Abstract. The prognosis of patients suffering from glioblastoma [also referred to as glioblastoma multiforme (GBM)] is dismal despite multimodal therapy. Chemotherapy with temozolomide may suppress tumor growth for a certain period of time (a few months); however, invariable tumor recurrence suggests that glioblastoma initiating cells (GICs) render these tumors persistent. Thus, the understanding of the molecular mechanisms of action of GICs as regards their role in the progression of GBM is important as such knowledge will be helpful in the discovery of novel drug targets, as well as in the design of novel therapeutic strategies for more effective treatment of the disease. In this study, we found that tumor suppressor candidate 3 (TUSC3) was downregulated in temozolomide-resistant U87MG cells (U87MG-res cells) and its restoration sensitized U87MG-res cells to temozolomide. TUSC3 was able to inhibit the formation of GIC phenotypes in the U87MG-res cells. The overexpression of microRNA (miR)-132 inhibited TUSC3 protein expression in the U87MG cells. However, its overexpression did not degrade TUSC3 mRNA expression in the cells. miR-132 was upregulated in the U87MG-res cells and its overexpression induced temozolomide resistance and the formation of cancer stem cell phenotypes in the U87MG cells. Thus, our data indicate that miR-132 induces temozolomide resistance and promotes the formation of cancer stem cell phenotypes by targeting TUSC3 in glioblastoma.

Introduction

Glioblastoma [also referred to as glioblastoma multiforme (GBM)] is the most prevalent and lethal type of primary brain tumor with a median survival rate of <15 months (1,2). Despite recent therapeutic developments in the treatment of cancers, current therapies for GBM remain largely ineffective due to drug resistance and rapid tumor recurrence (3). There is now compelling evidence to indicate that the bulk of malignant cells in GBM is generated by a rare fraction of self-renewing, multi-potent tumor cells, termed glioma stem cells (GSCs) or glioma-initiating cells (GICs) (4,5). The GIC hypothesis suggests that tumors consist of a cellular hierarchy with a subpopulation of cells able to maintain and propagate the tumor due to their capacity of self-renewal and resistance to chemotherapy and radiotherapy (6). Thus, the understanding of the molecular mechanisms of action of GICs as regards their role in the progression of GBM is important as such knowledge will be helpful in the discovery of novel drug targets, as well as in the design of novel therapeutic strategies aimed at developing effective treatments for the disease.

Tumor suppressor candidate 3 (TUSC3 or N33), is located on chromosomal band 8p22 and was identified as a potential tumor suppressor gene, which is frequently downregulated or deleted in several tumor types, including breast, prostate, ovarian and pancreatic cancer (7-12). Recently, it has been reported that the expression levels of TUSC3 are downregulated in both GBM tissues and cells (13). The overexpression of TUSC3 inhibits GBM cell proliferation and invasion (13). In addition, the effects of increased levels of methylation on the TUSC3 promoter are responsible for the decreased expression of TUSC3 in GBM (13). Its restoration can inhibit the proliferation and invasion of GBM cells by inhibiting the activity of the Akt signaling pathway (13). Further elucidating the roles of TUSC3 and the molecular mechanisms regulating its expression in GBM will further enyance our understanding of the pathogenesis and progression of the disease, and offer new targets for the discovery of novel drugs.

MicroRNAs (miRNAs or miRs) are endogenous, non-coding small RNAs 19-25 nucleotides in length, which have been recognized as critical post-transcriptional regulators of gene expression (14-16). miRNAs regulate both normal stem cells and tumor-initiating cells (TICs), as well as miRNA dysregulation and have been implicated in tumorigenesis (17-21). Recently, it has been reported that miR-132 is dysregulated in a range of human malignancies (22-24). The level of miR-132 in glioma has been shown to be increased compared to normal brain tissue, and a high level of miR-132
has been shown to correlate with a significantly shorter overall survival in patients with GBM treated with radiotherapy plus concomitant and adjuvant temozolomide chemotherapy (25). However, its roles in GBM have not yet been fully elucidated.

