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# Mir-338-3p Inhibits Malignant Biological Behaviors of Glioma Cells by Targeting MACC1 Gene

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**Background:** Human brain glioma is the most common endocranial tumor; its mortality and morbidity are very high. The objective of this study was to determine whether miR-338-3p can regulate malignant biological behaviors of glioma cells by targeted silencing of MACC1.

**Material/Methods:** The expression of miR-338-3p was detected by quantitative real-time PCR in brain glioma tissues and cell lines. Bioinformatics software was used to predict some potential target genes of miR-338-3p. Luciferase activities assay was used to verify the combination between target genes and miR-338-3p. And MACC1 protein expression was detected by Western blot. The apoptosis and proliferation ability were analyzed by MTT and flow cytometry assay.

**Results:** Compared with normal brain tissues and cells, miR-338-3p in glioma tissues and cell lines was confirmed to be expressed at low levels, and down-regulation of miR-338-3p tended to be correlated with worse histological grade. Up-regulation of miR-338-3p promoted apoptosis and sharply inhibited cell proliferation ability of U251 and U87 cells. The luciferase activities assay, biotin-avidin pull-down assay, and western blot analysis verified that MACC1 was a specific target gene of miR-338-3p. Subsequent experiments found that up-regulation of MACC1 significantly inhibited the apoptosis and increased the cell proliferation ability of U251 and U87 cells. The regulation effects of miR-338-3p on malignant biological behaviors of glioma cells can be partly reversed by up-regulation of MACC1.

**Conclusions:** Down-regulation of miR-338-3p was an independent prognostic biomarker associated with poor prognosis in glioma patients; miR-338-3p acted as a tumor-suppressing gene whose silencing can inhibit malignant biological behaviors of glioma cells. MACC1 was a specific target gene of miR-338-3p, which regulates malignant biological behaviors of glioma cells partly through directly silencing MACC1 expression.

**MeSH Keywords:** **Apoptosis • Cell Proliferation • Glioma • MicroRNAs**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/897055>



## Background

Human brain glioma is the most common endocranial tumor; it has very high mortality and morbidity rates. The prognosis of glioma is poor because glioma shows significant malignant proliferation and invasion [1]. Therapy for glioma includes surgery, chemotherapy, radiation therapy, and biological therapy. Surgery is the preferred strategy; radiation therapy and chemotherapy following surgical excision are considered as effectual supplementary therapy to prevent metastasis and relapse of glioma [2]. Nevertheless, these therapies are not effective for everyone, and only help about half of glioma patients. The median survival time of anaplastic astrocytoma is about 2–3 years, and the prognosis of high-grade glioma is even worse, with a 2-year median survival rate of only 30% [3].

Noncoding RNAs include lncRNA and short non-coding RNAs, such as microRNA, siRNA, and piRNA. MicroRNAs can function as an oncogene or tumor-suppressing gene in many kinds of malignant cancers, with anomalous expression and functions [4,5]. MiR-338-3p is a tumor-suppressing gene expressed at low levels in some kinds of malignant tumors, including hepatocellular carcinoma and gastric cancer [6,7]. To date, there have been no published studies on anomalous expression and function in glioma. In analysis of the regulatory mechanism of MACC1, bioinformatics analysis predicted MACC1 is a potential target gene of miR-338-3p. In our previous research, the metastasis-associated in colon cancer-1 (MACC1) gene was confirmed to be over-expressed in glioma tissue and silencing of MACC1 promoted the chemosensitivity of U251 cells to DDP [8]. However, the cause of MACC1 over-expression and its effects in regulating miR-338-3p to display malignant biological behaviors of glioma cells are unclear. In this study, we investigated the molecular mechanism by which miR-338-3p regulates malignant biological behaviors of glioma, as well as exploring the function of MACC1 in this regulation.

