miR-873 acts as a novel sensitizer of glioma cells to cisplatin by targeting Bcl-2

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Abstract. Treatment with cisplatin, a chemotherapeutic agent commonly used in glioma patients, often results in chemoresistance. Increasing evidence has shown that microRNAs (miRNAs) are implicated in the drug resistance of gliomas. However, the function of miR-873 in cisplatin resistance of gliomas remains unknown. In this study, we found that many miRNAs, including miR-873, are differentially expressed in cisplatin-resistant glioma cells compared to wild-type glioma cells. Moreover, cisplatin reduced the expression of miR-873 in a time-dependent manner. Overexpression of miR-873 decreased the cell proliferation, migration and invasion while increased apoptosis of cisplatin-resistant glioma cells and sensitized the cells to cisplatin-induced cell growth arrest and apoptosis. Furthermore, miR-873 was downregulated when Bcl-2 was upregulated in the tissues of twelve high-grade glioma patients compared to seven normal brain tissues, and the miR-873 level was negatively correlated with the Bcl-2 protein level. A luciferase reporter assay further confirmed that Bcl-2 was a direct target of miR-873, and miR-873 decreased the level of the Bcl-2 protein in cisplatin-resistant glioma cells. Notably, re-expression of Bcl-2 attenuated the function of miR-873 in cisplatin-resistant glioma cells and the sensitivity of the cells to cisplatin. Taken together, these data suggest that miR-873 might be a potential marker for cisplatin resistance and a promising sensitizer in cisplatin treatment.

Introduction

Gliomas represent the most common primary brain tumors in adults (1). The average survival time for high-grade glioma patients is ~14 months (2). Even with successful surgical resection, the prognosis for a patient whose tumor is not sensitive to chemotherapeutic drugs is poor. Cisplatin is well established for use as a chemotherapeutic drug in gliomas (3). However, the molecular mechanisms underlying the chemoresistance of gliomas are largely unclear.

MicroRNAs (miRNAs) are a class of small endogenous noncoding RNAs of 18-25 nucleotides that negatively regulate their target genes by interacting with the 3'-untranslated region (3'UTR) of the mRNAs (4). The differential expression of miRNA between cancers and their normal counterparts have been explored in various cancers, including glioma (5-9), and these results suggest critical roles for miRNAs in the pathogenesis of the cancers. Recently, accumulating evidence has indicated that miRNAs are implicated in the chemoresistance of gliomas. Let-7b was found to be significantly downregulated in cisplatin-resistant U251 cells compared to wild-type cells and has the ability to improve the therapeutic effect of cisplatin in glioma cells (10). Knockdown of miR-106a enhanced the temozolomide-induced apoptosis in glioma cells (11). Overexpression of miR-136 sensitizes glioma cells to cisplatin by targeting E2F1 and Bcl-2 (12,13). To date, only a few studies have reported the function of miR-873 (14-16). In particular, the effect of miR-873 on the chemoresistance of glioma cells has not been reported.

In this study, we found that hsa-miR-873-5p (miR-873) was downregulated in cisplatin-resistant glioma cells compared to wild-type cells. Furthermore, exposing wild-type cells to cisplatin decreased their expression of miR-873. Further experiments demonstrated that miR-873 decreased the activity of cisplatin-resistant glioma cells and sensitized cisplatin-resistant glioma cells to cisplatin by directly targeting Bcl-2, an anti-apoptotic protein expressed in a wide variety of cancers. Ectopic expression of Bcl-2 in cisplatin-resistant glioma cells could prevent the cisplatin-sensitizing effect of miR-873. These results indicated that miR-873 might be a promising therapeutic strategy to overcome cisplatin resistance in gliomas.

