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Research Article

miR-6743-5p, as a direct upstream regulator of GRIM-19, enhances proliferation and suppresses apoptosis in glioma cells

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Gene associated with retinoid-interferon-induced mortality-19 (GRIM-19) has been recognized as a tumor suppressor protein, which regulates cell growth, apoptosis, and migration by signal transducer and activator of transcription 3 (STAT3) signaling pathway and non-STAT3 pathway in glioma cells. Here, we investigated the molecular mechanisms that regulated GRIM-19 expression in glioma cells. By the TargetScan algorithm, four miRNAs, hsa-*miR-17-3p*, hsa-*miR-423-5p*, hsa-*miR-3184-5p*, and hsa-*miR-6743-5p*, were identified with the potential to bind with 3′-UTR of GRIM-19. Further miRNA inhibitor transfection and luciferase assays revealed that *miR-6743-5p* was able to directly target the 3′-UTR of GRIM-19. Additionally, *miR-6743-5p* expression was inversely related with GRIM-19 expression in glioma specimens and cell lines. Moreover, the inhibition of *miR-6743-5p* caused a significant inhibition of cell proliferation and a marked promotion of cell apoptosis in glioma cells, and this phenotype was rescued by GRIM-19 knockdown. Finally, the inhibition of *miR-6743-5p* expression suppressed the phosphorylation of STAT3, and the mRNA expression of CyclinD1 and Bcl-2, two target genes of STAT3, while *miR-6743-5p* mimic had the inversed effects. Treatment with STAT3 inhibitor AG490 partially rescued the proliferation-promoting and anti-apoptosis effects of *miR-6743-5p* overexpression or GRIM-19 knockdown. Collectively, *miR-6743-5p* may act as an oncomiRNA in glioma by targetting GRIM-19 and STAT3.

Introduction

Glioma is the most frequent type of primary malignant brain tumor, arising from the brain or spinal cord tissues [1]. According to the levels of malignancy, the World Health Organization (WHO) classification divides gliomas into grade I to grade IV [2]. Glioblastoma multiforme (GBM, grade IV) is the most malignant and frequent type, accounting for approximately 70% of all diagnosed gliomas [3]. The prognosis of glioma remains poor because of the characteristic malignant proliferation and diffuse invasion [4]. Therefore, a better understanding of the molecular mechanisms of the occurrence and development of glioma may provide new therapeutic target for glioma and improve the prognosis of this disease.

Gene associated with retinoid-interferon-induced mortality-19 (GRIM-19, also known as NDUFA13) was initially identified as a novel cell death-regulatory molecule in a breast carcinoma cell line by the antisense knockout technique [5]. Subsequent studies showed that GRIM-19 plays essential role in normal embryonic development, cell apoptosis, and cell growth [6-10]. Recent evidence has suggested that GRIM-19 may serve as a tumor suppressor protein in various human cancers. For example, GRIM-19 mutations have been detected in some thyroid carcinomas [11]. Loss of expression of GRIM-19 has been reported in renal cell carcinoma [12], cervical cancer [13], colon cancer [14], and glioma [15]. Several

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reports have shown that GRIM-19 can negatively regulate the activity of signal transducer and activator of transcription 3 (STAT3) and the expression of STAT3 downstream genes, thus inhibiting cell transformation [9,13-15]. It has been stated that GRIM-19 exerted inhibitory effects on cell proliferation and migration, and promotion effects on cell apoptosis of glioma cell lines.

miRNAs, small non-coding RNA molecules (approximately 20–23 nts), function in the degradation of mRNA and post-transcriptional regulation of target gene expression through binding to the 3′-UTRs of the target gene [16]. Numerous studies have revealed altered expression of miRNAs in a wide spectrum of human cancers, including glioma [17]. These researches also have established that many miRNAs can elicit oncogenic or tumor suppressor activities in various malignancies [17,18].

In the present study, the TargetScan algorithm was used to predict the miRNAs targetting GRIM-19, and four miRNAs were predicted to target the 3′-UTR of GRIM-19. The luciferase assay confirmed that one of the four identified miRNAs *miR-6743-5p* directly targetted the 3′-UTR of GRIM-19 and regulated its expression in U251 glioma cells. GRIM-19 expression level was inversely correlated to *miR-6743-5p* expression in glioma specimens and cell lines. Further cell proliferation and apoptosis assays showed that knockdown of the expression of GRIM-19 partially rescued the effects of *miR-6743-5p* inhibitor in glioma cells. Additionally, we investigated the roles of STAT3 pathway in the functions of *miR-6743-5p*/GRIM-19.

Materials and methods

Cell lines

The human glioma cell lines (T98G, U251, U373, U87, and SHG44) were supplied by the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). T98G, U373, and U87 were grown in Eagle's minimum essential medium (MEM; HyClone, Logan, UT, U.S.A.), while other two cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone). Both culture media were supplemented with 10% FBS (Gibco, Carlsbad, CA, U.S.A.) and antibiotics. All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Tissues' samples

The study protocol was approved by the Clinical Research Ethics Committee from the First People's Hospital of Zunyi and the First Affiliated Hospital of Zunyi Medical College. Written informed consent was obtained from each patient. A total of 90 glioma and 15 normal brain samples were obtained from the First People's Hospital of Zunyi and the First Affiliated Hospital of Zunyi Medical College between January 2009 and December 2015. Tissue samples were snap-frozen in liquid nitrogen and then stored at −80°C.