## Material and Methods

### Clinical specimens

We studied a total of 39 glioma and 17 adjacent normal brain tissues (NBT) obtained from Shengjing Hospital of China Medical University from February 2011 to November 2011. The Ethics Committee of China Medical University approved this study, and we obtained patient permission before surgery. After surgery, the tissue samples were stored at  $-80^{\circ}\text{C}$ , and the pathological information was obtained soon after the operation according to the 2007 WHO classification. Follow-up was performed every 3 months for the first 2 postoperative years, and every 6 months thereafter. The age (mean  $\pm$ SD) was  $53.8 \pm 6.9$  years (range, 46 to 61 years) with 24 men and

15 women. Among the glioma patients, there were 7 cases of grade I, 10 cases of grade II, 13 cases of grade III, and 9 cases of grade IV according to the histology.

### Cell culture

Human brain glioma U251 and U87 cell lines and HEK 293T cells were obtained from the China Academy of Chinese Medical Sciences. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (Life Technologies Corporation, Carlsbad, CA), and culture condition was 5%  $\text{CO}_2$  at  $37^{\circ}$ . Primary normal human astrocytes (NHA) were purchased from the Sciencell Research Laboratories (Carlsbad, CA) and cultured according to the manufacturer's instructions.

### Quantitative real time -PCR (qRT-PCR)

Trizol reagent was used to obtain total RNA and the Reverse Transcription Kit (Applied Biosystems, USA) was used to synthesize cDNA. Expression of miR-338-3p and MACC1 was detected with SYBR (Applied Biosystems, Foster City, CA). GAPDH and U6 were used as endogenous controls. The Ct value of detected genes was obtained from 3 independent experiments and quantified with the  $2^{-\Delta\Delta\text{Ct}}$  method.

### Transfection

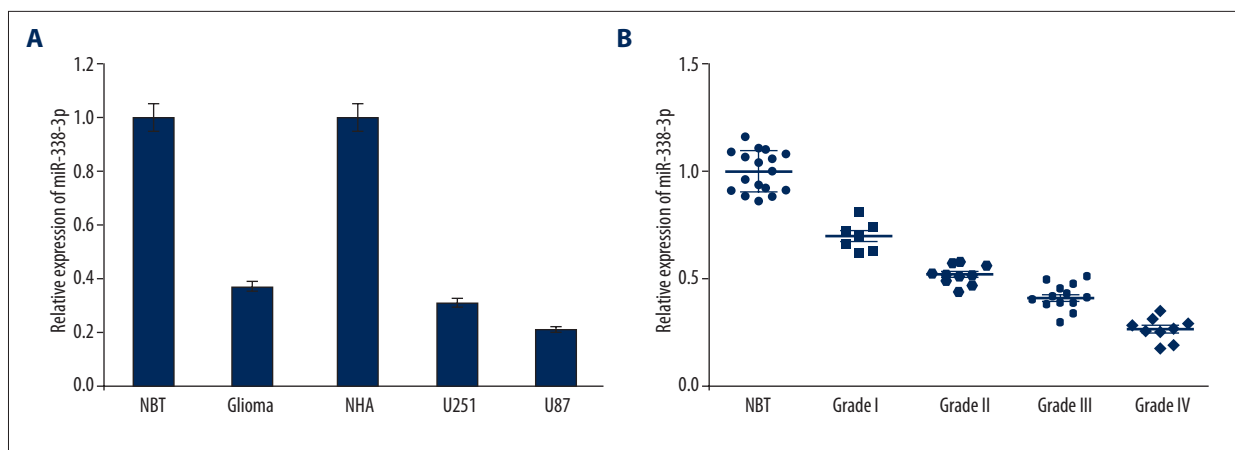
Cells at 70–80% concentration were seeded into 6-well plates before transfection. The precursor of miR-338-3p (pre-miR-338-3p), pre-NC, inhibitor of miR-338-3p (anti-miR-338-3p), and anti-NC were constructed by the Genescript company (Nanjing, China). The miRNAs were transfected to cells with Lipofectamine™ 3000 Reagent (Invitrogen, USA), the final concentration of miRNAs was 60 nM. After 4 h, cells were cultured with normal media and harvested 48 h later.

