

miR-501-3p sensitizes glioma cells to cisplatin by targeting MYCN

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Abstract. Cisplatin, a commonly used chemotherapeutic agent for glioma patients, treatment often leads to chemoresistance. Accumulating evidence has demonstrated that microRNA (miRNA/miR) is involved in drug resistance of glioma cells. Nevertheless, the role of miR-501-3p in glioma cell resistance to cisplatin is unclear. In the present study, it was revealed that miR-501-3p expression was decreased in glioma tissues and further underexpressed in cisplatin-resistant glioma cells compared with wild-type (WT) glioma cells. Furthermore, cisplatin treatment inhibited the level of miR-501-3p in a time-dependent way. Ectopic expression of miR-501-3p suppressed glioma cell growth and invasion, but increased cisplatin-resistant glioma cell apoptosis. Furthermore, miR-501-3p sensitized glioma cells to cisplatin-induced proliferation arrest and death. Mechanistically, it was demonstrated that miR-501-3p targeted MYCN in glioma cells. In addition, it was revealed that miR-501-3p inhibited MYCN expression by a luciferase reporter assay and reverse transcription-quantitative polymerase chain reaction. Notably, restoration of MYCN reversed the effects of miR-501-3p in cisplatin-resistant glioma cells. In conclusion, these results suggested that miR-501-3p may serve a promising marker for cisplatin resistance.

Introduction

Glioma has become the most frequent primary brain cancer among adults worldwide and contributes to a large amount of cancer-related deaths every year (1). Surgery combined with chemotherapy and radiotherapy is the major approach for glioma patients. However, resistance to chemoradiotherapy greatly attenuated the outcome. The five-year overall survival in glioma patients was very unsatisfactory (2). Most glioma patients died within 2 years after diagnosed (3). Therefore, it

is urgently required to understand the molecular mechanism underlying chemoresistance of gliomas cells.

microRNAs (miRNAs/miRs) belong to a group of small noncoding RNAs with a length of about 18-24 nucleotides (4). Evidences show that miRNAs could regulate gene expression via binding to target complementary site in the 3'-UTR region of specific mRNAs (5). More and more studies indicate that miRNAs possess extremely important functions in various biological processes, such as proliferation, death and migration (6,7). Differential expression of miRNAs in tumor and matched normal tissues has been observed in many cancers, such as glioma (8). In the past years, increasing reports show that miRNAs take part in the chemoresistance of glioma. For instance, miR-21 contributes to the resistance of glioma cells to camustine through inhibiting Spry2 mRNA levels (9). Both miR-324-5p and miR-524-5p target EZH2 to facilitate the growth and temozolomide resistance of glioma cells (10). miR-136 was reported to inhibit cisplatin chemosensitivity of glioma cells by suppressing E2F1 expression (11). Additionally, Let-7b regulates glioblastoma cell sensitivity to chemotherapy by targeting Cyclin D1 (12). Thus, it is critical to understand the correlation between miRNA and glioma chemoresistance.

miR-501-3p has been shown to regulate tumor development in hepatocellular carcinoma (13) and cervical cancer (14). However, the function of miR-501-3p in glioma is elusive. In the present study, we found that miR-501-3p expression was significantly downregulated in glioma tissues compared to matched normal tissues. Functionally, we found that miR-501-3p targeted MYCN, which contributes to the cisplatin-resistance of glioma cells. Overexpression of MYCN reversed the effects of miR-501-3p mimics on glioma cell resistance to cisplatin. In sum, our study demonstrated that miR-501-3p might be a potential target to solve cisplatin resistance in glioma.

Materials and methods

Patient tissues. A total of 31 pairs of glioma tissues and adjacent normal tissues were obtained between 2014 and 2016 from The Third People's Hospital of Linyi (Linyi, China). All samples were frozen in liquid nitrogen at -80°C until use. This study was approved by the ethics committee of The third People's Hospital of Linyi. Written informed consent was obtained from each enrolled patient.