Transfection of the miRNA mimic, miRNA inhibitor, and siRNA

The miRNA inhibitor of hsa-*miR-17-3p*, hsa-*miR-423-5p*, hsa-*miR-3184-5p* and hsa-*miR-6743-5p*, hsa-*miR-6743-5p* mimic as well as miRNA inhibitor scramble (anti-miR-control) and controls of the miRNA mimic scramble (miR-control) used in our study were synthesized by Genepharm Technologies (Shanghai, China). Glioma cells at approximately 70% confluence were transfected with a final concentration of 30 nM for miRNA inhibitor or 5 nM for miRNA mimic using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) in accordance with protocols suggested by the manufacturer. At 48 h post transfection, cells were harvested for RNA extraction and real-time PCR analysis.

Real-time PCR

Total RNA and miRNA was extracted from tissue samples or glioma cell lines by the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Stem-loop real-time RT-PCR was carried out to analyze miRNA expression. U6 RNA was used as an miRNA internal control. Briefly, extracted RNAs were converted into cDNAs with stem-loop reverse-transcription primers with cDNA synthesis kit (Thermo Fisher Scientific, Rockford, IL, U.S.A.). Real-time PCR was then performed using Maxima SYBR Green qPCR Master Mixes (Thermo Fisher Scientific) with miRNA or U6-specific forward primer and a universal reverse primer on ABI 7300 system (Applied Biosystems, Foster City, CA, U.S.A.) as the manufacturer suggested. The primers used here are listed in Table 1.

Real-time PCR was performed to determine the mRNA levels of interested genes. In brief, the extracted total RNA was treated with DNase I (Roche, Indianapolis, IN, U.S.A.) to remove possible genomic DNA contamination and reverse-transcribed into cDNA with cDNA synthesis kit (Thermo Fisher Scientific). GAPDH was served as an internal control for real-time PCR. The PCR primers are listed in Table 2. Target gene expression was calculated using the $2^{-\Delta\Delta C}{}_t$ method.

Table 1 Primers for the detection of miRNAs

Target	Primer	Primer sequence
hsa-miR-17-3p	RT primer	5′- CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGGATTC-3′
(MIMAT0000071)	Senses primer	5′- ACACTCCAGCTGGGACTGCAGTGAAGGCAC-3′
hsa- miR-423-5p	RT primer	5′- CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAGTC-3′
(MIMAT0004748)	Senses primer	5′- ACACTCCAGCTGGGTGAGGGGCAGAGAGCGA -3′
hsa-3184-5p	RT primer	5′- CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAAGC -3′
(MIMAT0015064)	Senses primer	5′- ACACTCCAGCTGGGTGAGGGGCCTCAGACCGA -3′
hsa-miR-6743-5p	RT primer	5′- CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGCCA-3′
(MIMAT0027387)	Senses primer	5′- ACACTCCAGCTGGGAAGGGGCAGGGACGGG -3′
U6	RT primer	5′- AACGCTTCACGAATTTGCGT -3′
	Senses primer	5′-CTCGCTTCGGCAGCACA-3′
Universal reverse primer		5′- TGGTGTCGTGGAGTCG-3′

Table 2 Primers for the detection of mRNA expression

Primer	Primer sequence	Size (bp)
GRIM-19	F: 5′- GGCCCATCGACTACAAACGG -3′	121
(NM_015965)	R: 5′- CGCTCACGGTTCCACTTCATT -3′	
CyclinD1	F: 5′- TGGAGCCCGTGAAAAAGAGC -3′	135
(NM_053056)	R: 5′- TCTCCTTCATCTTAGAGGCCAC -3′	
Bcl-2	F: 5′- GGTGGGGTCATGTGTGTGG -3′	89
(NM_000657)	R: 5′- CGGTTCAGGTACTCAGTCATCC -3′	
GAPDH	F: 5′- CACCCACTCCTCCACCTTTG -3′	110
(NM_001256799)	R: 5′- CCACCACCCTGTTGCTGTAG -3′	

Luciferase reporter assay

The human GRIM-19 3′-UTR carrying a wild-type (WT) or mutant (MUT) binding sequence of *miR-6743-5p* was inserted into the pGL3 vector (Promega, Madison, WI, U.S.A.) to make pGL3-GRIM-19-WT or pGL3-GRIM-19-MUT, respectively. The sequence of constructs was confirmed by DNA sequencing. Glioma cells were seeded into 24-well plates and co-transfected with *miR-6743-5p* mimic or inhibitor, and pGL3-GRIM-19-WT or pGL3-GRIM-19-MUT using Lipofectamine 2000. The luciferase activity was determined 24 h after transfection using a Dual-Glo® Luciferase assay kit (Promega), normalizing to *Renilla* luciferase activity.

Knockdown of GRIM-19 expression

To knock down the expression of GRIM-19, the oligonucleotides of shRNA targetting human GRIM-19 (shGRIM-19, 5′-CCGGCCATCGACTACAAACGGAATTCTCGAGGAATACCTCATCTTTCCTCTTTTTTTC-3′ and 5′-AATTGAAAAAAAGAGGAAAGATGAGGTATTCCTCGAGAATT CCGTTTGTAGTCGATGGGGCCT-3′) and control shRNA (shNC) were synthesized by Generay (Shanghai, China). The oligonucleotides were annealed and subcloned into AgeI/EcoRI sites of the lentiviral vector PLKO.1. Lentivirus was produced by transfecting HEK293T cells with the lentiviral construct and lentiviral packaging vectors with Lipofectamine 2000 based on the manufacturer's instructions. At 48 h post transfection, viruses were collected and infected glioma cells in the presence of 8 μg/ml polybrene.

