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Regulation of mTOR by miR-107 to facilitate glioma cell apoptosis and to enhance cisplatin sensitivity

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Abstract. – OBJECTIVE: The aberrant increasing expression of mammalian target of rapamycin (mTOR) participates in tumor occurrence and drug resistance. It has been found elevation of mTOR expression but reducing miR-107 expression in glioma tissues. Thus, we investigated the regulatory role of miR-107 on mTOR expression as well as glioma cell proliferation, apoptosis and cisplatin (DDP) resistance.

PATIENTS AND METHODS: Dual luciferase reporter gene assay was applied to confirm targeted regulation between miR-107 and m[illegible]. Tumor tissues were collected from glio[illegible]tients, in parallel with normal tissues af[illegible]rain contusion surgery. Expressions of m[illegible]07, mTOR and p-mTOR were compared. DDP-r[illegible]tant cell line U251/DPP was generated. U[illegible] DPP cells were further treated with miR-1[illegible] mimic or si-mTOR to exami[illegible]hange o[illegible] miR-107, mTOR, p-mTOR [illegible] su[illegible] levels. Flow cytometry was used [illegible] quantif[illegible]e effect of DDP treatment on ce[illegible]liferati[illegible] apoptosis.

RESULTS: Bioinf[illegible]atics [illegible]s revealed complementary bi[illegible]ng sites b[illegible]n miR-107 and 3'-UTR of m[illegible] mRNA. Dual [illegible]se assay confirmed [illegible]egulation bet[illegible]en miR-107 and mTO[illegible] Com[illegible] to control group, in glioma tissues, mTOR a[illegible]TOR expressions were sig[illegible]cantly elevate[illegible]le the level of miR-107 [illegible]xpression was ma[illegible]ly decreased. Of no[illegible] U251/DDP cells presented weakened apo[illegible]is com[illegible]ed to U251 cells, with high leve[illegible] mTO[illegible], p-mTO[illegible] and survivin and reductio[illegible]R-107 e[illegible]ession. However, the transfecti[illegible]miR-[illegible] mimic and/or si-mTOR [illegible]kably [illegible]d expressions of mTOR, p-[illegible]R and su[illegible]n in U251/DPP cells, weakene[illegible]ell proliferation and enhanced apoptosis.

C[illegible]CLUSIONS: We demonstrated that the le[illegible]7 was correlated with DDP resis[illegible]ce in glioma cells. Over-expression of miR-[illegible]decreased DPP resistance of glioma cells vi[illegible]ition of mTOR, which provides academic ba[illegible] for the future anti-glioma therapy.

Key Word[illegible]
MiR-1[illegible] mTOR, [illegible] Glioma.

Introduction

[illegible]lioma is a co[illegible]non type of malignant tumor fo[illegible]in central [illegible]rvous system (CNS) and occup[illegible]0% of all intracranial cancers[1]. Glioma p[illegible]nts the highest incidence among all [illegible]racranial tumors, and is mainly in age groups [illegible]nd 30-40 years[2]. Cisplatin (DDP) has [illegible]ely used in chemotherapy of glioma, but the existence of drug resistance severely affects treatment efficacy and prognosis. Therefore, the investigation of detailed mechanisms for onset, progression and drug resistance of glioma, and the identification of abnormal molecular change and novel treatment target in glioma, are of critical importance for early diagnosis, improvement of treatment efficacy and patient's prognosis. Mammalian target of rapamycin (mTOR) represents the serine/threonine protein kinase, and plays critical role in response to various signal stimuli from mitogen, cytokine, nutritional status and cellular energy level. Its major functions and activities are under the regulation of PI3K/protein kinase B (PKB) signal pathway, and mTOR exerts its signal transduction function at downstream of PI3K/AKT pathway. Previous work showed that abnormality of mTOR expression or functional activity resulted in occurrence, progression and resistance acquirement in multiple tumors[3-5]. Previous study indicated the correlation between mTOR expression/function enhancement and occurrence, drug resistance and unfavorable prognosis in glioma[6,7]. MicroRNA is a type of small molecule non-coding RNA with the length of 22-25 nucleotides. It

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can regulate more than one third of human gene expression by degrading or inhibiting translation of target gene mRNA, via complementary binding with 3'-untranslated region (3'-UTR) of mRNA of target gene [8]. Increasing evidence[11,12] illustrated that miRNAs played as oncogene[9,10] or tumor suppressor gene in tumor pathogenesis. Notably, various researches revealed that miR-107 expression was significantly down regulated in glioma tumor tissues[13,14]. We thus determined the role of miR-107 in mediating mTOR expression, and even glioma cell proliferation, apoptosis or DDP resistance.

