Let-7g-5p inhibits epithelial-mesenchymal transition consistent with reduction of glioma stem cell phenotypes by targeting VSIG4 in glioblastoma

XIN-HUA ZHANG[1], YUN QIAN[1], ZHENG LI[1], NING-NING ZHANG[1] and YUN-JIE XIE[2]

[1]Department of Neurosurgery, The Affiliated Hospital of Taishan Medical University, Tai'an, Shandong 271000; [2]Department of Neurosurgery, Jining No. 1 People's Hospital, Jining, Shandong 272011, P.R. China

Received April 27, 2016; Accepted September 2, 2016

DOI: 10.3892/or.2016.5098

Abstract. Epithelial-mesenchymal transition (EMT) and stem-like glioma cells display hallmark therapeutic resistance. Understanding of the mechanisms underlying these properties will be vital for the development of effective therapies. In this study, we found that VSIG4 protein is upregulated in glioblastoma. Overexpressing VSIG4 induced EMT and significantly promoted invasion and migration in glioblastoma U-87MG cells. Moreover, we showed that its overexpression promoted formation of glioma stem cell phenotypes in U-87MG cells. P4HB, VAMP8 and Connexin 43 (CX43) can promote temozolomide (TMZ) resistance in human glioma cells. We showed that P4HB, VAMP8 and CX43 protein were upregulated by VSIG4 in U-87MG cells, implying its upregulation might be a cause for temozolomide resistance. We found that let-7g-5p can inhibit VSIG4 protein expression, but it cannot degrade VSIG4 mRNA in U-87MG cells. Contrary to VSIG4, we demonstrated that overexpressing let-7g-5p promoted mesenchymal-epithelial transition (MET) and significantly inhibited invasion and migration consistent with the reduction of glioblastoma stem cell phenotypes in U-87MG cells. Thus, we concluded that let-7g-5p inhibits epithelial-mesenchymal transition (EMT) consistent with reduction of glioma stem cell (GSC) phenotypes by targeting VSIG4 in glioblastoma.

Introduction

Glioblastoma multiforme (GBM) is the most common form of human primary malignant brain tumors and it accounts for >60% of all primary brain tumors in adults (1,2). Because of resistance to conventional therapies, the prognosis of GBM remains dismal with median survival of ~14 months and 5-year survival only ~3% (3). Understanding molecular mechanisms underpinning resistance of conventional therapies of glioblastoma will offer novel targets for effective therapies.

MicroRNAs (miRNAs) are small, non-coding RNAs that post-transcriptionally regulate gene expression (4) and play significant roles in maintaining normal cellular functions (5). Deregulation of miRNA expression leads to diverse disease types, including cancers (6) as exemplified by their differential expression in carcinomas (7), sarcomas (8,9), and hematologic tumors (10). Let-7g-5p is significantly downregulated in the serum of GBM patients and it has been proposed as a tumor suppressive gene (11,12).

Glioma stem cells (GSCs) or glioma initiating cells (GICs) have been identified and shown to constitute a primitive cell population capable of self-renewal and differentiation that has the unique capacity to give rise to new tumors upon serial transplantation (13-16). Cancer stem/initiating cells are believed to play an essential role in tumor recurrence after therapeutic intervention (17), and their high chemo-resistance and radiation resistance (18) require the identification of alternative therapeutic strategies that could effectively lead to their functional or physical eradication. Although a few signaling pathways, including Sonic-Hedgehog (19), and the bone morphogenic proteins BMP4 and BMPR1B (20,21) have been shown to be implicated in GSCs maintenance, the mechanisms underlying GSCs generation, and propagation have yet to be elucidated.

Epithelial to mesenchymal transition (EMT) is an essential process for driving plasticity during development, but is also an unintentional behavior of cells during progression of malignant tumor (22-24). EMT confers mesenchymal properties on epithelial cells and has been closely associated with the acquisition of aggressive traits by carcinoma cells (25). Disturbance of a controlled epithelial balance is triggered by altering several layers of regulation, including the transcriptional and translational machinery, expression of non-coding RNAs, alternative splicing and protein stability (26-28).

In this study, we found that VSIG4 protein is upregulated in glioblastoma. Overexpressing VSIG4 induced epithelial-mesenchymal transition (EMT) and significantly promoted invasion and migration in glioblastoma U-87MG cells.

Correspondence to: Dr Yun-Jie Xie, Department of Neurosurgery, Jining No. 1 People's Hospital, 6 Jiankang Road, Jining, Shandong 272011, P.R. China
E-mail: xieyunjiejn@163.com

Key words: epithelial-mesenchymal transition, glioma stem cells, VSIG4, let-7g-5p, glioblastoma

Moreover, we showed that its overexpression promoted formation of glioma stem cell phenotypes in U-87MG cells. We found that let-7g-5p can downregulate VSIG4 protein expression, but it cannot degrade VSIG4 mRNA in U-87MG cells. Contrary to VSIG4, we demonstrated that overexpressing let-7g-5p promoted mesenchymal-epithelial transition (MET) and significantly inhibited invasion and migration consistent with the reduction of glioblastoma stem cell phenotypes in U-87MG cells.

