RESEARCH ARTICLE

MicroRNA-100 Resensitizes Resistant Chondrosarcoma Cells to Cisplatin through Direct Targeting of mTOR

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Abstract

Chondrosarcomas are malignant cartilage-forming tumors of bone which exhibit resistance to both chemotherapy and radiation treatment. miRNAs have been well demonstrated to regulate gene expression and play essential roles in a variety of biological processes, including proliferation, differentiation, migration, cell cycling and apoptosis. In this study, we obtained evidence that miR-100 acts as a tumor suppressor in human chondrosarcomas. Interestingly, cisplatin resistant chondrosarcoma cells exhibit decreased expression of miR-100 compared with parental cells. In addition, we identified mTOR as a direct target of miR-100. Overexpression of miR-100 complementary pairs to the 3’ untranslated region (UTR) of mTOR, resulted in sensitization of cisplatin resistant cells to cisplatin. Moreover, recovery of the mTOR pathway by overexpression of S6K desensitized the chondrosarcoma cells to cisplatin, suggesting the miR-100-mediated sensitization to cisplatin dependent on inhibition of mTOR. In summary, the present studies highlight miR-100 as a tumor suppressor in chondrosarcoma contributing to anti-chemoresistance. Overexpression of miR-100 might be exploited as a therapeutic strategy along with cisplatin-based combined chemotherapy for the treatment of clinical chondrosarcoma patients.

Keywords: Chondrosarcoma - mTOR - microRNA - cisplatin - chemoresistance

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Introduction

Chondrosarcoma is the second most frequent primary malignant type of primary bone malignancy after osteosarcoma (Fiorenza et al., 2002). About 30% of skeletal system cancers are chondrosarcomas (Onishi et al., 2011; Van Oosterwijk et al., 2013). Although chemotherapy and radiation have been tested for efficacy, they are not considered as active treatments since these tumors are notoriously resistant to both chemotherapy and radiation treatment (Gelderblom et al., 2008; Onishi et al., 2011). Therefore, developments of effective and low toxic therapeutic approaches are needed to improve chondrosarcoma clinical management.

Cisplatin are employed for the treatment of a variety of tumors, including testicular (Juliachs et al., 2013), ovarian (Mir et al., 2013), head and neck (Pendleton et al., 2013), colorectal (Germani et al., 2013), bladder (Sternberg et al., 2013) and lung cancers (Shin et al., 2013), as a single agent or in combination with other anticancer agents. The prominent mechanism that cisplatin exerts for anticancer effects involves the generation of DNA lesions followed by the activation of the DNA damage response and the induction of mitochondrial apoptosis (Galluzzi et al., 2012). However, despite a consistent rate of initial responses, cisplatin resistance limits its utilizations in cancer patients because some cancer cells develop acquired cisplatin resistance eventually (Negoro et al., 2007; Galluzzi et al., 2012; Barr et al., 2013). The mechanisms of cisplatin resistance are not fully understood yet. It has been reported that altered expression and activation of genes involved in cell death pathways were involved in the cisplatin resistance (Galluzzi et al., 2012). A recent study showed the activity of BCL-2 which is an anti-apoptosis protein contributed to the cisplatin resistance in chondrosarcoma (Wang et al., 2009). Overexpression of BCL-2 rendered chondrosarcoma cells resistant to cisplatin treatments, while BCL-2 inhibition can reverse chemoresistance of chondrosarcoma in vitro. Moreover, recombinant human PDCD5 (rhPDCD5) was also shown to sensitize chondrosarcoma cells to cisplatin-based chemotherapy, with inhibition of cell growth and apoptosis (Li et al., 2012).

The Akt/mTOR signaling pathway plays a central role in protein transnational control and other diverse cellular functions, including proliferation, growth, survival, and metabolism (Laplante et al., 2012). Activated mTOR pathway phosphorylates ribosomal protein S6 kinases (S6K) and 4E-BP1, leading to increased protein translation (Laplante et al., 2012). It is established that dysregulated
Akt/mTOR pathway contributes to cell survival and apoptosis resistance (Caron et al., 2011; Laplante et al., 2012). In addition, the hyperactive mTOR pathway in cancer cells increases drug resistance (Steelman et al., 2011; Yardley et al., 2013). However, the mechanism by which the Akt/mTOR survival pathway in cisplatin resistance in chondrosarcoma is not fully understood.

