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Participation of tumor suppressors long non-coding RNA MEG3, microRNA-377 and PTEN in glioma cell invasion and migration

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ABSTRACT

Purpose: Glioma is a common and fatal intracranial tumor. Both miR-377 and lncRNA MEG3 are tumor suppressors. This study was performed to investigate the association between miR-377 and lncRNA MEG3 in glioma cells.

Methods: U118 and U251 cell lines were incubated in Dulbecco's modified Eagle's medium supplemented with miR-377 mimics, MEG3 siRNA (si-MEG3) and/or MEG3 overexpression plasmids (pc-MEG3) for 48 h. Cell migration, invasion, apoptosis, cell cycle distribution and the expression of E26 tansformation-specific-1 (ETS-1), phosphatase and tensin homologue (PTEN), E-cadherin, N-cadherin and β-catenin were detected.

Results: MiR-377 mimics increased MEG3 expression and decreased the number of migrated and invaded U118 and U251 cells, without influence on apoptosis in both cell lines. Si-MEG3 transfection increased U118 cell migration and invasion and rescued miR-377 mimics-induced inhibitory in cell migration and invasion. Si-MEG3 decreased U118 cell apoptosis and induced G0/G1 cell cycle arrest, and pc-MEG3 increased U251 cell apoptosis via arresting cell cycle at G2/M phage. MiR-377 mimics and si-MEG3 increased the relative expression level of N-cadherin mRNA, and both si-MEG3 and pc-MEG3 increased E-cadherin in glioma cells. MiR-377 mimics increased ETS-1 mRNA in U118 cells, but decreased it in U251 cells. PTEN was increased by miR-377 mimics and si-MEG3 and decreased by pc-MEG3 in glioma cells.

Conclusions: These results suggested the link interaction of MEG3 with miR-377 and PTEN, but not functioning as the competing endogenous RNA. MiR-377 mimics and MEG3 were tumor suppressors in glioma cells through regulating PTEN expression.

1. Introduction

Glioma, a common and fatal intracranial tumor in children and adult, accounts for 80% of intracranial tumor [17]. Glioma presents a low 5-year survival rate of ~ 25%, and the most common glioma, glioblastoma presents a ~5% 5-year survival rate [6,17]. The incidence rate of glioma increases with age and differs between races, with an overall age-adjusted incidence rate of 4.67~5.73 per 100 000 persons [17,18,24].

The known risk factors of glioma are few, including age, body weight and height and ionizing radiation [5,10,21]. In addition, numerous genetic molecules with or without clinical values have been identified to be associated with the pathogenesis, development and prognosis of glioma [11,31,33]. For instance, a meta-analysis showed that p53 immuno-positivity positively correlates with high-grade glioma and poor 5-year overall survival rate [11]. In addition, the TP53 variant rs78378222 and rs557505857, respectively, confers a two and six-fold relative risk of primary adult glioma [19]. The expression of long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3), a tumor suppressor lncRNA, is negatively correlated with the WHO grade, IDH status, tumor recurrence and overall survival of glioma [31].

LncRNA MEG3, an epigenetic determinant of oncogenic signaling, inhibits the proliferation and promotes the apoptosis of glioma cells via the p53 and PI3K/Akt pathways by sponging miRNAs [13,30,31]. A tumor suppressor microRNA, miR-377, regulates glioma cell proliferation and apoptosis [7]. miR-377 functions in human cells via regulating

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p53, phosphatase and tensin homologue (PTEN) and other multiple tumor-suppressor genes [25], including DNA methyltransferase 1 (DNMT1) [2,26] and proto-oncogene E26 tansformation-specific-1 (ETS-1) [7]. The regulation of MEG3 on PTEN as well as p53 pathway has been validated in cancer cells, including glioma [13,16,32]. However, the association between miR-377 and MEG3 has not been reported in glioma.

We performed this study to identify the molecular mechanism of lncRNA MEG3 and miR-377 in glioma development *in vitro*. Glioma cells were transfected with siRNA or expression plasmids of MEG3 as well as miR-377 mimics, and then their effect on the cell migration, invasion and apoptosis were determined. Potential association between MEG3 and miR-377 in glioma was identified, which provided more information for understanding the mechanism of glioma pathogenesis or development.

2. Materials and methods

2.1. Cell lines and culture

Human glioma cell lines (U118 and U251; ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Corp., Grand Island, NY, USA) at 37 °C with 5% CO_2.

2.2. Cell transfection

MiR-377 mimics, scrambled sequences (used as negative control, NC), MEG3 siRNAs and scrambled controls (NC) were purchased from Genechem Co. Ltd. (Shanghai, China). The construction of MEG3 expressing plasmid (pc-MEG3) was implemented by cloning the open reading frame into pcDNA3.1 vector (Genechem Co. Ltd.). The pcDNA3.1 vector (empty) was used as the NC for MEG3 expressing plasmid transfection. Lipofectamine 2000 regents (Invitrogen Corp.) were used for cellular transfections following the manufactures' instructions. Cells were harvested for further analyses after transfection for 48 h.

2.3. Transwell assay

Cellular migration and invasion abilities were detected using transwell assay. For cell migration determination, transwell chambers (Coring, NY, USA) with naked filter membrane were filled with cells (1×10^5) combined with DMEM without serum (upper chamber) or completed DMEM (lower chamber) and maintained at 37 °C with 5% CO_2 for 48 h. For cell invasion determination, transwell chambers (Coring) with filter membrane coated with BD Matrigel (BD Biosciences, San Jose, CA, USA) were supplemented with aforementioned fillers (cells combined serum-free DMEM in upper chamber, and completed DMEM in lower chamber) for 48 h at 37 °C with 5% CO_2. Then, the uppersurface of filter membranes were cleaned and attached cells (non-invaded or non-migrated cells) were removed used sterile cotton swab. The migrated or invaded cells adhered to the lowersurface of the filter membranes were fixed and stained using crystal violet (Invitrogen Corp.). Olympus light microscope was used for capturing images and cell counting. Digital photographs of five non-overlapped arbitrarily fields were used for the calculation of average migrated and invaded cell numbers.