Materials and methods

Glioblastoma tissues. Glioblastoma tissues and adjacent normal tissues were obtained from the Department of Neurosurgery, The Affiliated Hospital of Taishan Medical University, Shandong, China. All tissues were examined histologically, and pathologists confirmed the diagnosis. Medical ethics committee approved the experiments. The use of human's tissue samples follows internationally recognized guidelines as well as local and national regulations. Informed consent was obtained from each individual.

Glioblastoma U-87MG cell line, VSIG4 expressing plasmids/empty vectors, pre-let-7g-5p/control miR and transfection. Human glioblastoma cell line U-87MG was obtained from American Type Culture Collection. Briefly, cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. VSIG4 expressing plasmids/empty vectors (pcDNA3.1) were purchased from Tiangen (Beijing, China). Pre-let-7g and control miR were purchased from Ambion, Inc. (Ambion, Austin, TX, USA). For transfection experiments, the cells were cultured in serum-free medium without antibiotics at 60% confluence for 24 h, and then transfected with transfection reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 6 h, the medium was removed and replaced with normal culture medium for 48 h, unless otherwise specified.

Western blot analysis. Western blot analysis was performed as described before (29). Briefly, after incubation with primary antibody anti-VSIG4 (1:500; Abcam, Cambridge, MA, USA), anti-CD133 (1:500; Abcam), anti-EZH2 (1:500; Abcam), anti-c-Met (1:500; Abcam), anti-P4HB (1:500; Abcam), anti-VAMP8 (1:500; Abcam), anti-CX43 (1:500; Abcam), anti-E-cadherin (1:500; Abcam), anti-TGFB1 (1:500; Abcam), anti-vimentin (1:500; Abcam), anti-SNAIL (1:500; Abcam), anti-Notch1 (1:500; Abcam), anti-TLR9 (1:500; Abcam), anti-EphA2 (1:500; Abcam), anti-MLK4 (1:500; Abcam) and anti-β-actin (1:500; Abcam) overnight at 4˚C, IRDye™-800 conjugated anti-rabbit secondary antibodies (LI-COR, Biosciences, Lincoln, NE, USA) were used for 30 min at room temperature. The specific proteins were visualized by Odyssey™ Infrared Imaging System (Gene Co., Lincoln, NE, USA).

Sphere growth. Cells (10^3/ml) in serum-free RPMI-1640/1 mM Na-pyruvate were seeded on 0.5% agar precoated 6-well plates. After 1 week, half the medium was changed every third day. Single spheres were picked and counted.

Immunofluorescence analyses. For U-87MG cell immunofluorescence analyses, U-87MG cells were plated on glass coverslips in 6-well plates and transfected as indicated. At 48 h after transfection, coverslips were stained with CD44 (1:500; Abcam) or antibody anti-VSIG4 (1:500; Abcam). Alexa Fluor 488 goat anti-rabbit IgG antibody was used as secondary antibody (Invitrogen). Coverslips were counterstained with DAPI (Invitrogen-Molecular Probes, Eugene, OR, USA) for visualization of the nuclei. Microscopic analysis was performed with a confocal laser-scanning microscope (Leica Microsystems, Bensheim, Germany). Fluorescence intensities were measured in a few viewing areas for 300 cells per coverslip and analyzed using ImageJ 1.37v software (http://rsb.info.nih.gov/ij/index.html).

Wound healing assay. Wound healing assay was performed as described before (30).

Migration and invasion assay. Migration and invasion assay was performed as described before (29).

Methods of bioinformatics. The analysis of potential microRNA target site using the commonly used prediction algorithms - miRanda (http://www.microrna.org/).

Real-time PCR for microRNAs. Total RNA from cultured cells, with efficient recovery of small RNAs, was isolated using the mirVana miRNA Isolation kit (Ambion). Detection of the mature form of miRNAs was performed using the mirVana qRT-PCR miRNA Detection kit and qRT-PCR Primer Sets, according to the manufacturer's instructions (Ambion). The U6 small nuclear RNA was used as an internal control.

Reverse transcription-polymerase chain reaction. It was performed as described before (31). Primers for VSIG4: forward, 5'-GTGTCCAGTTTGGCTAGTGCC-3'; reverse, 5'-GACTGGAGAACAGAAGCAGGC-3'. Primers for GAPDH: forward, 5'-CGGAGTCAACGGATTTGGTCG TAT-3'; reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Northern blot analysis. Northern blot analysis for miRNAs were performed as described previously (32). Probes were labeled with [γ-^{32}P]-ATP complementary to let-7g-5p and U6 snRNA.

Statistical analysis. Data are presented as mean ± SEM. Student's t-test (two-tailed) was used to compare two groups ($P<0.05$ was considered significant), unless otherwise indicated (χ^2 test).