### Biological behavior assay in glioma cells

Cell proliferation ability detected by MTT was performed as previously described [9]. Apoptosis rate was determined with dual-color flow cytometric method. Cells were harvested, and we detected apoptosis level with the Annexin V-FITC apoptosis detection kit (KeyGEN, China) following the manufacturer's protocol. Flow cytometry (BD, USA) and CELLQuest 3.0 software (BD, USA) were used to obtain and analyze apoptosis data. Each experiment was performed 3 times.

### MicroRNA targets prediction and dual-luciferase reporter assay

The target gene of miR-338-3p was predicted by TargetScan (<http://www.targetscan.org>). The dual-luciferase reporter plasmids, pmiR-MACC1-wt (containing the wild-type binding site



**Figure 1.** (A) Compared with normal brain tissues (NBT) and NHA cells, miR-338-3p was under-expressed in human glioma tissues and cell lines. (B) Glioma tissues with worse histological grade tended to have lower miR-338-3p expression. \*  $P < 0.05$  vs. NBTs or NHA group.

of MACC1 3'UTR in pmiR-RB-REPORTM luciferase reporter plasmid [RiboBio Co. Ltd., China] and pmiR-MACC1-mut (containing the mutant binding site of MACC1 3'UTR) were constructed by the Genescript Company (Nanjing, China). For the luciferase assay, pre-miR-338-3p and constructed plasmid were cotransfected into HEK 293T cells using Lipofectamine™ 3000 Reagent (Invitrogen, USA). Then the luciferase activity was detected with the dual-luciferase reporter assay system (Promega, USA) from 3 independent experiments after cotransfection of HEK 293T cells for 48 h following the manufacturer's protocol.

#### Pull-down assay

MiR-338-3p, miR-338-3p-Mut, and miR-338-3p-NC were biotinylated to be bio-miR-338-3p-wt, bio-miR-338-3p-mut, and bio-NC, respectively, by GenePharma Company (Shanghai, China), and transfected into glioma cells. At 48 h later, cells were harvested and lysed. The samples were incubated with Dynabeads M-280 Streptavidin (Invitrogen, CA) following the manufacturer's protocol. Biotinylated miR-338-3p was incubated with beads for 10 min, and then treated with washing buffer. The bound RNAs were obtained and analyzed by qRT-PCR. Each experiment was performed 3 times.

#### Western blot analysis

A total of 20 mg of protein was used for Western blotting. After gels electrophoresis, samples were transferred to PVDF membranes. After blocking, membranes were incubated with primary polyclonal antibody against MACC1 (Cell Signaling Technology, USA) at a 1/800 dilution for 12 h at 4°C, then incubated with secondary antibody (Cell signaling Technology, USA) at a 1/4000 dilution for 2 h at room temperature. Proteins were enhanced using a chemiluminescence detection kit. The

protein quantification was performed using ImageJ software. Each experiment was performed 3 times.

#### Statistical analysis

Statistical analysis was completed with SPSS 13.0 software (IBM, IL). The data are presented as mean  $\pm$ SD of 3 independent experiments and compared using the *t* test and one-way ANOVA.  $P < 0.05$  indicates a significant difference.