Cell proliferation assay

The cell growth was determined by using a cell-counting kit (CCK)-8 (CCK-8) (Dojindo Lab, Kumamoto, Japan). Briefly, cells were seeded in 96-well plates at 5000 cells per well. Cells were transfected with indicated miRNA mimic/inhibitor, infected with shGRIM-19 or shNC lentivirus, and/or treated with 10 μM AG490 (Selleck Chemicals, Houston, TX, U.S.A.) or vehicle (DMSO) as indicated. After incubation for indicated time periods, cells were incubated with the CCK-8 reagent for 1 h and optical density (OD) values at 450 nm were determined. Triplicates were performed at each time point.

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Evaluation of apoptosis by flow cytometry

The percentages of apoptotic cells were determined by Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). Cells were seeded in six-well plates at 300000 cells per well and treated as described above. At 48 h after treatment, cells were harvested, washed with ice-cold PBS, and double-labeled with Annexin V-FITC and PI in the dark according to the protocols suggested by the manufacturer. Cells were immediately analyzed using a flow cytometer (BD Biosciences, San Jose, CA, U.S.A.).

Western blotting

Total protein was extracted by using RIPA lysis buffer, resolved on SDS/polyacrylamide gel, and transferred on to nitrocellulose membranes (Millipore, Bedford, U.S.A.). Following blocking with 5% skim milk, the membranes were probed with antibodies against GRIM-19, p-STAT3, STAT3 (Abcam, Cambridge, MA, U.S.A.), and GAPDH (Cell Signaling, Danvers, MA, U.S.A.). After incubation with the horseradish peroxidase-conjugated secondary antibody (Beyotime), the blots were developed with ECL system (Bio–Rad, Richmond, CA, U.S.A.).

Statistical analysis

Statistical analyses were conducted with GraphPad Prism software Version 6.0 (San Diego, CA, U.S.A.). Student's *t* test and ANOVA test were carried out to determine the statistical significance between two groups and amongst more than two groups, respectively. Pearson correlation analysis was applied to evaluate the relationship between GRIM-19 and *miR-6743-5p* expression. All *in vitro* experiments were done in triplicates and repeated at least three times. The criterion for statistical significance was set at $P<0.05$.

Results

GRIM-19 is targetted by *miR-6743-5p*

The TargetScan algorithm (targetscan.org) was applied to predict the miRNAs targetting GRIM-19. We found that hsa-*miR-17-3p*, hsa-*miR-423-5p*, hsa-*miR-3184-5p*, and hsa-*miR-6743-5p* had the potential to bind with GRIM-19. The predicted interaction between the miRNAs and the target sequence within the GRIM-19 3′-UTR is shown in Figure 1A.

To clarify whether these miRNAs could regulate *GRIM-19* mRNA expression in glioma, we knocked down the expression of these miRNAs in a glioma cell line, U251, and then assessed GRIM-19 expression levels. The levels of *miR-17-3p*, *miR-423-5p*, *miR-3184-5p*, and *miR-6743-5p* dropped to 7.7, 9.1, 17.6, and 7.5% of the normal levels when the cells were treated with corresponding miRNA inhibitor, respectively (Figure 1B). Negative control inhibitor (anti-miR-control) had no effects on the expression of these miRNAs. As indicated by real-time PCR analysis, the *GRIM-19* mRNA expression was significantly enhanced by the treatment of *miR-6743-5p* inhibitor (anti-*miR-6743-5p*), whereas inhibitors for other three miRNAs had no obvious effect on GRIM-19 expression (Figure 1C).

To confirm that GRIM-19 was a direct target gene of *miR-6743-5p*, we constructed GRIM-19 WT and MUT GRIM-19 3′-UTR luciferase reporter plasmids and performed the 3′-UTR luciferase assay. As shown in Figure 1D, the luciferase activity of WT GRIM-19 3′-UTR reporter was significantly reduced after the co-transfection of *miR-6743-5p* mimic in U251 cells. On the contrary, *miR-6743-5p* inhibitor (anti-*miR-6743-5p*) remarkably enhanced the luciferase activity of WT 3′-UTR reporter. The *miR-6743-5p* mimic or inhibitor had no effects on the luciferase activity of MUT 3′-UTR reporter. These data suggested that *miR-6743-5p* suppressed *GRIM-19* mRNA expression by targetting the 3′-UTR.

Correlation analyses in glioma tissues and cell lines

The expression of *miR-6743-5p* and *GRIM-19* mRNA were assessed in 15 normal brain tissues (NB) and 90 glioma tissues by real-time PCR. Decreased *GRIM-19* mRNA expression (Figure 2A, $P<0.001$) and increased *miR-6743-5p* expression (Figure 2B, $P<0.01$) were observed in glioma tissues as compared with normal tissues.

Pearson correlation analysis revealed a statistically significant negative correlation between the expression of GRIM-19 and *miR-6743-5p* in 90 glioma tissue samples (Figure 2C). Furthermore, an inverse correlation was also in five glioma cell lines between the expression of GRIM-19 and *miR-6743-5p* (Figure 2D). These data further supported that GRIM-19 was regulated by *miR-6743-5p* in glioma.

