miR-186 reverses cisplatin resistance and inhibits the formation of the glioblastoma-initiating cell phenotype by degrading Yin Yang 1 in glioblastoma

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Abstract. Glioblastoma multiforme (GBM) is among the most devastating types of cancer, with a median survival of <1 year. Despite the development of new surgical and radiation techniques, and the use of multiple anti-neoplastic drugs, effective treatment strategies for malignant gliomas have not yet been developed. The limited efficacy of current treatments reflects the resistance of glioblastoma cells to cytotoxic agents. In this study, using western blot analysis, we found that Yin Yang 1 (YY1) expression was increased in cisplatin-resistant glioblastoma U87MG cells (U87MG-CR). We observed that the silencing of YY1 sensitized the U87MG-CR cells to cisplatin and that the overexpression of YY1 promoted the resistance of LN-229 glioblastoma cells to cisplatin, as shown by MTT assay. Using sphere formation assay, we also found that the silencing of YY1 inhibited the formation of the glioblastoma-initiating cell (GIC) phenotype in the U87MG-CR cells. In addition, the results of RT-qPCR revealed that miR-186 expression was decreased in U87MG-CR cells. Using RT-PCR and western blot analysis, we observed that overexpression of miR-186 inhibited YY1 expression in U87MG-CR cells. The overexpression of miR-186 also reversed cisplatin resistance and the formation of the GIC phenotype in glioblastoma cells. On the whole, the findings of this study demonstrate that miR-186 reverses cisplatin resistance and inhibits the formation of the GIC phenotype by degrading YY1 in glioblastoma.

Introduction

Glioblastoma is the most frequent primary malignant brain tumor among adults. The median survival is generally <1 year from the time of diagnosis, and even in the most favorable situations, the majority of patients succumb to the disease within 2 years (1-3). Standard therapy consists of surgical resection if that is safely feasible, followed by radiotherapy. However, the 5-year survival rate is <3% (4). One of the reasons for the dismal prognosis is that current treatment strategies cannot eliminate glioblastoma-initiating cells (GICs) (5-7). A comprehensive understanding of the molecular basis of GICs may contribute to the identification of novel therapeutic targets.

Yin Yang 1 (YY1) is an ubiquitously expressed zinc finger transcription factor encoded by the 23 kb YY1 gene (8-12). Comprised of 414 amino acids, YY1 carries out various cellular functions, including transcriptional regulation, cell proliferation, chromatin remodeling and apoptosis (12-16). YY1 regulates multiple targets, including Erb-B2 receptor tyrosine kinase 2 (ERBB2), p53, caspases and histone deacetylases (HDACs), which have been implicated in cancer progression (15). YY1 expression has been shown to be increased in many types of cancer, including metastatic breast cancer (17,18), colon cancer (19), gastric cancer (20) and prostate cancer (21). However, its roles have not yet been fully elucidated as regards the formation of GICs.

MicroRNAs (miRNAs or miRs), which are single-stranded long non-coding RNAs of 19-25 nucleotides in length, play important roles in the regulation of drug resistance and GICs (22,23). miR-186 has been demonstrated to play a significant role as a tumor suppressor in many types of cancer (24-26). For example, miR-186 is a novel tumor suppressor miRNA that functions to inhibit tumorigenesis in glioblastoma multiforme (GBM) both *in vitro* and *in vivo*, by targeting both FGF2 and RelA (27); miR-186 may be a molecular target of glioblastoma (27). However, the role of miR-186 in GIC and drug resistance remains elusive. In this study, we observed that miR-186 reversed cisplatin resistance and inhibited the formation of the GIC phenotype by degrading YY1 in glioblastoma.

Materials and methods

Human glioblastoma cell lines. U87MG cells (glioblastoma of unknown origin) and LN-229 glioblastoma cells were purchased from then Biochemistry and Cell Biology Institute of Shanghai, Chinese Academy of Sciences (Shanghai, China),

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within 3 months of the experiments. Of note, it has been reported that the U87MG cell line has been misidentified (28). The U87 cell line used has been authenticated by STR profiling; thus, misidentification is not likely to affect the outcomes of this study. To obtain cisplatin-resistant glioblastoma U87MG cells (U87MG-CR cells), the U87MG cells were treated with escalating concentrations of cisplatin from 10^7 to 10^5 M as previously reported (29). The established U87MG-CR cells grew at a similar rate in the presence or absence of 10^5 M cisplatin for 3 days (data not shown). The half maximal inhibitory concentration (IC_{50}) of the U87MG-CR cells increased by 12-fold, as compared with that of the U87MG cells (data not shown). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics (100 mg/ml penicillin/100 U/ml streptomycin (Invitrogen) in a 5% CO_2 incubator at 37˚C.

shYY1 plasmids and pre-miR-186 and control miR. The shYY1 plasmids and scramble control were purchased from Tiangen (Beijing, China). Pre-miR-186 and control miR were purchased from Ambion, Inc. (Ambion, Austin, TX, USA).

