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MiR-205 enhances cisplatin sensitivity of glioma cells by targeting E2F1

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Abstract. – OBJECTIVE: miR-205 has been previously identified as a diagnostic and prognostic factor in glioma. However, its exact functions in glioma remain unclear. The current research aimed to decipher the role of miR-205 in the development of cisplatin resistance in glioma cells.

MATERIALS AND METHODS: miR-205 expressions in both cisplatin sensitive and resistant cell lines were compared by the Real-time PCR method. The dose-response to cisplatin of U87/DDP cells was determined by MTT assay. Cell cycle and apoptosis were determined by flow cytometry, caspase 3/7 activity assay and Western blot assay. The direct repression of E2F1 by miR-205 was confirmed by luciferase assay and Western blot assay.

RESULTS: miR-205 expression was decreased in cisplatin resistant glioma cell lines, and cisplatin treatment led to a decrease of miR-205 in glioma cells. Overexpression of miR-205 in U87/DDP restored its cisplatin sensitivity by enhancing apoptosis and G1/S cell cycle arrest; notably, all these effects were then partially abrogated by E2F1 overexpression. Luciferase assay and Western blot assay confirmed E2F1 as the direct target of miR-205 in U87/DDP cells.

CONCLUSIONS: These findings suggest that down-regulation of miR-205 confers the cisplatin resistance in glioma cells via upregulation of E2F1. It might serve as a candidate for glioma therapy.

Key Words:
Glioma, miR-205, E2F1, Cell cycle, Apoptosis, Drug resistance.

Introduction

Glioma, the most prevalent primary tumor in the central nervous system, is one of the most deadly malignant diseases[1]. There is no effective treatment for glioma currently. Patients suffering from high grade glioma can only have a median survival of approximately 15 months[2]. Chemotherapy is conventionally used to eliminate cancer cells in combination with surgery and radiotherapy. However, glioma cells have strong ability to acquire drug resistance during treatment course[3-5]. Understanding the molecular basis underlying the drug resistant phenotype is, therefore, imperative and important for the establishment of new strategies for chemoresistance in its clinical treatment. MicroRNAs, which belong to a class of short non-coding RNAs, are important regulators implicated in cell proliferation, cell differentiation and cell death[6]. Deregulation of microRNA expression is a common feature in multiple types of cancer. Changes of subsets of important microRNAs alter cancer cell properties, thus, profoundly influencing the cancer development and progression[7,8]. Although it has been previously shown that microRNAs are implicated in the various aspects of tumor biology of glioma including angiogenesis, invasion, and cell metabolism[9], the functional significance of microRNAs in chemoresistance of glioma has not been fully addressed. It has been previously shown that a putative tumor suppressive microRNA, miR-205, is downregulated in the serum of patients suffering from glioma[10]. Particularly, miR-205 expression is associated with glioma progression and predicts its prognosis[10,11]. These findings strongly suggest a potential role of miR-205 in drug resistance, a likely cause for the aggressive phenotype. The current study aimed to test whether the decreased level of miR-205 in glioma tissues has a functional link to tumor cell behaviors such as drug resistance. To this end, we assessed miR-205 expression in cisplatin resistant glioma cells; additionally, multiple experimental approaches were utilized to explore its function and mechanism. Our data support that cisplatin resistant glioma cells have a signature of down-regulated miR-205 level, and exogenous miR-205 is sufficient to induce cell cycle arrest and apoptosis in these drug resistant cells to restore cisplatin sensitivity. Moreover, we provide evidence that

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these effects are mediated by the repression of E2F1, a target gene of miR-205. These findings uncover miR-205 as key element in determining cisplatin sensitivity in glioma. Thus, it may serve as an attractive candidate for glioma therapy.

Materials and Methods

Cell Culture, Treatment and Transfection

The cisplatin sensitive and resistant cell lines for glioma, U87 and U87/DDP, were purchased from KeygeneBio (Nanjing, China), and the U251 glioma cell line was purchased from 4ABio (Beijing, China). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) HyClone (South Logan, UT, USA) with 10% fetal bovine serum (FBS) HyClone (South Logan, Utah, USA), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Beyotime, Shanghai, China). Cells were cultured at 37°C in a humidified atmosphere. To ectopically express miR-205 in U87/DDP cells, miR-205 mimic (Genepharma, Shanghai, China) was transfected into cells (70% confluence) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the specification at 150 nM. 48 h after transfection, cells were subjected to cisplatin treatment as indicated. pCMV-E2F1 plasmid and the empty vector control were purchased from Origene (Rockville, MD, USA), and transfected with miR-205 mimic at 0.5 μg/ml.