Results

VSIG4 promotes formation of stem cell-like population in glioblastoma U-87MG cells. In an attempt to identify VSIG4 protein expression between glioblastoma tissues and adjacent normal tissues, we performed western blotting in tumor tissues versus normal tissues. Protein was isolated from 6 pairs of glioblastoma tissues and normal tissues (patient nos. 1-6). We found that VSIG4 protein was significantly increased in cancer

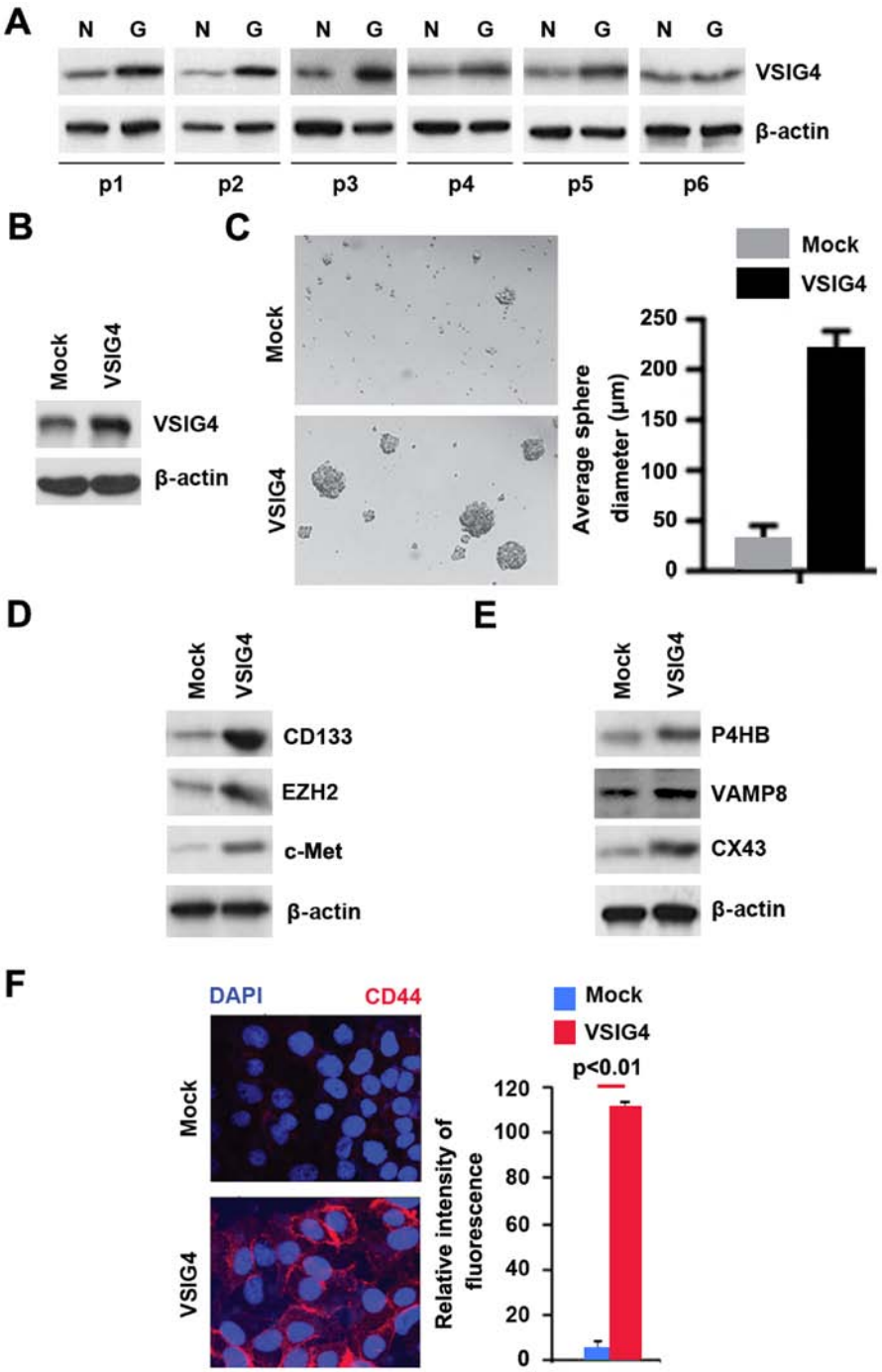

Figure 1. VSIG4 promotes formation of stem cell-like population in glioblastoma U-87MG cells. (A) Western blotting for VSIG4 in glioblastoma tissues (C) and adjacent normal tissues (N). Patients were numbered 1-6. All the 6 patients were diagnosed with glioblastoma. β-actin was a loading control. n=6. (B) Western blotting for VSIG4 in U-87MG cells transfected with VSIG4 expressing plasmids or empty vectors (mock). β-actin was a loading control. n=3. (C) Sphere growth for U-87MG transfected with VSIG4 expressing plasmids or empty vectors (mock). (D) Western blotting for CD133, EZH2 and c-Met in U-87MG cells transfected with VSIG4 expressing plasmids or empty vectors (mock). β-actin was a loading control. n=3. (E) Western blotting for P4HB, VAMP8 and CX43 in U-87MG cells transfected with VSIG4 expressing plasmids or empty vectors (mock). β-actin was a loading control. n=3. (F) Immunofluorescence analyses for CD44 in U-87MG cells transfected with VSIG4 expressing plasmids or empty vectors (mock). n=3.

tissues, compared with adjacent normal tissues (Fig. 1A). It implied that VSIG4 could be an oncogene in glioblastoma.

