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ORIGINAL ARTICLE

TUSC3 suppresses glioblastoma development by inhibiting Akt signaling

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Abstract Glioblastoma multiform is one of the most common and most aggressive brain tumors in humans. The molecular and cellular mechanisms responsible for the onset and progression of GBM are elusive and controversial. The function of tumor suppressor candidate 3 (TUSC3) has not been previously characterized in GBM. TUSC3 was originally identified as part of an enzyme complex involved in N-glycosylation of proteins, but was recently implicated as a potential tumor suppressor gene in a variety of cancer types. In this study, we demonstrated that the expression levels of TUSC3 were downregulated in both GBM tissues and cells, and also found that overexpression of TUSC3 inhibits GBM cell proliferation and invasion. In addition, the effects of increased levels of methylation on the TUSC3 promoter were responsible for decreased expression of TUSC3 in GBM. Finally, we determined that TUSC3 regulates proliferation and invasion of GBM cells by inhibiting the activity of the Akt signaling pathway.

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Introduction

Glioblastoma multiform (GBM) is the most prevalent type of primary brain tumor in humans, accounting for more than 20,000 cases annually in the USA. One of the most important hallmarks of GBM is its aggressiveness. The pathology of GBM involves abnormal proliferation and migration of glial cells [1]. Despite the progress of modern diagnostics and treatments, the prognosis of GBM remains poor. This situation is caused both by the difficulty of completely removing tumor tissue by surgery and tumor resistance to chemotherapy drugs. On the molecular and cellular levels, the mechanisms responsible for the onset and progression of GBM are elusive and controversial. A series of recent studies suggested that, similar to malignant tumors in other tissues, dysfunction of signaling pathways that regulate cell proliferation and apoptosis is involved in GBM. A better understanding of these pathways could lead to improved diagnosis and targeted therapies that improve the efficacy of current chemotherapeutics and GBM outcome [2–5].

Tumor suppressor candidate 3 (TUSC3), also known as N33, was originally described and identified as a homolog of the yeast Ost3p subunit of the oligosaccharyltransferase (OST) complex that is involved in N-glycosylation of proteins in *Saccharomyces cerevisiae* [6–8]. A recent structural study revealed that N33/Tusc3 increases protein glycosylation efficiency by slowing glycoprotein folding [9]. TUSC3 mutations have been found in families with non-syndromic autosomal recessive mental retardation [10–16]. Additionally, TUSC3 is required for cellular magnesium uptake and vertebrate embryonic development [17]. Recently, the loss of TUSC3 was

found to be associated with endoplasmic reticulum stress and is also a potential tumor suppressor gene in prostate cancer [18–20]. Homozygous deletions of the chromosomal region where TUSC3 resides have been detected in pancreatic cancer, and recently, downregulated TUSC3 was reported to promote pancreatic cancer proliferation, invasion, and metastasis [21–23]. In ovarian cancer, TUSC3 was found to be significantly downregulated. TUSC3 regulates proliferation, migration, and epithelial-to-mesenchymal transition, and the methylation status of TUSC3 can be a prognostic factor in ovarian cancer [24–27]. The molecular mechanism of the tumor-suppressor effect(s) of TUSC3 on cancer cells is not well understood, but recent studies have suggested that alterations of protein N-glycosylation could be associated with carcinogenic properties such as invasion and metastasis [28–30]. Cell surface growth factor receptors [31, 32], modulators of immune responses [33], and extracellular matrix receptors [34] are all targets for differential N-glycosylation in tumor cells. Interestingly, it also appears that the methylation status of the TUSC3 locus contributes to its tumor suppressor role. Hypermethylated TUSC3 was found in colorectal cancer and was age-related in normal colorectal mucosa [35]. Epigenetic silencing of TUSC3 by promoter methylation was demonstrated to correlate with the survival of ovarian cancer patients [27]. In addition, the presence of *Helicobacter pylori*, a primary risk factor for gastric cancer, was significantly associated with higher levels of TUSC3 methylation [36].

The function of TUSC3 has not previously been characterized in GBM. In this study, we believe we are the first to demonstrate that the expression levels of TUSC3 are downregulated in GBM tissues and cells. We also report that overexpression of TUSC3 inhibits GBM cell proliferation and invasion. In addition, the increased levels of methylation of the TUSC3 promoter were responsible for decreased expression of TUSC3 in GBM. Finally, we determined that TUSC3 regulates the proliferation and invasion of GBM cells by inhibiting the activity of the Akt signaling pathway.