Real-time PCR

Cells grown in 6-well plates were harvested with TRIzol reagents (Invitrogen, Carlsbad, CA, USA), followed by a standard procedure provided by the manufacture to isolate total RNA. The RNA samples were then washed with ethanol and dissolved in DEPC treated ddH_2O. The reversed transcription was conducted using the ReverTra Ace qPCR RT Master Mix (Toyobo, Tokyo, Japan), and cDNAs were amplified on an ABI 7000 system (Applied Biosystems, Forster City, CA, USA). U6 expression was used for normalization, and primers were purchased from Ribobio (Guangzhou, China).

Cell Viability Assay

Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, after being transfected with oligonucleotides and/or plasmids, U87/DDP cells were subjected to cisplatin treatment for 48 h. MTT solution 5 mg/ml was added to each well of the 96-well plates for 4 h. After incubation, 100 μl dimethyl sulfoxide (DMSO) were added to each well. The formazan was then quantified by measuring the 570 nm absorbance. Cells were plated at 5×104 per milliliter.

Luciferase Assay

PCR was used to amplify an approximate 600 bp 3' untranslated region (UTR) sequence in E2F1 mRNA, which contains the predicted binding site of miR-205. The sequence was then subcloned into pmiRGLO (Promega, Madison, WI, USA) plasmid. Mutant luciferase plasmid was constructed with the same manner, except that the insertion sequence was mutated with the Quik-Change™ Site-Directed Mutagenesis kit (Stratagene, Berkeley, CA, USA) as we designed. For luciferase assay, U87/DDP cells were grown at 5×104 per milliliter in 24-well plates, and transfected with miR-205 or negative control and wild type or mutant luciferase plasmid. 48 h after transfection, luciferase activities were measured using the Dual luciferase assay system (Promega, Madison, WI, USA).

Western Blot Analysis

U87/DDP or U87 cells were grown in 6-well plates; after the treatment as indicated above, cells were harvested with Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime, Shanghai, China). The cell lysates were sonicated and centrifuged at 15000 g for 15 min. The insoluble materials were discarded. Proteins were degenerated in Sodium dodecyl sulfate (SDS) sample buffer, followed by separating on a SDS-polyacrylamide gelelectrophoresis (PAGE) gel using electrophoresis. After that, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich, St. Louis, MO USA), and incubated with primary antibodies against E2F1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Cleaved-PARP (Cell Signaling Technology, Danvers, MA USA) and β-actin (Sigma-Aldrich, St. Louis, MO USA) overnight at 4°C. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. Protein expression was then determined using an ECL kit (CWBio, Beijing, China).

Cell Cycle Analysis

Cell cycle was determined using flow cytometry, treated U87/DDP cells were fixed in cold