In order to assess the role of VSIG4 in glioblastoma, we transfected U-87MG cells with VSIG4 expressing plasmids and then western blotting was performed. We found that VSIG4 protein was significantly increased in the cells transfected with VSIG4 expressing plasmids (Fig. 1B). To determine whether VSIG4 can affect GSCs, we performed sphere forming assay to assess formation of stem cell-like population. We found that formations of spheres were increased by VSIG4 in U-87MG cells (Fig. 1C). CD133, EZH2, c-Met and CD44 are robust markers and are of functional importance for GSC for tumor initiation (33-36). In order to detect whether CD133, EZH2, c-Met and CD44 protein expression can be affected by VSIG4,

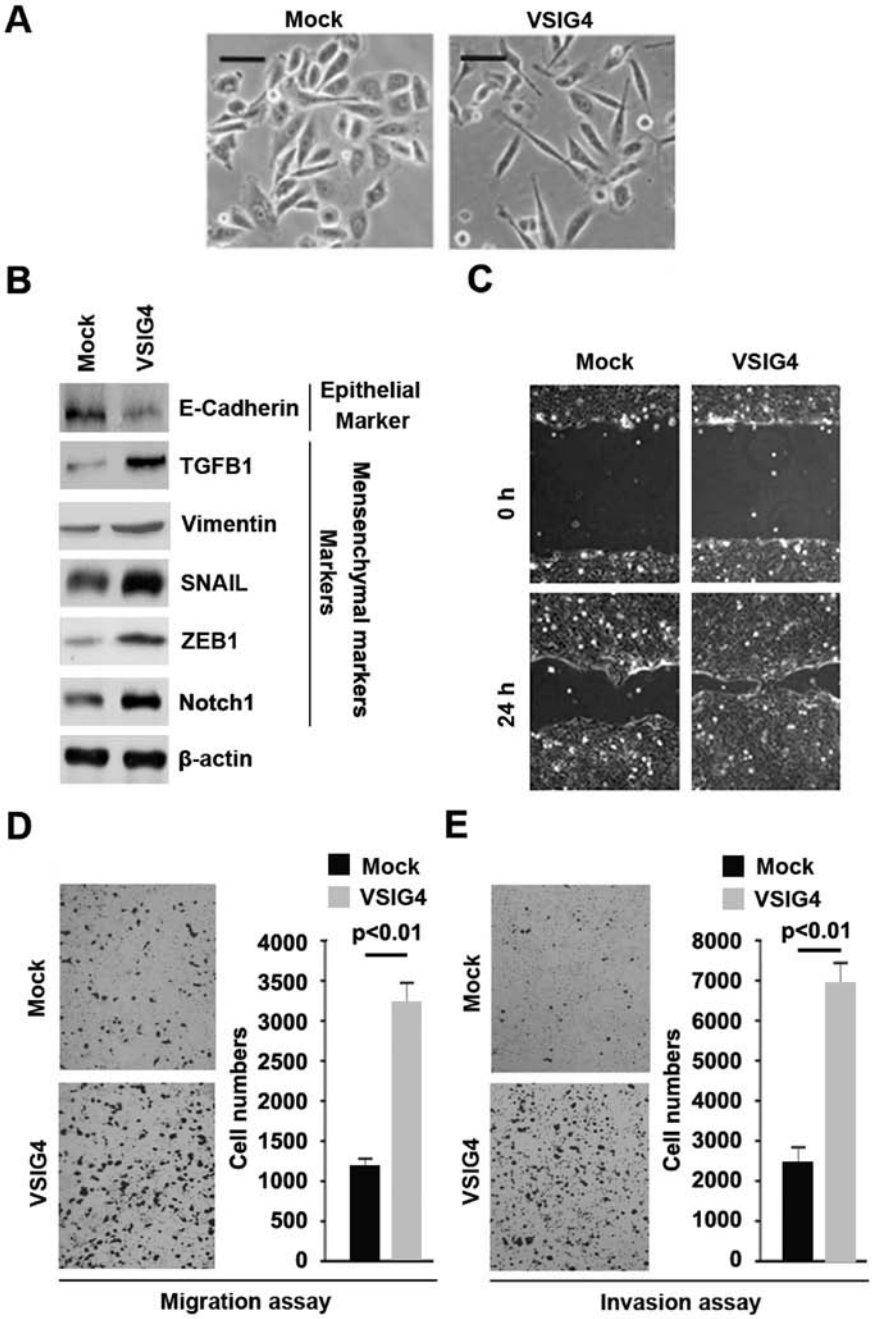

Figure 2. Overexpressing VSIG4 promotes EMT in glioblastoma U-87MG cells. (A) U-87MG cells were transfected with VSIG4 expressing plasmids or empty vectors (mock). Cells were photographed after 72 h of transfection. n=3. (B) Western blotting for E-cadherin, TGFB1, Vimentin, SNAIL, ZEB1 and Notch1 in U-87MG cells transfected with VSIG4 expressing plasmids or empty vectors (mock). β-actin was a loading control. n=3. (C) Wound-healing assays for U-87MG cells transfected with VSIG4 expressing plasmids or empty vectors (mock). The cell layer was photographed. n=3. (D) Migration assays for U-87MG cells transfected with VSIG4 expressing plasmids or empty vectors (mock). n=3. (E) Invasion assays for U-87MG cells transfected with VSIG4 expressing plasmids or empty vectors (mock). n=3.

we performed western blotting and immunofluorescence. The results showed that CD133, EZH2, c-Met (Fig. 1D) and CD44 protein were upregulated by VSIG4 (Fig. 1F).