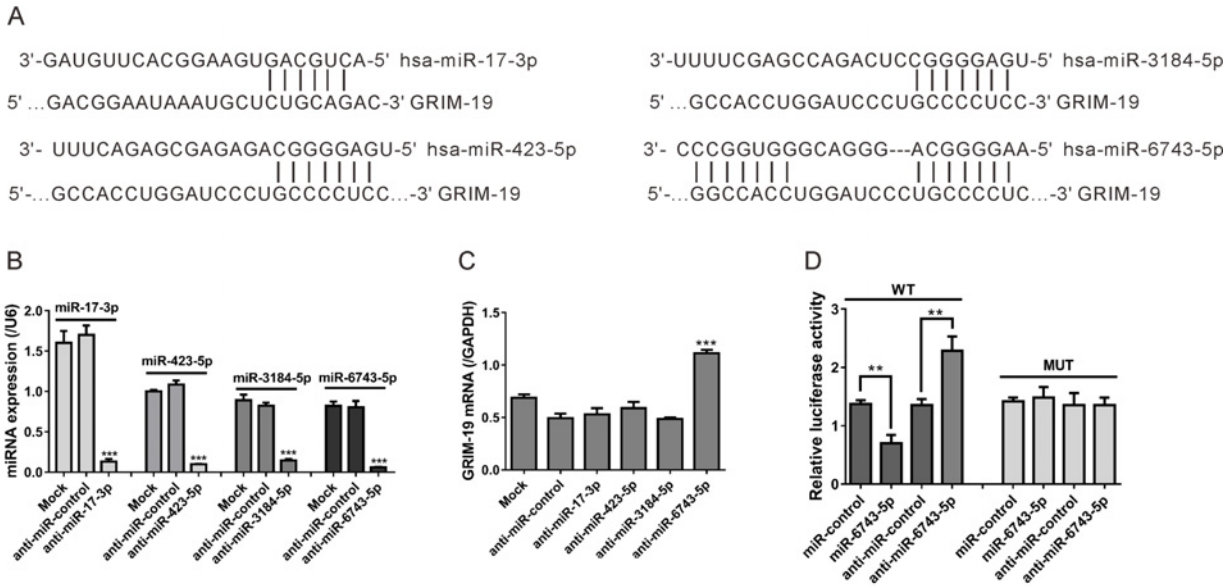

Figure 1. GRIM-19 is targetted by *miR-6743-5p*

(**A**) The putative binding sites of the miRNAs (hsa-*miR-17-3p*, hsa-*miR-423-5p*, hsa-*miR-3184-5p*, and hsa-*miR-6743-5p*) in GRIM-19 3′-UTR as predicted by TargetScan algorithm. (**B**, **C**) U251 cells were transfected with various miRNA inhibitors (anti-*miR-17-3p*, anti-*miR-423-5p*, anti-*miR-3184-5p*, or anti-*miR-6743-5p*) or miRNA inhibitor scramble (anti-miR-control). Cells without any treatment were set as a negative control (Mock). At 48 h post transfection, real-time PCR analysis was performed to assess the effects of indicated miRNA inhibitor transfections on the expression of indicated miRNA (B) and *GRIM-19* mRNA (C). ***$P<0.001$ compared with Mock and anti-miR-control. (**D**) U251 cells were co-transfected with WT or MUT GRIM-19 3′-UTR plasmid and *anti-miR-6743-5p* mimic, miR-control, anti-*miR-17-3p*, or anti-miR-control. The relative luciferase activities were determined at 24 h post transfection, normalizing to *Renilla* luciferase activity; **$P<0.01$.

Opposite effects of *miR-6743-5p* and GRIM-19 on glioma cell proliferation and apoptosis

To investigate the biological effects of *miR-6743-5p*/GRIM-19 in glioma, we analyzed the proliferation and apoptosis of U251 cells after *miR-6743-5p* inhibitor treatment or GRIM-19 knockdown. GRIM-19 expression was verified by real-time PCR analyses (Figure 3A). The exposure of the *miR-6743-5p* inhibitor decreased the proliferation rate, whereas the cells with GRIM-19 knockdown showed an increased proliferation rate (Figure 3B). Similarly, the percentage of apoptotic cells was significantly higher in the cells treated with the *miR-6743-5p* inhibitor, whereas the knockdown of GRIM-19 exhibited an inverse effect as defined by Annexin V/PI staining (Figure 3C) and TUNEL staining (Supplementary Figure S1). These data indicated that *miR-6743-5p* and GRIM-19 had opposite effects on glioma cell proliferation and apoptosis. Furthermore, the cells treated with both the *miR-6743-5p* inhibitor and GRIM-19 shRNA showed significantly higher proliferation and lower apoptotic rate than the cells treated with the *miR-6743-5p* inhibitor only (Figure 3B,C, D), indicating that *miR-6743-5p* may induce cell proliferation and inhibit cell apoptosis by down-regulating GRIM-19.

miR-6743-5p regulates the activity of STAT3 via GRIM-19

A previous study has shown that GRIM-19 exerted functions in glioma cells partially through STAT3-dependent pathway [15]. To explore the effect of *miR-6743-5p* on the activity of STAT3, Western blot analyses were performed in U251 cells. The *miR-6743-5p* inhibitor significantly suppressed the phosphorylation of STAT3, while the knockdown of GRIM-19 had the opposite effect (Figure 4A). Further, we analyzed the expression of STAT3 target genes, CyclinD1 and Bcl-2, at mRNA level. The mRNA expression of CyclinD1 and Bcl-2 was inhibited by the treatment of *miR-6743-5p* inhibitor, but increased by GRIM-19 knockdown (Figure 4B,C). Additionally, when compared with cells treated with the *miR-6743-5p* inhibitor, the glioma cells treated with both the *miR-6743-5p* inhibitor and shGRIM-19 exhibited higher levels of p-STAT3 as well as mRNA levels of CyclinD1 and Bcl-2 (Figure 4B), indicating that *miR-6743-5p* may active STAT3 pathway by down-regulating GRIM-19.