Materials and methods

Patients and samples. Twelve high-grade glioma tissues and seven normal brain tissues were collected between 2011 and
were calculated using the 2-
relative expression levels
miRNA qRT-PCR Primer kits (Ribobio, Guangzhou, China)
miR-873, the Bulge-Loop™
Reverse transcription was performed using
TRIZol reagent (Invitrogen, San Diego, CA, USA). Reverse transcription was performed using
Total RNA was extracted from
DDP) cell lines were established by prolonged exposure
The cisplatin-resistant U87 (U87/DDP) and U251 (U251/ DDP) cell lines were established by prolonged exposure to increasing concentrations of cisplatin (Sigma-Aldrich, St. Louis, MO, USA). The cisplatin concentrations were increased stepwise from 0.1 to 10 µg/ml when the cells resumed growth similar to the untreated cells. U87 cells with the ability to grow in 5 µg/ml of cisplatin and U251 cells with the ability to grow in 0.5 µg/ml of cisplatin were obtained 4 months after the initial drug exposure and were designated U87/DDP and U251/DDP, respectively. The MTT assay results demonstrated that the drug sensitivity of these cells was changed.
RNA extraction and qPCR. Total RNA was extracted from human tissues using TRIZol reagent (Invitrogen, San Diego, CA, USA). Reverse transcription was performed using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA). To detect miR-873, the Bulge-Loop™ miRNA qRT-PCR Primer kits (Ribobio, Guangzhou, China) were utilized following the manufacturer's instructions, and qPCR was performed using SuperReal PreMix (SYBR Green) (Tiangen, Beijing, China) with an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The relative expression levels were calculated using the 2^ΔΔCt method after normalization to the expression of U6 snRNA.
MTT assay. The cells were seeded into 96-well plates (3x10^4 cells/well) and incubated for 24 h. The cells were transfected with vectors for another 24 h and then treated with various concentrations of cisplatin. At 24, 48 and 72 h, the cell viability was determined using the MTT solution (0.5 mg/ml) (Sigma-Aldrich). The membrane was blocked with 5% skim milk for at least 2 h at room temperature. Monoclonal antibodies against Bcl-2 (1:1,000, Cell Signaling, Danvers, MA, USA), PARP (1:3,000, Cell Signaling), and β-actin (1:5,000, Sigma-Aldrich) were added in 3% BSA and incubated at 4˚C overnight. The membrane was washed and then incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:1,000, Sigma-Aldrich) at room temperature for 1.5 h. Then, the membrane was washed, and the proteins were visualized using an ECL kit (Millipore, Billerica, MA, USA).
Statistical analysis. The data are shown as the means ± SD (standard deviation) and were analyzed using SPSS 17.0 software. Statistical significance was determined using Student's t-test, and P<0.05 was considered to be significantly different. The Spearman rank correlation coefficients were calculated to determine the bivariate correlations between study variables.
Results
miR-873 was downregulated in glioma tissues and cisplatin-resistant glioma cells. Cluster analysis has revealed that several miRNAs including miR-873 are differentially expressed in glioma tissues (17). Therefore, we compared the expression of miR-873 in the tissues from twelve high-grade glioma patients
with those of seven normal brains by qPCR. The results showed that miR-873 was significantly downregulated in the high-grade glioma tissues compared with the normal tissues (Fig. 1A). In addition, differential expression of miRNA was frequently related to drug resistance in gliomas (10,11,18). Therefore, we generated the cisplatin-resistant glioma cells designated U87/DDP and U251/DDP to determine the correlation of dysregulated miR-873 with cisplatin resistance. We first determined the IC$_{50}$ for the wild-type cells and resistant cells. We found that the IC$_{50}$ for U87/DDP (Fig. 1B) or U251/DDP (Fig. 1D) was significantly higher than that in the respective wild-type cells (U87 or U251). Then, we found that several miRNAs that had been identified in the previous cluster analysis were dysregulated in the U87/DDP or U251/DDP cells (Fig. 1C and E). Among these, miR-873 was significantly downregulated in U87/DDP or U251/DDP cells compared to the respective wild-type cells (Fig. 1C and E).

Next, we assessed the effects of cisplatin on the expression of miR-873 in U87 and U251 cells. The expression of miR-873 was downregulated in a time-dependent manner after treatment with the IC$_{50}$ value of cisplatin (U87 cells were treated with 6.47 µg/ml and U251 cells were treated with 1.89 µg/ml) (Fig. 1F). These data suggested that miR-873 might influence the susceptibility of glioma cells to cisplatin.