Patients and Methods

Patients

A total of 22 glioma patients who received treatment in the fifth Affiliated Hospital of Harbin Medical University from September 2016 to December 2016 were recruited in this study. All patients received confirmed diagnosis by pathological examination. There were 12 males and 10 females in the patient cohort, with the av[illegible] age at 58.1 ± 12.9 years. Another coh[illegible] normal brain tissue samples was collect[illegible]om contusion traumatic surgery and was rec[illegible]d as the control group. There were 8 males a[illegible] females in the control group (average age = 5[illegible] ± 13.2 years). No significant [illegible]e of ag[illegible] or sex ratio was observed [illegible]ween [illegible] groups. This study was approved [illegible] Ethics [illegible]mmittee in the fifth Affiliated H[illegible] of H[illegible] cal University and al[illegible] the [illegible] signed informed co[illegible]nt.

Major Reage[illegible] Materials

Glioma U[illegible] cel[illegible] was purchased from Yanyu Bi[illegible] (Beijing, [illegible]). High-glucose DMEM [illegible]dium, fetal bo[illegible] serum (FBS) and pe[illegible]llin-streptomycin w[illegible]e bought from Gib[illegible] Walthar[illegible] MA, USA). RNA extraction bu[illegible]PLIT[illegible]NA Extraction Kit was collected [illegible]xogen ([illegible]nna, Austria). Transfection [illegible] FuG[illegible]E6 was provided by [illegible] (Base[illegible]land). QuantiTech SYBR Gr[illegible] qRT-PCR [illegible]t was offered from Qiagen (Hil[illegible], Germany). MiR-107 mimic, miR-107 inh[illegible]R-NC were obtained from Rio-[illegible] (Wuhan, Hubei, China). Rabbit anti-human [illegible]OR, p-mTOR and HRP conjugated second-a[illegible]ibody were acquired from Abcam (Cambrid[illegible] MA, USA). Rabbit anti-human survivin and β-actin antibody were from CST (Danvers, MA, USA). Si-NC and si-mTOR [illegible] chased from Santa Cruz Biotechn[illegible]gy (Sa[illegible] Cruz, CA, USA). EdU cell proli[illegible]tion kit was got from Molecular Probes [illegible]ltham, MA, USA). pMIR luciferase report[illegible]mid and Dual-luciferase Reporter [illegible]ssay [illegible] were from Promega (Madis[illegible] WI, USA)[illegible] assay kit and apopto[illegible] kit were bought [illegible] Beyotime (Shangha[illegible] China). [illegible]splatin (D[illegible]P) was provided by [illegible] P[illegible]maceuti[illegible]l (Lianyungang, Jia[illegible]su, C[illegible]

Cell Cultu[illegible]

U251 g[illegible]s were cultu[illegible] in high-glucose D[illegible]becco[illegible]dified Eagle Medium (DMEM) medium c[illegible]ing 10% fetal bovine seru[illegible]BS) and 1% [illegible]cillin-streptomycin. C[illegible] were passed at the ratio of 1:4 for further [illegible]periments.

[illegible]eration o[illegible]251/DDP Cell Model

[illegible] cells w[illegible] initially treated with 1 mg/L DD[illegible] original culture medium was changed [illegible]DDP-free medium. Cells were con[illegible]uously incubated until stable growth of cell [illegible] in DDP-containing medium. The [illegible]ug [illegible]centration was then gradually elevated to 2 mg/L, 4 mg/L, and 8 mg/L. Those cells that can normally grow at 8 mg/mL DDP were maintained for repeated passage to establish DDP-resistant cancer cell line U251/DDP. U251 and U251/DDP cells were treated for 48 h with gradient concentrations of DDP (0, 1, 2, 4, 8, 16, 32, 64 and 128 mg/L). Five replicates were recruited at each concentration. 10 μL CCK-8 reagent were then added into the culture medium. After 4 h incubation, absorbance values at 450 nm (A450) of each well were measured on a micro-plate reader. Inhibition rate (%) = (1-A450 (drug treatment group))/A450(control group) X 100%. IC_{50} value was calculated as the drug concentration inhibited 50% cell growth. Resistance index (RI) = IC_{50} of U251/DDP cells / IC_{50} of parental U251 cells.

Dual Luciferase Activity Assay

Using HEK293T cell genome as the template, full-length or mutant fragment of 3'-UTR of mTOR gene was amplified and was sub-cloned into pMIR plasmid for transforming to DH5α competent cells. Positive clones with correct sequences were screened out by sequencing and were named as pMIR-mTOR-

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UTR-wt and pMIR-mTOR-UTR-mut, respectively. FuGENE6 was used to co-transfect into HEK293T cells with pMIR-mTOR-UTR-wt (or pMIR-mTOR-UTR-mut) or miR-107 mimic (or miR-107 inhibitor or miR-NC). After 48 h incubation, cells were rinsed twice in phosphate-buffered saline (PBS). Passive Lysis Buffer from Dual Luciferase Reporter Assay System was added. 10 µL lysate were then added into 96-well plate for mixture with Stop&Go buffer. Dual luciferase was measured at 560 nm wave length using a micro-plate reader.