Transfection experiment. Cell transfection was performed as previously described (30). For the transfection experiments, the cells were cultured in serum-free medium without antibiotics at 60% confluence for 24 h, and then transfected using FuGENE HD transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Following incubation for 6 h in a 5% CO_2 incubator at 37˚C, the medium was removed and replaced with normal culture medium (serum-free medium without antibiotics) for 24 h. Subsequently, western blot analysis, MTT assay, immunostaining assay, PCR and immunofluorescence staining were performed as described below.

Western blot analysis. This was performed as previously described (30,31). Total protein was prepared using extraction buffer comprising NaCl/P_i containing 0.5% Triton X-100, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, and complete protease inhibitors (Roche, Shanghai, China). The concentration of each protein lysate was determined using a BCA™ protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of total protein were subjected to 12% SDS/PAGE. The samples were then transferred onto nitrocellulose membranes and blocked for 60 min at room temperature in 5% skim milk powder (w/v) in NaCl/P_i and protein was probed with antibodies against human YY1 (ab109228; 1:500) mouse double minute 2 homolog (ab38618; 1:500), ATPase copper transporting beta (ab124973, 1:500), integrinα6 (ab235905, 1:500), signal transducer and activator of transcription 3 (ab68153, 1:500) or β-actin (ab8227, 1:500) (all from Abcam, Cambridge, MA, USA) and then with IRDyeTM-800 conjugated anti-rabbit secondary antibodies (1:10,000; ab150077; Abcam) all for 30 min at room temperature. The specific proteins were visualized using the Odyssey™ Infrared Imaging System (Gene Company, Lincoln, NE, USA).

MTT assay. To monitor the resistance to cisplatin, the U87MG, U87MG-CR and LN-229 cells were treated with 20 μM cisplatin or DSMO for 24 h. MTT assay was performed as previously described (32). Data were analyzed using software origin 7.5 (OriginLab, Northampton, MA, USA) to fit the sigmodial curve.

Sphere formation assay. The cells (10^3/ml) in serum-free RPMI-1640/1 mM Na-pyruvate were seeded on 0.5% agar pre-coated 6-well plates. After 1 week, half the medium was exchanged every 3rd day. Single spheres were selected and counted by an inverted microscope (TE2000-E2, Nikon Corporation, Tokyo, Japan).

Immunostaining assay for YY1 and CD133 in glioblastoma spheres. Single cell suspensions of glioblastoma cells transfected as indicated above were prepared and plated using ultra low adherent wells of 6-well plate at 5,000 cells/well in sphere formation medium (serum-free RPMI-1640/1 mM Na-pyruvate; Invitrogen), as described above. Following 7 days of treatment, the spheres were collected by centrifugation (10,00 x g, 10 min, 4˚C), washed with 1X PBS, and fixed with 3.7% parformaldehyde for immunofluorescence staining. Anti-YY1 (ab109228; 1:500; Abcamand anti-CD133 antibodies (ab19898, 1:500) were used for immunostaining assay following the manufacturer's instructions and as previously described (33,34). The coverslips were counterstained with 4'6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) for visualization of the nuclei. Microscopic analysis was performed with a confocal laser-scanning microscope (Leica Microsystems, Bensheim, Germany). The fluorescence intensities were measured in a few viewing areas for 300 cells per coverslip and analyzed using ImageJ 1.37v software (http://rsb.info.nih.gov/ij/index.html).

Real-time PCR for miRNA expression. Total RNA was isolated from the cells using the mirVana miRNA Isolation kit (Ambion, Austin, TX, USA). The detection of the mature form of miRNAs was performed using the mirVana qRT-PCR miRNA Detection kit and qRT-PCR Primer Sets, according to the manufacturer's instructions (Ambion). For the quantification PCR of miR-186, the forward primer was as follows: 5'-GCGGCGCAAAGAATTCTCCT-3', and the reverse primer was as follows: 5'-GTGCAGGGTCCGAGGT-3'. The quantification of PCR performed was performed using the ΔΔCq method (35). The U6 small nuclear RNA was used as an internal control.