Overexpression of miR-873 attenuated the tumorigenic properties of cisplatin-resistant glioma cells. To assess the function of miR-873 on cisplatin-resistant glioma cells, 50 nM miR-873 mimics or 50 nM negative control (NC) mimics, which was used as a control, was transfected into U87/DDP and U251/DDP cells. Forty-eight hours after the transfection, the qPCR results indicated that miR-873 expression was significantly upregulated in the miR-873 mimics transfected cells compared to the NC mimics transfected cells (Fig. 2A). Then, we examined cell proliferation by MTT assay for indicated time and the results showed that miR-873 significantly inhibited cell proliferation at 48 and 72 h (Fig. 2B and C). The colony formation assay gave the same conclusion as the MTT assay in the U87/DDP and U251/DDP cells (Fig. 2D). Moreover, we tested the effects of exogenous miR-873 expression on cell migration and invasion. Overexpression of miR-873 decreased both the migration (Fig. 2E) and invasiveness (Fig. 2F) of U87/DDP and U251/DDP cells as determined by Transwell assay. Furthermore, miR-873 obviously increased apoptosis of U87/DDP and U251/DDP cells as determined by caspase-3/7 activity analysis (Fig. 2G). Collectively, these findings indicated that miR-873 acts as a tumor suppressor and might relate to cisplatin resistance of glioma cells.

Overexpression of miR-873 sensitized glioma cells to cisplatin. To further investigate the function of miR-873 on the cisplatin resistance, the miR-873 or NC mimics was transfected into U87/DDP and U87 cells. At first, we performed the MTT and colony formation assays to determine the effect of miR-873 on the proliferation of glioma cells. After being transfected with the miR-873 or NC mimics for 24 h, the glioma cells were exposed to cisplatin for another 24-72 h.
Then, the absorbance of glioma cells was measured using a microplate reader at 570 nM. The results of the MTT assay showed that miR-873 could enhance the anti-proliferative effect of cisplatin in both the cisplatin-resistant and wild-type glioma cells (Fig. 3A and B). The colony formation assay gave the same results as MTT assay in the U87/DDP and U87 cells (Fig. 3C). Later, we used flow cytometry to analyze the effects of miR-873 on cisplatin-induced apoptosis of the U87/DDP and U87 cells. These results also showed that miR-873 was able to significantly increase cisplatin-induced apoptosis of the U87/DDP and U87 cells after 48 h of cisplatin treatment (Fig. 3D). Moreover, western blotting showed a decreased expression of Bcl-2 and an increased expression of cleaved PARP, while the caspase-3/7 activity assay showed an increased activity of caspase-3/7 in the miR-873 mimic-transfected U87/DDP and U87 cells when exposed to cisplatin treatment (Fig. 3E and F). Thus, overexpression of miR-873 could decrease the resistance of the glioma cells to cisplatin.

Bcl-2 is a functional target of miR-873. It is known that miRNAs perform their functions by regulating the expression of their target genes (19). Thus, the targets of miR-873 were predicted by miRanda and TargetScan, two publicly available prediction tools. We selected several putative targets that were related to cisplatin resistance from the predictions. The 3'UTR containing the potential binding site were cloned into pMiR-Reporter vector and luciferase assays were performed by transfection into U87/DDP cells. After co-transfection with 3'UTRs and the miR-873 or NC mimics for 24 h, luciferase assays showed that the activity of Bcl-2 3'UTR was strongest inhibited by miR-873 (Fig. 4A). Furthermore, we cloned a mutated 3'UTR into the pMiR-Reporter vector to create a pMiR-Bcl-2-mu vector (Fig. 4B). After co-transfection of the U87/DDP cells with the miR-873 or NC mimics and the pMiR-Bcl-2-wt vector or pMiR-Bcl-2-mu vector for 24 h, a luciferase assay was performed. The results showed that the luciferase activity was decreased by transfection with the miR-873 mimic (Fig. 4C). However, no significant difference
was found when the cells were transfected with the miR-873 or NC mimics combined with the pMiR-Bcl-2-mu vector (Fig. 4C). Subsequently, western blotting was performed to analyze the effect of miR-873 on Bcl-2 protein expression. The results showed that Bcl-2 expression was significantly lower in the cells transfected with the miR-873 mimics for 72 h compared with the NC mimics both in U87/DDP and U251/DDP cells (Fig. 4D and E). Furthermore, we examined the Bcl-2 levels in twelve high-grade glioma patient tissues and those of seven normal brains tissues by western blotting. The results showed that Bcl-2 was upregulated in the high-grade glioma tissues (Fig. 4F and G). There was an obvious negative correlation between these signals (r=-0.731, P<0.01; Fig. 4H). Therefore, miR-873 could downregulate the expression of Bcl-2 by directly targeting the Bcl-2 3'UTR.