In this study, we found that TUSC3 was downregulated in temozolomide-resistant U87MG cells (U87MG-res cells) and its restoration sensitized U87MG-res cells to temozolomide. TUSC3 inhibited the formation of cancer stem cell phenotypes in the U87MG-res cells. The overexpression of miR-132 inhibited TUSC3 protein expression in the U87MG cells. However, its overexpression did not degrade TUSC3 mRNA expression in the cells. miR-132 was upregulated in the U87MG-res cells and its overexpression induced temozolomide resistance and the formation of GIC phenotypes in the U87MG cells. Thus, our data indicated that miR-132 induces temozolomide resistance and promotes the formation of GIC phenotypes by targeting TUSC3 in GBM.

Materials and methods

**Human GBM cell lines: U87MG and U87MG-res cells.** U87MG cells were purchased from the Biochemistry and Cell Biology Institute of Shanghai, Chinese Academy of Sciences, Shanghai, China. To obtain temozolomide-resistant U87MG cells (U87MG-res cells), we treated the U87MG cells with escalating concentrations of temozolomide from 10^7 to 10^8 M as previously described (26). The established U87MG-res cells grew at a similar rate in the presence or absence of 10^8 M temozolomide for 3 days (data not shown). The half maximal inhibitory concentration (IC_{50}) in the U87MG-res cells increased 12-fold, as compared with that in the U87MG cells (data not shown). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 mg/ml penicillin; 100 U/ml streptomycin) in a 5% CO_2 incubator at 37°C.

**Western blot analysis.** Total proteins in cells were extracted using protein lysis solution (Tiangen Biotech, Beijing, China). The protein concentration was measured with a bicinchoninic acid kit (Tiangen Biotech). The protein extracts were resolved through sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes (Bio-Rad, Berkeley, CA, USA), which were blocked with 5% non-fat milk solutions for 1 h at room temperature. The target proteins were then detected using primary antibodies against human TUSC3 (1:500; ab77660), signal transducer and activator of transcription 3 (STAT3; 1:1,000; ab109085), mouse double minute 2 homolog (MDM2; 1:500; ab170880), p53 (1:500; ab26242), MET (1:1,000; ab68141), CD133 (1:500; ab19898), zinc finger protein, X-linked (ZF/X; 1:500; ab15998) and β-actin (1:500; ab8227) (all from Abcam, Cambridge, MA, USA) at 4°C overnight. The blots were then washed with 0.1% Tween-PBS and incubated with goat anti-rabbit secondary antibodies (1:500; ab218695; Abcam) for antibody for 1 h at room temperature. The blots were then detected by enhanced chemiluminescence (ECL).

**Clonogenic assay.** The U87MG-res cells were collected after trypsinization, and re-suspended in complete medium (same as culture medium described above). Single cell suspensions were plated in regular 10 cm in diameter Petri dishes at the clonal density of 1,000 cells/dish. Following 2-3 weeks of culture, colonies were fixed with 4% paraformaldehyde for 10 min, stained with crystal violet (Tiangen Biotech) for an additional 10 min, and washed with 1X phosphate-buffered saline (PBS). The colonies were then photographed using a CX21 Olympus microscope (Olympus Corp., Tokyo, Japan). The colony numbers were counted using software image analysis program Scion Image downloaded from NIH website (http://www.scioncorp.com). The Particle Analysis program was used for counting the colony numbers. Data are presented as the relative colony number.

**Sphere formation assay.** The cells (10^4/ml) in serum-free RPMI-1640/1 mM Na-pyruvate were seeded on 0.5% agar pre-coated 6-well plates. After 1 week, the half the medium was exchanged every 3rd day with fresh serum-free RPMI-1640/1 mM Na-pyruvate. Single spheres were selected and counted.