Immunofluorescence staining. This was performed as previously described (36). Following transfection, the cells were fixed in 4% paraformaldehyde for 15 min, and then blocked with goat serum blocking solution for 20 min at room temperature. Subsequently, rabbit antibody against YY1 (ab109228; 1:500; Abcam) were added, and the mixtures were incubated in a humid chamber overnight. After washing 3 times with NaCl/Pi, the cells were incubated with appropriate secondary antibodies (1:10,000; ab150077; Abcam) for 30 min at 37˚C. After washing with NaCl/Pi, the samples were observed under a laser scanning confocal microscope (Olympus, Tokyo, Japan). DAPI staining (blue) was used to highlight the nuclei.

Reverse transcription-quantitative polymerase chain reaction PCR (RT-qPCR) for mRNA expression. Total RNA was isolated

from the cells using TRIzol reagent (Invitrogen/Thermo Fisher Scientific). cDNA was synthesized from 1 μg of total RNA in a 20 μl reverse transcription (RT) system followed by PCR amplification in a 50 μl PCR system performed using an RT-PCR kit (Cat no. A3500, Promega, Madison, WI, USA). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as the RNA loading control. The PCR primer sequences were as follows: YY1 forward, 5'-CAG AAGCAGGTGCAGATCAAG-3' and reverse, 5'-GACCAC ATGGTGACCGAGAAC-3'; and GAPDH forward, 5'-ATT CAACGGCACAGTCAAGG-3' and reverse, 5'-GCAGAA GGGGCGGAGATGA-3'. PCR was conducted according to the manufacturer's instructions: The thermal cycle profile was as follows: Denaturation for 30 sec at 95 ˚C, annealing for 45 sec at 52-58˚C depending on the primers used, and extension for 45 sec at 72˚C. Each PCR reaction was performed for 28-32 cycles. The PCR products were analyzed by agarose gel electrophoresis. Gels were photographed and densities of the bands were determined with a computerized image analysis system (Alpha Innotech, San Leandro, CA, USA). The area of each band was calculated as the integrated density value (IDV). qPCR for YY1 was performed using Power SYBR-Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantification of PCR performed was performed using the ΔΔCq method (35).

Methods of bioinformatics. The analysis of potential miRNA target sites was carried out using the commonly used prediction algorithm, miRDB (http://mirdb.org/).

Northern blot analysis. Northern blot analysis of miRNAs, was performed as previously described (37). Probes were labeled with [γ-^{32}P] ATP complementary to miR-186 and U6 snRNA.

Statistical analysis. Data are presented as the means ± SEM. The Student's t-test (two-tailed) was used for comparisons between 2 groups. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

YY1 expression is increased in U87MG-CR cells and the silencing of YY1 sensitizes the U87MG-CR to cisplatin. In order to determine whether cisplatin resistance is associated with YY1 expression, we examined the YY1 protein concentrations in the U87MG and U87MG-CR cells. We observed that YY1 protein expression was increased in the U87MG-CR cells (Fig. 1A). To identify the role of YY1, we examined whether transfection with shYY1 plasmid would downregulate YY1 protein expression in the U87MG-CR cells. The results revealed that YY1 protein expression was inhibited by transfection with the shYY1 plasmid (Fig. 1B). To further determine whether YY1 affects the sensitivity of glioblastoma cells to cisplatin, we transfected the U87MG-CR cells with shYY1 plasmid or the scramble control and then performed MTT assay. We found that the silencing of YY1 transformed the U87MG-CR to cells to cisplatin-sensitive cells (U87MG cells), as evidenced by the decreased viability of the shYY1-transfected cells (Fig. 1C). We then examined