2.4. Flow cytometric analysis

Cell apoptosis and cell cycle distribution of the glioma cell lines were determined using flow cytometry. At 48 h post transfection, cells were harvested, fixed and then subjected into propidium iodide (PI; BD Biosciences) for the analysis of cell cycle distribution. For cell apoptosis analysis, fixed cells were treated with Annexin V-FITC and PI solutions (BD Biosciences) according to the manufacturers' instructions. A FACS Calibur flow cytometry (BD Biosciences) was employed for the analysis of cell cycle distribution and apoptosis percentage. Average numbers were calculated for each experiment of 3 replicates.

2.5. RNA isolation, reverse transcription, and quantitative PCR

Total cellular RNA was isolated from glioma cells using Trizol reagents (TaKaRa, Tokyo, Japan), followed with the synthesis of the first-strand cDNA using Taq DNA polymerase (TaKaRa). PCR amplification was performed using 1 μg DNA in 20 μL volume using SYBR Green Master mix kit (TaKaRa) following the manufacturers' instructions. Applied Biosystems (ABI) 7900 Fast Real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) was used for PCR amplification, following reaction system: 95 °C for 4 min; 40 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s; and 65 °C for 4 min. The $2^{-\triangle\triangle Ct}$ methods were used to calculate the relative expression level of genes, with normalization to the internal reference gene GAPDH.

2.6. Western blot analysis

Cellular proteins were isolated from glioma cells using RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), following with quantification using a BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). Protein separation and immunoblotting was performed using 10% SDS-PAGE (Invitrogen) and Millipore poly vinylidene fluoride (PVDF) membrane (Millipore, Whatman, Germany). PVDF membranes carrying protein peptides were incubated with 5% nonfat milk (Beyotime) and specific primary antibody against ETS-1 (1: 1000), N-cadherin (1: 1500), β-catenin (1: 1000) and PTEN (1: 1000) at room temperature for 60 min. All primary antibodies were purchased from Boster Biotechnology Ltd., (Wuhan, China). β-actin (1: 2000) or α-tubulin (1: 1500, Boster Biotechnology) was used as control. Secondary antibody of HRP goat anti-rabbit IgG (1: 20000, Boster Biotechnology) was used. Target protein peptides in the membranes were processed using enhanced chemiluminescence (ECL) system (Millipore). Image processing software (image-Pro Plus 6.0; Media Cybernetics, Inc., Buckinghamshire, UK) was used for the analysis of the relative abundance.

2.7. Statistical analysis

GraphPad Prism 6.0 was used for statistical analysis. All data were represented as mean ± standard deviation (SD) of at least 3 replicates. Statistical difference between and among groups was performed using unpaired *t* test and one-way ANOVA, respectively. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. MiR-377 increases MEG3 expression in glioma cells

The addition of miR-377 mimics into U118 cells increased the expression of both miR-377 and MEG3 by 1.85- and 1.70-times fold (Fig. 1**A and B**), respectively; and the transfection of miR-377 mimics into U251 cells increased miR-377 and MEG3 expression by 2.26- and 1.26-times fold (Fig. 1**C and D**), respectively.

3.2. MiR-377 expression inhibits U118 and U251 cell migration and invasion

Transwell assay determined that the expression of miR-377 in U118 cells as increased by mimics transfection decreased the number of migrated (6.25 ± 0.98 vs. 11.25 ± 4.03 in blank) and invaded (52.00 ± 8.60 vs. 121.25 ± 13.30 in blank) cells ($p < 0.01$ or all, Fig. 2**A and B**). MiR-377 mimics transfection induced similar results in

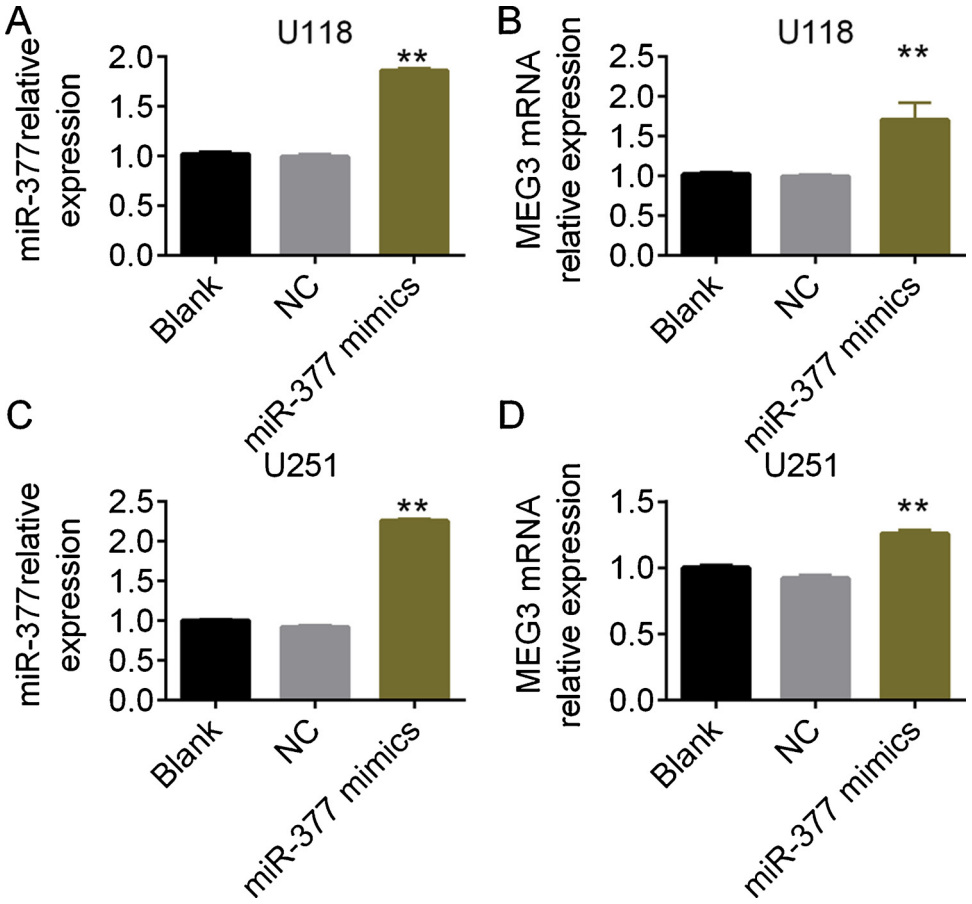

Fig. 1. Expression of miR-377 in glioma cells. A and B, the relative expression level of miR-377 and MEG3 in U118 cells, respectively. C and D, the relative expression level of miR-377 and MEG3 in U251 cells, respectively. **p < 0.01 vs. Blank and/or NC.