MicroRNA (miRNA) is non-coding, single-stranded RNA of about 22 nucleotides. Hundreds of miRNAs have been well-studied to regulate gene expression and play essential roles in a variety of biological processes including proliferation, differentiation, migration, cell cycle, and apoptosis (Croce et al., 2009; Ameres et al., 2013). It has been reported that miRNAs are involved in drug resistance (Zheng et al., 2010), and acting as potential oncogenes or tumor suppressors (Ma et al., 2010; Xu et al., 2013). MiR-100 has been reported play a tumor suppressor role through multiple pathways. However, the precise role of miR-100 in chondrosarcoma has not been elucidated. In this study, we examined the expressions of miR-100 in chondrosarcoma cells and reported miR-100 was tumor suppressor in chondrosarcoma. In addition, we explored the inverse expression levels of miR-100 in cisplatin resistant chondrosarcoma cells. We identified mTOR as the direct target of miR-100 and inhibition of mTOR pathway by either mTOR inhibitor or overexpression of miR-100 significantly resensitized cisplatin resistant cells to cisplatin.

Materials and Methods

Cell lines and Patient samples
CHON-001 and C-28/I2 cells were normal human chondrocyte cell line. All cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM)/F12 (Gibco BRL, Germany) with 10% FBS (fetal bovine serum; Gibco BRL, Germany) in humidified atmosphere 5% CO2 in air at 37°C. Cisplatin resistant clone 1, 2 (CDDP CR1, CR2) and CDDP RP (pooled clone) were developed from the parental cell line. Cisplatin resistant cells were seeded on 10cm dish with regular cell culture conditions for up to two months, several resistant cell clones were developed from the parental cell line. Cisplatin resistant clone #1, #2 and pooled clones were used for subsequent experiments in this study. The resistant cells were selected by cisplatin treatments each month.

Primary human normal chondrocytes were purchased from PromoCell (C-12710) and cultured under the conditions according to the manufacturer recommended. All primary Human chondrosarcoma patient specimens were obtained from patients undergoing surgery for chondrosarcoma tumor during 2009 to 2012 at the Zaozhuang Municipal Hospital Cancer Center and stored in liquid nitrogen until analysis. All patients provided written informed consent. The study was approved by the Ethics Committee of the Zaozhuang Municipal Hospital, Zaozhuang, Shandong, China

Antibodies and reagents
Antibodies used from this project were purchased from: mTOR pathway and substrate antibody sampler kit (Cell Signaling #9862/#9964); β-actin (Cell Signaling #4967); AKT (Cell Signaling: #2966); Cisplatin was purchased from Sigma-Aldrich (Hong-Kong, China). BEZ235 (BioVision: #1626-25).

Pre-miRNA or Anti-miRNA Transfection
miRNA precursors (pre-miRNAs) and miRNAs antisense RNAs (anti-miRNAs) were purchased from Applied Biosystems. Pre-miR negative and anti-miR-negative were used as negative controls. Lipofectamine 2000 (Invitrogen) was used for the transfection of pre-miRNAs or anti-miRNAs. Forty-eight hours after transfection, the expression of miR-100 was detected by Real-time-PCR, and the expression of mTOR, a target of miR-100, was tested by Western blotting.

Plasmid DNA transfections
Transfection was performed using the Lipofectamine 2000 Transfection reagent (Invitrogen) according to the manufacturer’s protocol. Overexpression vectors containing wild type S6K (Myc-DDK-tagged) (RC217324) was purchased from www.origene.com. Forty-eight hours after transfection, cells were collected or whole-cell lysates were prepared for further analysis.

Clonogenic assay
For chondrosarcoma cells foci formation assay, 500 cells were seeded on 10cm dish with regular cell culture medium. Cisplatin resistant cells and parental cells without or with treatment of cisplatin were grown for 2 weeks and the surviving colonies were stained with gentian violet after methanol fixation, and visible colonies (>50 cells) were counted. Colonies from randomly-selected image areas of three replicate wells were enumerated.