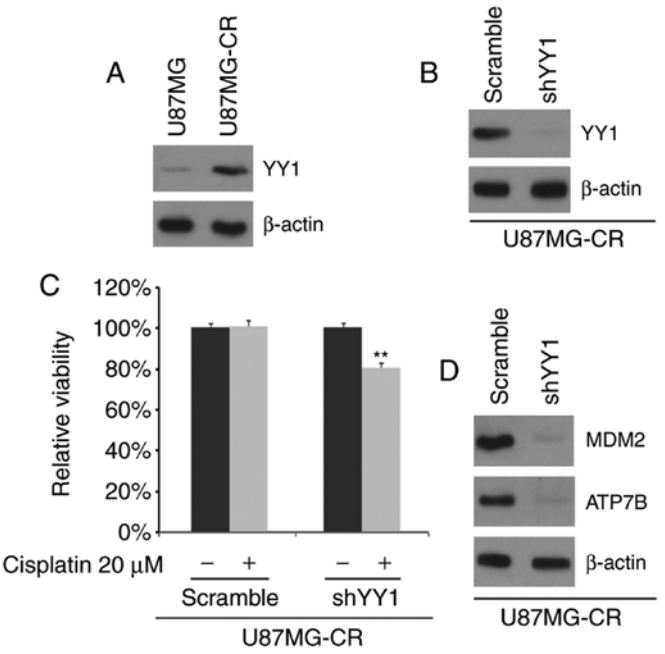

Figure 1. YY1 expression is increased in cisplatin-resistant U87MG cells (U87MG-CR cells) and its silencing sensitizes U87MG-CR cells to cisplatin. (A) Western blot analysis of YY1 expression in U87MG and U87MG-CR cells. (B) Western blot analysis of YY1 expression in U87MG-CR cells transfected with shYY1 plasmids or the scramble control. (C) MTT assay for the viability of the U87MG-CR cells. Cells transfected with the shYY1 plasmid or the scramble control were untreated or treated with cisplatin. (D) Western blot analysis of MDM2 and ATP7B expression in U87MG-CR cells transfected with shYY1 plasmid or the and scramble control. β-actin was used as a loading control; n=3. $^{**}P<0.05$, compared with the shYY1-transfected cells not treated with cisplatin. YY1, Yin Yang 1; MDM2, mouse double minute 2 homolog; ATP7B, ATPase copper transporting beta.

the expression of MDM2 and ATP7B as MDM2 protein can confer the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis and ATP7B is associated with cisplatin resistance (38,39). In this study, we observed that the MDM2 and ATP7B protein expression levels were decreased in the U87MG-CR cells following transfection with shYY1 (Fig. 1D).

Overexpression of YY1 promotes the resistance of LN-229 cells to cisplatin. To examine the effects of YY1, we examined whether YY1 protein expression was increased by YY1-expressing plasmids in LN-229 cells (cisplatin-sensitive cells). We observed that YY1 protein expression was increased following transfection with YY1-expressing plasmids (Fig. 2A). To identify whether the responses to cisplatin can be altered by YY1, we transfected the LN-229 cells with YY1-expressing plasmids and we then performed MTT assay. We found that the overexpression of YY1 promoted the resistance of LN-229 cells to cisplatin, as no marked difference in cell viability was observed between the cisplatin-treated or untreated LN-229-expressing cells (Fig. 2B).

Silencing of YY1 inhibits the formation of the GIC phenotype in U87MG-CR cells. To determine whether the silencing of YY1 affects the GIC phenotype of the U87MG-CR cells, we performed a sphere formation assay to assess the formation of GICs in the U87MG-CR cells. We observed that the cells

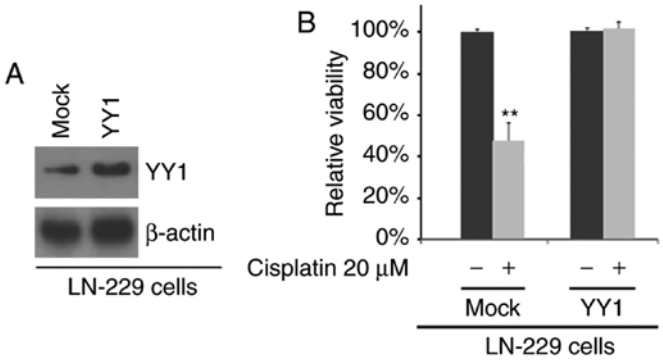

Figure 2. Overexpression of YY1 promotes the resistance of LN-229 cells to cisplatin. (A) Western blot analysis of YY1 in the LN-229 cells transfected with the YY1-expressing plasmid or the empty vector (mock). (B) MTT assay for the viability of LN-229 cells. Cells transfected with the YY1-expressing plasmid or the empty vector (mock) were untreated or treated with cisplatin. **P<0.05, compared with the mock-transfected cells not treated with cisplatin. YY1, Yin Yang 1.

transfected with shYY1 formed much smaller spheres after 14 days of culture as compared with the control cells (Fig. 3A). As CD133 expression is associated with the GIC phenotype in glioblastoma (40), in this study, we examined whether YY1 regulates CD133 protein expression. We performed immunostaining assay in the spheres isolated from the U87MG-CR cells transfected with the shYY1 plasmid or the scramble control. The results revealed that CD133 protein expression was decreased in the spheres isolated from the U87MG-CR cells transfected with the shYY1 plasmid (Fig. 3B). In addition, as integrinα6 regulates GICs and targeting integrinα6 in GICs inhibits self-renewal, proliferation and tumor formation capacity (41); thus, in this study, we also examined the expression of integrinα6. Moreover, as STAT3 is required for the proliferation and maintenance of multi-potency in glioblastoma stem cells (42), we also examined its expression. We found that the silencing of YY1 downregulated integrinα6 and STAT3 protein expression in the U87MG-CR cells (Fig. 3C).