**Soft agar assay.** The U87MG-res cells were harvested and suspended in culture medium. To make the bottom layer, 1 ml of 0.5% agarose (Invitrogen, Carlsbad, CA, USA) was added to 6-well plates, and allowed to gel at room temperature. To prepare the top layer (0.25% agarose), 500 µl of 0.5% agarose was mixed with 500 µl cell suspension containing 5,000 cells. This mixture was overlaid above the bottom layer and allowed to solidify at room temperature. An additional 2 ml of culture medium was added after solidification to the top layer, and the cells were incubated for 3 weeks at 37°C. After 3 weeks of growth, the colonies were photographed (magnification, x40) as a CX21 Olympus microscope (Olympus Corp.). The colony numbers were counted under a phase contrast CX53 type Olympus phase microscope (magnification, x40). Data are presented as colony numbers/field.

**MTT assay.** To monitor resistance to temozolomide, the U87MG and U87MG-res cells were treated with temozolomide at various concentrations for 24 h. MTT assay was performed as previously described (27). Data were analyzed with software origin 7.5 (OriginLab, Northampton, MA, USA) to fit sigmoidal curve. The IC_{50} value was the temozolomide concentration that reduced proliferating cells by 50%.

**Cell transfection.** The TUSC3 expression plasmid was obtained from Tiangen Biotech. An empty vector was also purchased and used to transfect cells in the mock group. As described above, to obtain temozolomide-resistant U87MG cells (U87MG-res cells), we treated the U87MG cells with temozolomide. As we found that (IC50) value in the U87MG-res cells increased 12-fold, as compared with that in the U87MG cells (data not shown). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 mg/ml penicillin; 100 U/ml streptomycin) in a 5% CO_2 incubator at 37°C.

**CONCLUSION.** miR-132 was upregulated in the U87MG-res cells and its overexpression induced temozolomide resistance and the formation of GIC phenotypes in the U87MG cells. Thus, our data indicated that miR-132 induces temozolomide resistance and promotes the formation of GIC phenotypes by targeting TUSC3 in GBM.
Real-time PCR for miRNAs. Total RNA from the cultured cells, with the efficient recovery of small RNAs, was isolated using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA). The 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.

Northern blot analysis. For northern blot analysis, samples of total RNA (20 µg) were denatured by treatment with formamide and separated by electrophoresis using 1.3%-denaturing agarose gels. The RNA was transferred to Hybond-N+ nylon membranes (Amersham Biosciences, Little Chalfont, UK) and cross-linked by exposure to UV radiation. Hybridization was performed using hybridization buffer (Ambion). All probes were labeled with gamma [32P]-ATP using the MEGALABEL kit (Takara-Shuzo, Kyoto, Japan). Following overnight hybridization at 65˚C, the membranes were washed twice with 2X SSC-0.1% sodium dodecyl sulfate (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 5 min at room temperature, followed by a wash with 1X SSC-0.1% sodium dodecyl sulfate for 15 min at 60˚C. The signals were then visualized using a BAS-3000 image-analyzer (GE Healthcare, Pittsburgh, PA, USA).

Immunofluorescence staining. Immunofluorescence staining was performed as previously described (28). Following transfection, the cells were fixed in 4% paraformaldehyde, and then blocked with blocking solution at room temperature. Subsequently, anti-TUSC3 antibody (1:200 dilution; ab77600; Abcam, Cambridge, MA, USA) was added, and the mixture was incubated in a humid chamber overnight. After washing 3 times with PBST, the cells were incubated with the appropriate secondary antibody [goat-anti-rabbit IgG (ab:150079; Abcam) for 30 min at 37˚C. After washing, the samples were observed under a laser scanning confocal microscope (Olympus Corp.).