Materials and methods

Human tissue samples

This study was approved by the institutional review board at Harbin Medical University. Thirty fresh glioblastoma (GBM) and five normal brain tissue samples were obtained from the Department of Neurosurgery at the First Affiliated Hospital of Harbin Medical University. Written informed consent was obtained from all patients. Diagnosis of GBM was according to World Health Organization guidelines. None of the patients in the study received chemotherapy or radiation therapy prior to surgery.

Cell lines, transfection, and reagents

Human GBM cell lines U87, U138, LN229, and U251 were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA), supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). The normal human astrocyte (NHA) cell line was obtained from the Lonza group (Lonza, Basel, Switzerland) and cultured according to the manufacturer's instructions. All cells were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C.

TUSC3 complementary DNA (cDNA) was amplified from total U87 DNA by PCR and subcloned into the pcDNA3 vector (Invitrogen). X-tremeGENE (Roche, Mannheim, Germany) was used for plasmid transfection according to the manufacturer's protocol.

5-aza-2′-deoxycytidine (5-Aza) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and MK-2206 was purchased from Selleck Chemicals (Houston, TX, USA).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total messenger RNA (mRNA) from cultured cells and GBM tissues was extracted using the TRIzol reagent (Life Technologies) according the manufacturer's instructions. The cDNA was synthesized from 2 μg of total RNA from each sample, using the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. qRT-PCR were performed using SYBR Premix Ex Taq (TaKaRa). The sequences of the primers were as follows:

Human *TUSC3* forward 5′-GAACGGATGT TCATATTCGGGT-3′ and
Reverse 5′-CGCTTAAAGCAAACCTCCAACAA-3′;
GAPDH forward 5′-TGGACTCCACGACGTA CTCAG-3′ and
Reverse 5′-CGGGAAGCTTGTCATCAATGGAA-3′.

Immunohistochemistry (IHC)

IHC was performed as previously described [37]. TUSC3 primary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Western blot

Cells were lysed in a RIPA buffer with proteinase inhibitors (Santa Cruz Biotechnology). Protein concentrations were determined using the BCA method (Thermo Scientific, Rockford, IL). Protein lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) by electroblotting. Primary

antibodies anti-TUSC3, anti-p-Akt, anti-Akt, anti-BAD, anti-caspase 9, anti-p27, anti-GSK3-β, anti-MMP9, and anti-GAPDH were from Santa Cruz Biotechnology. The immune complexes were detected using the enhanced chemiluminescence (ECL) method.

MTT assay

Cells (3×10^3 per well) were seeded into 96-well plates overnight to allow cells to adhere to the plate bottom. MTT solution (15 μl; 5 mg/mL) was prepared in phosphate buffer solution (PBS) and added to each well and cultured at 37 °C for 4 h. The MTT was then aspirated, and the formazan crystals resulting from mitochondrial enzymatic activity on the MTT substrate were dissolved with 150 μL of DMSO; absorbance at 570 nm was recorded by the multi-well plate reader.

Bromodeoxyuridine (BrdU) assay

The BrdU assay was performed as previously described [38]. Anti-BrdU was obtained from Life Technologies.

Caspase-3 activity assay

Caspase-3 activity was quantified with the Caspase-3 Colorimetric Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 10^6 GBM cells were collected, washed, and lysed with cell lysis buffer. Each sample was centrifuged, and the supernatant was collected and mixed with a solution containing colorimetric substrate (acetyl-Asp-Glu-Val-Asp-p-nitroanilide), then incubated at 37 °C for 90 min in a water bath. Absorbance was measured at 405 nm.

Wound healing assay

Transfected GBM cells were grown to 80–90 % confluence in 24-well plates. The cellular layer was wounded using a sterilized tip (200 μl). Twenty-four hours later, cell migration was monitored and microscopically photographed. Migration ability was assessed by measuring changes in sizes of wounded areas of six fields.