miR-186 inhibits YY1 protein expression in U87MG-CR cells. To confirm whether YY1 is regulated by miRNAs, we used a commonly used prediction algorithm, miRDB (http://mirdb.org/) to analyze the 3'UTR of YY1. A total of 38 miRNAs were found by the algorithm. However, we were interested in miR-186, as miR-186 is a tumor suppressor gene by inhibiting oncogene expression (24,26,43). Moreover, miR-186 may sensitize cancer cells to paclitaxel and cisplatin (25). The target sites on the 3'UTR of YY1 are shown in Fig. 4A. In an attempt to identify the role of miR-186 in regulating YY1 expression in glioblastoma, we transfected the U87MG-CR cells with pre-miR-186 and control miR. Following transfection, miR-186 expression was detected by real-time PCR and the results revealed that miR-186 expression was increased by transfection with pre-miR-186 (Fig. 4B). We then performed western blot analysis to detect YY1 protein expression in the U87MG-CR cells transfected with pre-miR-186 or control miR. We found that YY1 protein expression was inhibited by miR-186 (Fig. 4C). We then performed immunofluorescence analyses of the U87MG-CR cells transfected with pre-miR-186 anord control miR. We observed that YY1

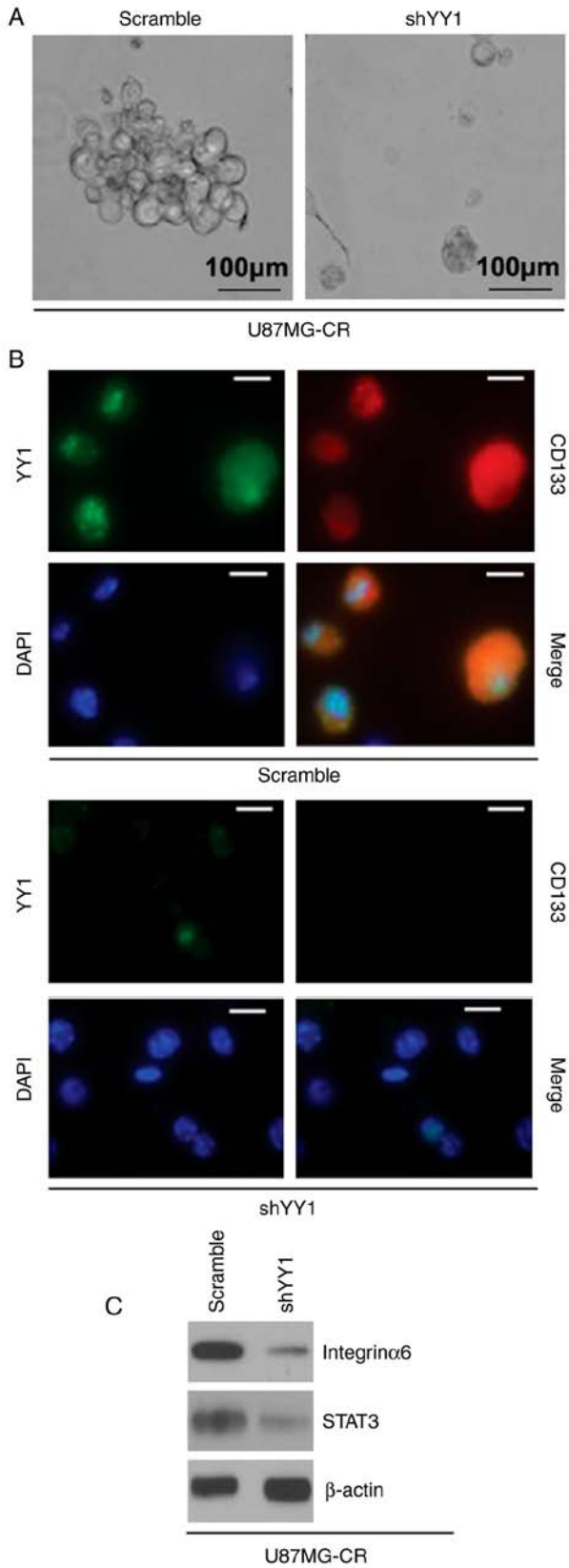

Figure 3. Silencing of YY1 inhibits the formation of the GIC phenotype in U87MG-CR cells. (A) Sphere growth for U87MG-CR cells transfected with the shYY1 plasmid of the scramble control. (B) Representative images of YY1 and CD133 immunofluorescence staining of spheres isolated from U87MG-CR cells transfected as indicated. Nuclei were counterstained with DAPI. Green indicates YY1 staining, red indicates CD133 fluorescence, and blue indicates DAPI (scale bars, 10 μm). (C) Western blot analysis of integrinα6 and STAT3 in U87MG-CR cells transfected with the shYY1 plasmid or the scramble control. β-actin was a loading control; n=3. YY1, Yin Yang 1; STAT3, signal transducer and activator of transcription 3.