Generation of cisplatin resistant cell line
CH-2879 cells were treated with gradually increasing concentrations of cisplatin in regular cell culture conditions for selection of resistant cells. After successive treatments for up to two months, several resistant cell clones were developed from the parental cell line. Cisplatin resistant clone #1, #2 and pooled clones were used for subsequent experiments in this study. The resistant cells were selected by cisplatin treatments each month.

Quantitative RT PCR (qRT-PCR)
RNA was isolated from cultured cells using the RNaseasy mini-kit (Qiagen, Germany) (with an on-column DNAse digestion step according to the manufacturer’s instructions). Briefly, lysates of cells were passed through a Qiashredder (Qiagen, Germany) and the eluted lysates mixed 1:1 with 70% ethanol. The lysates were applied to a Qiagen spin column, and the column was washed with ethanol. The RNA was eluted with Tris buffer. The RNA quantity and quality of total RNA samples was checked by agarose-gel-electrophoresis and using the Bioanalyzer RNA 6000 Nano assay (Agilent, Waldbronn, Germany). For miRNA expression analysis, qRT-PCR was done by using the TaqMan microRNA reverse transcription kit (Applied Biosystems) and TaqMan microRNA assays kit (Applied Biosystems) following the manufacturer’s protocols.

All reactions were performed in triplicate. Human U6 served as an internal control. The relative amounts of mRNA were calculated by using the comparative CT method.

Luciferase Reporter Assay

The pMIR-reporter luciferase vector containing the wild type 3'-UTR and 3'-UTR with binding site mutations of mTOR and the empty vector were constructed according to the methods previously described (24). For the luciferase assay, cells at the density of 2x10^5 per well in 24-well plates were cotransfected with pMIR-REPORT luciferase reporters with 3'-UTR of wild type mTOR or binding site mutant mTOR, pre-miR-100, or pre-miR-negative using Lipofectamine 2000 reagent. Forty-eight hours later, cells were harvested and lysed with passive lysis buffer (Promega). Luciferase activity was measured by using a dual luciferase reporter assay (Promega). The pRL-TK vector (Promega) was used as an internal control. The results were expressed as relative luciferase activity (firefly Luc/Renilla Luc).

Cell Viability Assay

A total of 1x10^4 cells for each well were seeded in 48-well plates for overnight. The medium was replaced with fresh medium with or without cisplatin at the indicated concentrations and incubated for 48 hrs. Cell viability was measured using the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Absorbance was measured spectrophotometrically at 570 nm by the Universal Microplate Reader EL800 (BIO-TEK instruments, Inc., Vermont, MA, USA).

Western Blot analysis

Whole cells were lysed in 1x SDS sample buffer and resolved by electrophoresis using SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies overnight, and then incubated with appropriate horseradish peroxides–conjugated secondary antibodies for 3 hrs followed by detection with a Super Signal Enhanced Chemiluminescence kit (Pierce, Rockford, IL). For sequential blotting, the membranes were stripped with Stripping Buffer (Pierce) and re-probed with proper antibodies.

Statistical Analysis

Statistical evaluation for data analysis was determined by unpaired Student’s t test. All data were shown as the means. A statistical difference of p<0.05 was considered significant.

Results

miR-100 is down regulated in human chondrosarcoma cells and patient samples

Since miR-100 has been studied to act as a tumor suppressor in multiple tumor types, we first examined the expression of miR-100 in human chondrosarcoma cells and normal chondrocyte cell. As we expected, miR-100 was significantly down regulated in multiple chondrosarcoma cell lines compared with normal chondrocyte cell (Figure 1A), suggesting miR-100 might be a tumor suppressor in human chondrosarcoma. To investigate whether the putative tumor suppression function of miR-100 was clinically applicable, multiple tumor samples from chondrosarcoma patients were excised and analyzed by Western blotting. Consistently, our data showed similar results that the expression of miR-100 was decreased in chondrosarcoma patient samples.