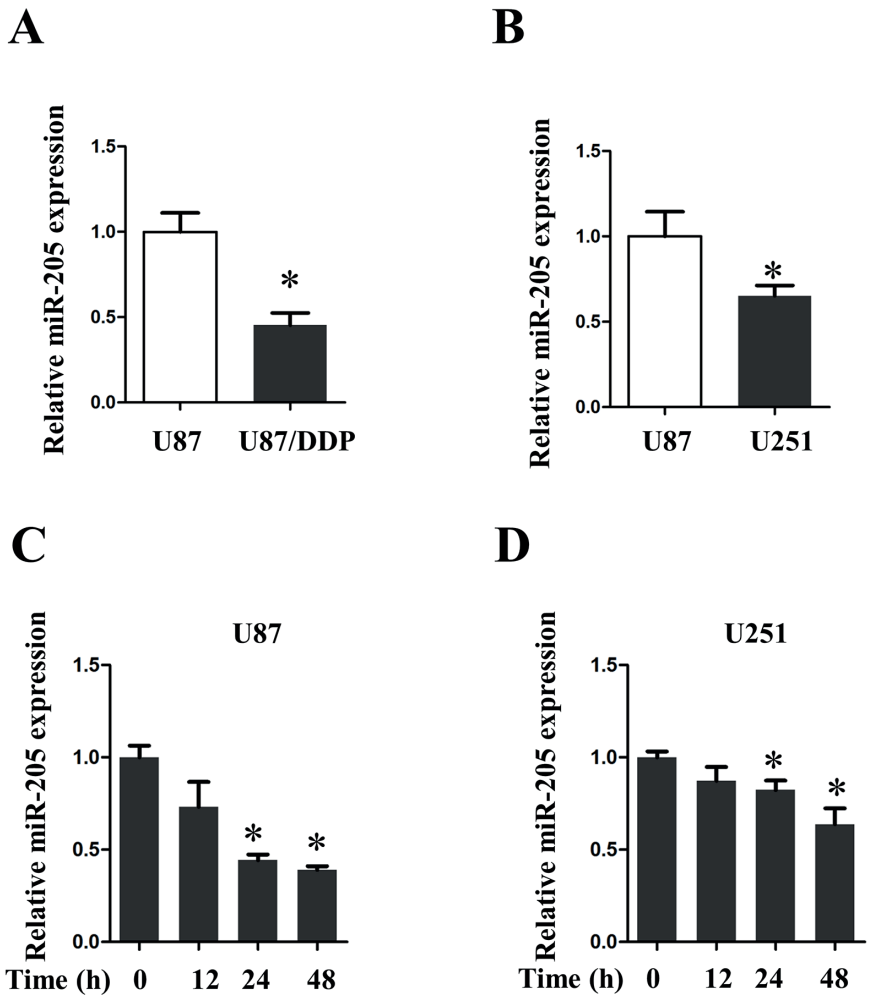

Figure 1. MiR-205 expression is lower in cisplatin resistant glioma cells and cisplatin treatment decreases its expression. ***(A)*** The expression of miR-205 in U87 and U87/DDP cells. $^{*}p<0.05$ vs. U87. ***(B)*** The expression of miR-205 in U87 and U251 cells. $^{*}p<0.05$ vs. U87. ***(C)*** The expression of miR-205 in U87 cells after cisplatin treatment (5 μg/ml) at 12, 24 and 48 h. $^{*}p<0.05$ vs. 0 h. ***(D)*** The expression of miR-205 in U251 cells after cisplatin treatment (5 μg/ml) at 12, 24 and 48 h. $^{*}p<0.05$ vs.0 h.

ethanol before staining with propidium iodide (PI) solution (50 μg/ml, Beyotime, Shanghai, China) which contains 500 μg/ml RNase A (Beyotime, Shanghai, China). Cell cycle was analyzed on a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ, USA).

Apoptosis Assay

Apoptosis was determined using a kit for measuring the Caspase-3/7 activity (Invitrogen, Carlsbad, CA, USA) according to the instruction.

Statistical Analysis

Data were shown as means± Standard Error Mean (SEM), and pairwise comparisons were analyzed by Student's *t*-test; a two tailed $p<0.05$ was considered statistically significant. All the statistics and graphs were performed and plotted by GraphPad Software 5.0 (La Jolla, CA, USA).

Results

MiR-205 Expression is Lower in Cisplatin Resistant Glioma Cells and Cisplatin Treatment Decreases its Expression

Previous studies have proposed a critical diagnostic and prognostic function of miR-205 in glioma. We, therefore, asked whether miR-205 plays a role in drug resistance in glioma cells. By Real-time PCR analysis, we revealed that miR-205 expression was significantly lower in U87/DDP cells compared with that in its parental cells, U87 (Figure 1A). It was previously shown