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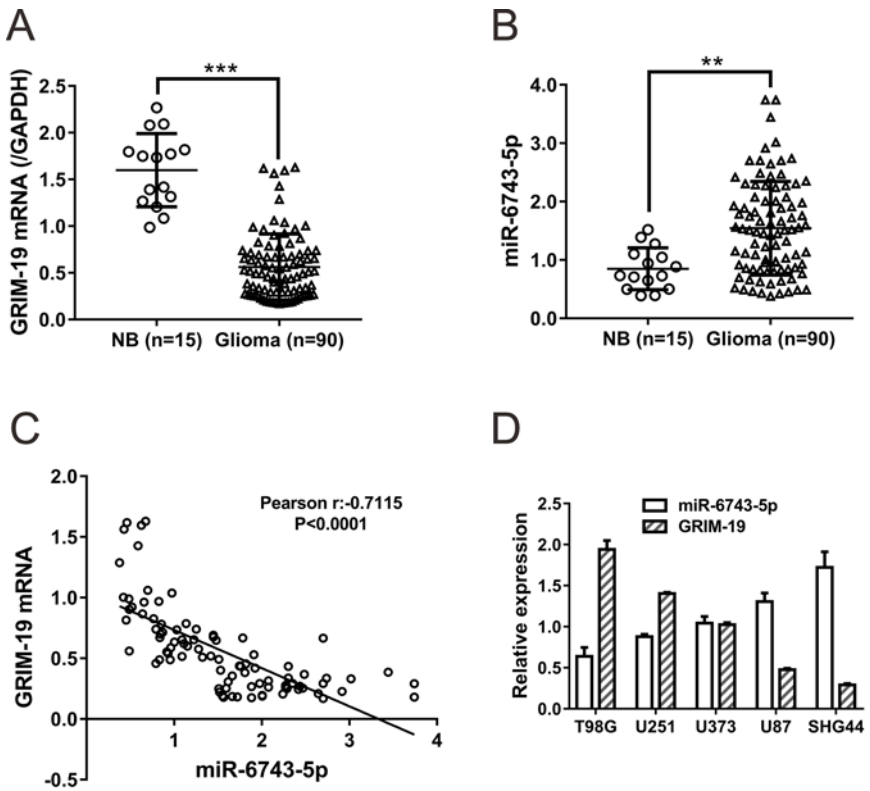

Figure 2. Correlation analyses in glioma tissues and cell lines

(**A**, **B**) Real-time PCR analysis was performed to evaluate the expression of *miR-6743-5p* (B) and *GRIM-19* mRNA (A) in 90 glioma tissues and 15 NB. $^{**}P<0.01$ and $^{***}P<0.001$. (**C**) Pearson correlation scatter plots of *miR-6743-5p* and *GRIM-19* mRNA in glioma tissues ($n=90$). (**D**) The expression of *miR-6743-5p* and *GRIM-19* mRNA were detected by real-time PCR analysis in five glioma cell lines.

miR-6743-5p/GRIM-19 regulates glioma cell proliferation and apoptosis via STAT3 pathway

To further confirm the involvement of STAT3 in the functions of *miR-6743-5p*/GRIM-19, a STAT3 inhibitor AG490 was used. AG490 exposure in U251 cells had no effects on the mRNA and protein expression of GRIM-19 (Figure 5A,B), but significantly decreased the p-STAT3, and the mRNA expression of CyclinD1 and Bcl-2 (Figure 5B,C). Overexpression of *miR-6743-5p* or GRIM-19 knockdown activated STAT3 pathway, which was notably suppressed by AG490 treatment (Figure 5B,C). Consistently, AG490 exposure partially rescued the proliferation-promoting and anti-apoptosis effects of *miR-6743-5p* overexpression or GRIM-19 knockdown (Figure 5D,E and Supplementary Figure S2). These data indicated that *miR-6743-5p*/GRIM-19 regulated the proliferation and apoptosis of glioma cells through regulating STAT3 activity.

Discussion

GRIM-19 was first identified by Angell et al. [5] as a novel cell death-regulatory molecule. Since its identification, GRIM-19 is considered to actively function in embryonic development, cell growth, and apoptosis [6-10]. Besides, it may exert tumor-suppressive role in human cancers [11-14]. The present study focussed on the regulation of GRIM-19 expression in glioma. To the best of our knowledge, there are three major findings of the present study that have not been reported. First, we have shown for the first time that *miR-6743-5p* directly targetted GRIM-19 and down-regulated its expression in glioma cells. Second, the suppression of *miR-6743-5p* caused a significant inhibition of cell proliferation and a marked induction of cell apoptosis in glioma cells, and this phenotype was rescued by GRIM-19 knockdown. Last, treatment with STAT3 inhibitor AG490 partially rescued the proliferation-promoting and anti-apoptosis effects of *miR-6743-5p* overexpression or GRIM-19 knockdown.