U251 cells, as indicated by decreased number of migrated (52.50 ± 8.23 vs. 119.25 ± 20.00 in blank) and invaded cells (159.00 ± 16.83 vs. 520.00 ± 9.83 in blank, p < 0.01 or all, Fig. 2**C and D**).

3.3. Effect of miR-377 on glioma cell apoptosis and cell cycle distribution

Fig. 3 shows that the overexpression of miR-377 does not change the percent of apoptotic glioma cells (p > 0.05, Fig. 3**A and B**). The cell cycle distribution of U118 cells was not affected by miR-377 mimics, while miR-377 mimics decreased the percentages of U251 cells at G0/G1 phage (20.17 ± 0.62% vs. 45.51 ± 5.46% in blank) and S phage (4.23 ± 0.22% vs. 12.55 ± 0.42% in blank), and increased the percentage of cells at G2/M phage (68.67 ± 2.91% vs. 34.33 ± 2.67% in blank) significantly (p < 0.01 or all, Fig. 3C).

3.4. MEG3 expression inhibits cell invasion and migration

We then transfected glioma cells with siRNA against MEG3 (si-MEG3) and MEG3 overexpression plasmids (pc-MEG3) to investigate the effect of MEG3 expression on cell migration and invasion. Fig. 4 shows the significant decrease and increase in the expression of MEG3 by si-MEG3 (p < 0.01, Fig. 4A) and pc-MEG3 (p < 0.01, Fig. 4B), respectively. Si-MEG3 transfection into U118 cells promoted cell migration (66.34 ± 16.74 vs. 21.25 ± 4.03 in blank) and invasion (127.25 ± 14.01 vs. 87.00 ± 12.58 in blank) significantly (p < 0.01 or all, Fig. 5**A and B**), while pc-MEG3 transfection into U251 cells inhibited cell migration (63.00 ± 10.60 vs. 133.25 ± 14.73 in blank) and invasion (207.75 ± 23.00 vs. 488.25 ± 16.83 in blank, p < 0.01 or all, Fig. 5**C and D**). The rescue experiment showed that the inhibition of MEG3 rescued miR-377-suppressed U118 cell migration (30.50 ± 4.20 vs. 5.00 ± 0.82 in mimics and 12.75 ± 2.22 in blank) and invasion (134.75 ± 24.10 vs. 49.50 ± 13.43 in mimics and 134.00 ± 6.38 in blank; Fig. 6**A and B**). These results suggested that MEG3 inhibition enhanced glioma cell invasion and migration.

3.5. MEG3 expression inhibits cell apoptosis

We then confirmed that the inhibition of MEG3 by siRNA decreased cell apoptosis percent in U118 cells (52.46 ± 1.24% vs. 59.58 ± 6.71% in blank, p < 0.01) and the overexpression of MEG3 increased cell apoptosis percent in U251 cells (26.57 ± 0.89% vs. 20.51 ± 3.46% in blank, p < 0.01 or all, Fig. 7A). Cell cycle distribution analysis showed si-MEG3 treatment increased the percentage of U118 cells at the G0/G1 phage (72.83 ± 1.62% vs. 68.38 ± 1.00% in blank) and decreased that at G2/M phage (16.15 ± 0.54% vs. 25.41 ± 0.69% in blank, p < 0.01 for all, Fig. 7B), while the overexpression of MEG3 reversed it in U251 cells (p < 0.01).

3.6. Effect of miR-377 and MEG3 expression on epithelial-mesenchymal transition (EMT)-related factors

Since both miR-377 and MEG3 expression affected the invasion and migration of glioma cells, we then investigated the expression of EMT-related proteins in response to miR-377 and MEG3 expression. Results in Fig. 8 shows that miR-377 mimics increases N-cadherin gene expression in U118 and U251 cells significantly (p < 0.01, Fig. 8**A and B**); decreases E-cadherin gene expression in U118 cells (p < 0.01), but not in U251 cells (p > 0.05); and increases β-catenin gene in expression U251 cells, but not in U118 cells. Si-MEG3 increased the relative