Cell Transfection and Grouping

In vitro cultured U251/DDP cells were assigned into four transfection groups: miR-NC transfection group, miR-107 mimic transfection group, si-NC transfection group, and si-mTOR transfection group. Cells were cultured into 6-well plate to reach 60-70% confluence before transfection. During transfection, 100 µL serum-free basic medium was used to dilute 10µL FuGENE6. After gentle mixture, 30 nmoL miR-NC, miR-107 mimic, si-NC or si-mTOR was added for gentle mixture and 20 min incubation at room temperature. Culture medium was then changed into serum-free and antibiotics-free DMEM medium. Transfection complex was then added into cells with serum-free and antibiotics-free medium for [illegible] incubation. Next, normal DMEM medium containing serum and dual antibiotics was replaced for further 72 h incubation. After treatment in IC_{50} concentration of DDP for 48 h [illegible] collected for evaluation a[illegible]n or apoptosis.

qRT-PCR for [illegible] Expression

QuantiTech SYBR [illegible] RT-PCR Kit was used to test relative expression level of target genes by using one-step qRT-PCR using RNA with S[illegible]T RNA extraction kit. In a 20 µL reaction system, 10 µL 2X QuantiTech SYBR Green Master Mix, 1.0 µL forward and reverse primer [illegible]m/L each, 2 µg RNA template, 0.5 µL QuantiTech RT Mix, and distilled water were used. Reverse transcription conditions were: 50[illegible] for 30 min. PCR conditions were: 95°C 15 min pre-denature, followed by 40 cycles each co[illegible] 15 s for denature, 60°C 30 s for annealing, and 72°C 30 s for elongation. Gene expression was measured on Applied Biosystems QuantStudio type 3 Real-time fluorescent qPCR cycler.

Western Blot

Cells were digested by trypsin [illegible] collected. After rinsing twice in PBS, RIPA lysis buffer was added. Protein concentration was quantified from supernatant after centrifugation. 50 µg samples were loaded and were separated in 1[illegible]% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to [illegible] membrane, which was blocked in 5% defatted milk powder at room temperature. Primary antibody (mTOR at 1:[illegible], p-mTOR at 1:1000, survivin at 1:[illegible]0, and β-actin at 1:[illegible]00) was added for 4[illegible] incubation, 12 [illegible] membrane was rinsed in PBST for three times, and horseradish peroxidase (HRP) conjugated secondary antibody (1:25[illegible]) was incubated for 60 min at room temperature. With three times of PBST rinsing, ECL approach was used to test protein expression.

Cell Apoptosis Assay

Cells were digested by trypsin and were collected. After re-suspending in Binding Buffer, 5 µL Annexin V-FITC and 5 µL PI were sequentially added. Cell apoptosis was measured by Beckman CytoFLEX flow cytometry (Brea, CA, USA).

Flow Cytometry for Cell Proliferation

Cells were re-suspended in complete medium. Cell proliferation was measured by Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kits. In brief, after incubation in 10 µM EdU for 2 h, cells were continuously incubated for 48 h, and were digested by trypsin and were collected. After centrifugation, fixation and permeabilization, reaction buffer with Alexa Fluor 488 labels was added for 30 min dark incubation at room temperature. By centrifugation and washing, Beckman CytoFLEX flow cytometry was used to measure positive rate of EdU cells, for reflecting cell proliferation potency.

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean±standard deviation (SD). Student *t*-test was used to compare measurement data between groups. A statistical significance was defined when $p < 0.05$.

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Results

Targeted Regulation Between miR-107 and mTOR

Online *in silico* prediction (microRNA.org) showed the existence of complementary binding sites between miR-107 and 3'-UTR of mTOR mRNA (Figure 1A). Furthermore, dual luciferase gene reporter assay via the transfection of miR-107 mimic showed that the relative luciferase activity in HEK293T cells was significantly suppressed ($p < 0.05$) (Figure 1B), indicating the targeted regulation between miR-107 and mTOR.