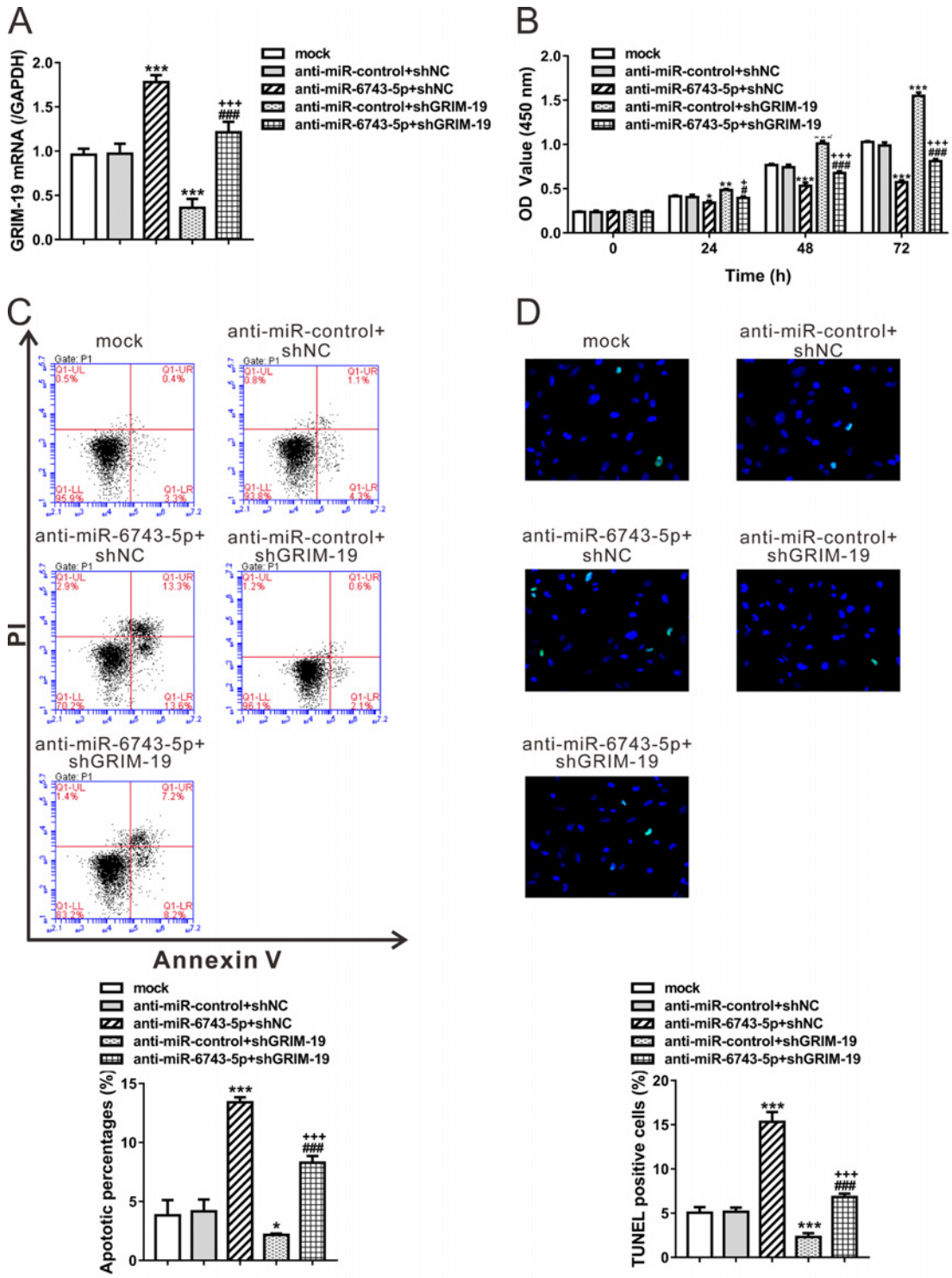

Figure 3. Opposite effects of *miR-6743-5p* and GRIM-19 on glioma cell proliferation and apoptosis

U251 cells were transfected with anti-miR-control/anti-*miR-6743-5p*, and infected with shGRIM-19 or shNC lentivirus as indicated. Cells without any treatment were set as a negative control (Mock). (**A**) The mRNA levels of GRIM-19 were assessed at 48 h after treatment. (**B**) The proliferation curves of U251 cells at 0, 24, 48, and 72 h after treatment as determined by CCK-8 assay. (**C, D**) Cell apoptosis was determined by Annexin V/PI staining and flow cytometry analysis (**C**) and TUNEL assay (**D**) at 48 h after treatment. The representative images and the quantitative analysis are shown. The lower right quadrant (Annexin V+/PI–) represents the apoptotic cells. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with Mock and anti-miR-control + shNC; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ compared with anti-*miR-6743-5p* + shNC; $^{+}P<0.05$ and $^{+++}P<0.001$ compared with anti-miR-control + shGRIM-19.