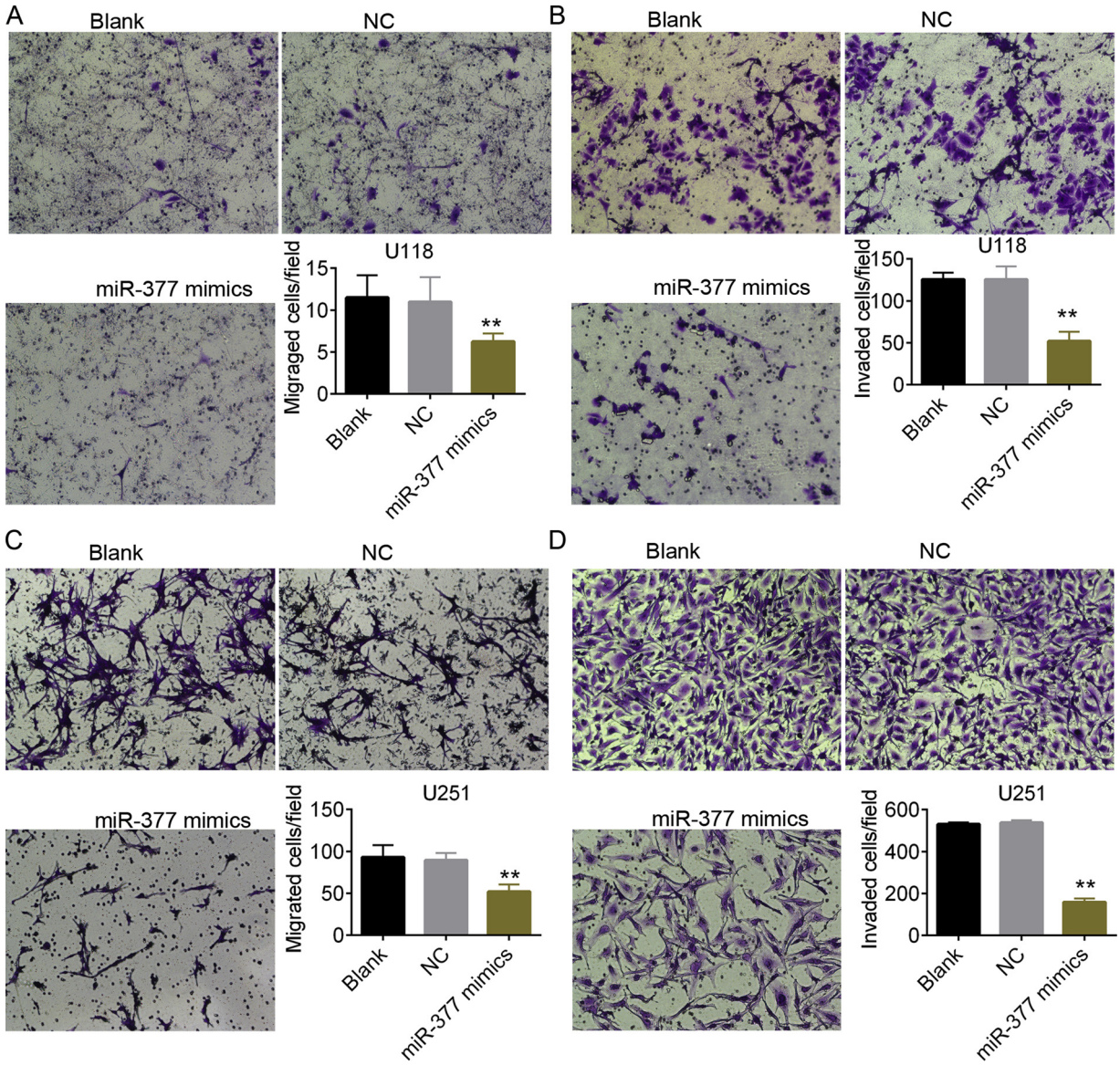

Fig. 2. MiR-377 expression reduced glioma migration and invasion. A and B, the number of migrated and invaded U118 cells, respectively. C and D, the number of migrated and invaded U251 cells, respectively. **p < 0.01 vs. Blank and/or NC.

expression levels of N-cadherin, E-cadherin and β-catenin mRNA in U118 cells ($p < 0.05$, Fig. 8A). Pc-MEG3 increased E-cadherin gene expression, but decreased β-catenin gene expression in U251 cells. Western blot assay demonstrated that miR-377 mimics, si-MEG3 and pc-MEG3 transfection did not change the expression of N-cadherin and β-catenin proteins in U118 cells, but decreased β-catenin protein in U251 cells (Fig. 8C). These results suggested that there might be different mechanisms of MEG3- and miR-377-mediated glioma cell inhibition.

3.7. Expression of ETS-1 and PTEN

Fig. 9 shows that miR-377 mimics insignificantly increased ETS-1 gene expression in U118 cells ($p > 0.05$) and significantly decreased ($p < 0.01$) it in U251 cells (Fig. 9**A and B**), respectively. MiR-377 mimics increased PTEN gene expression in both cell lines. Si-MEG3 increased both ETS-1 and PTEN gene expression in U118 cells ($p < 0.01$, Fig. 8A), and pc-MEG3 increased ETS-1 gene expression but decreased PTEN gene expression in U251 cells, respectively ($p < 0.01$, Fig. 9 **B**). Both miR-377 mimics and si-MEG3 did not change the expression of ETS-1 and PTEN proteins in U118 cells (Fig. 9C). In U251 cells, PTEN protein was decreased by miR-377 mimics and pc-MEG3; ETS-1 expression was decreased by pc-MEG3 (Fig. 9C).

4. Discussion

Both MEG3 and miR-377 are tumor suppressors [2,13,26,30,31]. MEG3 regulates the proliferation and apoptosis of cancer cells via sequestering oncogenic miRNAs. Our present study showed that both the overexpression of lncRNA MEG3 and miR-377 inhibited the invasion and migration of U118 and U251 glioma cells. The inhibition of MEG3 rescued miR-377 mimics-induced inhibitory effect on glioma cell invasion and migration. MEG3 expression promoted cell apoptosis as well as G2/M arrest, suggesting the tumor suppressive effect of MEG3 and miR-377 in glioma cells.

MEG3 functions as a tumor suppressor via regulating tumor suppressor gene p53, sponging oncogenic miRNAs, or inhibiting angiogenesis-related factor [1,4,13]. The expression of MEG3 elevates p53 protein and then stimulates p53 targets, including the MDM2, a major inhibitor of p53 [4]. MEG3 also functions in human cancer cells in p53