Decreased miR-107 and Elevated mTOR Expression in Glioma Tissues

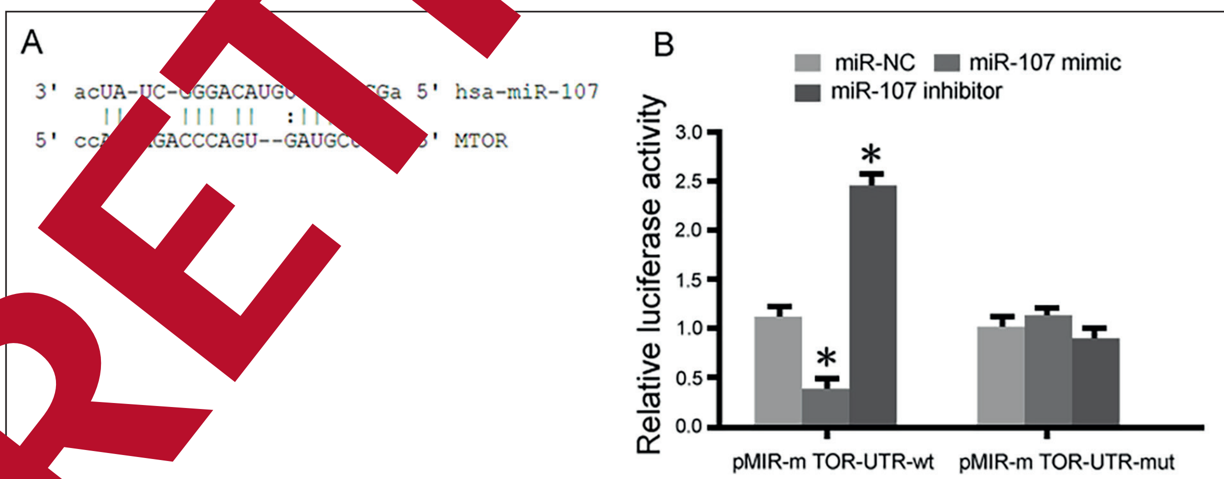
qRT-PCR showed that, compared to brain contusion tissues without malignant lesion, miR-107 expression was statistically reduced, while mTOR mRNA expression in glioma tissues was significantly elevated ($p < 0.05$) (Figure 2A, 2B). Western blot results indicated significantly higher expressions of mTOR and p-mTOR proteins in glioma tissue compared to that in control group ($p < 0.05$) (Figure 2C).

MiR-107 Down-Regulation Was Correlated With mTOR Up-Regulatio[illegible] and Drug Resistance

Under treatment of different concentratio[illegible] of DDP, the inhibitory effect[illegible] viabilit[illegible] were more significant in U[illegible] cells [illegible]n U251/DDP cells (Figure 3A). R[illegible]ts show[illegible]hat IC_{50} value of U251 cells w[illegible] mg/[illegible] whilst IC_{50} value of U251/[illegible] reached [illegible] 53.29 mg/L. RI of U251/DDP cells against parental cell line U251 was 10.97. Flow [illegible] showed relatively higher apoptoti[illegible] of U2[illegible] cells under treatment of 4.86 m[illegible], whilst lower apoptotic rate of U251/DD[illegible]s was found under the same concentration [illegible]P (Figure 3B). qRT-PCR results rev[illegible]led tha[illegible]ession of miR-107 in U251/DP[illegible]lls was sig[illegible] decreased compared t[illegible]at of U251 cells, [illegible] mTOR and survivi[illegible]RNA [illegible]ressions were significantly elevat[illegible]) (Fig[illegible] 3C). Western blot d[illegible] exh[illegible]hat the[illegible]vels of mTOR, p-mT[illegible] and Surv[illegible] in U251/DDP cells [illegible] remarkably [illegible] compared to that in [illegible] (Figure 3D).

Over-Expression [illegible]iR-107 Suppressed DD[illegible]istance and [illegible]ilitated Cell A[illegible]ptosis of U251/DD[illegible] Cells

Under the treatment of 8 mg/L DDP, U251/[illegible]DP cells was [illegible]htly affected, with relatively [illegible] apoptosis, c[illegible]pared to U251 cells (Figure 4[illegible]). Howeve[illegible]n order to determine the regulat[illegible]m of miR-107, miR-107 mimic was trans[illegible]ed and our data presented that cel[illegible]ar expressions of mTOR, p-mTOR and Sur[illegible] significantly decreased ($p < 0.05$) ([illegible]igu[illegible]4A and 4B), cell proliferation potency was weakened (Figure 4D), and DDP-induced inhibitory effect on cell proliferation activity was re-enhanced (Figure 4C). Moreover, the inhibi[illegible]ion of mTOR by si-mTOR also showed similar effects by miR-107 over-expression, suggesting that miR-107 decreased DDP resistance of U251/DDP cells via suppressing mTOR (Figure 4).



Fi[illegible]. Targeted regulation between miR-107 and mTOR. *(A)* Binding sites between miR-107 and 3'-UTR of mTOR mRN[illegible]*B)* Dual luciferase reporter gene assay. *, $p < 0.05$ compared to miR-NC group.