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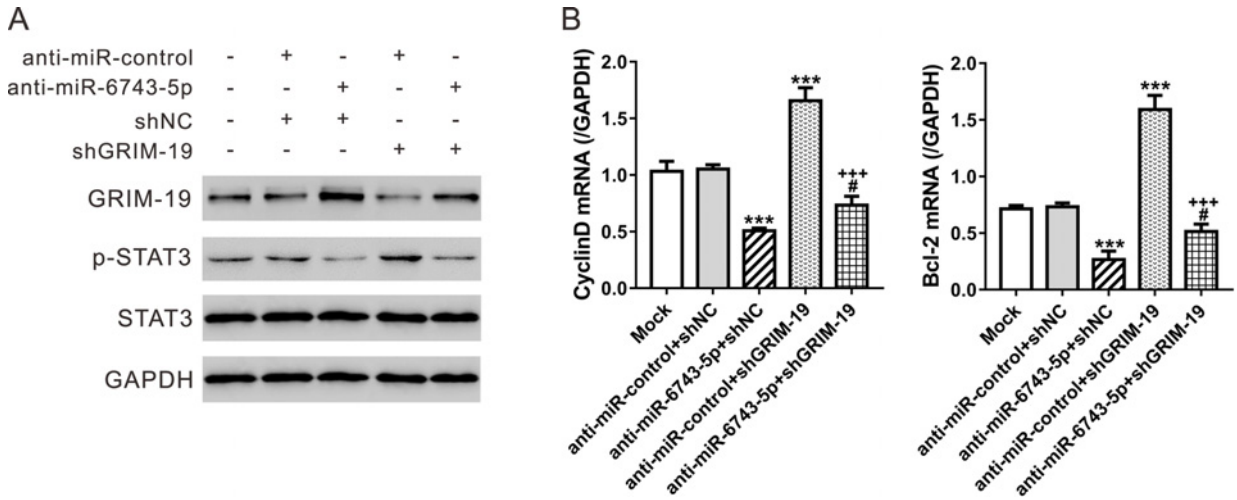

Figure 4. miR-6743-5p regulates the activity of STAT3 via GRIM-19

U251 cells were treated as described in Figure 3. (**A**) Protein levels of GRIM-19, p-STAT3, and STAT3 were assessed by Western blot analyses at 48 h after indicated treatment. GAPDH was detected as loading control. (**B**) The mRNA levels of CyclinD1 and Bcl-2 were detected by real-time PCR analysis at 48 h post treatment. $^{***}P<0.001$ compared with Mock and anti-miR-control + shNC; $^{\#}P<0.05$ compared with anti-*miR-6743-5p* + shNC; $^{+++}P<0.001$ compared with anti-miR-control + shGRIM-19.

Aberrant expression of hundreds of miRNAs has been reported in GBM tissues [17]. A number of miRNAs, such as *miR-9* [19], *miR-21* [20], *miR-125b* [21], and *miR-221/222* [22], play important roles in the progression and development of glioma via repressing the expression of tumor suppressor genes. Considering the post-transcriptional regulatory role of miRNAs, we supposed that miRNAs may be involved in GRIM-19 regulation. By bioinformatics tools, we predict several miRNAs that targetted GRIM-19. Then, by specific miRNA inhibitor transfection and luciferase assays, we demonstrated that *miR-6743-5p*, a miRNA with unknown function, directly bound to the 3′-UTR of GRIM-19, thus reducing the mRNA and protein levels of GRIM-19 (Figure 1). *miR-6743-5p* was observed to be up-regulated in human glioma tissues compared with normal brain tissues. The lower expression of GRIM-19 in glioma tissues was consistent with the previous study [15]. Moreover, an inverse correlation between the expression of *miR-6743-5p* and GRIM-19 was observed in glioma tissues and cell lines (Figure 2). Furthermore, the inhibition of *miR-6743-5p* expression suppressed the proliferation and induced apoptosis of U251 glioma cells (Figure 3), while *miR-6743-5p* mimic had the inversed effects (Figure 5). In addition, knockdown of GRIM-19 expression could reverse the effects of *miR-6743-5p* inhibitor (Figure 3), suggesting that *miR-6743-5p* exerts oncogenic function via targetting GRIM-19.

STAT3 plays a critical role in cell growth, anti-apoptosis, and cell differentiation. It is a tumor suppressor and constitutively active in glioma [23,24]. A previous study has reported that p-STAT3 expression is inversely correlated to GRIM-19 expression in gliomas and that GRIM-19 negatively regulates STAT3 activity in glioma cells [13]. Here, the inhibition of *miR-6743-5p* expression suppressed the phosphorylation of STAT3, and the mRNA expression of CyclinD1 and Bcl-2, two target genes of STAT3 (Figure 4), while *miR-6743-5p* mimic had the inversed effects (Figure 5). Furthermore, GRIM-19 knockdown could rescue the effects of *miR-6743-5p* inhibitor on STAT3 (Figure 4). The exposure of STAT3 inhibitor AG490 could partially abolish the proliferation-promoting and anti-apoptosis effects of *miR-6743-5p* overexpression or GRIM-19 knockdown (Figure 5). These findings suggested that STAT3 was involved in the functions of *miR-6743-5p*/GRIM-19 in glioma.

In conclusion, the current study explored the molecular mechanisms accounting for the dysregulation of GRIM-19 in glioma cells and identified that *miR-6743-5p* directly targetted GRIM-19. Furthermore, *miR-6743-5p* plays an essential role in the proliferation and apoptosis of glioma cells through modulating GRIM-19/STAT3.