Reverse transcription-quantitative polymerase chain reaction for mRNAs. Total RNA was isolated from the cells or tissues using TRIzol reagent (Invitrogen). cDNA was synthesized from 1 µg of total RNA in a 20 µl reverse transcription (RT) system followed by PCR amplification in a 50 µl PCR system performed using an RT-PCR kit (Promega, Madison, WI, USA). Quantitative PCR (qPCR) for TUSC3 was performed using Power SYBR-Green PCR Master Mix (from Applied Biosystems, Austin, TX, USA). Quantitative PCR (qPCR) for TUSC3 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.

Bioinformatics analysis. The analysis of potential miRNA target sites were identified using the commonly used prediction algorithm, miRanda (http://www.microrna.org/).

Statistical analysis. Data are presented as the means ± SEM. The Student's t-test (two-tailed) was used to compare 2 groups. A value of p<0.05 was considered to indicate a statistically significant difference.

Results

TUSC3 is downregulated in temozolomide-resistant U87MG cells (U87MG-res cells) and its restoration sensitizes U87MG-res to temozolomide. In order to determine whether temozolomide resistance is associated with TUSC3 protein expression, we examined TUSC3 protein expression in the U87MG and U87MG-res cells. The results revealed that TUSC3 protein expression was downregulated in the U87MG-res cells. To determine the role of TUSC3, we examined whether a TUSC3 expression plasmid could be used to stably express TUSC3 protein in the U87MG-res cells. The results revealed that TUSC3 protein expression was significantly increased following transfection with the TUSC3 expression plasmid in the cells (Fig. 1B). To further determine whether TUSC3 can mediate the effectiveness of temozolomide in GBM cells, we transfected the U87MG-res cells with TUSC3 expression plasmid. We then performed MTT assay in the U87MG-res cells transfected with the TUSC3 expression plasmid. The results revealed that TUSC3 transformed the U87MG-res into U87MG cells (cells resistant to temozolomide; Fig. 1C), as evidenced by the decrease in cell viability in the cells transfected with the TUSC3 expression plasmid. This suggested that TUSC3 overexpression reversed temozolomide resistance. Recently, it has been reported that STAT3, MDM2 and p53 expression are associated with temozolomide resistance in GBM (29,30). Thus, we examined the expression levels of these proteins in the U87MG-res cells. Our results revealed that the STAT3 and MDM2 levels were downregulated, and those of p53 were upregulated in the U87MG-res cells transfected with the TUSC3 expression plasmid (Fig. 1D).

TUSC3 inhibits the formation of GIC phenotypes in U87MG-res cells. In order to determine whether TUSC3 can affect GIC traits in U87MG-res cells, we performed sphere forming assay to assess the capacity of GIC or GIC-like cell self renewal in the U87MG-res cells. Sphere forming assay revealed that the TUSC3-overexpressing cells formed much smaller spheres after 14 days of culture as compared with the control cells, indicating markedly decreased GIC traits following transfection with the TUSC3 expression plasmid (Fig. 2A). CD133, MET and ZFX are positively associated with GICs, characteristics in GBM (31-33). Thus, to determine whether TUSC3 regulates CD133, MET and ZFX protein expression, we performed western blot analysis in the U87MG-res cells transfected with TUSC3 expression plasmid or empty vector. The results revealed that the CD133, MET
and ZFX protein levels were downregulated in the U87MG-res cells transfected with TUSC3 expression plasmid or empty vector (mock). β-actin was used as a loading control; n=3 experiments. (C) Western blot analysis for MET, CD133 and ZFX in U87MG-res cells transfected with TUSC3 expression plasmid or empty vector (mock). β-actin was used as a loading control; n=3 experiments. (D) Western blot analysis for STAT3, MDM2 and p53 in U87MG-res cells transfected with TUSC3 expression plasmid or empty vector (mock). β-actin was used as a loading control; n=3 experiments.