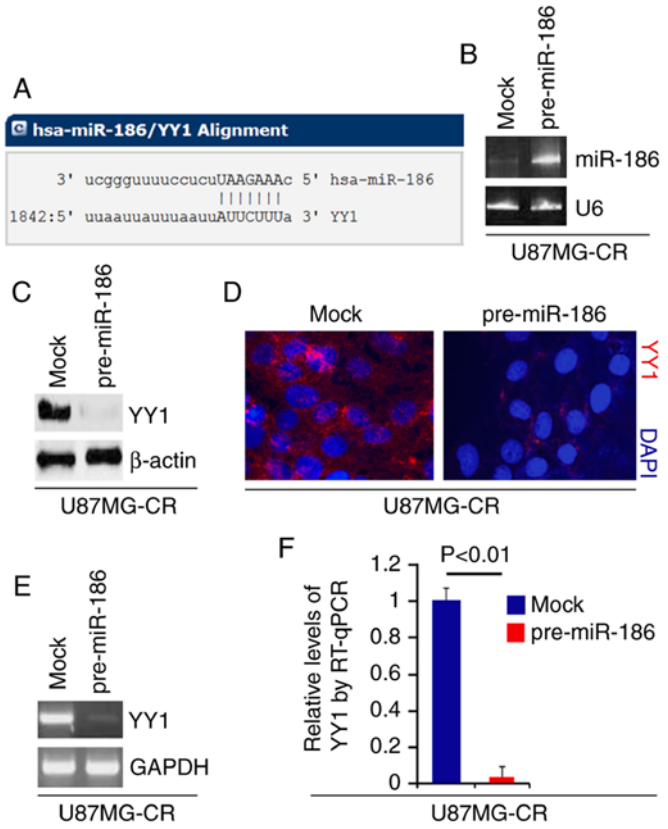

Figure 4. miR-186 inhibits YY1 expression in U87MG-CR cells. (A) Diagram demonstrating that YY1 is a target gene of miR-186, as predicted by miRanda. (B) Real-time PCR for miR-186 in U87MG-CR cells transfected with pre-miR-186 or the control miR (mock). U6 was used as a loading control. (C) Western blot analysis of YY1 expression in U87MG-CR cells transfected with pre-miR-186 or the control miR (mock). β-actin was used as a loading control. (D) Immunofluorescence analyses of YY1 in U87MG-CR cells transfected with pre-miR-186 or the control miR (mock). (E) RT-qPCR of YY1 in U87MG-CR cells transfected with pre-miR-186 or the control miR (mock). GAPDH was used as a loading control. (F) RT-qPCR of YY1 in U87MG-CR cells transfected with pre-miR-186 or the control miR (mock). GAPDH was used as a loading control; n=3. YY1, Yin Yang 1.

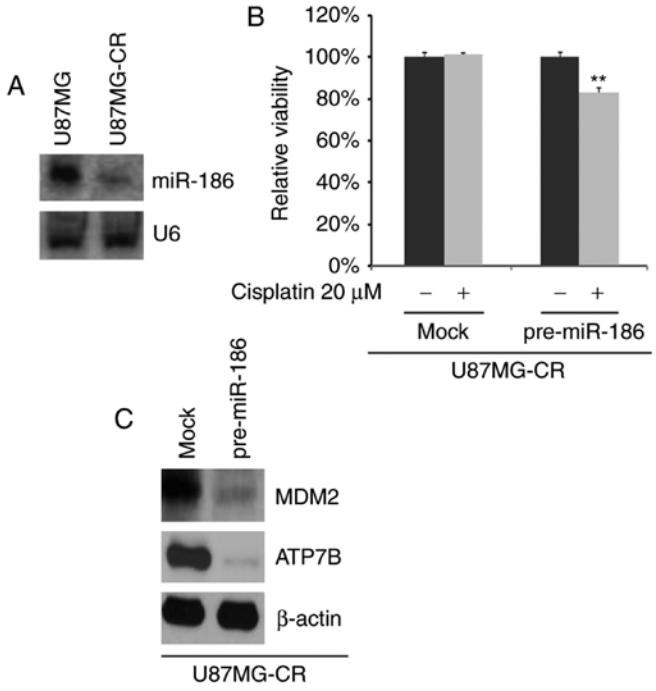

Figure 5. miR-186 is downregulated in U87MG-CR cells and its overexpression reverses cisplatin resistance. (A) Northern blot for miR-186 in U87MG and U87MG-res cells. U6 was used as a loading control. (B) MTT assay of the viability of U87MG-CR cells. Cells transfected with pre-miR-186 or the control miR (mock) were untreated or treated with cisplatin. (C) Western blot analysis of MDM2 and ATP7B in U87MG-CR cells transfected with pre-miR-186 or the control miR (mock). β-actin was used as a loading control; n=3. **P<0.05, compared with the pre-miR-186-transfected cells not treated with cisplatin. YY1, Yin Yang 1; MDM2, mouse double minute 2 homolog; ATP7B, ATPase copper transporting beta.