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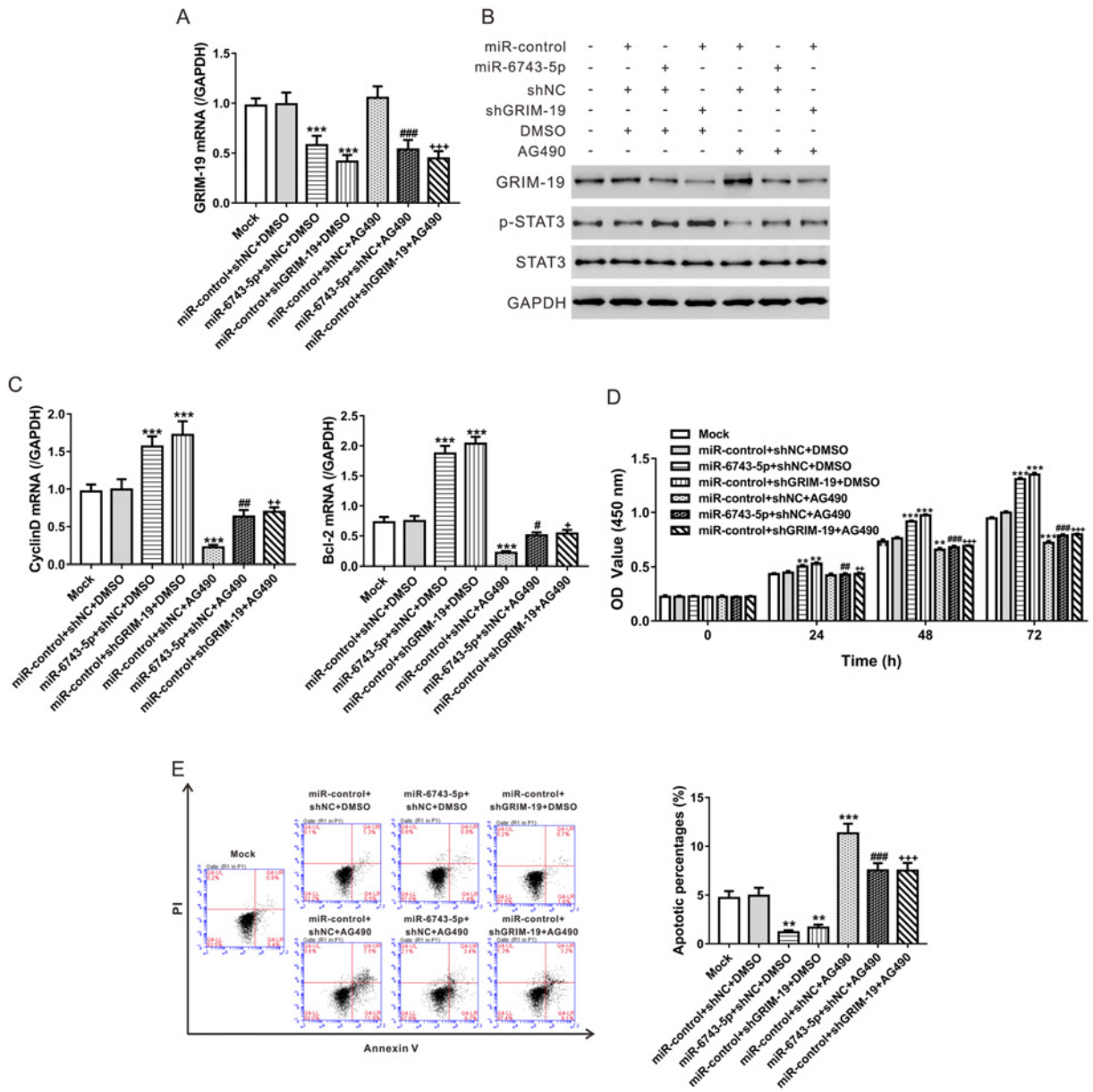

Figure 5. *miR-6743-5p*/GRIM-19 regulates glioma cell proliferation and apoptosis via STAT3 pathway

U251 cells were transfected with *miR-6743-5p* mimic/miR-control, infected with shGRIM-19 or shNC lentivirus, and/or treated with 10 μM of AG490 or vehicle (DMSO) as indicated. Cells without any treatment were set as a negative control (Mock). (**A**) The mRNA levels of GRIM-19 at 48 h after treatment. (**B**) The protein levels of GRIM-19, p-STAT3, and STAT3 at 48 h after indicated treatment. (**C**) The mRNA levels of CyclinD1 and Bcl-2 at 48 h post treatment. (**D**) The proliferation curves as determined by CCK-8 assay. (**E**) The representative image and the quantitative analysis of apoptosis assays. $^{**}P<0.01$ and $^{***}P<0.001$ compared with Mock and miR-control + shNC + DMSO; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ compared with *miR-6743-5p* + shNC + DMSO; $^{+}P<0.05$, $^{++}P<0.01$, and $^{+++}P<0.001$ compared with miR-control + shGRIM-19 + DMSO.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Author contribution

STY and FG designed the research; FG, QZ, WC, FZ and YG performed research; FG, QZ, QSR and YH analyzed data; FG, QZ and STY wrote the manuscript; All authors reviewed the manuscript.

Abbreviations

CCK-8, cell-counting kit-8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBM, glioblastoma multiforme; GRIM-19, gene associated with retinoid-interferon-induced mortality-19; MUT, mutant; PI, propidium iodide; RIPA, radio radioimmunoprecipitation assay; RT-PCR, reverse transcription PCR; STAT3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; WT, wild-type.

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