Cisplatin resistant chondrosarcoma cells exhibits lower expression of miR-100

As we discussed, chemoresistance in chondrosarcoma is the major obstacle for the clinical management. The correlation between dysregulated expression of the tumor
suppressor gene and chemoresistance of cancer cells has been wildly studied. To explore the function of miR-100 in chondrosarcoma, we generated cisplatin resistant chondrosarcoma cell line using CH-2879 cell by gradually treated CH-2879 cells at elevated concentration of cisplatin for two months. Three CH-2879 cisplatin resistant clones were picked and the rest of them were pooled as CDDP resistant clone1, clone2 and pool. Our results showed miR-100 could target mTOR in chondrosarcoma cells. To determine whether miR-100 could target mTOR in chondrosarcoma cells, we transfected the pre-miR-100 and anti-miR-100 into CH-2879 cells. The overexpression of miR-100 significantly down-regulated mTOR proteins and inhibition of miR-100 restored the expression of mTOR in CH-2879 cells (Figure 3A). Since the Akt/mTOR signaling pathway is a prototypic survival pathway that plays a central role in diverse cellular functions, we measured the downstream targets of mTOR, phosphorylation of S6K and phosphorylation of 4EBP1. Western blotting results in Figure 3B that the activities of two downstream effectors were both inhibited by overexpression of miR-100 and restored by the inhibition of miR-100.

miR-100 is a direct target of miR-100 in chondrosarcoma cells

To further explore the functions of the dysregulated miR-100 in chondrosarcoma, we searched miRNA databases for potential miR-100 targets that may possibly contribute to cisplatin resistance. The three public miRNA databases (TargetScan, Pictar, and MicroRNA) all predicted that mTOR might be a target for miR-100, and the 3' UTR of mTOR contains a highly conserved binding site for miR-100 (Figure 3A). So far, no publication reported that mTOR is a miR-100 direct target in chondrosarcoma cells. To determine whether miR-100 could target mTOR in chondrosarcoma cells, we transfected the pre-miR-100 and anti-miR-100 into CH-2879 cells. The overexpression of miR-100 significantly down-regulated mTOR proteins and inhibition of miR-100 restored the expression of mTOR in CH-2879 cells (Figure 3A). Since the Akt/mTOR signaling pathway is a prototypic survival pathway that plays a central role in diverse cellular functions, we measured the downstream targets of mTOR, phosphorylation of S6K and phosphorylation of 4EBP1. Western blotting results in Figure 3B that the activities of two downstream effectors were both inhibited by overexpression of miR-100 and restored by the inhibition of miR-100.

The expressions of total levels of S6K, 4EBP1 and AKT were not changed as miR-100 specifically targeted on mTOR suppression. The results were expressed as relative luciferase activity (firefly LUC/Renilla LUC). Columns, mean of three independent experiments; bars, SE. **P < 0.01

Figure 3. mTOR is a Direct Target of miR-100 in Chondrosarcoma. (A) Target prediction from Targetscan.org, the position 295-301 of mTOR 3' UTR contains putative binding sites for miR-100. (B) CH-2879 cells were transfected with 100 nM pre-miR-negative (Ct), pre-miR-100 or anti-miR-100 for 48 hrs. Cell lysates were prepared for Western blotting analysis. β-actin was used as a loading control. (C) CH-2879 cells were co-transfected with luciferase reporter plasmids with wild type 3'-UTR of mTOR or mutant 3'-UTR of mTOR and pre-miR-100, or pre-miR-negative (Control miR) by using Lipofectamine 2000 reagent. Forty-eight hours post transfection, cells were harvested or pre-miR-negative (Control miR) by using Lipofectamine 2000 reagent. Forty-eight hours post transfection, cells were harvested and lysed with lysis buffer. Luciferase activities were measured by a dual luciferase reporter assay. The pRL-TK vector was used as an internal control. The results were expressed as relative luciferase activity (firefly LUC/Renilla LUC). Columns, mean of three independent experiments; bars, SE. **P < 0.01