## Results

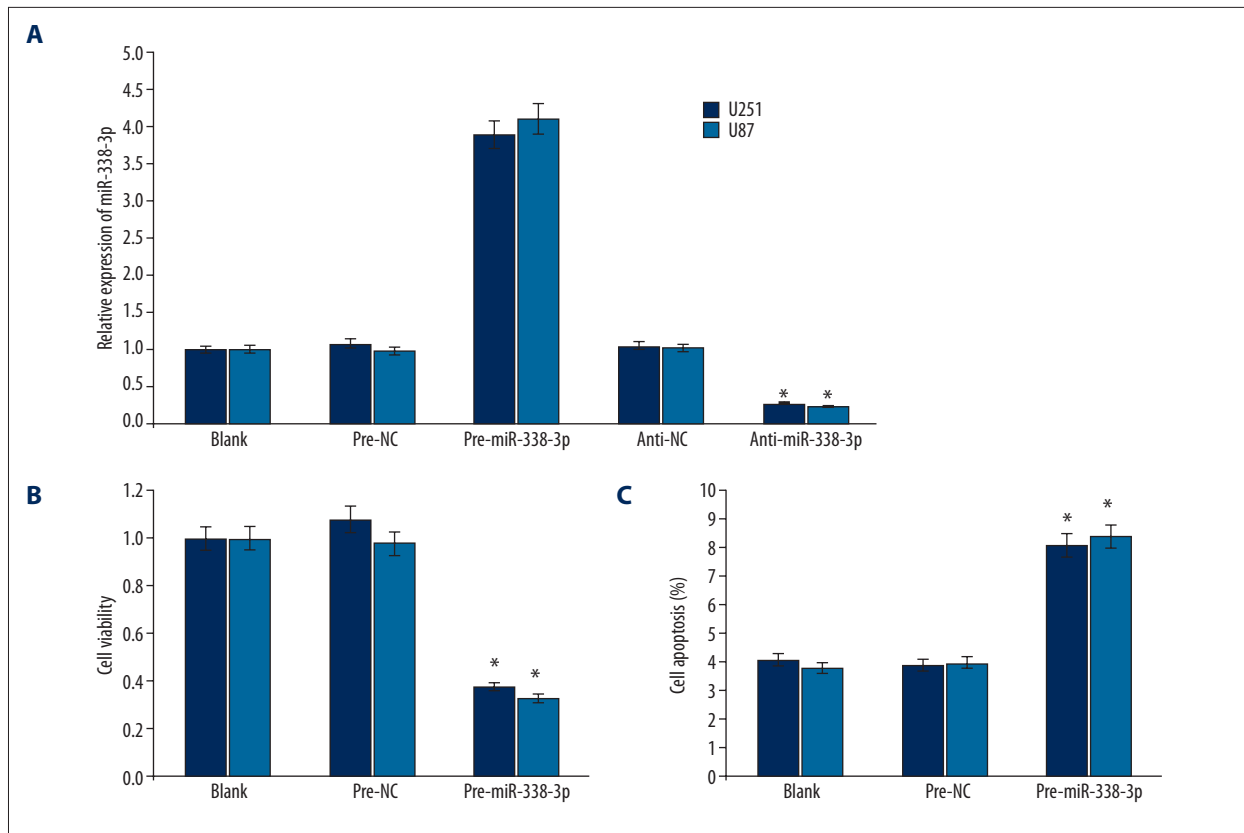
### MiR-338-3p was up-regulated in glioma tissues and cells

Our results show that miR-338-3p expression in glioma samples was much lower than in NBT samples, and the expression of miR-338-3p in U251 and U87 cells was also much lower than in NHA cells ( $p < 0.05$ ) (Figure 1A), which provides initial evidence that the depletion of miR-338-3p may play a role in glioma tumorigenesis.

Moreover, the correlation between miR-338-3p expression and specific clinical characteristics of glioma was studied. Glioma patients with lower miR-338-3p expression tended to be correlated with worse histological grade ( $p < 0.05$ ) (Figure 1B). However, miR-338-3p expression was not significantly correlated with other clinical characteristics such as sex and age ( $p > 0.05$ ).

### Up-regulation of miR-338-3p inhibits the malignant biological behaviors of glioma cells

We investigated whether miR-338-3p influences the malignant biological behaviors using glioma cells. Pre-miR-338-3p was transfected into U251 and U87 cells to increase the expression



**Figure 2.** (A) Compared with control groups, pre-miR-338-3p increased the expression of miR-338-3p in U251 and U87 cells, and anti-miR-338-3p can inhibit miR-338-3p expression. (B, C) MiR-338-3p up-regulation inhibited cell viability and advanced apoptosis of U251 and U87 cells. \*  $P < 0.05$  vs. control glioma cells.

of miR-338-3p (Figure 2A). In comparison to control cells, the cell viability was markedly decreased by MTT assay in U251 and U87 cells with miR-338-3p up-regulation (Figure 2B), but the apoptosis rate increased significantly in U251 and U87 cells with miR-338-3p up-regulation (Figure 2C).