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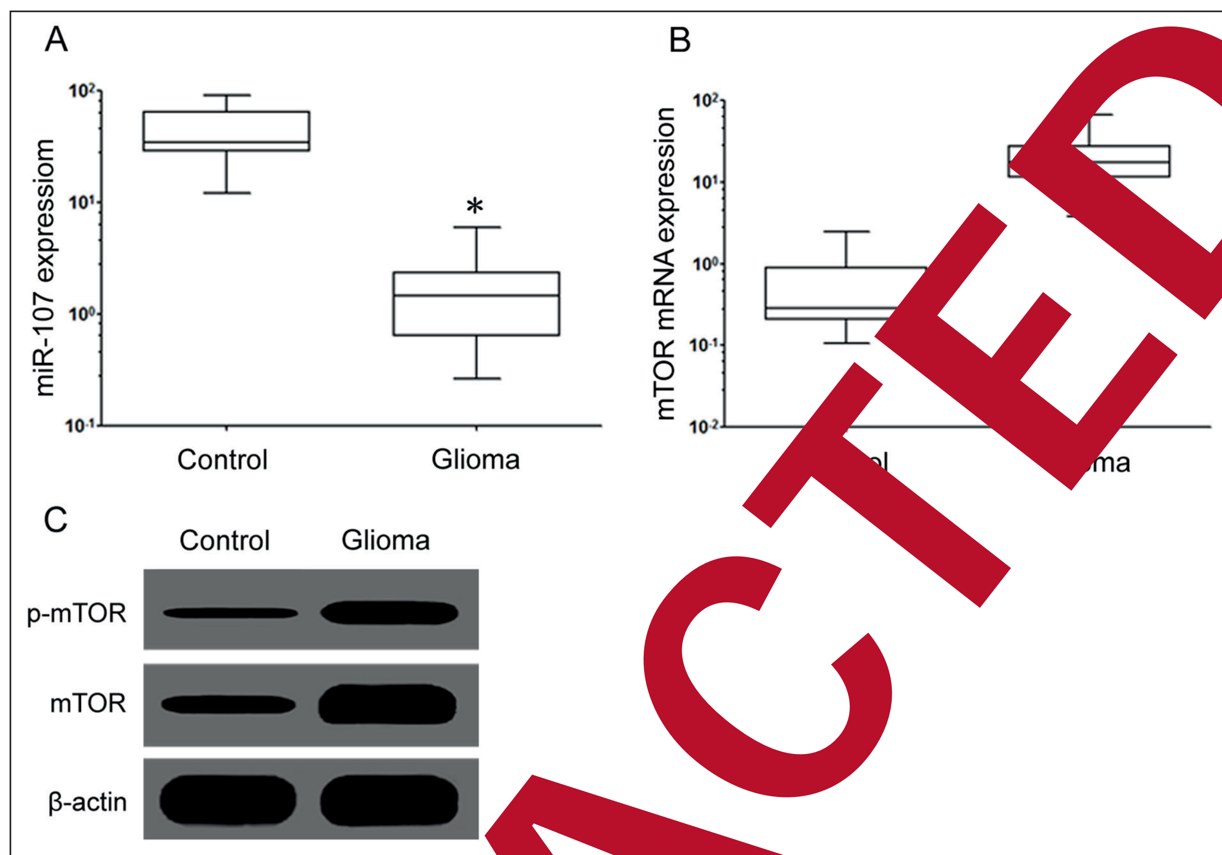

Figure 2. Down-regulation of miR-101 and up-regula[…]f mT[…]n glio[…] tissues. ***(A)*** qRT-PCR for Gli1 mRNA expression; ***(B)*** qRT-PCR for miR-132 expression; ***(C)*** Western blo[…] Gl[…]otein expression. *, $p < 0.05$ compared to control group.

Discussi[…]