Figure 4. Overexpression of miR-100 in Chondrosarcoma Cells Increases the Sensitivity to Cisplatin. (A) CH-2879 parental cells (upper) and cisplatin resistant cells (lower) were treated with cisplatin alone, BEZ235 alone and the combination of cisplatin and BEZ235 for 48 hrs followed by cell viability analysis. (B) Overexpression of miR-100 in parental (upper) and cisplatin resistant CH-2879 cells (lower). (C) CH-2897 parental cells (left) and cisplatin resistant cells (right) were transfected with pre-miR-100 for 48 hrs, then cells were re-placed into 12-well plates under the treatment of cisplatin at indicated concentrations. Columns, mean of three independent experiments; bars, SE. *P < 0.05
Overexpression of miR-100 in chondrosarcoma enhances the sensitivity to cisplatin

Previous study revealed that Akt/mTOR survival pathway plays an important role in cisplatin resistant ovarian cancer cells. To explore the mechanisms accounting for the miR-100 mediated cisplatin sensitivity, we first examined whether inhibition of mTOR pathway by specific inhibitor sensitized chondrosarcoma cells to cisplatin. BEZ235 has been reported to effectively inhibit chondrosarcoma cell growth (Zhang et al., 2013). We treated CH-2879 parental and resistant cells with CDDP alone, BEZ235 alone and the combination of them. Both CH-2879 parental and CDDP resistant cells showed synergistic inhibitory effects on the combination of BEZ235 and Cisplatin (Figure 4A). Treatments of CDDP at 1uM or BEZ235 at 5nM on CH-2879 parental cells only slightly inhibited cell viabilities, but when combined cisplatin with BEZ235, the cell viabilities were significantly suppressed (Figure 4A upper). Similar results were observed in CH-2879 CDDP resistant cells (Figure 4A lower), suggesting inhibition of mTOR pathway might be an efficient adjuvant in addition to cisplatin treatment for chondrosarcoma cells. Our above results revealed that miR-100 contributed to the sensitivity of cisplatin (Figure 2C). To investigated the roles of miR-100 in cisplatin resistant chondrosarcoma cells, we transfected miR-100 and control microRNA into CH-2879 parental and cisplatin resistant cells (Figure 4B), then treated the cells with indicated concentrations of cisplatin for 48 h. We observed overexpression of miR-100 in both parental and cisplatin resistant chondrosarcoma cells promoted the susceptibility to cisplatin (Figure 4C). Taken together, our results suggested overexpression of miR-100 sensitized chondrosarcoma cells to cisplatin through the inhibition of mTOR pathway.

Restoration of the activity of mTOR pathway renders miR-100 overexpressing chondrosarcoma cells resistant to cisplatin

To examine whether the sensitization to cisplatin by overexpression of miR-100 was through the inhibition of mTOR pathway, we transfected overexpression vector containing wild type S6K into miR-100 pre-transfected chondrosarcoma cells. Exogenous overexpression of S6K restored the phosphorylation of S6K to the original level (Figure 5A), indicating the activities of mTOR pathway was recovered by overexpression of S6K in miR-100 overexpressing cells. Restoration of the activity of mTOR pathway in miR-100 overexpressing cells led to a significant resistance compared with the transfection with control vector in both parental and cisplatin resistant cells (Figure 5B), suggesting overexpression of miR-100 sensitized chondrosarcoma cells to cisplatin by the inhibition of mTOR pathway.

Discussion

Since chondrosarcomas was proved to be resistant to conventional chemo- and radiotherapy, very limited chemotherapeutic agent can be offered for high-grade and metastatic tumors (Van Oosterwijk et al., 2013; Onishi et al., 2011). The most current hypotheses regarding the molecular mechanisms mediating chemotheraphy and radiation resistance of chondrosarcoma therapy have been discussed. The most prominent mechanism of chemoresistance is the abnormal expression of P-glycoprotein in chondrosarcoma. In addition, the transcription factor hypoxia inducible factor-1α (HIF-1α) induced angiogenesis under hypoxia (Lin et al., 2004); the telomerase activity-mediated chondrosarcoma cell Immortality (Parsch et al., 2008) and the loss of tumor suppressor p16 in chondrosarcoma (Schrage et al., 2009) have been reported to leads to chemo- and radioresistance. A recent study reported that the growth of chondrosarcoma cells can be inhibited by mTOR inhibitor in an in vivo syngeneic rat model (Zhang et al., 2013), suggesting a putative chemotherapeutic approach for clinical applications.

