous studies have demonstrated that miRNAs also are involved in tumorigenesis (6-8). In osteosarcoma, miR-21 was revealed to be highly upregulated (9). The neutralization of miR-21 suppressed invasion and migration in MG63 cells (10). The expression profile of miR-34a was also significantly altered in osteosarcoma (11). As a regulated target of p53, miR-34a inhibits p53-mediated cell cycle arrest, proliferation, apoptosis and migration in osteosarcoma cells (12). Compared with osteoblasts, in osteosarcoma cell lines, miR-199a-3p was overexpressed and associated with a decrease in cell growth, and with G_1 phase cell cycle arrest in a p53-independent manner (13). Notably, miRNAs are also involved in the induction of chemoresistance. miR-132 and miR-140 have previously been revealed to serve critical roles in the induction of chemoresistance, which suggests the necessity of furthering current understanding of the molecular mechanisms underlying this disease (14,15).

miR-133b has previously been reported to be a muscle-specific miRNA that serves a regulatory role in

the development of skeletal muscle (16). In a recent study, miR-133b was identified to be downregulated in osteosarcoma tissues, compared with the adjacent tissue (17). Subsequently, further research demonstrated its critical role in promoting cell proliferation, migration, invasion and apoptosis (18). However, the role of miR-133b in the chemoresistance of osteosarcoma remains unclear.

Based on previous results, the present study hypothesized that miR-133b may serve critical roles in regulating the chemoresistance of osteosarcoma. In order to validate this hypothesis, the present study evaluated the expression levels of miR-133b in cisplatin-resistant MG63 cells and normal MG63 cells using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The present study aimed to reveal miR-133b as a novel therapeutic target for treating chemoresistance.

Materials and methods

Cell culture and induction of the cisplatin-resistant sub-line. The MG63 human osteosarcoma cell line (no. CRL-1427; American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The cisplatin-resistant sub-line of MG63 (MG63-DDP) was derived from the original MG63 cell line by continuous exposure to cisplatin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The initial dose of cisplatin was 0.1 μM; after 72 h, the media was removed and cells were allowed to recover for a further 72 h. This continuous period lasted for six months. Cells were subsequently maintained in the presence of a half-concentration of the inducing dose, 0.05 μM cisplatin.

Proliferation assay (MTT). Cells ($5x10^3$) were seeded into 96-well plates and allowed to attach to the plate overnight at 37˚C. Following 0.2 μM cisplatin treatment, the MTT reagent was added to cells and incubated for 4 h at 37˚C according to the manufacturer's instructions. Subsequently, dimethylsulphoxide was added to the cells and mixed for 5 min according to manufacturer's instructions. Absorbance at 595 nm was measured using a BioTek Synergy (BioTek Instruments, Inc., Winooski, VT, USA).

Clonogenic survival assay. The clonogenic assay, which is used to determine the effectiveness of cytotoxic agents, including chemotherapeutic agents, was performed to determine the sensitivity of cells to cisplatin. Cells ($1x10^5$) were seeded in a 6-well plate and allowed to attach overnight at 37˚C, and maintained with 0.25, 0.5, 0.75, 1, 1.5 or 2 μM cisplatin for 14-21 days. Subsequently, the colonies were observed by fixation with 4% paraformaldehyde and staining with methanol (25% v/v) substituted with crystal violet (0.05% w/v) for 30 min, prior to being washed with 1X PBS. Colonies >40 μm in diameter were counted using a X71 (U-RFL-T) fluorescence microscope (Olympus, Melville, NY, USA).

Evaluation of cisplatin-DNA adducts. Cells treated with the half maximal inhibitory concentration (IC_{50}) cisplatin (~1.36 μM) were fixed using methanol at room temperature for 30 min, and then subjected to proteolytic digestion with 100 μg/ml pepsin and 50 μg/ml proteinase K at 37˚C for 10 min. To block the non-specific binding sites, fixed cells were incubated with PBS supplemented with 5% (w/v) bovine serum albumin and 5% FBS for 30 min. Subsequently, PBS was removed from the cells using a pipette without washing. The antibody (MABE416; Millipore, Billerica, MA, U.S.A.) against cisplatin-GpG DNA adducts, was added at dilution of 1:2,000 and incubated at 37˚C for 3 h. The primary antibody-DNA adducts complex was detected using an anti-rat Cy5-labeled secondary antibody, goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody (cat. no. A10525, 1:5,000 dilution; Thermo Fisher Scientific, Inc.). Cells were subsequently incubated in 1 μg/ml DAPI and PBS for 10 min at room temperature for nuclear counterstaining. Images were acquired using an Olympus X71 fluorescence microscope (Olympus, Tokyo, Japan).