mTOR, as a serine/t[…]e pr[…] belongs to phosphati[…]ylino[…]ase ([…]) protein kinase like […]ily[15]. m[…]ne locates on human chrom[…]me 1q36.2 an[…]produce 289kDa protei[…]RNA transla[…][16]. The function and […]ivity […]TOR is mainly under regulation of PI3K/prote[…]se B (PKB) signal pathway[17…] PI3K/AKT is […]ost important signal t[…]sduction molecule up[…]eam of mTOR, such […]3K/AKT/mTOR signal pathway is the ma[…]pend[…] route for mTOR to exert regulator[…]ns. Und[…]he synergistic effects of related […]s su[…]s eukaryotic translation […]ing fac[…]nding protein 1 (4E-BP1), m[…] can reg[…]e transcription and expression […] multiple target genes, and exert critical fu[…]l division, growth, proliferation, […]ptosis a[…] drug resistance[19-21]. When PI3K/[…]T/mTOR signal pathway is activated, PI3K u[…]es self change of conformation for activatio[…] and further accelerates phosphorylation of phosphatidylinositol-(4,5)-bisphosphate (PIP2) into phosphatidylinositol-(3, 4, 5)-trisphosphate (PIP3), which acts on AKT for its phosphorylation under PDK1 and PDK2. The activated AKT kinase directly activates mTOR by phosphorylation. It can also phosphorylate tuberous sclerosis complex-2 (TSC-2) protein to suppress the formation of TSC-1/TSC-2 complex. The Ras homolog enriches in brain (Rheb) and indirectly potentiates mTOR activation[4,22]. As an important anti-apoptotic factor, Survivin serves as an important target gene at downstream of PI3K/AKT/mTOR signal pathway, and is closely correlated with tumor pathogenesis[23]. Scholars[13,14] showed that, compared to normal brain tissues, glioma patients had significantly lower miR-107 expression in tumor tissues, indicating possibly tumor suppressor gene role of miR-107 in glioma. He et al[24] showed the role of abnormally decreased miR-107 expression in facilitating cell proliferation, antagonizing apoptosis and accelerating glioma pathogenesis. Ji et al[14] showed lower miR-107 expression in glioma tissues compared to