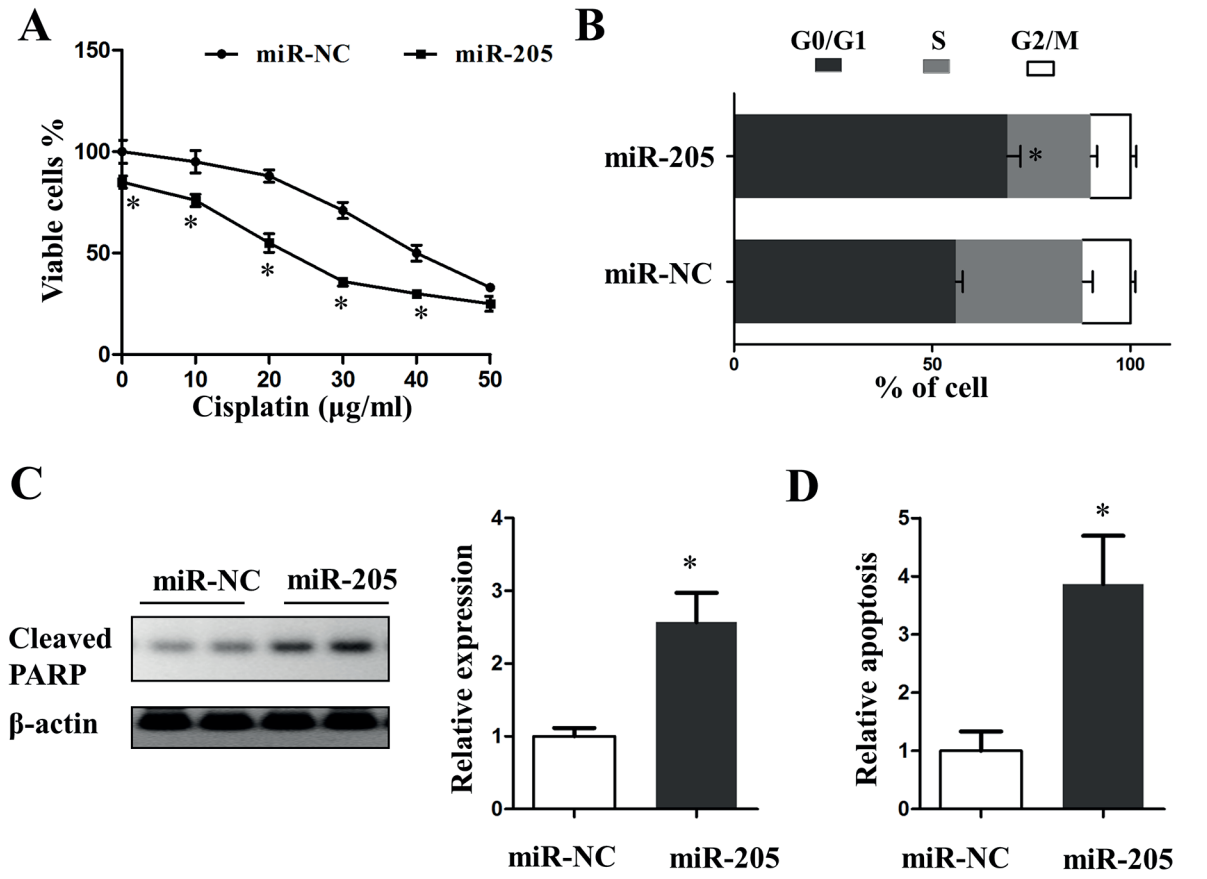

Figure 2. Overexpression of miR-205 restores cisplatin resistance in U87/DDP cells by enhancing apoptosis and cell cycle arrest. ***(A)*** The cell viability of U87/DDP cells transfected with miR-NC and miR-205, cells were exposed to cisplatin at various doses for 48 h. $^{*}p<0.05$ vs. miR-NC. ***(B)*** Cell cycle distribution of U87/DDP cells transfected with miR-NC and miR-205, cells were treated with 20 μg/ml cisplatin for 48 h. $^{*}p<0.05$ vs. miR-NC for G0/G1 phase. ***(C)*** Expression of cleaved-PARP in U87/DDP cells transfected with miR-NC and miR-205 was examined by Western blot, cells were treated with 20 μg/ml cisplatin for 48 h. $^{*}p<0.05$ vs. miR-NC. ***(D)*** Apoptosis in U87/DDP cells transfected with miR-NC and miR-205 was measured by caspase 3/7 activity assay. Cells were treated with 20 μg/ml cisplatin for 48 h. $^{*}p<0.05$ vs. miR-NC.

that U251 glioma cells are relatively more resistant to cisplatin compared with U87 cells[12]. We thus compared the miR-205 expression in these two cells, and we observed that miR-205 expression in U251 was lower than that in U87 cells (Figure 1B). Importantly, treatment of cisplatin in U87 and U251 cells time dependently reduced miR-205 expression (Figure 1C and D). These data suggest a positive correlation with miR-205 expression and cisplatin sensitivity, and miR-205 may have important function in regulating the cisplatin responsiveness in glioma cells.