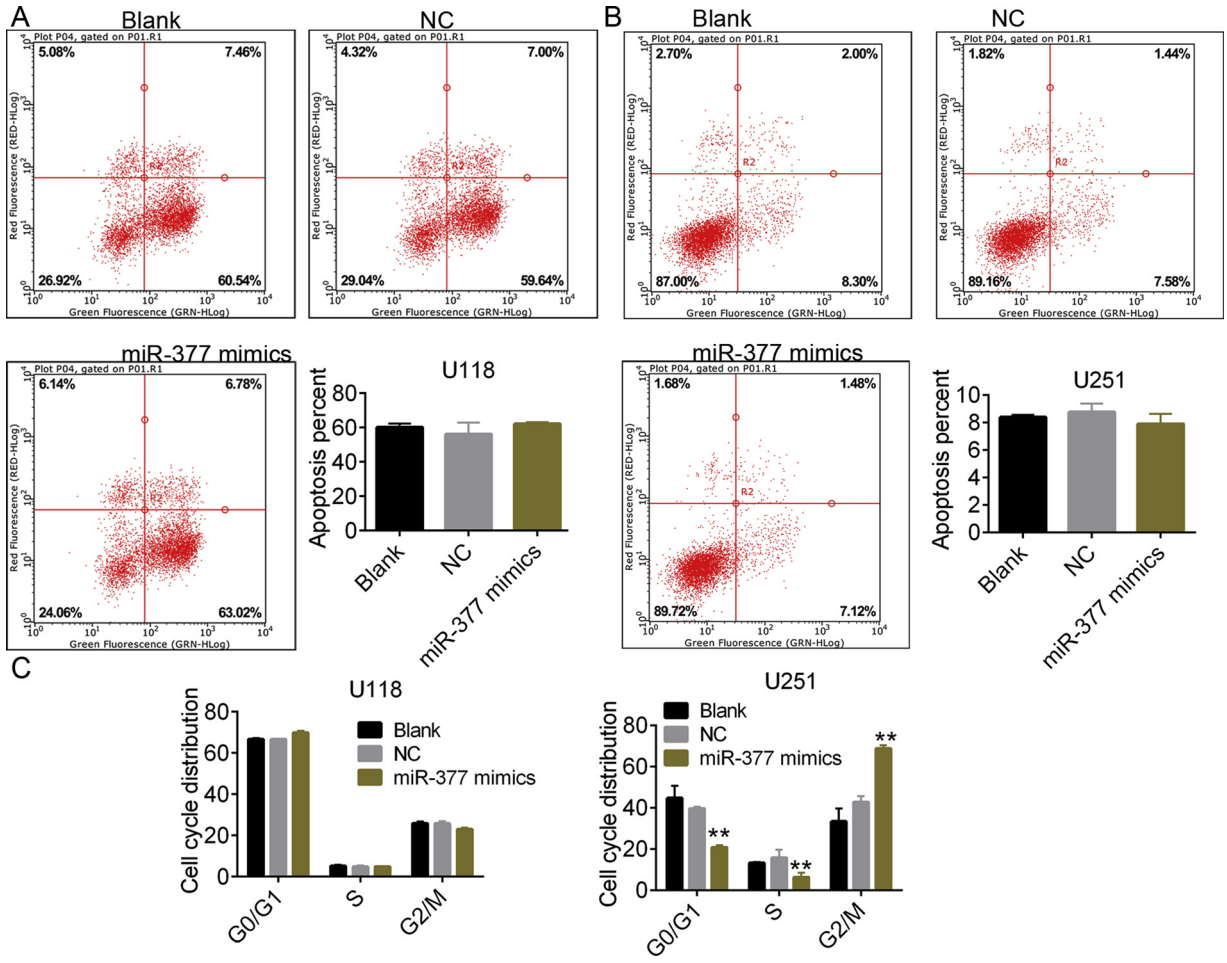

Fig. 3. Flow cytometric analysis for glioma cells. A and B, the apoptosis of U118 and U251 cells, respectively. C, the cell cycle distribution of U118 (left) and U251 (right) cells. **p < 0.01 vs. Blank and/or NC.

independent manner [29]. Both p53 and PTEN are tumor suppressors [8]. PTEN governs cell survival, proliferation, migration, invasion and other biological processes [28]. It has been reported that PTEN was targeted and inhibited by tumor suppressor miR-1297, and MEG3 overexpression abolished this inhibitory effect through sponging miR-1297, therefore inactivating Akt and inhibiting the growth of testicular germ cell tumor (TGCT) [27]. Li et al reported that the expression of DNMT1, an activator of epigenetic regulation of MEG3 promoter, induced MEG3 inhibition and p53 activation, and then promoted U87 and U251 glioma cell proliferation and inhibited apoptosis [13]. Our present study revealed that MEG3 inhibition in U118 cells inhibited glioma cell apoptosis, arrested cell cycle at G0/G1 phage, promoted cell invasion and migration, while MEG3 overexpression in U251 cells reversed them and arrested cell cycle at G2/M phage. These results suggested that MEG3 functioned as a tumor suppressor in glioma cells *in vitro*.

Both p53 and PTEN are regulated by miR-21 [12], miR-19a [22], miR-1297 [27] and miR-377 [25], suggesting they play crucial roles in

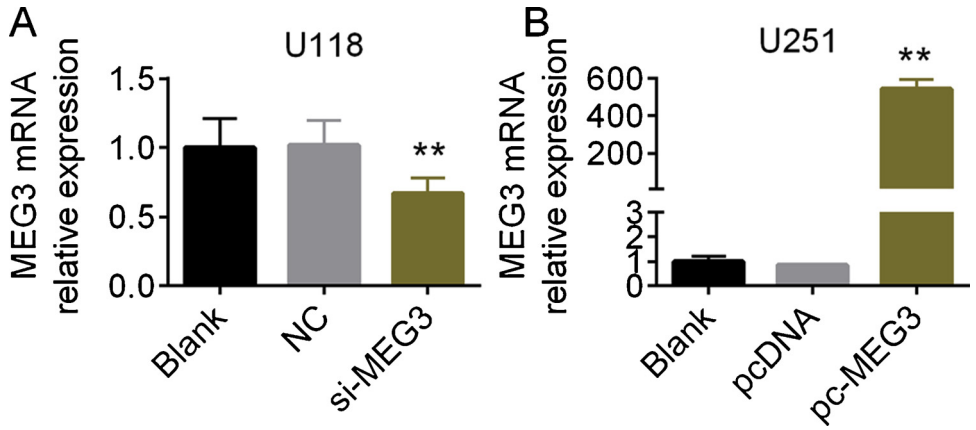

Fig. 4. The expression of MEG3 in glioma cells. A and B, the relative expression level of MEG3 in U118 and U251 cells, respectively. **p < 0.01 vs. Blank and NC or pcDNA.