Cell culture and transfection. The human glioma cell line U251 was purchased from American Type Culture Collection

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(ATCC). The cisplatin-resistant U251 cell line (U251/DDP) was obtained through adding increasing concentrations (from 0.1 to 10 $\mu\text{g/ml}$) of cisplatin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to the culture medium until the cell proliferation ability was similar to wild-type U251 cells. All cells were cultured in DMEM medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 $\mu\text{g/ml}$ streptomycin and 100 U/ml penicillin according to the manufacturer's instruction and maintained at 37°C in a 5% CO₂ humidified incubator.

miR-501-3p mimics and negative controls (NC) were purchased from GenePharma (Shanghai, China). MYCN coding sequence was cloned into pCDNA3 vector. miR-501-3p mimics, pCDNA2-MYCN and corresponding controls were transduced into glioma cells using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

Cell proliferation. For cell viability analysis, 3×10^3 U251 or U251/DDP cells transfected with miR-501-3p or control per well were seeded into 96-well plate and cultured for indicative times. Then MTT solution (0.5 $\mu\text{g/ml}$) (Sigma-Aldrich; Merck KGaA) was added for cellular viability analysis and incubated for 4 h at 37°C, followed by addition with 150 μl DMSO (Sigma-Aldrich; Merck KGaA). Immediately, the plate was vortexed for 30 min and absorbance at 570 nm was measured by spectrophotometer.

In vitro invasion assays. For transwell invasion assay, 1×10^4 U251 or U251/DDP cells in 200 μl DMEM medium were seed into the upper Matrigel-coated chamber with 8 μm pores in diameter (Corning Incorporated, Corning, NY, USA) of the 24-well plate. The lower chamber contains 600 μl DMEM medium supplemented with 10% FBS. After culture for 24 h, the cells in upper chamber were scrapped. Then the invasive cells in the lower chamber was fixed with methanol for 1 h and stained with 0.1% crystal violet for 15 min. Invasive cells were photographed using a light microscope (Olympus Corporation, Tokyo, Japan) at 100x magnifications.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Then 1 μg RNA templates were added for cDNA generation using a Reverse Transcription System kit (Takara Biotechnology Co., Ltd., Dalian, China). miR-501-3p and MYCN levels were analyzed utilizing SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) and the TaqMan miRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.), respectively. We used GAPDH as a normalized control for MYCN and U6 as a control for miR-501-3p. The results were calculated according to the $2^{-\Delta\Delta C_t}$ method (15).

Caspase-3/7 activity analysis. We used the Caspase-Glo3 assay kit (Promega Corporation, Madison, WI, USA) to analyze Caspase-3/7 activity in U251 and U251/DDP cells according to the manufacturer's instructions.

Luciferase reporter assay. To obtain the MYCN-wild-type (MYCN-WT) reporter, we cloned the 3'-UTR sequence of MYCN containing the binding site for miR-501-3p into pmiR-Reporter vector (Promega Corporation). As for the MYCN-mutant (MYCN-Mut) reporter, the putative binding site for miR-501-3p was mutated using Quik-Change™ Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). For dual luciferase reporter assay, 50 ng WT or mutant reporter plasmid and miR-501-3p mimics or negative control were transduced into U251/DDP cells in the 24-well plate using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. 24 h later after transfection, cells were harvested and assayed with the Dual-Luciferase Reporter Assay kit (Promega Corporation) according to the manufacturer's instruction.

Statistical analysis. All statistical analyses were performed utilizing SPSS 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test and one-way ANOVA followed by Tukey's post hoc test were used to analyze 2 or multiple groups, respectively, for statistical significance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-501-3p expression patterns in glioma. To analyze the function of miR-501-3p in glioma for drug resistance, we first checked the levels of miR-501-3p in pairs of glioma tissues and matched normal tissues by RT-qPCR. The results indicated that miR-501-3p expression was significantly downregulated in glioma tissues compared to adjacent normal tissues (Fig. 1A). Then, we generated a cisplatin-resistant glioma cell line named U251/DDP. We measured the IC₅₀ for WT U251 and U251/DDP cells. The results indicated that the IC₅₀ in U251/DDP cells was significantly higher than that in WT cells (Fig. 1B). We measured the expression of miR-501-3p in U251 and U251/DDP cells by RT-qPCR and found that miR-501-3p expression was lower in U251/DDP cells than that in U251 cells (Fig. 1C), suggesting miR-501-3p might regulate the resistance to cisplatin. Thus, we further assessed the effects of cisplatin on miR-501-3p expression in U251 cells. miR-501-3p expression was significantly decreased in a time-dependent manner after treatment with cisplatin (5 $\mu\text{g/ml}$) (Fig. 1D). Above data indicated that miR-501-3p might regulate glioma cell resistance to cisplatin.