Transwell assay

Transwell chambers (pore size 8 μm; BD Biosciences) were used to measure cell invasive ability. The membranes of filters were coated with basement membrane Matrigel (50 μl per filter) (BD Biosciences). Transfected cells were then collected and added to the upper chamber in 1 % FBS medium. The lower chamber was filled with complete growing medium. After incubation for 24 h, the cells on the upper surface of the membrane were removed with a cotton swab. The migrating cells on the lower membrane were stained with calcein acetoxymethyl ester (4 μg/ml; BD Biosciences) in PBS for 30 min and were observed by fluorescence microscopy (Olympus). We imaged six fields per filter and counted the average numbers of cells per field as a measure for cell invasion.

Methylation-specific PCR (MSP)

MSP of TUSC3 was performed as previously described [27].

Statistical analysis

Data were expressed as mean ± SD of three independent experiments. Comparison was performed using one-way analysis of variance (ANOVA) or Student *t* test. GraphPad Prism software was used to perform statistical analysis (Version 5.0; GraphPad Software, Inc., San Diego, CA, USA). A difference was considered statistically significant if the p value was <0.05.

Results

Expression of TUSC3 is downregulated in GBM

To determine the involvement of TUSC3 in GBM development, we first examined the expression levels of TUSC3 in a microarray data set (GDS1813/14565/TUSC3, containing 41,421 cDNA elements) of 30 GBM and four normal brain tissue samples [39]. We found that TUSC3 expression was significantly downregulated in GBM tissues (Fig. 1a). We further analyzed the expression levels of TUSC3 by qRT-PCR in 30 GBM tissues and five normal brain tissues from clinical samples and found that the levels of TUSC3 were significantly decreased (Fig. 1b). We also examined TUSC3 protein levels by IHC staining. The signal of TUSC3 staining was markedly weaker in GBM than that in normal brain tissues (Fig. 1c). Protein levels of TUSC3 were consistently decreased in all four independent GBM cell lines (U87, U138, LN229, and U251) compared to those in normal human astrocytes (NHA), as determined by Western blot analysis (Fig. 1d). Taken together, these data indicated that the expression of TUSC is downregulated in GBM.

TUSC3 regulates GBM cell proliferation and invasion

Downregulation of TUSC3 in GBM suggests that TUSC3 might play roles in regulating GBM development. We therefore examined the functions of TUSC3 in proliferation and invasion of GBM cells. We overexpressed TUSC3 by plasmid transfection in two independent GBM cell lines, U87 and U251 (Fig. 2a), then subjected these cells to MTT cell proliferation assays and found that TUSC3 overexpression