#### MACC1 is a specific target gene of miR-338-3p in glioma

As for the important effects of miR-338-3p gene in glioma, further mechanism analysis is becoming necessary. Using the publicly available databases, we found a conserved binding site in 3'-UTRs of MACC1 (1932–1938 bp) for miR-338-3p (Figure 3A).

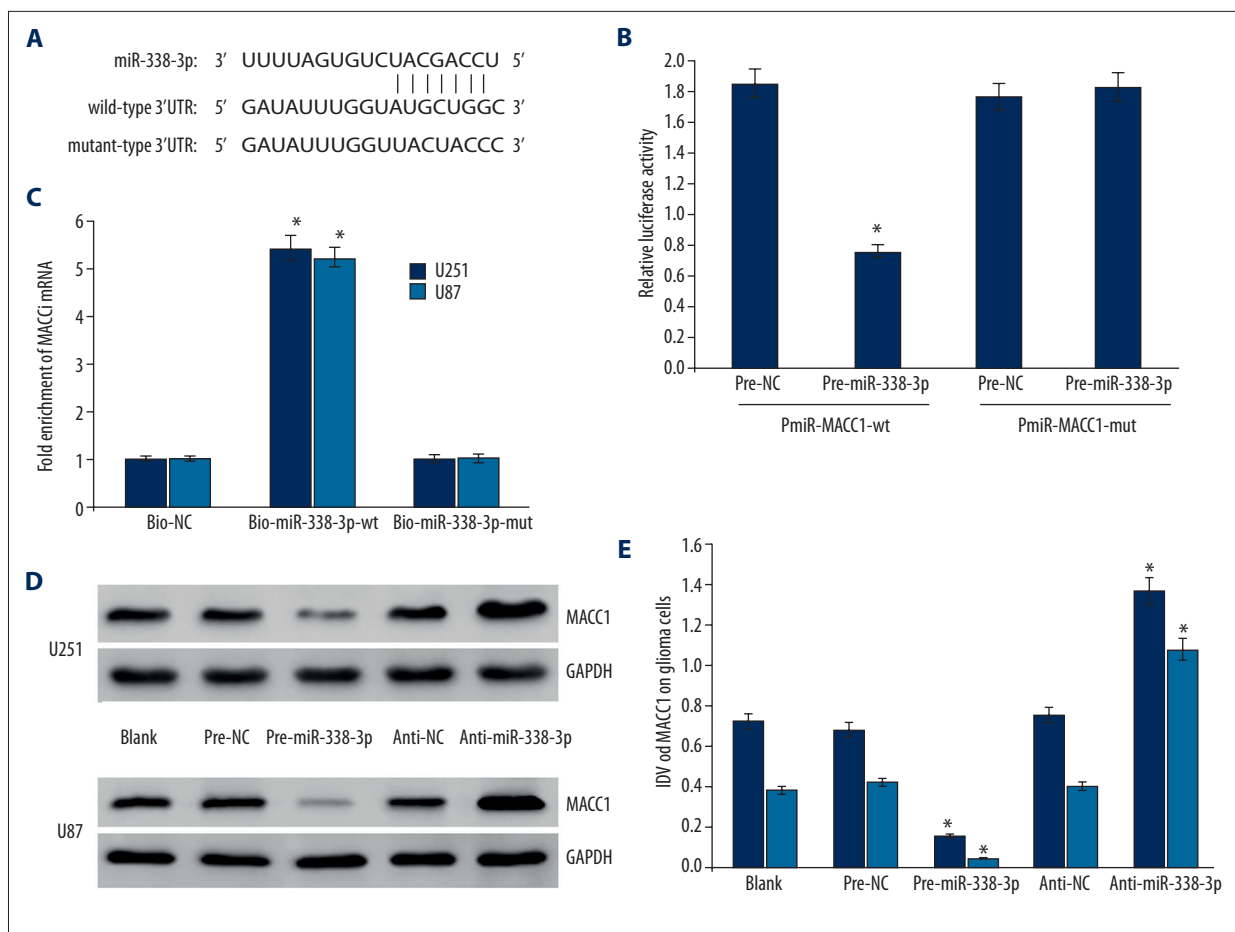
The luciferase reporter assay was affirmed to identify the direct interaction between miR-338-3p and MACC1. As shown in Figure 3B, the relative luciferase activity was much lower in glioma cells cotransfected with wild-type vector and pre-miR-338-3p than in other transfected groups. Those results indicate that pre-miR-338-3p specifically binds to the seed zone of MACC1 3'UTR to inhibit luciferase expression, while mutant-type vector does not combine with miR-338-3p to decrease the relative luciferase activity. MACC1 is a specific target gene of miR-338-3p.