miR-501-3p inhibits proliferation and invasion of U251/DDP cells while promoting apoptosis. To determine the function of miR-501-3p on cisplatin-resistant glioma cells, we transfected U251/DDP cells with 50 nM miR-501-3p mimics or negative control (NC). 48 h later, we measured miR-501-3p expression and found that miR-501-3p was significantly upregulated in U251/DDP cells transfected with miR-501-3p mimics (Fig. 2A). Then, we examined cell proliferation by MTT assay. The results illustrated that miR-501-3p overexpression significantly inhibited cell proliferation (Fig. 2B). Besides, miR-501-3p ectopic expression promoted the apoptosis of U251/DDP cells as shown by increased caspase-3/7 activity (Fig. 2C). Moreover, we examined the effect of miR-501-3p on

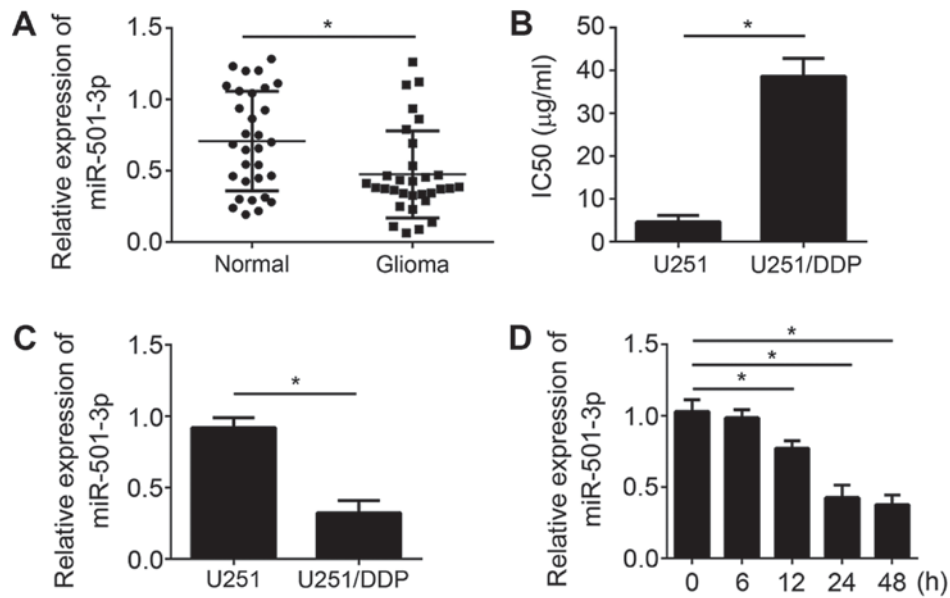


Figure 1. miR-501-3p expression patterns in glioma. (A) miR-501-3p expression was downregulated in glioma tissues compared to adjacent normal tissues. (B) The IC50 values of U251 and U251/DDP cells were measured. U251/DDP cells show higher IC50 value. (C) qPCR detection for miR-501-3p expression in U251 and U251/DDP cells. U251/DDP cells showed lower level of miR-501-3p. (D) Cisplatin (5 μg/ml) administration decreased miR-501-3p expression in U251 cells. *P<0.05.