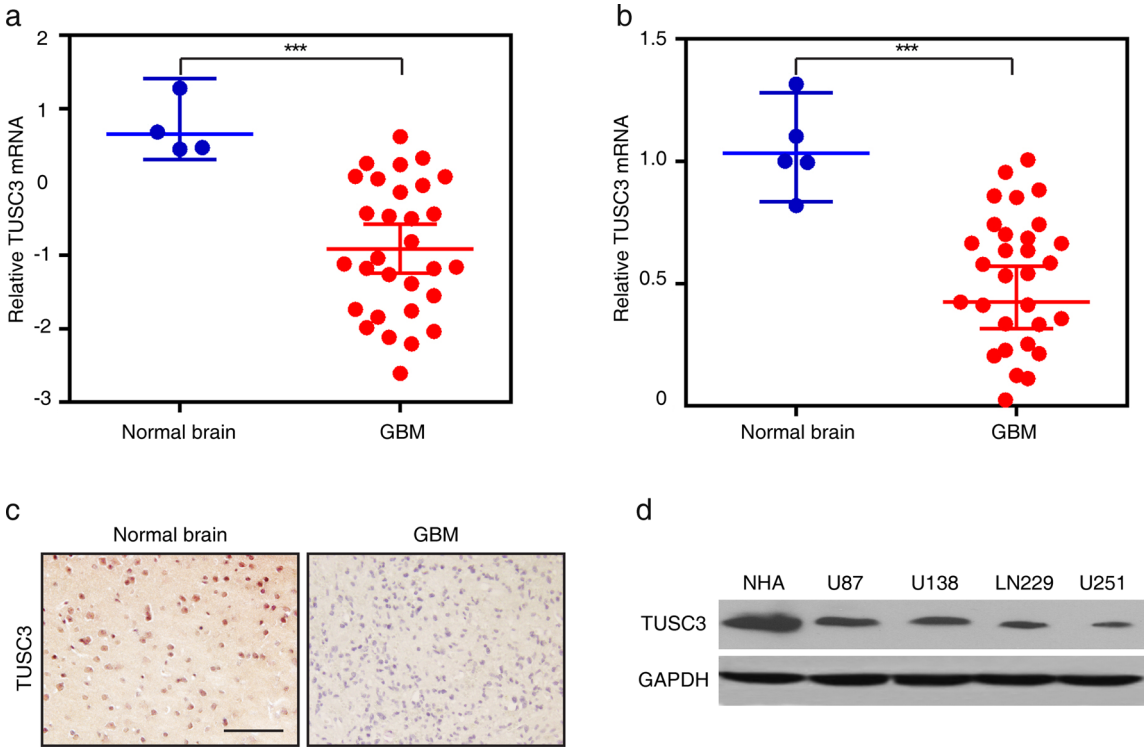

Fig. 1 Expression of TUSC3 is downregulated in GBM tissues and cell lines. **a** Comparison of TUSC3 mRNA levels in GBM ($n = 30$) versus normal brain tissues ($n = 4$) based on gene expression data from the microarray data set (GDS1813/14565/TUSC3) ($p < 0.01$). **b** Relative TUSC3 mRNA levels in clinical GBM ($n = 30$) and normal brain tissues ($n = 5$) ($p < 0.01$) determined by qRT-PCR. **c** Representative image of immunohistochemistry staining of TUSC3 in GBM and normal brain tissue. *Scale bar*: 100 μm. **d** Western blot of TUSC3 protein in normal human astrocytes (*NHA*) and four GBM cell lines (U87, U138, LN229, and U251). GAPDH was used as a loading control

significantly inhibited cell proliferation in both cell lines (Fig. 2b). BrdU assays consistently indicated that significantly smaller proportions of cells underwent active proliferation in TUSC3-overexpressing cells (Fig. 2c). To determine whether TUSC3 affects GBM cell survival and apoptosis, we performed MTT assays on TUSC3-transfected GBM cells cultured in serum-free medium for 48 h. We found that TUSC3 overexpression led to significantly higher levels of cell death upon serum withdrawal (Fig. 2d). Moreover, TUSC3 overexpression induced higher levels of caspase-3 activity in GBM cells, indicating increased cell apoptosis (Fig. 2e). We also examined the functions of TUSC3 on GBM cell migration and invasion and found that its overexpression in U87 and U251 cells significantly suppressed both cell migration in wound healing assays (Fig. 3a) and cell invasion (Fig. 3b) in transwell invasion assays. These results collectively support an inhibitory role of TUSC3 in GBM cell proliferation and invasion.

The promoter of TUSC3 is methylated in GBM

A recent study suggested that methylation of the TUSC3 promoter contributes to its deregulation in cancers [27, 35, 36]. We therefore determined the methylation status of the TUSC3 promoter in GBM tissues and cells by performing methylation-specific PCR on six GBM samples and two normal brain tissue samples. We detected methylation in all six GBM samples, but no methylation in normal brain tissues (Fig. 4a). We also examined the methylation status of the TUSC3 promoter in four GBM cell lines (U87, U138, LN229, and U251) and detected methylation in all four cells lines, while no methylation was detected in normal human astrocytes (NHA) (Fig. 4b). Furthermore, treatment with the DNA methylation transferase inhibitor, 5-Aza, partially suppressed methylation of the TUSC3 promoter (Fig. 4c), and this treatment also resulted in increased levels of TUSC3 expression in GBM cells (Fig. 4d). These findings suggest that the decreased expression levels of TUSC3 in GBM tissues and cells could be attributed to the increased levels of methylation of the TUSC3 promoter.

TUSC3 regulates GBM proliferation and invasion via the Akt signaling pathway

It has been reported that TUSC3 regulates endoplasmic reticulum structure and stress response by modulating the activity of the Akt signaling pathway [20]. This discovery prompted us to investigate whether the Akt signaling pathway mediates the regulation of GBM cell