Overexpression of miR-205 Restores Cisplatin Resistance in U87/DDP Cells by Enhancing Apoptosis and cell Cycle Arrest

To assess the role of miR-205 in cisplatin sensitivity of glioma cells, we overexpressed miR-205 in U87/DDP cells, and analyzed how cells responded to cisplatin. Cell viability assay has shown that the anti-proliferation effect of cisplatin was greatly improved in cells transfected with miR-205 mimic, compared with negative control (miR-NC) transfected cells (Figure 2A). To confirm this effect in detail, we employed flow cytometry to analyze cell cycle. We found that overexpression of miR-205 resulted in more cells in G0/G1 phase and fewer cells in S phase when treated with cisplatin, suggesting a G1/S cell cycle arrest (Figure 2B). Western blot analysis for cleaved PARP expression and the data of caspase 3/7 activities revealed that the apoptosis rate is increased in miR-205 transfected U87/DDP cells (Figure 2C and D). These data indicate that miR-205 is involved in the regulation of cisplatin sensitivity in glioma. Increasing its expression enhances apoptosis and cell cycle arrest in cisplatin resistant glioma cells.

E2F1 is a Target of miR-205 in Glioma Cells

The effects of microRNAs on cell behaviors are achieved by their direct repression on target genes. Next, we searched for the seed sequence matching 3'UTRs of miR-205 with the online tool miRanda and found a free energy favorable sequence in the 3'UTR region of E2F1 mRNA (Figure 3A). We tested the possibility of miR-205-E2F1-3'UTR binding using a luciferase assay. We constructed luciferase plasmid containing wild type E2F1 3'UTR or mutant E2F1 3'UTR that had the putative seed sequence binding site mutated (Figure 3A). U87/DDP cells co-transfected with miR-205 and wild type E2F1-3'UTR exhibited a much lower luciferase activity compared with those co-transfected with miR-NC and wild type E2F1-3'UTR. However, miR-205 did not change the luciferase activity in mutant E2F1-3'UTR transfected cells (Figure 3B). We also found that U87 has a higher E2F1 expression compared with its parental cell line (Figure 3C), and transfection of miR-205 indeed decreased E2F1 expression in U87/DDP cells (Figure 3D). These data strongly suggest that E2F1 is a direct target of miR-205 in glioma.

E2F1 Mediates the Effect of miR-205 on Cisplatin Resistance in Glioma Cells

We re-expressed E2F1 in U87/DDP cells to assess whether E2F1 mediates the effect of miR-205 on cisplatin resistance. Cell viability assay revealed that E2F1 partially abrogated the increase of cisplatin sensitivity by miR-205 (Figure 4A). Importantly, the enhancement of cell cycle arrest and apoptosis by miR-205 was also attenuated by E2F1 transfection (Figure 4B-D). These data suggest that miR-205 restores cisplatin sensitivity in U87/DDP glioma cells by down-regulating E2F1.

Discussion

Cisplatin is one of the commonly used chemotherapeutic agents in the treatment of glioma. Drug resistance confers a considerable part of the poor therapeutic effect for this malignancy3-5. However, changes of the genetic or epigenetic that underlie the acquirement of drug resistance are not fully addressed. In this work, we identified a novel regulator of cancer, miR-205, to be involved in the cisplatin resistance of glioma. We demonstrate that miR-205 expression is po-