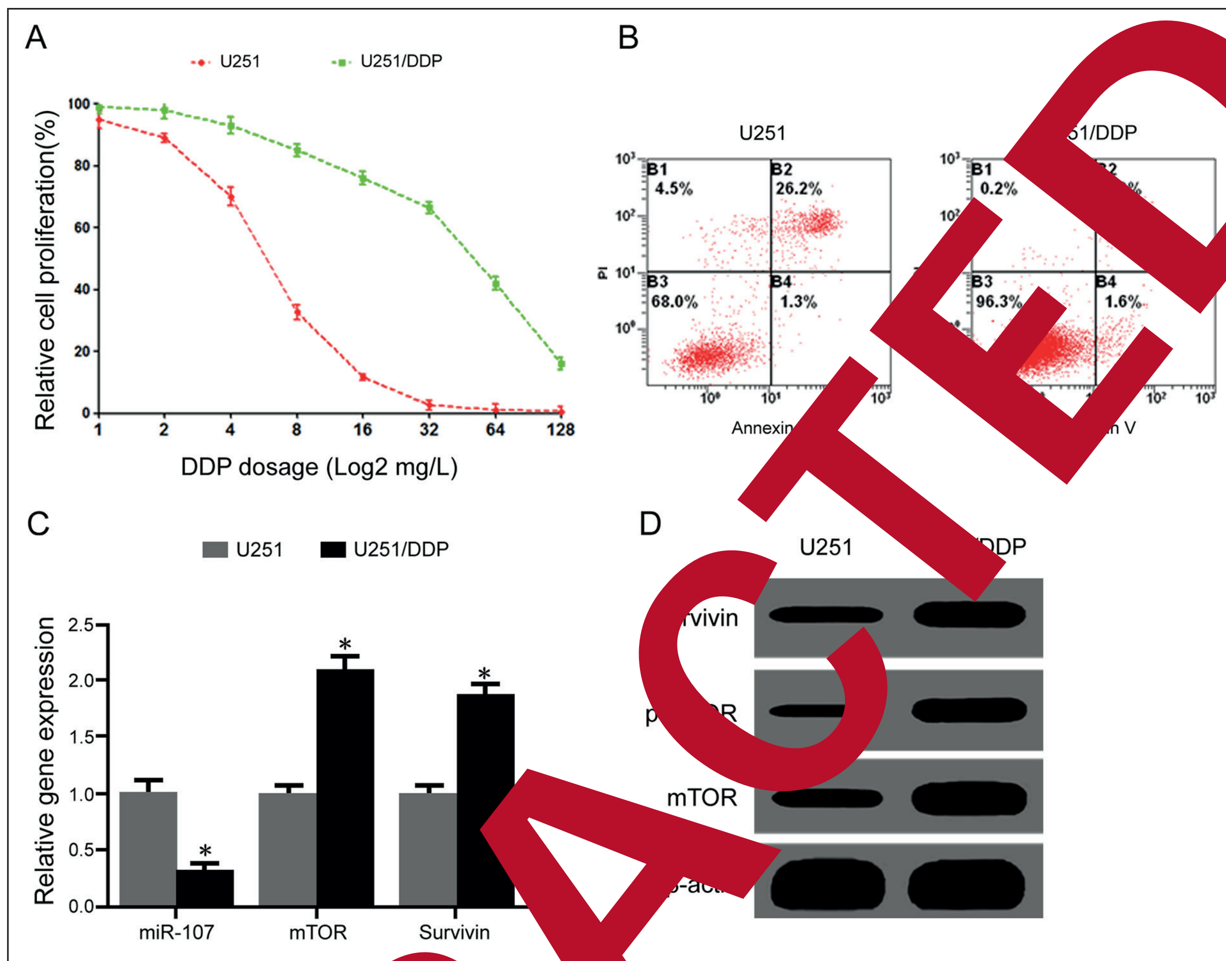

Figure 3. MiR-107 down-regulat[illegible] was [illegible]ated with [illegible]R up-regulation and drug resistance. ***(A)*** CCK-8 assay for inhibitory effects on cell viabili[illegible]y DDP; [illegible] flow cytom[illegible]y for cell apoptosis; ***(C)*** qRT-PCR for gene expression; ***(D)*** Western blot for protein expre[illegible] *, $p < 0.0$[illegible]mpared to U251 cells.

normal brain tissue[illegible]us wors[illegible]ival span or prognosis in thos[illegible]ith lower mi[illegible]expression. Chen et al[illegible] that compare[illegible] normal brain tissues[illegible]ioma[illegible]es had significantly decreased miR-107 expre[illegible] Compared to normal astr[illegible]e, glioma cell [illegible] including U87, U251 a[illegible]A172 had abnormally lower expression of m[illegible]07. Che[illegible] et al[13] also found that over-expre[illegible] of m[illegible]07 could inhibit clonal formation o[illegible]ells U87[illegible]d A172, and weakened migration[illegible]vasi[illegible] potency of glioma cells [illegible]rgeted [illegible] on NOTCH2 expression. Int[illegible]ingly, ou[illegible]sults of dual luciferase gene repo[illegible]r assay showed that transfection of miR-107[illegible]ificantly suppressed relative lu[illegible]ase ac[illegible]y in HEK293T cells, and transfec[illegible] of miR-107 inhibitor remarkably potentiated re[illegible]y luciferase activity in HEK293T cells. Furt[illegible]more, this study showed that compared to brain contusion tissues, the mTOR and p-mTOR levels were significantly elevated, whilst miR-107 expression was significantly decreased. Chen et al[25] observed abnormally decreased miR-107 in glioma tumor tissues and cell lines. In this study, we found abnormally lower miR-107 expression in glioma tissues, as similar with He et al[24], Ji et al[14] and Chen et al[13], indicating possible involvement of miR-107 down-regulation in glioma pathogenesis. Compared to U251 cells, in drug resistant U251/DDP cells, the miR-107 expression was reduced, with increasing levels of mTOR, p-mTOR and Survivin. Transfection of miR-107 mimic or si-mTOR remarkably decreased mTOR, p-mTOR and survivin expression in U251/DDP cells, weakened cell proliferation potency and enhanced cell apoptosis, thus enhancing inhibitory effects on cell proliferation by DDP. Chen et al[25] showed that over-expression of miR-107 could

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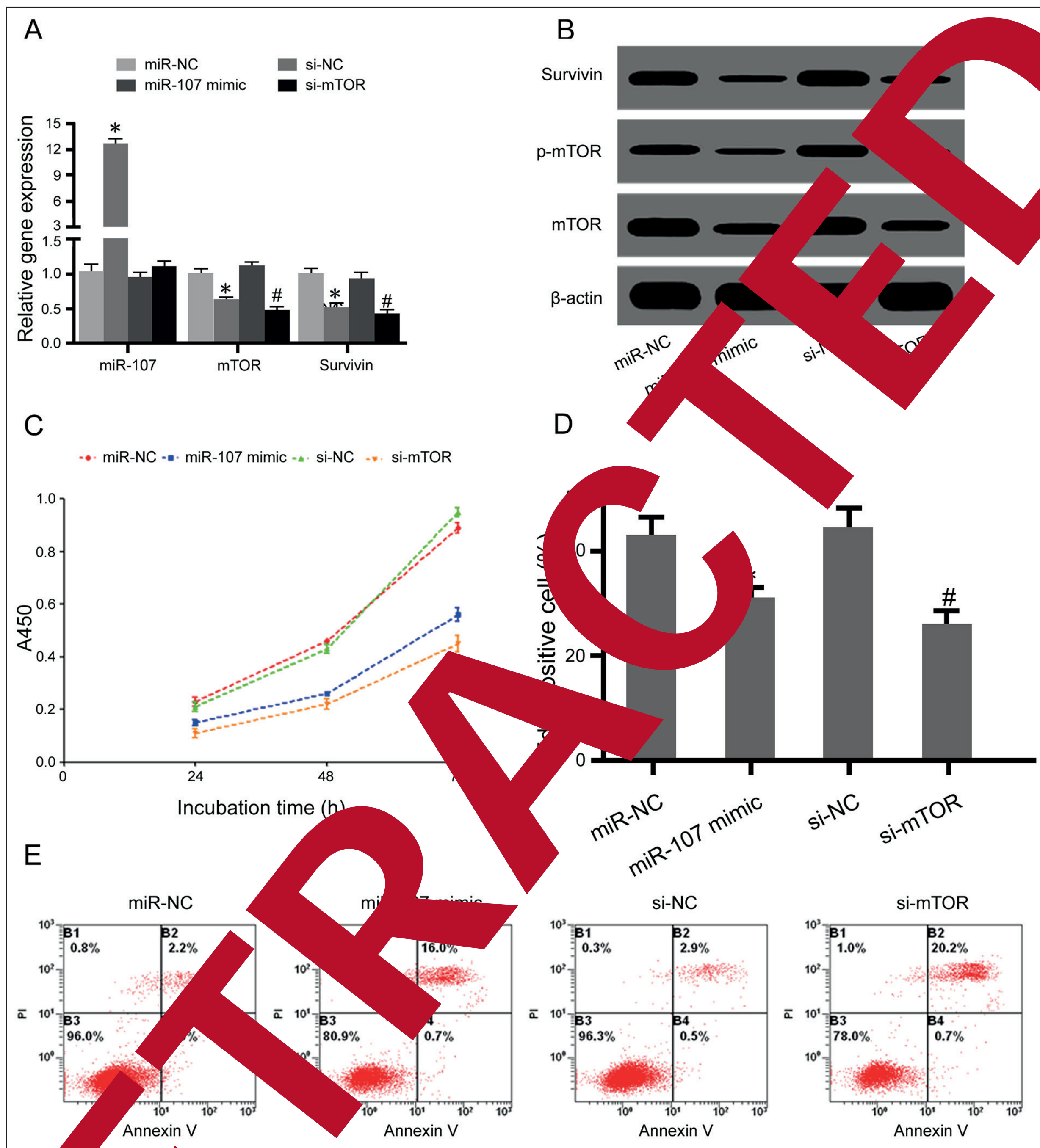