protein expression was inhibited in the cells transfected with pre-miR-186 (Fig. 4D). To examine whether miR-186 degrades YY1 mRNA, we performed RT-qPCR and real-time PCR and we found that the overexpression of miR-186 degraded YY1 mRNA expresoin (Fig. 4E and F).

miR-186 expression is decreased in U87MG-CR cells and its overexpression reverses cisplatin resistance. To determine whether cisplatin resistance is associated with miR-186 expression, we performed northern blot analysis to detect miR-186 expression in U87MG cells and U87MG-CR cells. We observed that miR-186 expression was markedly decreased in the U87MG-CR cells (Fig. 5A). To further identify whether miR-186 can affect the resistance/sensitivity of U87MG-CR cells to cisplatin, we transfected the U87MG-CR cells with pre-miR-186 or control miR. We then performed MTT assay with the U87MG-CR cells treated as indicated (Fig. 5B). We found that the overexpression of miR-186 reversed cisplatin resistance, evidenced by the decreased viability of the U87MG-CR cells treated with cisplatin and transfected with pre-miR-186 (Fig. 5B). We also performed western blot analysis to examine MDM2 and ATP7B protein expression in the U87MG-CR cells transfected with pre-miR-186 or control miR. The results revealed that MDM2 and ATP7B protein expression was inhibited by miR-186 (Fig. 5C).

miR-186 inhibits the formation of the GIC phenotype of U87MG-CR cells. To identify whether miR-186 can affect the GIC phenotype of U87MG-CR cells, we performed a sphere formation assay to assess the formation of GICs in the U87MG-CR cells. Sphere formation assay revealed that the overexpression of miR-186 inhibited the formation of GICs in U87MG-CR cells (Fig. 6A). Subsequently, to determine whether miR-186 regulates integrinα6 and STAT3 protein expression, we performed western blot analysis of the U87MG-CR cells transfected with pre-miR-186 or control miR. We observed that integerinα6 and stat3 protein expression levels were inhibited by miR-186 (Fig. 6B).

Silencing of miR-186 promotes the resistance of LN-229 cells to cisplatin. To determine whether miR-186 affects the sensitivity of the LN-229 cells to cisplatin, we transfected the LN-229 cells with anti-miR-186. We then performed real-time PCR to detect miR-186 expression in the LN-229 cells transfected with anti-miR-186 and scramble (mock). We observed that miR-186 expression was evidently decreased in the LN-229 cells transfected with anti-miR-186 (Fig. 7A). We then performed MTT assay of the LN-229 cells treated as indicated (Fig. 7B). The

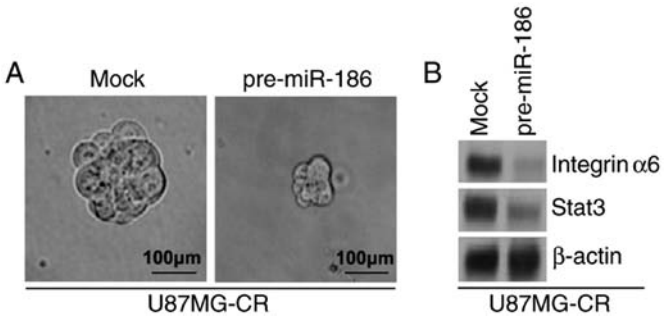

Figure 6. miR-186 inhibits formation of GICs phenotype in U87MG-CR cells. (A) Sphere growth of the U87MG-CR cells transfected with pre-miR-186 or the control miR (mock). (B) Western blot analysis of integrinα6 and STAT3 expression in U87MG cells transfected with pre-miR-186 or the control miR (mock). β-actin was used as a loading control; n=3. YY1, Yin Yang 1; STAT3, signal transducer and activator of transcription 3.