Transfection of pre-miR™ miRNA precursors (mimics) and anti-miR™ miRNA inhibitors into MG63 or MG63-DDP cells. Mimics and anti-miRNA mimics for miR-133b were purchased from Ambion; Thermo Fisher Scientific, Inc. MG63 cells were transfected with miR-133b mimics (MG63/miR-133b mimics) or scrambled miR-133b mimics (MG63/vector) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 100 nM miRNA was mixed with 8 μl Lipofectamine® 2000 and incubated at room temperature for 30 min. MG63-DDP cells were transfected with anti-miR-133b mimics (MG63-DDP/anti-miR-133b) or scrambled anti-miR-133b mimics (MG63-DDP/vector), following the aforementioned protocol.

RNA extraction and RT-qPCR. RNA was extracted from the original MG63, MG63/vector, MG63/miR-133b mimics, MG63-DDP, MG63-DDP/anti-miR-133b or MG63-DDP/vector cells treated in the previously described conditions, using the mirVana™ miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.) and was treated with DNase I to eliminate the contaminated genomic DNA. The detecting primers, M-MLV Reverse Transcriptase, and PowerUpä SYBR® Green Master Mix were also all supplied by Ambion; Thermo Fisher Scientifc, Inc. The primer sequences were as follows: miR-133b forward, 5'-AAAGGACCCCAACAACCAGCAA-3' and reverse, 5'-TTGCTGGTTGTTGGGGTCCTTT-3'; and U6 small nuclear (sn)RNA forward, 5'-CTCGCTTCGGCA GCACATATACT-3' and reverse, 5'-ACGCTTCACGAATTT GCGTGTC-3'. The relative expression level for each miRNA was determined using the comparative Cq method following normalization to the level of U6 RNA. Individual samples were run in triplicate on the Applied Biosystems ABI 7500 PCR system (Thermo Fisher Scientific, Inc.) (19).

Scratch wound assay. A total of $1x10^5$ cells were seeded in a 24-well plate and allowed to adhere overnight at 37˚C. On the second day, a scratch was made on a 100%-confluent monolayer of cells using a sterile 200 μl disposable pipette tip, and cells were washed with 3 ml PBS to remove debris. Images to evaluate cell proliferation were captured using the Olympus X71 microscope (Olympus) at 0 and 24 h following the scratch.

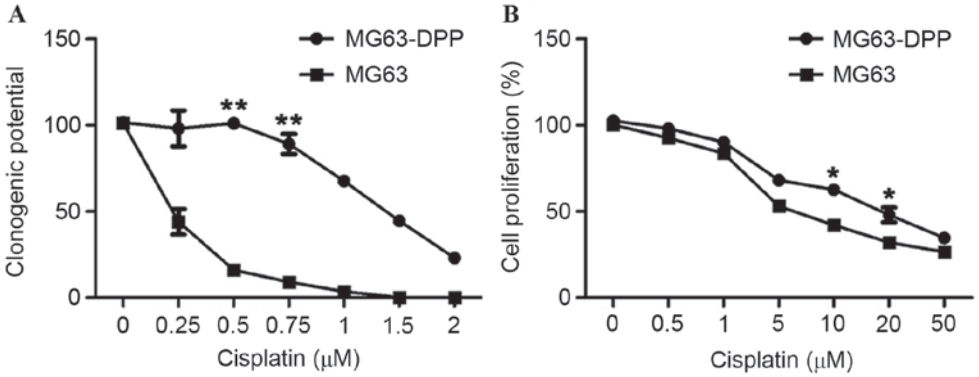

Figure 1. Cisplatin-resistant MG63 cells demonstrate a higher clonogenic ability and proliferation, compared with non-resistant cells. (A) A clonogenic assay 14 days post-treatment and (B) an MTT proliferation assay were performed with MG63 and MG63-DDP cells. *P<0.05 and **P<0.01 vs. MG63. miR, microRNA; DDP, cisplatin.

Invasion assay. To investigate the invasion ability of cells, $1x10^5$ cells were seeded into the upper chamber of a Transwell chamber with a Matrigel-coated membrane (Corning Inc., Corning, NY, USA). Medium without FBS but with cisplatin was added to the upper chamber, while medium supplemented with 2% FBS and cisplatin was applied to the lower chamber. The cells were subsequently incubated at 37˚C for 24 h. Cells that did not invade through the pores were removed using a cotton swab. Cells on the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 0.05% crystal violet for 15 min at room temperature and counted using an Olympus X71 microscope (Olympus).