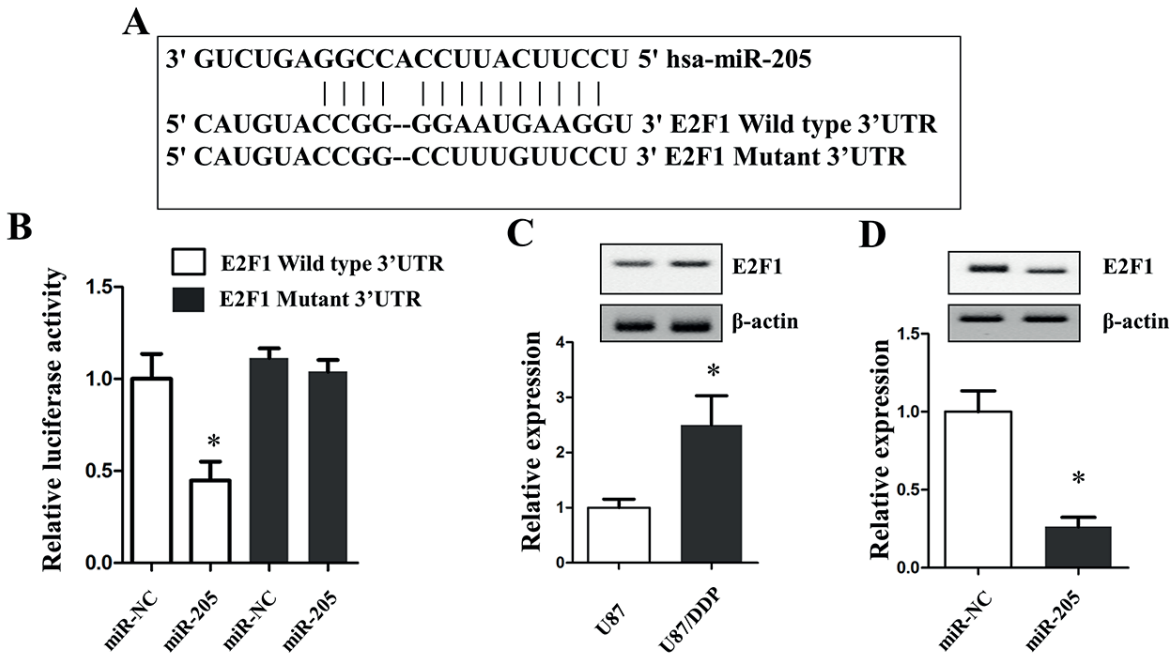

Figure 3. E2F1 is a target of miR-205 in glioma cells. ***(A)*** The predicted binding site of miR-205 in E2F1 3'UTR and the generation of mutagenesis in the seed sequence binding site. ***(B)*** Relative luciferase activity of the E2F1 -wild type -3'UTR and E2F1-mutant-3'UTR reporter in miR-NC or miR-205 transfected U87/DDP cells. $^{*}p<0.05$ vs. miR-NC+E2F1-wild type-3'UTR. ***(C)*** The expression of E2F1 in U87 and U87/DDP cells. $^{*}p<0.05$ vs. U87. ***(D)*** The expression of E2F1 in miR-NC and miR-205 transfected U87/DDP cells. $^{*}p<0.05$ vs. miR-NC.