P4HB, VAMP8 and Connexin 43 (CX43) can promote temozolomide (TMZ) resistance in human glioma cells (37-39). To identify whether VSIG4 could have potential to affect temozolomide (TMZ) resistance, we performed western blotting to detect P4HB, VAMP8 and Connexin 43 (CX43) protein. The results showed that P4HB, VAMP8 and Connexin 43 (CX43) protein were upregulated by VSIG4 in U-87MG cells (Fig. 1E).

Overexpressing VSIG4 promotes EMT in glioblastoma U-87MG cells. EMT has been shown to result in cancer cells with stem cell-like characteristics that have a propensity to invade surrounding tissue and display resistance to certain therapeutic interventions (40). In order to assess the role of VSIG4 in EMT of U-87MG, we transfected U-87MG cells with VSIG4 expressing plasmids and then we found that its overexpression caused significant changes in the cell morphology (EMT, phenotype from a cobblestone-like to a spindle-like morphology) (Fig. 2A). To further verify that the changes in

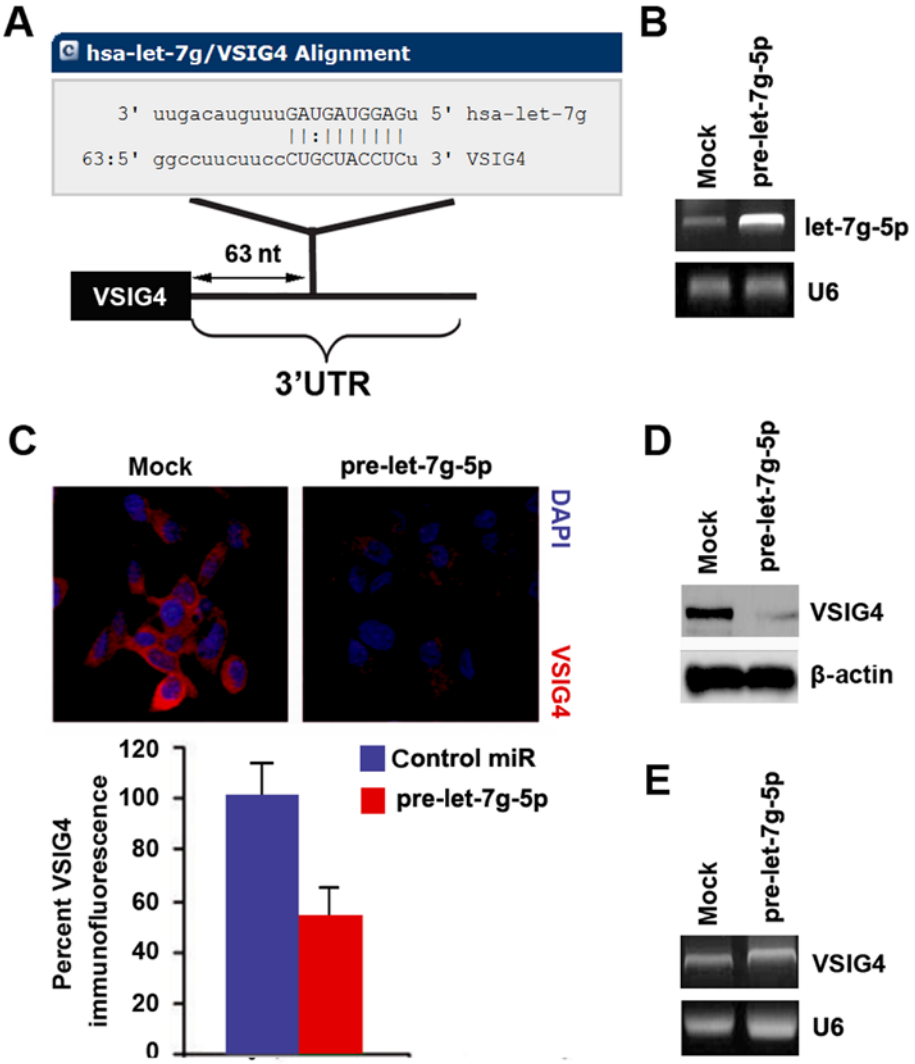

Figure 3. Let-7g-5p inhibits VSIG4 in glioblastoma cells. (A) Schematic of predicted let-7g-5p binding sites in the 3'UTR of VSIG4 mRNA by TargetScan. (B) Real-time RT-PCR for let-7g-5p in U-87MG cells transfected with pre-let-7g-5p or control miR (mock). U6 was a loading control. n=3. (C) Immunofluorescence analyses for VSIG4 in U-87MG cells transfected with pre-let-7g-5p and control miR (mock). Upper panel shows microscopic images of immunofluorescence staining of one representative experiment (x100 magnification). Bottom panel shows graphic presentation of mean fluorescence intensities. n=3. (D) Western blotting for VSIG4 in U-87MG cells transfected with pre-let-7g-5p and control miR (mock). β-actin was a loading control. n=3. (E) RT-PCR for VSIG4 in U-87MG cells. U-87MG cells were transfected with pre-let-7g-5p and control miR (mock). GAPDH was a loading control. n=3.

cell morphology are caused by EMT, we performed western blotting to detect expression of epithelial and mesenchymal markers in U-87MG cells transfected with VSIG4 expressing plasmids and the cells transfected with empty vectors. The results revealed that epithelial marker (E-cadherin) was inhibited and the mesenchymal markers (TGFB1, Vimentin, SNAIL, ZEB1 and Notch1) were induced by VSIG4 in U-87 MG cells (Fig. 1B).