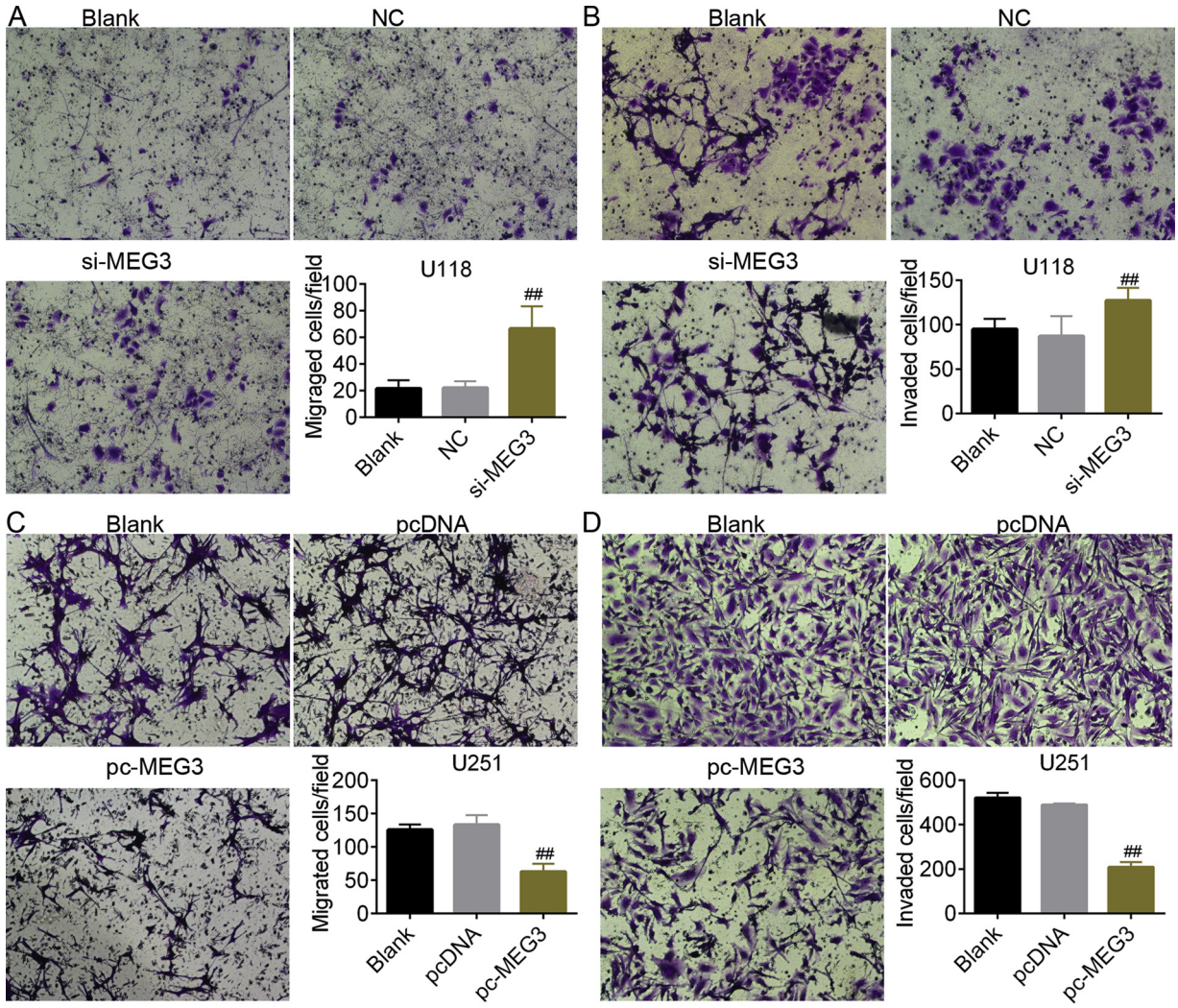

Fig. 5. MEG3 inhibits glioma cell invasion and migration. A and B, the migration and invasion of U118 cells, respectively. C and D, the migration and invasion of U251 cells, respectively. ## $p < 0.01$ vs. Blank and/or NC.

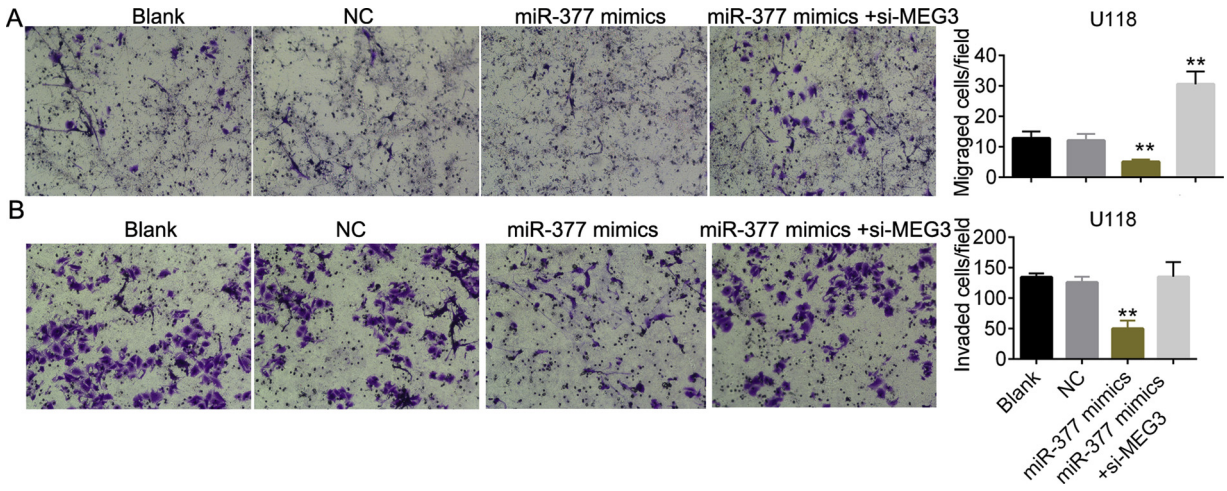

Fig. 6. The rescue assays of cell migratory and invasive ability. A and B, the cell migration and invasion ability of U118 cells transfected with miR-377 mimics or plus with si-MEG3. **$p < 0.01$ vs. Blank and/or NC.