We used biotin-avidin pull-down assay to discover that MACC1 mRNA could be pulled down by bio-miR-338-3p-wt (Figure 3C). However, MACC1 mRNA could not be pulled down by bio-miR-338-3p-mut, which was introduced with mutations in the putative recognition site between MACC1 mRNA and miR-338-3p. Those findings show that miR-338-3p can sequence-specifically combine with MACC1 mRNA.

Compared with control, miR-338-3p enhancement remarkably inhibited the expression of MACC1 protein, and miR-338-3p silencing induced by the inhibitor of miR-338-3p (anti-miR-338-3p) caused the up-regulation of MACC1 protein in U251 and U87 cells (Figure 3D, 3E). However, there was no significant alteration of MACC1 mRNA (data not shown). These results show that miR-338-3p can silence MACC1 expression at the post-translational level.

#### MiR-338-3p inhibits malignant biological behaviors of glioma cells by targeting MACC1 gene

Because miR-338-3p can inhibit malignant biological behaviors of glioma cells, and because MACC1 is a miR-338-3p targeting gene, we next studied the physiological role of



**Figure 3.** (A) Putative binding sites of miR-338-3p within the 3'-UTR region of MACC1 mRNA, and the sequences of wild-type and mutant-type vector. (B) The relative luciferase activities were inhibited in the HEK-293 cells cotransfected with wild-type vector and pre-miR-338-3p, not with the mutant-type vector. Firefly luciferase activity was normalized to Renilla luciferase. (C) Compared with the control group (bio-NC) and biotinylated mutational miR-338-3p (bio-miR-338-3p-mut), expression of MACC1 mRNA was much higher in the sample pulled down by biotinylated miR-338-3p (bio-miR-338-3p-wt). (D) The influence of transfection with pre-miR-338-3p and anti-miR-338-3p to MACC1 protein expression in U251 and U87 cells. (E) The relative quantitative analysis of MACC1 protein normalized to GAPDH protein. IDV is the abbreviation for "integrated density values" \**P*<0.05 vs. control glioma cells.

miR-338-3p-target MACC1 in glioma cells after up-regulation of MACC1. As shown in Figure 4A, 4B, expression vector of MACC1 (pc-MACC1) significantly increased the expression of MACC1 protein in U251 and U87 cells transfected with pre-miR-338-3p.