EMT can result in increased cell invasion and migration (41-43). Thus, we reasoned that VSIG4 could also affect invasion and migration in U-87 MG cells. To identify this reason, we performed would healing, invasion, and migration assays. We found that overexpressing VSIG4 resulted in enhanced migration (Fig. 1C and D) and invasion (Fig. 1E) in the cells.

Let-7g-5p inhibits VSIG4 in glioblastoma U-87MG cells. Having demonstrated that overexpressing VSIG4 promoted formation of stem cell-like population and EMT, next we studied the mechanisms regulating VSIG4 expression in the disease. MicroRNAs (miRs) are a class of small non-coding RNAs (~22 nucleotides) and negatively regulate protein-coding gene expression by targeting mRNA degradation or translation inhibition (44-46). To further confirm whether VSIG4 could be regulated by microRNA, we used the commonly used prediction algorithm - miRanda (http://www.microrna.org/microrna/home.do) to analyze 3'UTR of VSIG4. A dozen of microRNAs were found by the algorithm. Nonetheless, we are interested in let-7g-5p, because let-7g-5p has been proposed as a tumor suppressive gene (47,48). However, its role still keeps emerging in glioblastoma.

Target sites on 3'UTR of VSIG4 are shown in Fig. 3A. We reasoned that let-7g-5p could downregulate VSIG4 expression by targeting its 3'UTR in glioblastoma. Downregulation of let-7g-5p can contribute to upregulation of VSIG4 in glioblastoma. In an attempt to identify the role of let-7g-5p in regulating VSIG4 expression in glioblastoma, we transfected U-87MG

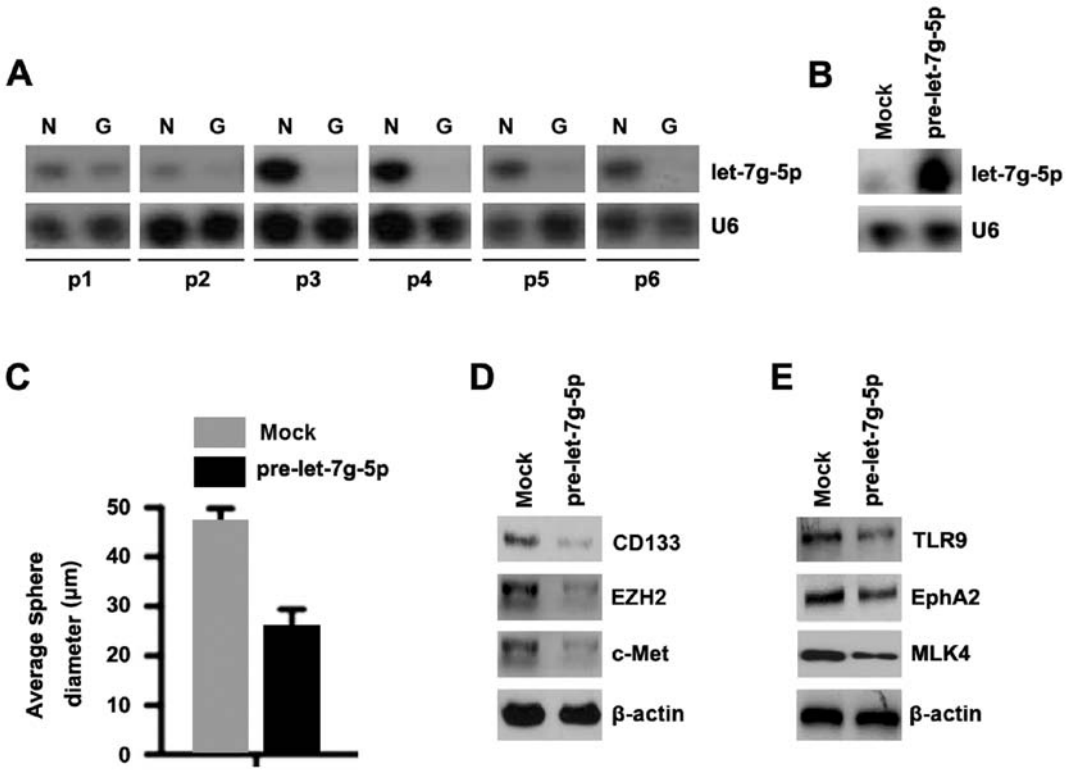

Figure 4. Let-7g-5p inhibits formation of stem cell-like population in glioblastoma U-87MG cells. (A) Northern blotting for let-7g-5p in glioblastoma tissues (C) and adjacent normal tissues (N). Patients were numbered 1-6. All the 6 patients were diagnosed as glioblastoma. U6 was a loading control. n=6. (B) Northern blotting for let-7g-5p in U-87MG cells transfected with pre-let-7g-5p and control miR (mock). U6 was a loading control. n=3. (C) Sphere growth for U-87MG cells transfected with pre-let-7g-5p or control miR. (D) Western blotting for CD133, EZH2 and c-Met in U-87MG cells transfected with pre-let-7g-5p and control miR (mock). β-actin was a loading control. n=3. (E) Western blotting for TLR9, EphA2 and MLK4 in U-87MG cells transfected with pre-let-7g-5p and control miR (mock). β-actin was a loading control. n=3.

cells with pre-let-7g-5p and control miR. After transfection, let-7g-5p expression was detected by real-time PCR and the results showed that let-7g-5p was significantly increased by pre-let-7g-5p in the cells (Fig. 3B).