Statistical analysis. Data in the present study are expressed as the mean ± standard deviation of a minimum of three independent experiments. Analyses were performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). An unpaired Student's t-test was used for comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

MG63 cells with a cisplatin-resistant phenotype demonstrate a higher miR-133b expression level, lower proliferation and clonogenic ability. In order to investigate the miR-133b expression profile in cisplatin-resistant osteosarcoma cells, cisplatin-resistant MG63 (MG63-DDP) cells were generated by long-term treatment with cisplatin, as mentioned previously. The clonogenic assay determined that MG63-DDP cells are significantly more resistant to cisplatin treatment compared with ordinary cells (Fig. 1A; IC_{50}, ~1.2 μM for MG63-DDP cells; IC_{50}, ~0.2 μM for MG63 cells). A short-term proliferation assay was also performed to evaluate the effects of cisplatin on cell division. Notably, a significant difference in the level of proliferation at 48 h was observed between MG63 and MG63-DDP cells (P<0.05); This indicated that the cisplatin-resistant phenotype induced a difference in clonogenic ability (Fig. 1B). According to this observation, 0.2 μM cisplatin was selected for further study. To determine the difference in expression between MG63-DDP cells and MG63 cells, semiquantitative and quantitative RT-PCR were performed. The results demonstrated that MG63-DDP cells expressed ~2.3-fold higher levels of miR-133b, compared with ordinary MG63 cells (Fig. 2). This suggests that the upregulation of miR-133b may have induced this difference.

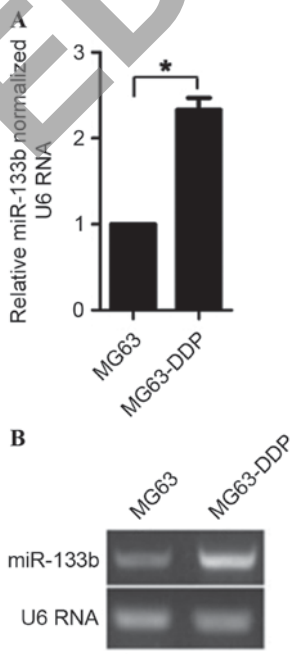

Figure 2. (A and B) miR-133b is significantly upregulated in MG63-DDP cells, compared with the original MG63 cells. *P<0.05. miR, microRNA; DDP, cisplatin.

miR-133b mimic is sufficient to induce a cisplatin-resistant phenotype in MG63 cells. As the aforementioned result demonstrated upregulation of miR-133b in MG63-DDP cells, miR-133b may serve as a potential effector for inducing a cisplatin-resistant phenotype. Thus, the present study determined the efficacy of using miR-133b mimics to induce a cisplatin-resistant phenotype, independent of long-term treatment with cisplatin. The expressing vectors containing the coding sequence of pre-miR-133b were transfected into MG63 cells for 72 h, and subsequently evaluated using a clonogenic assay. As hypothesized, promotion of the clonogenic ability of MG63/pre-miR-133b cells was observed, which confirmed

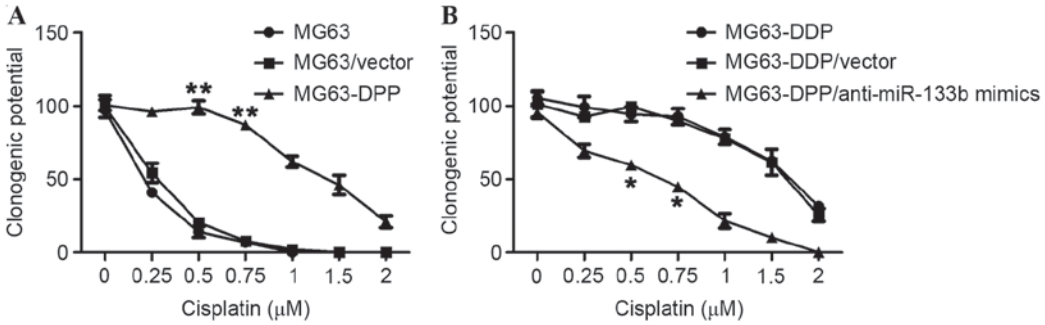

Figure 3. (A and B) miR-133b is sufficient and necessary for the induction of cisplatin resistance in MG63 cells. A clonogenic assay (14 days post-treatment) was performed to determine the clonogenic ability of MG63 cells transfected with scrambled miR-133b mimics (MG63/vector) or miR-133b mimics (MG63/miR-133b mimics), and MG63-DDP cells were transfected with scrambled anti-miR-133b mimics (MG63-DDP/vector) or anti-miR-133b mimics (MG63-DDP/anti-miR-133b mimics). *P<0.05, compared with MG63-DDP/vector; **P<0.01, compared with MG63-vector. miR, microRNA; DDP, cisplatin.