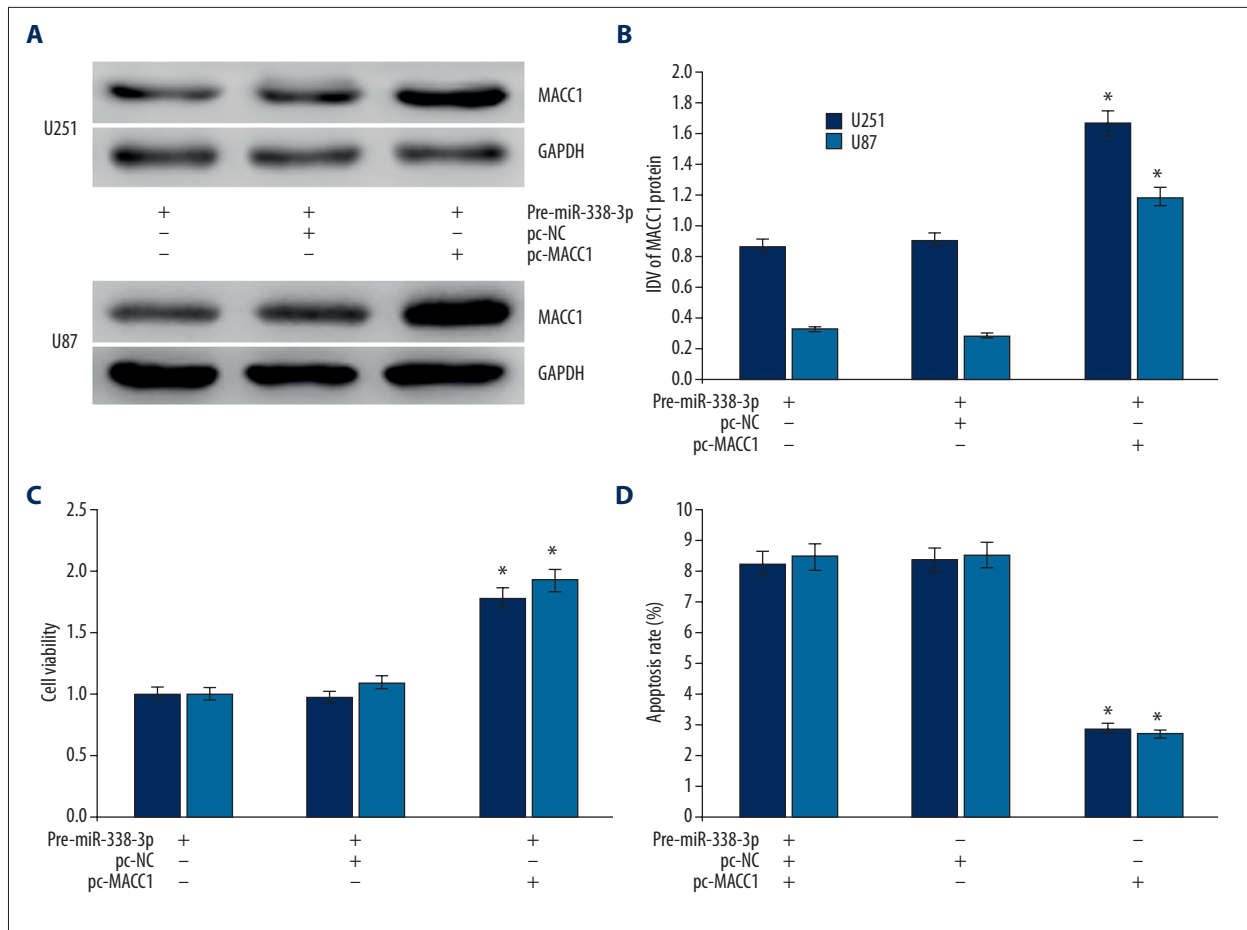
Compared to the glioma cells transfected with pre-miR-338-3p, the cell viability in glioma cells co-transfected with pre-miR-338-3p and pc-MACC1 was significantly upgraded (Figure 4C), and the cell apoptosis was markedly inhibited (Figure 4D). The regulation effects of miR-338-3p on malignant biological behaviors of glioma cells can be reversed to a certain degree by up-regulation of MACC1.

In summary, miR-338-3p was confirmed to control malignant biological behaviors of U251 and U87 cells, partly by targeting MACC1 gene.

## Discussion

It is widely accepted that glioma cells possess a powerful proliferation ability, low apoptosis rate, and vigorous resistance to chemotherapy and radiotherapy, which contribute to malignancy and recurrence of glioma [10–12].