To confirm the reason, we performed immunofluorescence analyses in U-87MG cells transfected with pre-let-7g-5p and control miR. The results showed that VSIG4 protein was evidently inhibited in the cells transfected with pre-let-7g-5p (Fig. 3C). We next performed western blotting and RT-PCR to detect VSIG4 expression in U-87MG cells transfected with pre-let-7g-5p and control miR. The results showed that VSIG4 protein (Fig. 3D) was significantly downregulated in the cells transfected with pre-let-7g-5p. However, we found that let-7g-5p did not degrade VSIG4 mRNA (Fig. 3E).

Let-7g-5p inhibits formation of stem cell-like population in glioblastoma U-87MG cells. In an attempt to identify let-7g-5p expression between glioblastoma tissues and adjacent normal tissues, we performed northern blotting in tumor tissues versus normal tissues. Protein was isolated from 6 pairs of glioblastoma tissues and normal tissues (patient nos. 1-6). We found that let-7g-5p was significantly decreased in glioblastoma tissues, compared with adjacent normal tissues (Fig. 4A). It indicated that let-7g-5p could be a tumor suppressive gene in glioblastoma. In order to assess the role of let-7g-5p in glioblastoma, we transfected U-87MG cells with pre-let-7g-5p and then northern blot analyses were performed. We found that let-7g-5p was significantly increased in the cells transfected with pre-let-7g-5p (Fig. 4B).

To determine whether let-7g-5p could affect stem-like cell characteristics, we performed sphere forming assay to assess the capacity of CSC or CSC-like cell self-renewal in this study. We found that formations of spheres were decreased by let-7g-5p in U-87MG cells (Fig. 4C). We also performed western blotting to detect whether GSCs markers, CD133, EZH2, c-MET, TLR9, EphA2 and MLK4 can be affected by let-7g-5p in the cells. The results showed that CD133, EZH2, c-MET, TLR9, EphA2 and MLK4 protein was significantly decreased by let-7g-5p in U-87MG cells (Fig. 5D and E).

Overexpressing let-7g-5p promotes MET in glioblastoma U-87MG cells. To assess the role of let-7g-5p in U-87MG cells, we transfected U-87MG cells with pre-let-7g-5p and control miR. We found that its overexpression caused slight changes in the cell morphology (MET, phenotype from a spindle-like morphology to a cobblestone-like) (Fig. 5A). To further verify that the changes in cell morphology are caused by MET, we performed western blotting to detect expression levels of epithelial and mesenchymal markers in U-87MG cells transfected with pre-let-7g-5p and the cells transfected with control miR. The results revealed that epithelial marker (E-cadherin) was induced and the mesenchymal markers (TGFB1, Vimentin, SNAIL, ZEB1 and Notch1) were inhibited by let-7g-5p in U-87 MG cells (Fig. 5B).