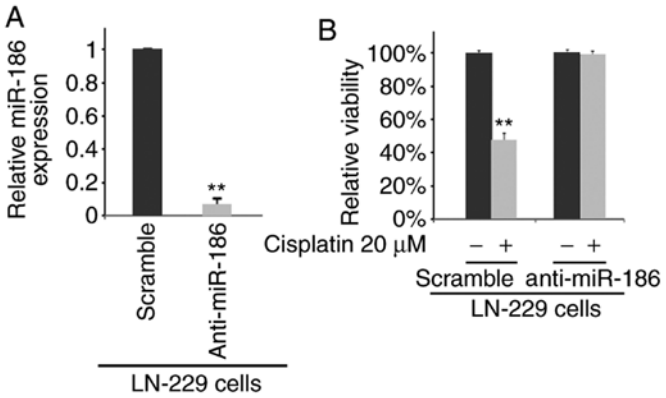

Figure 7. Silencing of miR-186 promotes the resistance of LN-229 cells to cisplatin. (A) Real-time PCR of miR-186 expression in LN-229 cells transfected with anti-miR-186 or the scramble control. GAPDH was used as a loading control. (B) MTT assay of the viability of the LN-229 cells. Cells transfected with anti-miR-186 or the scramble control were untreated or treated with cisplatin; n=3. $^{**}P<0.05$, compared with the anti-miR-186-transfected or mock-transfected (scramble) cells not treated with cisplatin.

results revealed that the overexpression of miR-186 promoted cisplatin resistance, as the cells transfected with anti-miR-186 and treated with cisplatin exhibited no marked difference in viability compared with the anti-miR-186-transfected cells not treated with cisplatin (Fig. 7B).

Discussion

Cisplatin is a neutral, square planar platinum (II) complex containing two chloride ligands oriented in a *cis* configuration. It has become one of the most effective chemotherapeutic agents for the treatment of glioblastoma (44). However, intrinsic or acquired resistance to cisplatin reduces its efficacy (45). The mechanisms of resistance include miRNA deregulation and the formation of GICs (46-48). miR-186 has been demonstrated to play a significant role as a tumor suppressor in many types of cancer (24-26). Nevertheless, its biological function in glioblastoma remains unknown. In the current study, we found that miR-186 played an important role in the formation of GICs and in the regulation of cisplatin resistance. These findings provide novel insight into the potential roles of miR-186 in promoting the formation of GICs and conferring chemoresistance in glioblastoma. MDM2 protein can confer the resistance of a human glioblastoma cell line to cisplatin (39). We demonstrated that the overexpression of miR-186 inhibited MDM2 protein expression. The ATP7B product, a protein of 1465 amino acids (ATP7B), is expressed pre-dominantly in the liver, kidneys and placenta in humans (49). ATP7B expression is associated with cisplatin resistance (38). In this study, we found that ATP7B expression was inhibited by miR-186 in U87MG-CR cells.

A number of studies have relied on the enrichment of GICs based on the expression of the cell surface protein CD133 (prominin-1) (50,51), which has also been used as a selection marker for neural stem cells (51). In this study, we demonstrated that miR-186 inhibited CD133 expression. Moreover, integrinα6 is co-expressed with conventional GIC markers (41); STAT3 is required for maintenance of multipotency in GICs (42). Herein, we observed that the overexpression of miR-186 significantly inhibited integrinα6 and STAT3 protein expression in the U87MG-CR cells.

YY1 plays an important role in the EGFR-Src-p38 signaling cascade in glioblastoma. However, its roles and regulatory mechanisms have not yet been fully elucidated. EGFR signaling plays an important role in drug resistance for the treatment of glioblastoma (52) and EGFR inhibitor can enhance cisplatin sensitivity of human glioma cells (52). We demonstrated herein that YY1 expression was increased in cisplatin-resistant U87MG cells and that the silencing of YY1 sensitized the U87MG-CR cells to cisplatin. In addition, we observed that YY1 expression was regulated by miR-186 in U87MG-CR cells.

Recently, the U-87 MG cell line from ATCC was reported to be contaminated or misidentified (28). It has been proposed as a glioblastoma cell line whose origin is unknown (28). However, the U-87 MG cell line is still widely used for glioblastoma research (25). In the present study, the U87MG and LN-229 cells were used. The results were same from the 2 cell lines. Thus, the contamination or misidentification may not affect the conclusions presented herein.

In conclusion, elucidating the mechanisms through which miR-186 reverses cisplatin resistance and inhibits the formation of the GIC phenotype by degrading YY1 in glioblastoma may enhance our understanding of the molecular mechanisms of cisplatin resistance in glioblastoma. As shown by our findings, the restoration of miR-186 expression may represent a promising therapeutic strategy with which to inhibit YY1-mediated cisplatin resistance. However, the roles of miR-186 and YY1 require further confirmation by *in vivo* studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and FG conceived the study, collected the experimental data and wrote a draft of the manuscript. JS contributed to the experimental work and data analysis. All authors edited and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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