Figure 2. Tumor suppressor candidate 3 (TUSC3) inhibits the formation of glioblastoma-initiating cell (GIC) phenotypes in U87MG-res cells. (A) Sphere growth in U87MG-res cells transfected with TUSC3 expression plasmid or and empty vector (mock). The data are reported as the number of spheres formed/1,000 seeded cells; n=3 experiments. (B) Western blot analysis for MET, CD133 and ZFX in U87MG-res cells transfected with TUSC3 expression plasmid or empty vector (mock). β-actin was used as a loading control; n=3 experiments. (C) Clonogenic assay for U87MG-res cells transfected with TUSC3 expression plasmid or and empty vector (mock); n=3 experiments.

Overexpression of miR-132 inhibits TUSC3 protein expression in U87MG cells. Having demonstrated that the overexpression of TUSC3 inhibited the formation of GIC phenotypes, we then examined the mechanisms regulating TUSC3 expression in U87MG cells. miRNAs are a class of small non-coding RNAs (approximately 22 nucleotides in length) that negatively regulate protein-coding gene expression by targeting mRNA degradation or translation inhibition (34-36).

In this study, to further confirm whether TUSC3 is regulated by miRNAs, we used the commonly used prediction algorithm, miRanda (http://www.microrna.org/microrna/home.do) to analyze the 3’UTR of TUSC3. A dozen miRNAs were found by the algorithm. However, we were interested in miR-132, as it has been reported that miR-132 upregulation is associated with an unfavorable clinical outcome in patients with primary GBM (25). The target sites on the 3’UTR of TUSC3 are shown in Fig. 3A. We hypothesized that miR-132 may downregulate
TUSC3 expression by targeting its 3'UTR in GBM cells. The upregulation of miR-132 may contribute to the downregulation of TUSC3 and to temozolomide resistance in GBM.

In an attempt to determine the role of miR-132 in regulating TUSC3 expression in GBM, we transfected the U87MG cells with pre-miR-132 and control miR. Following transfection, miR-132 expression was detected by real-time PCR and the results revealed that miR-132 was significantly increased by pre-miR-132 in the cells (Fig. 3B). To confirm the reason, we performed western blot analysis to detect TUSC3 protein expression in the U87MG cells transfected with pre-miR-132 or control miR. The results revealed that TUSC3 protein was significantly inhibited by miR-132 (Fig. 3C). We then performed immunofluorescence assay in the U87MG cells transfected with pre-miR-132 or control miR. The results indicated that TUSC3 protein expression was evidently inhibited in the cells transfected with pre-miR-132 (Fig. 3D). To examine whether miR-132 can degrade TUSC3 mRNA, we performed RT-PCR and real-time PCR and we found that miR-132 did affect the TUSC3 mRNA level (Fig. 3E and F).

**miR-132 is upregulated in U87MG-res cells and its overexpression induces temozolomide resistance.** In order to examine whether temozolomide resistance is associated with miR-132 expression, we performed northern blot analysis to detect miR-132 expression in the U87MG cells and U87MG-res cells. The results revealed that miR-132 was significantly upregulated in the U87MG-res cells. Using northern blot analysis, we also examined whether pre-miR-132 can be used to stably upregulate miR-132 in the U87MG-res cells. Consistent with the results of real-time PCR (Fig. 3B), the results revealed that miR-132 expression was significantly increased by transfection with pre-miR-132 in the cells (Fig. 4B).