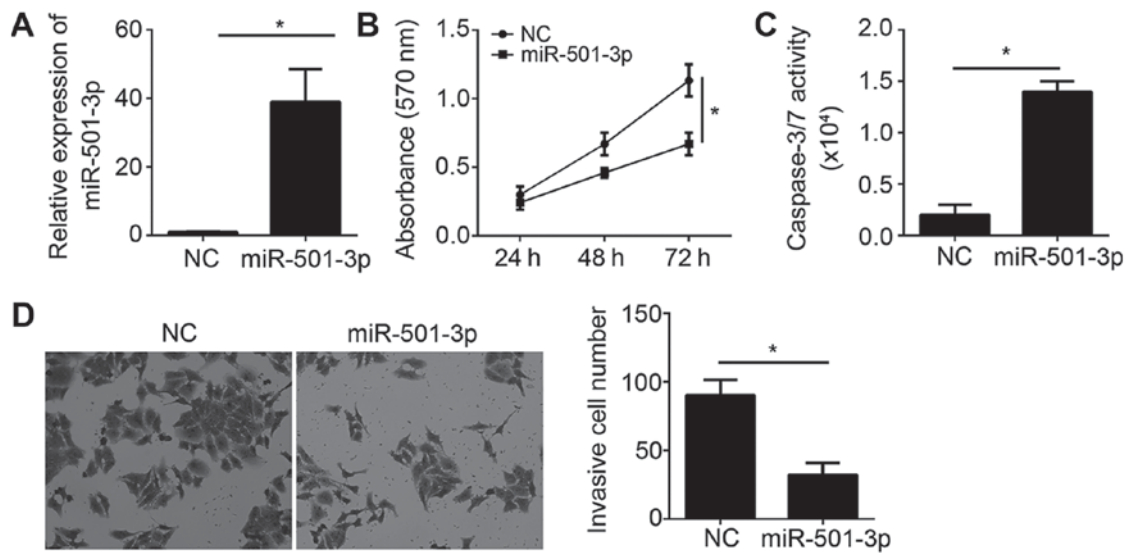


Figure 2. miR-501-3p inhibited proliferation and invasion of U251/DDP cells while promoting apoptosis. (A) miR-501-3p expression was elevated in U251/DDP cells after transfection with the miR-501-3p. (B) MTT assay showed that miR-501-3p overexpression inhibited the proliferation of U251/DDP cells. (C) miR-501-3p promoted caspase-3/7 activity in U251/DDP cells. (D) The effect of miR-501-3p on U251/DDP cell invasion was examined by Transwell assay (x200 magnification). *P<0.05.

cell invasion by transwell assay. The results showed that overexpression of miR-501-3p reduced the invasive cell number in U251/DDP cells (Fig. 2D). Collectively, our data indicated that miR-501-3p served as a tumor suppressor and might be related with cisplatin resistance of glioma cells.

miR-501-3p sensitizes glioma cells to cisplatin. To explore the role of miR-501-3p on cisplatin resistance, miR-501-3p mimics was transduced into U251 and U251/DDP cells. Firstly, we conducted MTT assay to assess the effect of miR-501-3p on glioma cell proliferation. After transfection with miR-501-3p for 24 h, cisplatin was added and incubated for another 24-72 h.

Then cell viability was measured. The results of MTT assay revealed that miR-501-3p significantly enhanced the suppressive effect of cisplatin on both U251 and U251/DDP cell proliferation (Fig. 3A and B). Moreover, the caspase-3/7 activity assay showed that an upregulated activity of caspase-3/7 in miR-501-3p mimic-transfected U251 and U251/DDP cells was observed when exposed to cisplatin (Fig. 3C and D). Taken together, these results suggested that miR-501-3p promoted the sensitivity of glioma cells to cisplatin.

MYCN is a target of miR-501-3p. It has been acknowledged that miRNAs play roles via regulating target gene expression (13).