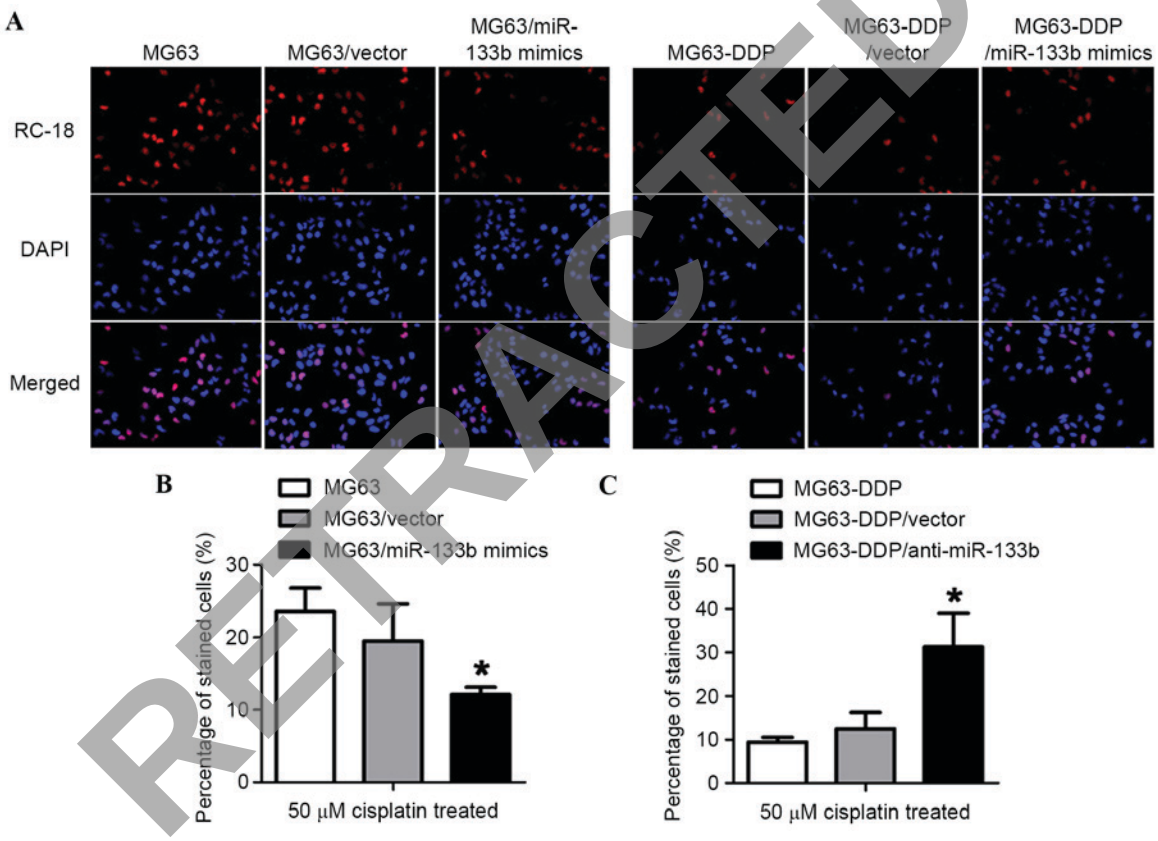

Figure 4. miR-133b expression inhibits cisplatin-DNA adduct formation and cell death. (A) Cells were treated with cisplatin for 24 h, fixed and stained using the RC-18 antibody or (B and C) stained with carboxyfluorescein succinimidyl ester/propidium iodide for cytometry assay to determine the rate of cell death. *P<0.05 vs. (B) MG63 and MG63/vector, or (C) MG63-DDP and MG63-DDP/vector. miR, microRNA; DDP, cisplatin.

the effect of miR-133b on the induction of a cisplatin-resistant phenotype in MG63 cells (Fig. 3).