In order to further examine whether miR-132 can affect the efficacy of temozolomide in the U87MG GBM cells, we transfected the U87MG cells with pre-miR-132. We then performed MTT assay in the U87MG cells treated as indicated. The results indicated that the overexpression of miR-132 transformed the U87MG cells into U87MG-res cells (Fig. 4C), suggesting that the overexpression of this miRNA induced temozolomide resistance. We then also performed western blot analysis to detect STAT3, MDM2 and p53 protein expression in the U87MG cells transfected with pre-miR-132 or control miR. The results demonstrated that STAT3 and MDM2 expression increased and p53 expression was downregulated by miR-132 (Fig. 4D).
miR-132 induces the formation of GIC phenotypes in U87MG cells. In order to determine whether miR-132 can affect GIC traits in U87MG cells, we performed sphere forming assay to assess the capacity of GICs or GIC-like cell self renewal in U87MG cells. Sphere forming assay revealed miR-132-over-expressing cells formed more spheres after 14 days of culture than the control cells, indicating markedly increased GIC traits by miR-132 (Fig. 5A). In addition, in order to examine whether TUSC3 can regulate CD133, MET and ZFX protein expression, we performed western blot analysis in the U87MG cells transfected with pre-miR-132 or control miR. The results revealed that the CD133, MET and ZFX protein levels were upregulated in the U87MG cells transfected with pre-miR-132 (Fig. 5B). To determine whether cells with GIC characteristics have an increased clonogenic ability, we performed clonogenic assay. We found that the clonogenic ability of the U87MG cells was significantly increased following transfection with pre-miR-132 (Fig. 5C).
miR-132 transcribed from an intergenic region on human chromosome 17, has been shown to regulate a host of central nervous system-specific processes, including neurogenesis, synaptic plasticity, neuroendocrine-modulated inflammation and the differentiation of dopamine neurons (37-40). It is dysregulated in several brain-related diseases, including Huntington's disease, Parkinson's disease and schizophrenia (41-43). Recently, it has been reported that miR-132 upregulation is associated with an unfavorable clinical outcome in patients with primary GBM treated with radiotherapy plus concomitant and adjuvant temozolomide chemotherapy, suggesting that miR-132 plays an important role in radiotherapy resistance or temozolomide resistance (25). Patients with GBM tend to suffer a relapse following radiation and chemotherapy, and this relapse is believed to be largely attributed to the stem cell-like properties of a fraction of cells (44,45). In the present study, we found that miR-132 may play an important role in the formation of GICs and in the regulation of temozolomide resistance (temozolomide is widely used to treat GBM; however, many patients exhibit acquired drug resistance). These findings provide new insight into the potential roles of miR-132 deregulation in promoting the formation of GICs and conferring chemoresistance in GBM.

A decreased TUSC3 expression was associated with higher pathological TNM staging and a poorer outcome and promotes colorectal cancer progression and epithelial-mesenchymal transition (EMT) in cancer (12,46). EMT in cancer cells can have cancer initiating cell-like features and can render cancer cells to become resistant to temozolomide (47,48). In line with that study, we demonstrated that the overexpression of TUSC3 reversed temozolomide resistance in GBM cells and inhibited the formation of GIC traits. The results further confirmed the hypothesis that eliminating GICs can inhibit resistance to chemotherapy in cancer. As previously demonstrated, STAT3 inhibitor or STAT3 knockdown potentiates temozolomide efficacy in temozolomide-resistant GBM cell lines and it has been proposed that STAT3 inhibitor may be one of the candidate reagents for combination therapy with temozolomide for patients with temozolomide-resistant GBM (29). In this study, we demonstrated that TUSC3 significantly inhibited STAT3 protein expression (Fig. 6). The modulation of the MDM2/p53-associated signaling pathways has been proposed as a novel approach for decreasing temozolomide resistance in GBM (30). We also demonstrated that TUSC3 can downregulate MDM2 and upregulate p53 protein expression in U87MG-res cells (Fig. 6). Moreover, we found that the overexpression of miR-132 inhibited TUSC3 protein expression, and it promoted the formation of GICs and conferred chemoresistance to U87MG cells (Fig. 6). In the future, we aim to determine whether the silencing of miR-132 can restore TUSC3 protein expression in U87MG-res cells. The restoration of TUSC3 may represent a novel therapeutic target for the elimination of GICs and may prevent temozolomide resistance.

Figure 6. microRNA-132 induces temozolomide resistance and promotes formation of cancer stem cell phenotypes by targeting tumor suppressor candidate 3 (TUSC3) in glioblastoma.

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