It is well known that microRNAs play important roles in critical biological behaviors, including proliferation, apoptosis, and invasion, and its anomalous expression and functions may advance the malignant development of glioma [13,14]. Recent studies report that some microRNAs have a relationship with prognosis and can be used as prognostic biomarkers of glioma [13,14].



**Figure 4.** (A) The influence of transfection with pre-miR-338-3p and pc-MACC1 to MACC1 protein expression in U251 and U87 cells. (B) The relative quantitative analysis of MACC1 protein normalized to GAPDH protein. (C, D) The influence of transfection with pre-miR-338-3p and pc-MACC1 on cell viability and apoptosis of U251 and U87 cells. \*  $P < 0.05$  vs control glioma cells. \*  $P < 0.05$  vs. glioma cells transfected with pre-miR-338-3p.

Our study found that miR-338-3p was under-expressed in glioma tissues and cells, and its expression level had a negative correlation with glioma histological grades. We found that miR-338-3p up-regulation can inhibit cell proliferation ability, and advance cell apoptosis of U251 and U87 cells. Accordingly, miR-338-3p plays important roles in tumorigenesis of glioma, and is a good biomarker for prognostic prediction in glioma.

MicroRNAs accommodate their target genes to exercise their functions, such as miR-21, FASLG, miR-200c, and ZEB2 [9,15]. Thus, bioinformatics software was used to predict the target gene of miR-338-3p. Among predicted candidate genes, MACC1 gene is the most conserved and recommended for use.

The MACC1 gene was first discovered by Stein in 2009 [16]. Studies have reported that MACC1 is up-regulated in many kinds of malignant tumors, such as colon cancer, gastric cancer, and glioma [17–19]. In our previous research, the metastasis-associated in colon cancer-1 (MACC1) gene was confirmed

to be over-expressed in glioma tissue and we found that silencing of MACC1 promoted chemosensitivity to DDP of U251 cells [8]. Previous studies reported MACC1 gene can activate the HGF/Met signal pathway to promote tumorigenesis, malignant development, and metastasis [20,21]. However, the cause of MACC1 up-regulation in glioma and its effects in the regulation of miR-338-3p to malignant biological behaviors of glioma cells are still unclear.

The conclusion that MACC1 is a target gene of miR-338-3p was confirmed by a series of gain-of-function experiments. First, luciferase reporter assay identified miR-338-3p could specifically combine with 3'-UTRs of MACC1 at 1932-1938 bp and can silence the expression of luciferase. Second, the biotin-avidin pull-down assay powerfully validated the specific and direct combination between MACC1 mRNA and miR-338-3p. Third, anti-miR-338-3p enhancement can negatively regulate MACC1 protein expression at the post-translational level, and miR-338-3p silencing displayed contradictory regulative effectiveness.

The targeted modulation of miR-338-3p to MACC1 gene was also reported in gastric cancer [18].

Because miR-338-3p over-expression inhibits malignant biological behaviors of glioma cells, and MACC1 is a pivotal target gene of miR-338-3p, we hypothesized that miR-338-3p might regulate malignant biological behaviors through directly down-regulating MACC1. Then, the pc-MACC1 was transfected to up-regulate the expression of MACC1 in U251 and U87 cells with miR-338-3p up-regulation. Our experiments found that up-regulation of MACC1 could sharply promote malignant biological behaviors of glioma cells. The regulatory effects of pre-miR-338-3p on malignant biological behaviors can be partly reversed by MACC1 up-regulation. Accordingly, the verification of MACC1 as a target gene of miR-338-3p offers a probable explanation of how the under-expression of miR-338-3p can function as a tumor-suppressing gene in glioma, and how

miR-338-3p might influence chemosensitivity of glioma cells to DDP through targeting the MACC1 gene.

## Conclusions

miR-338-3p acts as a tumor-suppressor gene whose silencing can inhibit malignant biological behaviors of glioma cells. MACC1 is a target gene of miR-338-3p, and miR-338-3p regulates malignant biological behaviors of glioma cells, partly through directly silencing MACC1 expression.

## Competing interests

The authors declare they have no competing interests regarding this study.

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