Epigenetic expression of miR-133b promoted a decrease in cisplatin-1,2-intrastrand d (GpG) crosslinking (GpG) DNA adduct formation and apoptotic ratio under cisplatin stress. To qualitatively determine the level of DNA adducts in the nuclear DNA of MG63-DDP cells, the cisplatin-treated cells were stained for Platinum-GpG (Pt-(GpG)) cross-links in DNA using the RC-18 antibody. It was revealed that the epigenic expression of miR-133b decreased cisplatin-DNA adduct formation compared with MG63 or MG63/vector cells (Fig. 4A, left panel). As hypothesized, the introduction of anti-miR-133b mimics markedly increased cisplatin-DNA adduct formation (Fig. 4A, right panel), which suggested that miR-133b inhibited the accumulation of Pt-DNA lesions. To further confirm the role of miR-133b in the cisplatin-resistant phenotype, miR-133b mimics and the antisense strand of miR-133b mimics (anti-miR-133b) were synthesized and introduced into the respective cells. To investigate death rate, CFSE/PI dual staining was performed. Compared with MG63 or MG63/vector cells, MG63 cells transfected with miR-133b mimics (MG63/miR-133b mimics) demonstrated a lower cell death ratio, indicating the cell's cisplatin resistance (Fig. 4A).

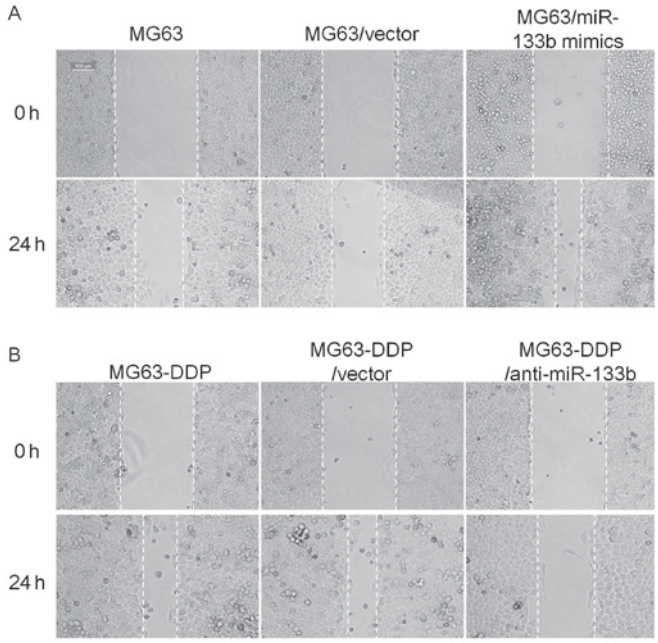

Figure 5. miR-133b affects wound healing. A scratch was created using a 200 μl sterile pipette in 100% confluent cells. The effects of miR-133b on migration of (A) orignial MG63 cells or (B) MG63-DDP cells were detected separately. miR, microRNA; DDP, cisplatin.

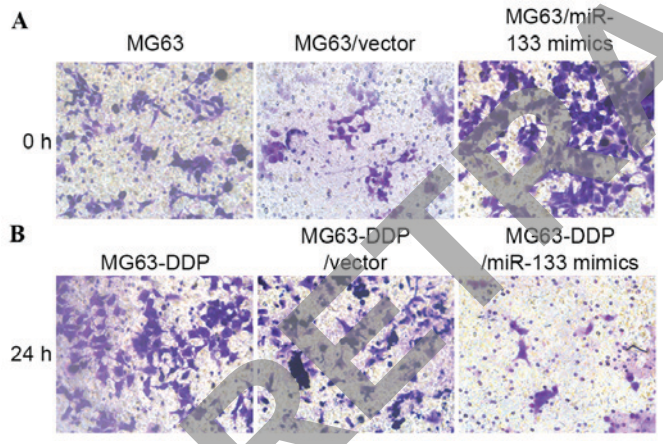

Figure 6. miR-133b promotes cell invasion in MG63 or MG63-DDP cells under cisplatin stress. The effects of miR-133b on invasion of (A) original MG63 cells or (B) DDP-MG63 cells were detected separately. miR, microRNA; DDP, cisplatin.