Many miRNAs have been identified as having an oncogenic or tumor suppressor-like function shown to be involved in cell proliferation, differentiation, apoptosis, and drug resistance. MiR-100 has been shown its tumor-suppressor functions in several cancers including ovarian cancer (Nagaraja et al., 2010), bladder cancer (Xu et al., 2013), hepatocellular carcinoma (Chen et al., 2013), cutaneous squamous cell carcinoma (Henson et al., 2009) and lung cancer (Liu et al., 2012). A recent miRNA array
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analysis described that miR-100 was down regulated in chondrosarcoma cells, indicating miR-100 might act as a tumor suppressor in human chondrosarcoma (Yoshitaka et al., 2013). However, little is known about their role in chemoresistance, such as to cisplatin. Currently, the explicit functions of miR-100 in human chondrosarcoma is still under investigation. Our results showed the expressions of miR-100 in multiple human chondrosarcoma cells were downregulated compared with normal human chondrocytes. Consistently, the expressions of miR-100 were decreased in clinical chondrosarcoma patient samples. Importantly, miR-100 was negatively correlated to cisplatin sensitivity. The cisplatin resistant chondrosarcoma cells exhibited decreased expression of miR-100 and overexpression of miR-100 sensitized the cisplatin resistant cells. Taken together, our study revealed miR-100 acts as a tumor suppressor in chondrosarcoma cells and might be a therapeutic target in the treatments of chondrosarcoma patients.

mTOR is frequently activated in multiple carcinoma and can be a promising therapeutic target in the treatments of clinical cancer patients. mTOR phosphorylates p70 S6 kinase (p70S6K) and the 4E-BP1 translational repressor, leading to translation of proteins required for cell proliferation (Caron et al., 2010; Laplante et al., 2012). It has been reported that AKT-mTOR signaling is frequently activated in epithelial ovarian cancer (Caron et al., 2010; Steelman et al., 2011). Moreover, mTOR inhibition by BEZ235 has been reported be efficacious for the inhibition of chondrosarcoma cells growth in vitro (Zhang et al., 2013). Recently, another mTOR inhibitor, everolimus (RAD001), has been shown to inhibit the proliferation of ovarian cancer cells and enhance sensitivity to cisplatin in vitro and in vivo (Mabuchi et al., 2007). However, the mechanism of cisplatin resistance in cancer cells is not fully understood. In this study, we showed that the inhibition of Akt/mTOR survival pathway resestizes cisplatin resistant chondrosarcoma cells to cisplatin by the treatment of BEZ235, suggesting the Akt/mTOR survival pathway plays an important role in cisplatin resistance in human chondrosarcoma cells.

Our data illustrated miR-100 possessed an inhibitory function in chondrosarcoma in this study since it negatively regulated mTOR pathway and promotes chemosensitivity to cisplatin. The downregulation of miR-100 in chondrosarcoma indicated miR-100 might be a tumor suppressor which triggered us explore the roles of miR-100 in chondrosarcoma cells chemosensitivity. Since it has been reported the activity of AKT/mTOR pathway was correlated with cisplatin resistance in ovarian cancer (Mabuchi et al., 2007), in this study, we identified mTOR as the direct target of miR-100 and first reported the overexpression of miR-100 sensitized chondrosarcoma cells to cisplatin through the inhibition of mTOR pathway. To test the specificity of the mechanism that miR-100 inhibited mTOR pathway which led chondrosarcoma cells sensitive to cisplatin, we restored the activity of mTOR pathway by overexpression of S6K which is a downstream effect of mTOR. Recovery of mTOR pathway desensitized the chondrosarcoma cells to cisplatin, suggesting the miR-100 mediated sensitization to cisplatin is through the inhibition of mTOR. In our next project, we will further verify our in vitro data using mice model and explore the detailed mechanisms accounting for the miR-100-mediated chemosensitivity. In summary, our study proposed a novel mechanism for the overcoming chemoresistance in chondrosarcoma and will provide new aspect for the development of new therapies for this difficult-to-treat cancer.

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