miRNA-mediated cell survival and growth. It has been reported that miR-19a [14,22], miR-183 [3,20] and miR-21 [15,20] are oncogenic miRNAs, while the function of miR-1297 [27]as tumor suppressor [9] or oncogene [27] is cancer type-dependent. For instance, Supic et al. reported that patients with tongue carcinoma with overexpression of miR-183 and miR-21, two promising biomarkers of poor outcomes, presented poorer outcomes and shorter overall survival times compared with patients with low expression of miR-183 and miR-21 [20]. Gao

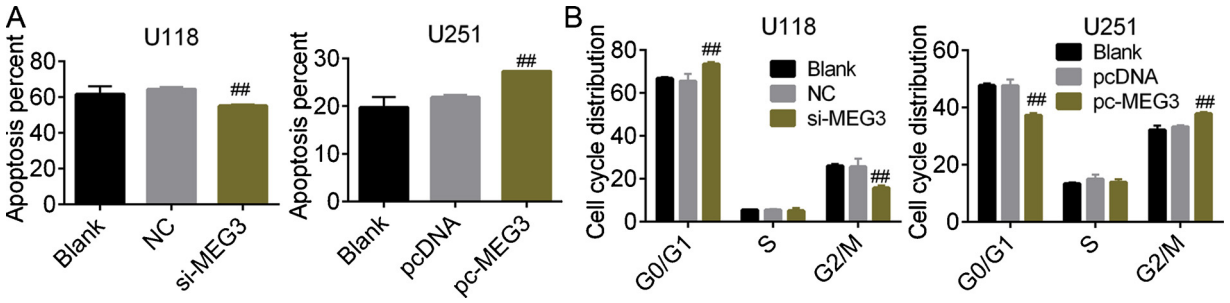

Fig. 7. Effect of MEG3 expression on the apoptosis and cell cycle distribution of glioma cells. A, the analysis of apoptotic U118 and U251 cells. B, the analysis of the cell cycle distribution in U118 and U251 cells. ##p < 0.01 vs. Blank and/or NC.

et al reported that the low expression of miR-1297 in gastric cancer tissues was positively associated with larger tumor size, advanced grade, lymph node metastasis, and poor survival [9]; and was significantly lower in esophageal squamous cell cancer compared with control [23]; suggesting a promising tumor suppressor. The oncogenic role of miR-1297 in TGCT has been mentioned as above in study of Yang et al. [27], functioning via inhibiting tumor suppressor PTEN which was abolished by MEG3 overexpression. These suggested the crucial and promising roles of miRNAs in tumor cell proliferation and inhibition.

Our present study confirmed that miR-377 was a tumor-suppressor in glioma cells, showing inhibitory effect on U118 and U251 cell migration and invasion, showing coincidence to the study of Wang et al. [7]. MiR-377 targets to DNMT1 [26], ETS-1 [7] and regulates p53, PTEN and other multiple tumor-suppressor genes [25]. Wang et al. reported that miR-377 regulated glioma cell proliferation and apoptosis via targeting proto-oncogene ETS-1 [7]. However, our present study detected that miR-377 mimics inhibited ETS-1 gene expression in U251 cells, but increased ETS-1 gene expression in U118 cells. ETS-1 protein was not influenced by miR-377 mimics. These results suggested that ETS-1 might not, at least not by directly, targeted by miR-377 in glioma cells. Further experiments showed PTEN was promoted by miR-377 mimics in both cell lines; and si-MEG3 and pc-MEG3 increased and decreased PTEN in U118 and U251 cells, respectively, suggesting the negative correlation between PTEN and MEG3 expression in glioma cells.

This correlation between PTEN and MEG3 expression has been widely provided in various cells, including liver cancer cells [32], human pulmonary artery smooth muscle cells [34] and TGCT [27]. It was aforementioned that DNMT1 is a target of miR-377 [26] and the expression of MEG3 is suppressed by DNMT1-mediated epigenetic modulation [13]. The fact that miR-377 overexpression induced MEG3 in both U118 and U251 cells might suggest the link between miR-377 and MEG3, but not by sponging miR-377. The inconsistent conclusions on EMT-related factors in the two glioma cell lines showed the complex mechanism underlying glioma invasion and migration surpassing MEG3