Fig[illegible] **Over-**[illegible]**ession of miR-107 suppressed DDP resistance and facilitated cell apoptosis of U251/DDP cells.** ***(A)*** qRT-PCR fo[illegible]ssion; ***(B)*** [illegible]stern blot for protein expression; ***(C)*** CCK-8 assay for the effect of DDP on cell proliferation activity; ***(D)*** [illegible]aining f[illegible]ell proliferation potency; ***(E)*** Flow cytometry for cell apoptosis. *, $p < 0.05$ comparing between [illegible]07 mim[illegible]iR[illegible], #, $p < 0.05$ comparing between si-mTOR and si-NC.

indu[illegible] G0/G1 cell cycle arrest of glioma cells and we[illegible]oliferation potency via targeted [illegible]bition of CDK6 and Notch-2 expression. He [illegible][24] demostrated that up-regulation of miR-1[illegible]ificantly suppressed target gene SALL4 expr[illegible]on, weakened glioma cell MO59K proliferation and induced cell apoptosis. Chen et al[26] found that up-regulation of miR-107 could suppress stem cell features of glioma cells, inhibit glioma stem cell proliferation, weaken stem cell invasion potency and suppress their tumorigenic potency inside the body. Zhu et al[6] revealed that

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down-regulation of mTOR weakened resistance of glioma cells against anti-tumor reagent temozolomide. Garros-Regulez et al[27] detected that inhibition of mTOR functional activity could significantly enhanced sensitivity of glioma stem cells on chemotherapy drug temozolomide. Wu et al[28] found that FK228 could enhance the sensitivity of glioma cells against temozolomide via suppressing PI3K/AKT/mTOR signal pathway. Consistently, this study also showed the correlation between mTOR expression and drug resistance of glioma cells, as down-regulation of mTOR decreased drug resistance of glioma cells, as similar with Zhu et al[6], Garros-Regulez et al[27] and Wu et al[28]. Accumulative evidence presented the promising effect of miRNAs in the treatment of glioma tumor[29-31]. Our data unraveled the similar role of miR-107 on the restriction of progression of glioma cancer cells. However, the limitation in this study still exists that further *in vivo* investigation on miR-107-mediated PI3K/AKT/mTOR pathway and cisplatin resistance in patients with glioma is required, and the clinical value of miR-107 needs evaluation based on a large cohort of patients.

Conclusions

We observed that miR-107 was correlated [illegible] the rise of DDP resistance of glioma cells. T[illegible] over-expression of miR-107 [illegible]press th[illegible] proliferation of DDP-resist[illegible] glio[illegible]ells via targeted inhibition of mT[illegible] express[illegible], which lays fundamental lead[illegible]e fu[illegible] practice.

Conflict of Int[illegible]

The Authors de[illegible] that [illegible]e no conflict of interests.

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