Re-expression of Bcl-2 attenuates the effect of miR-873 on cisplatin-resistant cells. To examine whether Bcl-2 expression mediates the phenotype associated with increased miR-873 expression, the CMV-MCS/Bcl-2 vector or the CMV-MCS empty vector as a control was transfected into U87/DDP cells. A western blot assay confirmed that the expression of the Bcl-2 protein was significantly upregulated in the U87/DDP cells (Fig. 5A). The MTT assay showed that re-expression of Bcl-2 decreased the antiproliferation effect of miR-873 on U87/DDP cells at 48 and 72 h (Fig. 5B). To further investigate the antitumor effect of miR-873 on U87/DDP cells, Transwell assay and caspase-3/7 activity analysis were performed. Consistent with MTT result, re-expression of Bcl-2 recovered the migration (Fig. 5C and D) and invasive (Fig. 5C and E) ability of U87/DDP cells and abrogated the pro-apoptotic effect of miR-873 (Fig. 5F). These data further suggested Bcl-2 might be a functional target of miR-873.

Re-expression of Bcl-2 decreases the effect of miR-873 on cisplatin sensitivity. Having demonstrated that Bcl-2 is a direct target of miR-873, we further investigated the role of Bcl-2 in cisplatin sensitivity of miR-873. After being co-transfected with the miR-873 or NC mimics and the CMV-MCS/Bcl-2 or CMV-MCS vector for 24 h, the U87/DDP cells were treated with IC50 value of cisplatin for another 24-72 h. The MTT assay showed that the proliferation was inhibited in the miR-873 mimic-transfected cells compared to the NC mimic-transfected cells at 48 and 72 h (P<0.01; Fig. 6A). However, co-transfection with the miR-873 mimics and CMV-MCS/Bcl-2 vectors decreased the miR-873-induced sensitivity of the U87/DDP cells to cisplatin (Fig. 6A). The colony formation assay gave the same result as the MTT assay in the U87/DDP cells (Fig. 6B). Furthermore, we determined whether overexpression of Bcl-2 could impair...
the effect of miR-873 on sensitivity to cisplatin using flow cytometry and the caspase-3/7 activity assay. In the U87/DDP cells, decreases in the caspase-3/7 activity and apoptosis rate were observed in cells co-transfected with the miR-873 or NC mimics compared to co-transfected with the miR-873 mimics for 48 h (Fig. 6C and D). Taken together, these results indicated that re-expression of Bcl-2 was able to reduce the sensitivity-enhancing effect of miR-873 to cisplatin.

**Discussion**

Resistance to chemotherapeutic agents is a vital challenge in the treatment of glioma patients. In this study, we investigated the function of miR-873 on the cisplatin resistance of glioma cells. To our knowledge, this is the first report that miR-873 significantly decreased tumor chemoresistance, as measured by MTT assay, colony formation assay, flow cytometry, western blotting and caspase-3/7 activity assay. Furthermore, we demonstrated for the first time that miR-873 enhanced the sensitivity of cisplatin by targeting the mRNA 3’UTR of Bcl-2 and that miR-873 and Bcl-2 were inversely dysregulated in glioma. Thus, these results indicated that miR-873 could serve as a molecular target for regulating the glioma cell sensitivity to cisplatin.

Previous studies have shown that aberrant miRNA expression was implicated in the progression of chemoresistance in gliomas. Guo et al demonstrated that Let-7b was significantly decreased in cisplatin-resistant glioma cells compared to wild-type cells, and overexpression of Let-7b significantly increased cisplatin-induced apoptosis (10). Moreover, it has been reported that miR-106a was significantly upregulated in cisplatin-resistant and gefitinib-resistant glioma cells and that knockdown of miR-106a enhanced the temozolomide-induced apoptosis in glioma cells (11). Furthermore, it has...
Figure 5. Ectopic expression of Bcl-2 partially abrogates the effect of miR-873. (A) Western blot detection of Bcl-2 protein after transfection with CMV-MCS/Bcl-2 vector or CMV-MCS empty vector for 72 h in U87/DDP cells. β-actin was assayed as a loading control. (B) Cell proliferation was assessed for miR-873 mimics and the CMV-MCS/Bcl-2 or CMV-MCS vector transfected U87/DDP cells by MTT assay. (C) The effects of miR-873 on U87/DDP cell migration and invasion were examined by Transwell assay after transfected with the miR-873 or NC mimics and the CMV-MCS/Bcl-2 or CMV-MCS vector. (D and E) A representative field of cells was available at a magnification of x200. *P<0.05, **P<0.01. (F) The caspase-3/7 activity was assayed using a caspase-3/7 activity assay kit.