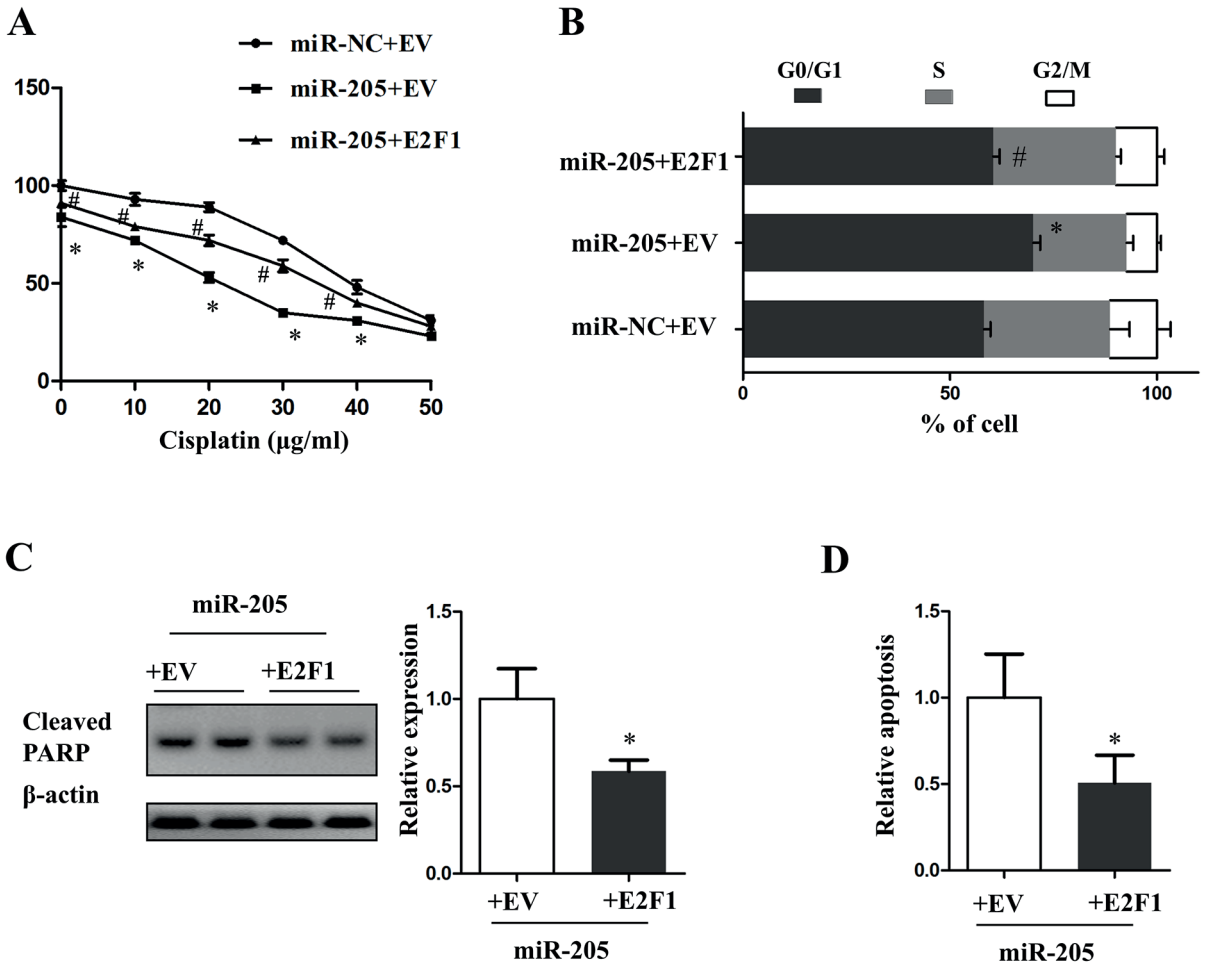

Figure 4. E2F1 mediates the effect of miR-205 on cisplatin resistance in glioma cells. ***(A)*** The effect of E2F1 on miR-205 regulated dose-response to cisplatin in U87/DDP cells. Cells were transfected with miR-NC+EV, miR-205+EV and miR-205+E2F1, and exposed to cisplatin at various doses for 48 h. $^{*}p<0.05$ vs. miR-NC+EV; #p<0.05 vs. miR-205+EV; EV, empty vector. ***(B)*** The effect of E2F1 on miR-205 enhanced cell cycle arrest. Cells were transfected with miR-NC+EV, miR-205+EV and miR-205+E2F1, and exposed to 20 μg/ml cisplatin for 48 h. $^{*}p<0.05$ vs. miR-NC+EV; #p<0.05 vs. miR-205+EV. ***(C)*** Expression of cleaved-PARP in U87/DDP cells transfected with miR-205+EV and miR-205+E2F1 was examined by Western blot. Cells were treated with 20 μg/ml cisplatin for 48 h. *p<0.05 vs. miR-205+EV. ***(D)*** Apoptosis in U87/DDP cells transfected with miR-205+EV and miR-205+E2F1 was measured by caspase 3/7 activity assay. Cells were treated with 20 μg/ml cisplatin for 48 h. *p<0.05 vs. miR-205+EV.