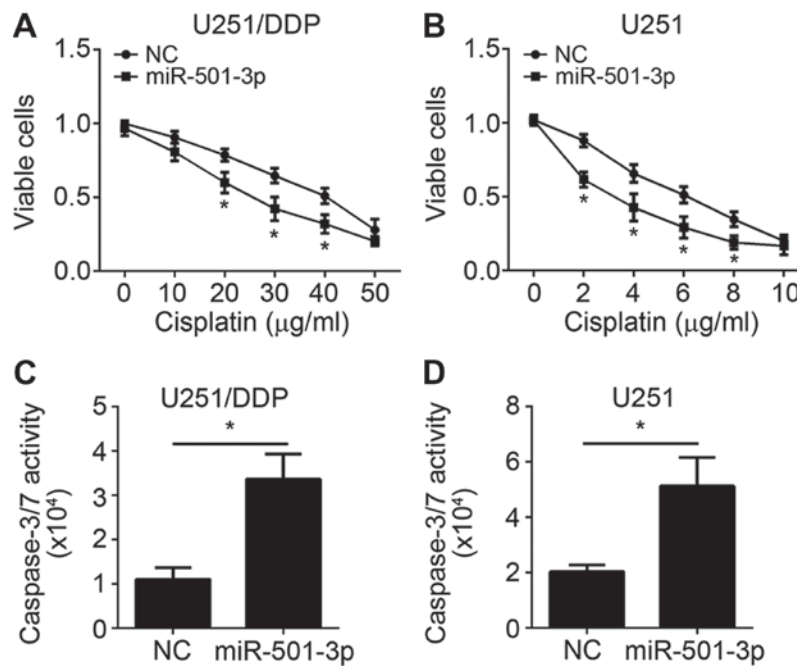


Figure 3. miR-501-3p sensitized glioma cells to cisplatin. After transfection with the miR-501-3p or NC for 48 h, dose response curves of cisplatin in (A) U251/DDP and (B) U251 cells were measured by MTT assay. Overexpression of miR-501-3p inhibited cell viability in both U251/DDP and U251 cells. miR-501-3p overexpression promoted the caspase-3/7 activity in (C) U251/DDP and (D) U251 cells. *P<0.05.

Hence, we predicted the targets of miR-501-3p by TargetScan. We identified MYCN as the most potential target of miR-501-3p. There was a potential binding site of miR-501-3p of MYCN in the 3'-UTR of MYCN (Fig. 4A). To validate it, we performed luciferase reporter assay. The 3'-UTR region containing the WT or mutant (Mut) potential binding site was cloned into pmiR-Reporter vector. The results showed that overexpression of miR-501-3p significantly repressed the luciferase activity of MYCN-WT in U251/DDP cells but not MYCN-Mut (Fig. 4B), suggesting that there was a direct interaction between MYCN and miR-501-3p. Furthermore, RT-qPCR analysis showed that overexpression of miR-501-3p inhibited the mRNA level of MYCN in U251/DDP cells (Fig. 4C). Consistently, western blot result indicated that ectopic expression of miR-501-3p led to reduced protein levels of MYCN in both U251 and U251/DDP cells (Fig. 4D).

Ectopic expression of MYCN reverses the effects of miR-501-3p. To explore whether MYCN expression is responsible for the function of miR-501-3p in glioma cells, we overexpressed MYCN in U251/DDP cells (Fig. 5A). Then we performed MTT assay and found that restoration of MYCN significantly increased cell proliferation in U251/DDP cells transfected with miR-501-3p mimics (Fig. 5B). Besides, re-expression of MYCN abrogated the pro-apoptotic effect of miR-501-3p on U251/DDP cells (Fig. 5C). Furthermore, transwell assay also showed that MYCN overexpression rescued the invasive ability of U251/DDP cells (Fig. 5D). Taken together, these results demonstrated that MYCN was a functional target of miR-501-3p in glioma.

Discussion

Resistance to chemotherapeutic agents remains a major problem for the treatment of glioma patients. The molecular

mechanism underlying resistance to chemotherapeutic agents is largely unknown. In the present study, we explored the role of miR-501-3p on cisplatin resistance in glioma cells. Our study is the first time to show that miR-501-3p was significantly downregulated in glioma tissues and involved in tumor chemoresistance. We found that miR-501-3p expression was further decreased by cisplatin treatment. Through MTT, caspase-3/7 activity and transwell assays, we showed that miR-501-3p overexpression suppressed cisplatin-resistant glioma cell proliferation and invasion while promoting apoptosis. Moreover, we for the first time illustrated that miR-501-3p enhanced the sensitivity of glioma cells to cisplatin by targeting the 3'-UTR region of MYCN mRNA. Taken together, these results demonstrated that miR-501-3p might be a promising target for overcoming the resistance of glioma to chemotherapeutic agents.