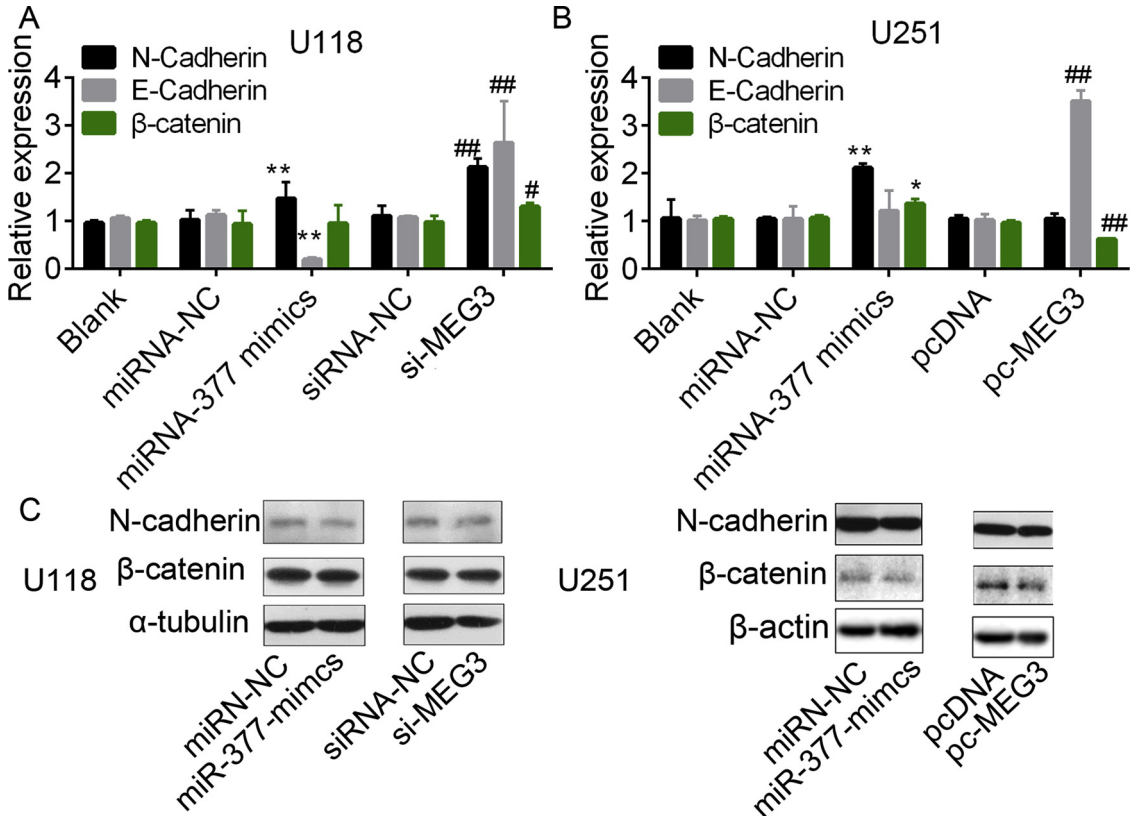

Fig. 8. Expression of EMT-related gene and proteins in glioma cells. A and B, the relative expression levels of EMT-related genes in U118 and U251 cells, respectively. C, western blot analysis of N-cadherin and β-catenin proteins in glioma cells. α-tubulin or β-actin is control. *p < 0.05 vs. Blank and/or miRNA-NC. **p < 0.01 vs. Blank and/or miRNA-NC. #p < 0.05 vs. Blank and pcDNA or siRNA-NC. ##p < 0.01 vs. Blank and pcDNA or siRNA-NC.

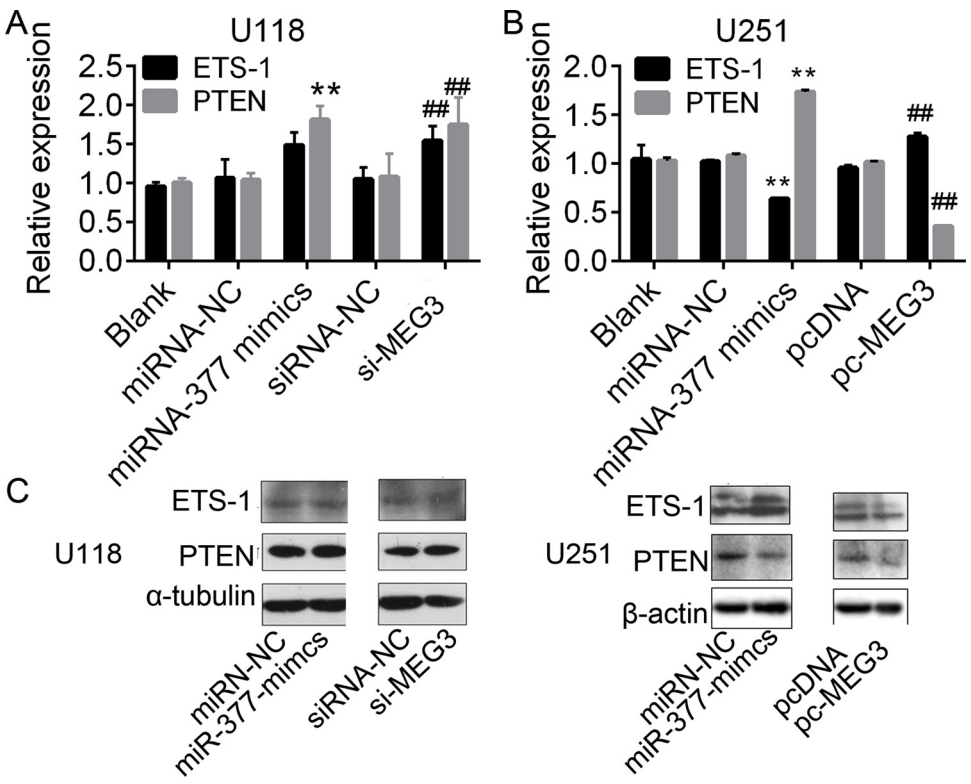

Fig. 9. Expression of ETS-1 and PTEN in glioma cells. A and B, the relative expression levels of ETS-1 and PTEN mRNA in U118 and U251 cells, respectively. C, western blot of ETS-1 and PTEN proteins in glioma cells. α-tubulin or β-actin is control. **p < 0.01 vs. Blank and/or miRNA-NC. ##p < 0.01 vs. Blank and pcDNA or siRNA-NC.

and miR-377 and their interactions.

5. Conclusions

In summary, our present study investigated the tumor suppressive effect of both miR-377 and MEG3 in glioma cells. The expression of both miR-377 and MEG3 promoted cell apoptosis and inhibited glioma cell invasion and migration via regulating cell cycle distribution (G2/M arrest). MiR-377 mimics and si-MEG3 promoted PTEN mRNA expression, suggesting the link between miR-377, MEG3 and PTEN. However, the cross talk between miR-377 and MEG3 as well as underlying mechanism of the mediated glioma cell migration and invasion should be validated.

Authors' contributions

Conception and design of the research: Wang D. Analysis, interpretation and statistical analysis: Wang D, Fan DQ and Fu CW. Drafting the manuscript: Wang D. Manuscript revision for important intellectual content: Fan DQ and Fu CW. All authors have read and approved the manuscript.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

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Declaration of Competing Interest

The authors have no competing interests to declare.

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