sitively correlated with the sensitivity to cisplatin in glioma cells. miR-205 has the ability to restore cisplatin sensitivity in U87/DDP cells by promoting apoptosis and inducing G1/S cell cycle arrest. Investigating the possible molecular mechanisms, we show that miR-205 targets and suppresses E2F1, an essential factor involved in cell cycle transition. In addition, exogenous E2F1 expression partially abrogated the drug sensitizing effect of miR-205. Our data reveal a significance novel role of the miR-205/E2F1 signal in cisplatin resistance of glioma cells, demonstrating the possible application of miR-205 as a cisplatin sensitizer in glioma treatment. Previous studies have highlighted the essential roles in the progression of glioma by affecting the tumor cell invasiveness, metabolism and angiogenesis[9]. Although the involvement of microRNAs in the drug resistance to chemotherapeutic agents has been less reported, they may exert indispensable effects in the phenotypic changes to these therapeutic agents. For example, the well-characterized oncogene, miR-21, is required for glioma cells to maintain the TRAIL- resistant phenotype13. miR-136 has been shown to be involved in the drug resistance of cisplatin and temozolomide[14,15]. It has also

been shown that the miR-9*/SOX2 pathway mediates the chemoresistance driven by ID4[16]. These observations indicated the broad implication of microRNAs in determining the cellular responses to chemotherapy. It was previously identified that miR-205 serves as either tumor suppressive or enhancing factors in cancer types from other systems[17-22]. There is still lack of experimental evidence for miR-205 in glioma. Yue et al[10] investigateded the serum samples collected from glioma patients and reported that miR-205 was lower in patient samples compared with that in healthy controls. Importantly, miR-205 expression was positively associated with the survival of patients with the advanced pathological grade glioma. Hou et al[11] analyzed the tissue samples and obtained similar results. To some extent, our work is consistent with the two clinical studies, since cisplatin resistant seen in glioma may represent a more aggressive phenotype, which might significantly influence the survival of these patients. Our investigation, which demonstrates the cisplatin sensitizing effect of miR-205 in glioma cells, has provided a new target for overcoming chemotherapy resistance. It is still unknown whether miR-205 regulated drug sensitivity can be applied to other therapeutic agents such as temozolomide, a first line treatment for glioblastoma multiforme. More investigation is needed to gain a comprehensive understanding on this question. Nevertheless, based on the high stability and specificity feature and the small size of chemically modified microRNAs, miR-205 could be a potential candidate to enhance chemotherapy in glioma treatment. Our finding has shown that the transcriptional factor E2F1 is a direct target of miR-205. Many of the E2F1 target genes, such as CYCLIN E/A and CDC2, are crucial for the G1/S transition[23]. E2F1 overexpression has been reported in glioma tissues[24]. In contrast with observations with miR-205, lower expression of E2F1 correlated with longer survival, which corroborates the miR-205/E2F1 pathway found in the current study[24]. We found that exogenous supplement of E2F1 with miR-205 greatly weakened cisplatin induced apoptosis compared with miR-205 treatment alone, indicating that E2F1 is a crucial mediator of miR-205 induced cisplatin-sensitizing effect. Intriguingly, the role of E2F1 in tumors seems to be contradictory depending on multiple factors. It has been suggested that E2F1 also induces p53 dependent apoptosis, and that E2F1 knockout mice develop lung adenocarcinoma and lymphoma[25-27]. On the contrary, GFAP-E2F1 transgenic mice are more likely to develop malignant glioma[28]. Studies[15,29,30] have identified that E2F1 is under the regulation of numbers of microRNAs including miR-136, miR-106a and miR-329 in glioma. Our research is consistent with these findings and supported an oncogenic role of E2F1 in promoting the cisplatin resistance of glioma cells. However, the cisplatin sensitizing action of miR-205 in glioma cells needs to be further confirmed by preclinical researches. Furthermore, various targets of miR-205 have been identified in other cancer types; it remains to be seen whether there are other factors regulated by miR-205 in the cisplatin resistant glioma cells. If so, how they might be involved in the biology of glioma deserves further investigation.

Conclusions

We demonstrate that down-regulation of miR-205 confers part of the cisplatin resistance of glioma cells by attenuating cell cycle arrest and apoptosis. E2F1 is a direct target of miR-205 and at least partially mediates these effects. Thus, miR-205 based precision therapy might aid in the treatment of glioma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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