Previous studies have demonstrated that aberrant expression of miRNAs is implicated in the process of chemoresistance in glioma. For instance, miR-223 influences glioblastoma cell growth and TMZ resistance by modulating PI3 K/Akt signaling (16). miR-101 regulates glioblastoma cell resistance to temozolomide through inhibiting GSK3β (17). miR-29c decreased the resistance of glioma cells to temozolomide via inhibiting O6-methylguanine-DNA methyltransferases (18). miR-873 sensitizes glioma cell to cisplatin by inhibiting Bcl-2 (1). miR-203 is involved in chemoresistance in human glioblastoma through targeting SNAI2 (19). Furthermore, other studies also indicated that miR-139 and miR-136 were under-expressed in glioma tissues and enhances cisplatin-induced or temozolomide-induced apoptosis of glioma (20). These evidences together showed that dysregulated miRNA expression might affect the response of glioma cells to chemotherapy. To date, the role of miR-501-3p is poorly understood. Luo *et al* showed that miR-501-3p inhibits liver cancer growth and metastasis via targeting LIN7A (13). Sanches *et al* indicated that

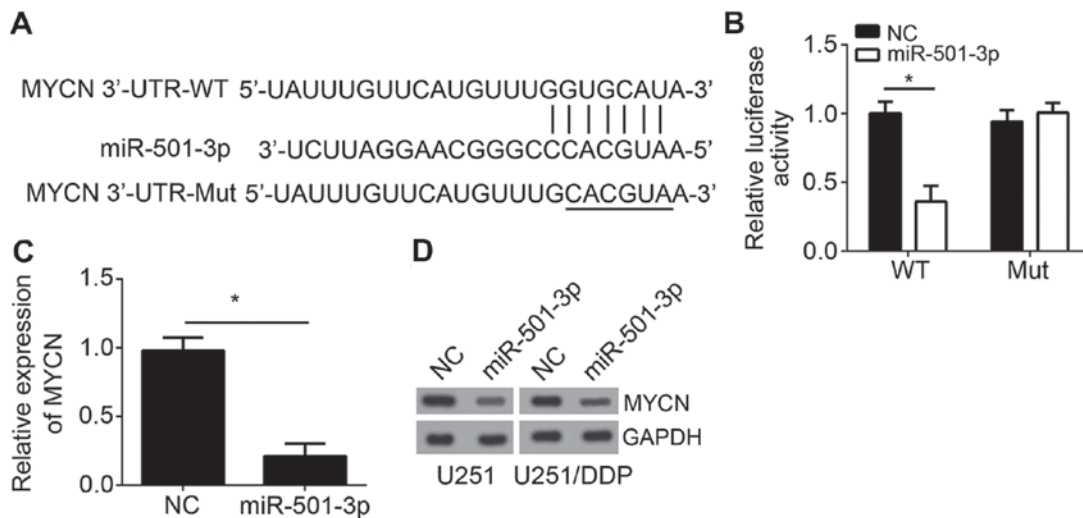


Figure 4. MYCN was a target of miR-501-3p. (A) Diagram of the seed sequence of miR-501-3p matched the 3'-UTR of MYCN (MYCN-3'-UTR-WT) and positions of the mutated nucleotides in the 3'-UTR of MYCN (MYCN-3'-UTR-Mut). (B) Luciferase reporter assay showed that miR-501-3p overexpression inhibited the activity of MYCN-WT reporter in U251/DDP cells (C) RT-qPCR indicated that miR-501-3p overexpression inhibited the mRNA level of MYCN in U251/DDP cells. (D) Western blot analysis indicated that miR-501-3p overexpression suppressed the protein level of MYCN in U251 and U251/DDP cells. *P<0.05.