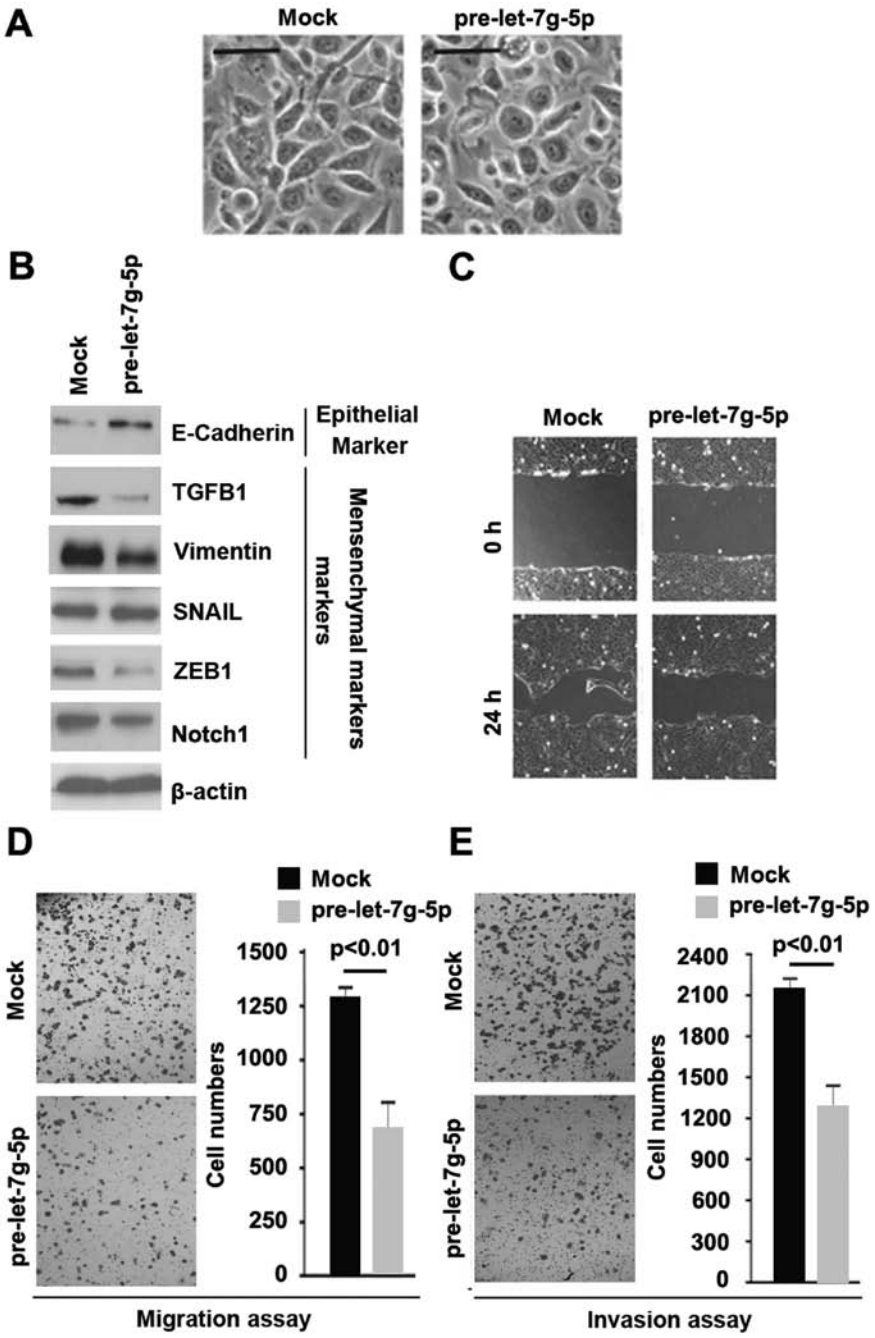

Figure 5. Overexpressing let-7g-5p promotes MET in glioblastoma U-87MG cells. (A) U-87MG cells were transfected with pre-let-7g-5p and control miR (mock). Cells were then photographed after 72 h of transfection. n=3. (B) Western blotting for E-cadherin, TGFB1, Vimentin, SNAIL, ZEB1 and Notch1 in U-87MG cells transfected with pre-let-7g-5p and control miR (mock). β-actin was a loading control. n=3. (C) Wound-healing assays for U-87MG cells transfected with pre-let-7g-5p and control miR (mock). The cell layer was photographed. n=3. (D) Migration assays for U-87MG cells transfected with pre-let-7g-5p and control miR (mock). n=3. (E) Invasion assays for U-87MG cells transfected with pre-let-7g-5p and control miR (mock). n=3.

To identify whether let-7g-5p could inhibit migration and invasion, we performed wound-healing, migration and invasion assays. We found that overexpressing let-7g-5p resulted in decreased migration (Fig. 5C and D) and invasion (Fig. 5E) in the cells.

Discussion

Recently, it was reported that VSIG4 is highly expressed in glioblastoma and correlated with poor prognosis of high-grade glioma patients (49). However, its role has not been reported in glioblastoma cells. Consistent with the previous report, we found that VSIG4 protein is upregulated in glioblastoma. Ionizing radiation represents the most effective therapy for glioblastoma (50), but radiotherapy remains only palliative (51) because of radio-resistance. Glioma stem cells can promote radio-resistance (18). We showed that overexpressing VSIG4 promoted glioma stem cell phenotypes in U87MG cells, implying that VSIG4 might play an important role in radio-resistance. The emerging role of VSIG4 in glioblastoma response to radiotherapy urges further investigation. Notch1 and Notch2 can promote radio-resistance of GSCs in glioma

(52). We found that VSIG4 can evidently promote Notch1 protein expression. The results further indicated that VSIG4 is a potential candidate to prevent radiotherapy resistance. Moreover, VAMP8 can promote temozolomide resistance in human glioma cells (37). Our results also found that VSIG4 can upregulate VAMP8 protein expression in glioblastoma cells, indicating its upregulation might be a cause for temozolomide resistance.

Although the cell origin of cancer stem cells (CSCs) remains to be fully elucidated, mounting evidence has demonstrated that epithelial-to-mesenchymal transition, induced by different factors, is associated with tumor aggressiveness and metastasis and these cells share molecular characteristics with CSCs (53). We found that VSIG4 induced epithelial-to-mesenchymal transition consistent with glioma stem cell phenotypes in glioblastoma cells.

Let-7g-5p is significantly downregulated in the serum of GBM patients and it has been proposed as a tumor suppressive gene in glioblastoma (11,12). Our results showed that its overexpression inhibited VSIG4 protein in glioblastoma cells. Contrary to VSIG4, overexpressing let-7g-5p promoted mesenchymal-epithelial transition and significantly inhibited invasion and migration consistent with the reduction of glioblastoma stem cells phenotypes in U87MG cells.

Elucidating the mechanism that let-7g-5p inhibits epithelial-mesenchymal transition consistent with the reduction of glioma stem cell phenotypes by targeting VSIG4 in glioblastoma will help us to better understand the molecular mechanism of epithelial-mesenchymal transition and glioma stem cells in glioblastoma. Thus, restoration of let-7g-5p may represent a promising therapeutic way to inhibit VSIG4-mediated EMT and GSCs regulation. However, the roles of let-7g-5p/VSIG4 need to be further confirmed *in vivo*.

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