Figure 6. Enforced expression of Bcl-2 decreased the effect of miR-873 on cisplatin sensitivity. (A) After transfection with the miR-873 mimics and the CMV-MCS/Bcl-2 or CMV-MCS vector for 48 h, dose response curves of cisplatin in U87/DDP cells were measured by MTT assay. (B) U87/DDP cells were co-transfected with the miR-873 or NC mimics and the CMV-MCS/Bcl-2 or CMV-MCS vector. As indicated, the cells were plated in triplicate and exposed to the IC50 value of cisplatin for 48 h. Then the cells were washed with PBS and the cell medium were changed to fresh 10% fetal bovine serum (FBS) and cultured for another 2 weeks. (C) After co-transfection with the miR-873 or NC mimics and the CMV-MCS/Bcl-2 or CMV-MCS vector for 24 h, the U87/DDP cells were treated with cisplatin for another 48 h. The cell apoptosis rate was determined using flow cytometry. (D) The caspase-3/7 activity was assayed using a caspase-3/7 activity assay kit.
been shown that miR-136 and miR-139 were downregulated in human glioma tissues and cells, and overexpression of miR-136 or miR-139 promotes cisplatin-induced or temozolomide-induced apoptosis in glioma (12,13,18). Based on these observations, dysregulated expression of miRNA may determine the response of glioma cells to chemotherapy. In this study, we found that miR-873 was downregulated in high-grade glioma tissues compared to normal tissues and deceased in cisplatin-resistant glioma cells compared with wild-type cells. These results indicated that miR-873 may affect the sensitivity of glioma cells to cisplatin. However, the cause of the low expression of miR-873 in the cisplatin-resistant glioma cells remains to be determined in further studies.

To date, few studies have reported on the function of miR-873. Liu et al demonstrated that when stimulated by IL-17, miR-873 promotes the activation of NF-κB and the production of inflammatory cytokines in human multiple sclerosis (16). It has also been reported that miR-873 is involved in the regulation of the Hedgehog signaling and has relevance to physiological cranial bone development (20). miR-873 is downregulated in tamoxifen-resistant breast tumor cells, while overexpression of miR-873 reverses the tamoxifen resistance by targeting cyclin-dependent kinase 3 (CDK3) (15). More importantly, cluster analysis revealed that miR-873 is significantly downregulated in glioblastomas (17) and that ectopic expression of miR-873 reduces cell proliferation, migration and invasion of glioma cells by targeting IGF2BP1 (14). Here, we demonstrated that overexpression of miR-873 sensitized glioma cells to cisplatin by arresting cell proliferation and inducing apoptosis as indicated by the results of the MTT assay, colony formation assay, flow cytometry, western blotting and caspase-3/7 activity assay. However, whether miR-873 could sensitize glioma cells to other chemotherapeutic drugs remains to be determined.

Bcl-2, a key anti-apoptotic protein, functions as an oncogene on the basis that it promotes migration and invasiveness of glioma cells (21,22). Moreover, inhibition of Bcl-2 expression enhances the chemosensitivity of glioma cells, including sensitivity to cisplatin (23). Many studies have confirmed that Bcl-2 is an important target for miRNA in the regulation of the chemosensitivity of glioma cells (13,24,25). In this study, we used the luciferase assay to demonstrate that Bcl-2 was a functional target of miR-873. Ectopic expression of Bcl-2 could offset the miR-873-induced sensitivity of the glioma cells to cisplatin. Nevertheless, many more genes are targeted by miR-873, and the additional target genes need to be explored further.

In conclusion, we demonstrated that miR-873 was downregulated in cisplatin-resistant glioma cells compared to wild-type cells and that miR-873 enhanced the sensitization to cisplatin by targeting Bcl-2. Our data suggest that miR-873 might be a potential biomarker and a promising therapeutic strategy for cisplatin-resistant glioma cells.

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