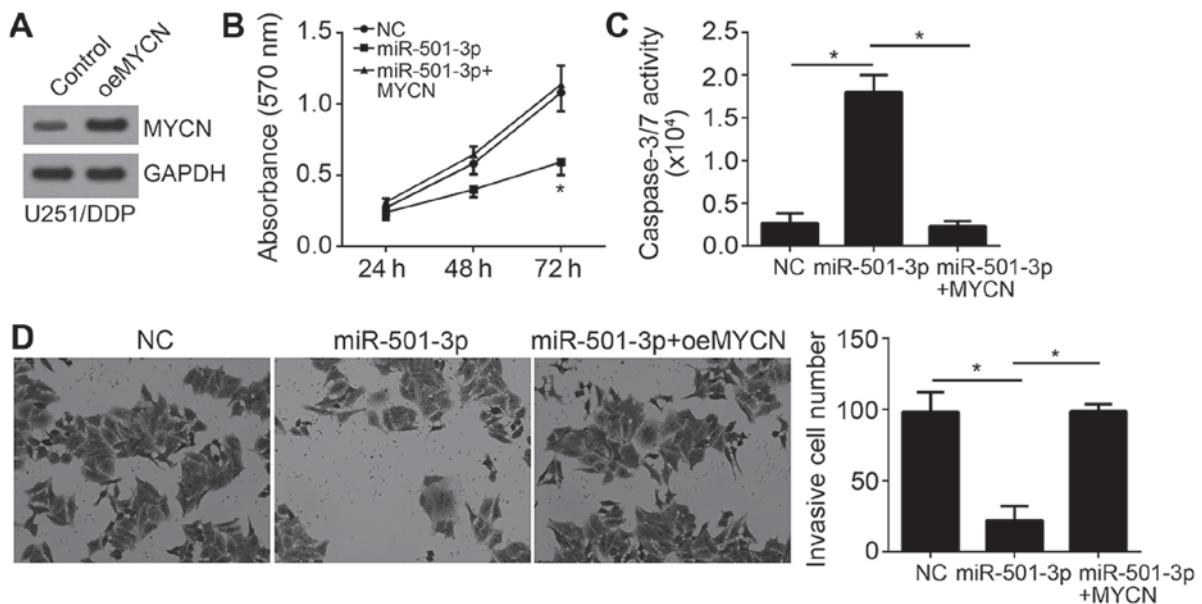


Figure 5. Ectopic expression of MYCN reversed the effects of miR-501-3p. (A) MYCN protein level was elevated after transfection with pCDNA3-MYCN for 72 h in U251/DDP cells. (B) Cell proliferation was assessed in U251/DDP cells transfected with miR-501-3p mimics and pCDNA3-MYCN vector by MTT assay. (C) MYCN overexpression decreased the caspase-3/7 activity in U251/DDP cells transfected with miR-501-3p. (D) MYCN overexpression promoted the invasion of U251/DDP cells transfected with miR-501-3p (x200 magnification). *P<0.05.

miR-501 overexpression enhances proliferation and metastasis of cervical cancer cells through inhibiting CYLD (14). However, the role of miR-501-3p in glioma requires investigation. In our study, we showed that miR-501-3p was downregulated in glioma tissues compared to adjacent normal tissues and further decreased in cisplatin-resistant glioma cell line. Our results also demonstrated that miR-501-3p regulates the sensitivity of glioma cells to cisplatin. Nevertheless, the mechanism regulating miR-501-3p downregulation during cisplatin treatment in glioma remains to be determined. Moreover, to better confirm the correlation between miR-501-3p expression and cisplatin resistance, it is meaningful to obtain more clinical data

of the glioma patients. Furthermore, whether every patient with low miR-501-3p expression shows cisplatin resistance requires investigation in future.

MYCN is a classical oncogene in various cancers, such as hepatocellular carcinoma (21) and prostate cancer (22). Besides, MYCN is also reported to be an oncogenic driver and serve as a worst prognostic biomarker in neuroblastoma (23,24). Bjerke *et al* showed that upregulation of MYCN promotes glioblastoma progression (25). In our study, we utilized luciferase reporter assay to validate that MYCN was a direct target of miR-501-3p. Overexpression of MYCN could significantly offset the miR-501-3p-induced inhibition of

cisplatin-resistant glioma cells on cell proliferation and invasion. However, more genes targeted by miR-501-3p require to be investigated further.

In conclusion, we for the first time demonstrated that miR-501-3p was underexpressed in cisplatin-resistant glioma cells and promotes the sensitization of cisplatin through targeting MYCN. Our findings suggest that miR-501-3p might serve as a promising biomarker and target for cisplatin-resistant glioma therapy.

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Availability of data and materials.

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CZ designed this study, performed the experiments, and analyzed and interpreted the results. XW conceived this study and wrote this manuscript. FY, YL, YS and XL performed certain experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

For the use of human samples, the protocol for the present study was approved by the Institutional Ethics Committee of The third People's Hospital of Linyi and all enrolled patients signed a written informed consent document.

Patient consent for publication

All patients within this study provide consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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