Consistently, in MG63-DDP cells, the removal of miR-133b by antisense strand miR-133b mimics revealed an increased sensitivity to cisplatin compared with MG63-DDP and MG63-DDP/vector cells (Fig. 4B and C). Taken together, the expression levels of miR-133b in MG63 cells was sufficient to induce cisplatin resistance, and disturbance of miR-133b expression was also sufficient to make cisplatin-resistant MG63 cells more sensitive to cisplatin.

miR-133b expression level promotes migration and invasion under cisplatin stress. To clarify the effect of miR-133b expression on the migration and invasion of MG63 or MG63-DDP cells, two experiments were performed. As presented in Fig. 5, the scratch wound assay demonstrated that cell migration was markedly increased in MG63/miR-133b mimics compared with the original MG63 or MG63/vector cells at 24 h, respectively. Conversely, in MG63-DDP cells, neutralization of miR-133b by the introduction of anti-miR-133b mimics inhibited their migration and invasion. Furthermore, the Transwell migration assay demonstrated that the MG63/miR-133b mimics and the MG63-DDP/anti-miR-133b cells traversed the matrix gel membrane further than the control groups (Fig. 6).

Discussion

Cisplatin is one of the most commonly used agents in chemotherapy due to its high efficiency, mild side effects and easy administration (20). However, the failure of cisplatin treatment is often observed due to chemoresistance, which motivates the search for novel strategies to enhance cell sensitivity to cisplatin (21). One strategy is to suppress the p38α MAPK pathway that is responsible for desensitizing cancer cells to cisplatin treatment (22). A previous study by Wang *et al* (23) demonstrated that downregulation of *P28GANK* gene expression may sensitize osteosarcoma cells to cisplatin treatment via the subsequent downregulation of multi-drug resistance gene 1 and B-cell lymphoma 2 (23). A previous study further confirmed that cisplatin resistance primarily resulted from an increase in the enzymatic activity of glutathione S-transferase P1 (24). The high mobility group box 1 protein-mediated autophagy is a signaling pathway that induces cisplatin-resistance in osteosarcoma cells (25). An alternative strategy is to stimulate the transcription start site pathway to enhance the efficacy, which is directly associated with the enhancement of chemotherapeutic effects (26).

miRNAs are known to be contributors to tumor malignancy. Emerging evidence has revealed that miRNAs also serve an important role in the induction of chemoresistance in osteosarcoma (27). It has previously been revealed that overexpression of miR-126 desensitizes osteosarcoma cells to cisplatin by inhibiting apoptosis under epigallocatechin-3-gallate treatment (28). Compared with adjacent tissues, osteosarcoma cells demonstrated a higher expression level of miR-33a, which promoted osteosarcoma cell resistance to cisplatin by downregulating TWIST protein *in vitro* (29). miR-221 has previously been reported to induce cell survival and cisplatin resistance via the phosphoinositide-3 kinase/protein kinase B signaling pathway in human osteosarcoma cells (30).

The present study demonstrated that overexpressing miR-133b in human MG63 osteosarcoma cells enhanced their resistance to cisplatin by inhibiting cell death induced by cisplatin, migration and invasion. Upregulation of miR-133b resulted from long-term cisplatin treatment. Taken together, these results indicate that miR-133b serves an important role in cisplatin-induced chemoresistance in osteosarcoma cells by supporting tumor cell survival and cisplatin resistance. Previous studies revealed that miR-133b is typically specifically expressed in muscle tissue (31); it has also been identified to be abnormally downregulated in numerous types of cancer cells, which indicates its potential role in tumorigenesis (32). By targeting the epidermal growth factor receptor, the expression level of miR-133b negatively regulates cell proliferation, migration and invasion in prostate cancer cell lines (33).

However, the biological roles of miR-133b in induction of chemoresistance in osteosarcoma are not yet clear. The present study demonstrated that cisplatin treatment induced the upregulation of miR-133b. Similar effects were observed when miR-133b mimics were delivered to MG63 cells during cisplatin treatment. Furthermore, the introduction of anti-miR-133b mimics sensitized the MG63 cells to cisplatin, also suggesting that miR-133b is sufficient for inducing cisplatin resistance in MG63 cells.

In conclusion, miR-133b expression levels are upregulated in human MG63 osteosarcoma cells following long-term cisplatin treatment. Cisplatin treatment induces the overexpression of miR-133b in MG63 cells, leading to the inhibition of cisplatin-induced cell death, and promotion of migration and invasion under cisplatin stress. The present study provided a novel insight into the underlying mechanism of chemoresistance to cisplatin, and miR-133b upregulation exhibits potential as a biomarker of chemoresistance in osteosarcoma.

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