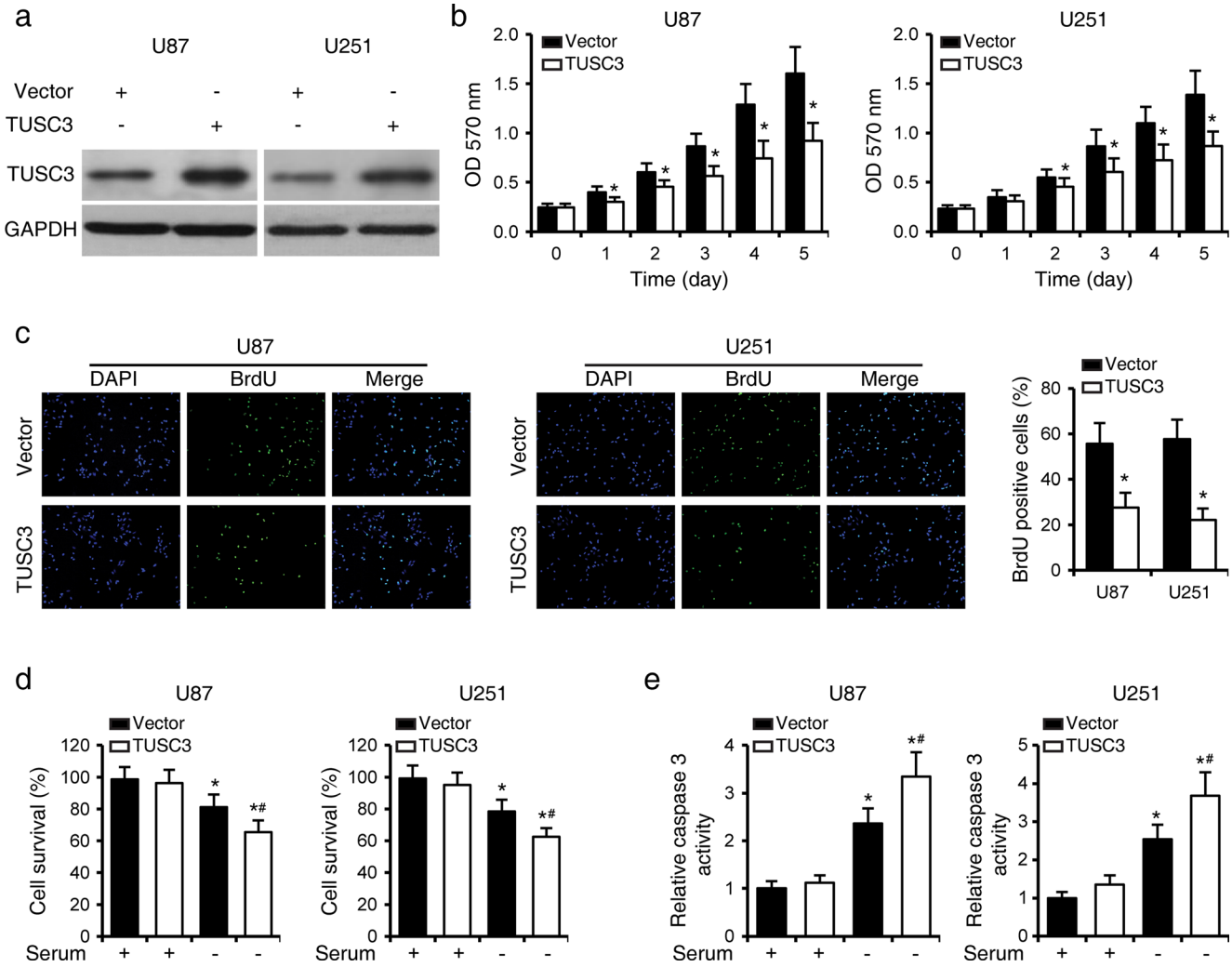

Fig. 2 TUSC3 inhibits GBM cell proliferation and survival. **a** Western blot analysis of TUSC3 protein in U87 and U251 cells transfected with a TUSC3-overexpressing plasmid for 48 h. GAPDH was used as a loading control. **b** MTT assays in U87 and U251 cells with TUSC3 overexpression. *$p < 0.05$ compared to vector control cells. **c** BrdU assays in U87 and U251 cells with TUSC3 overexpression. Quantification of BrdU-positive cells is shown in the *right panel*. *$p < 0.05$ compared to vector control cells. **d** MTT assays in U87 and U251 cells with TUSC3 overexpression cultured in medium with or without 10 % FBS for 48 h. *$p < 0.05$ compared to vector control cells with FBS. #$p < 0.05$ compared to vector control cells without FBS. **e** Assay for caspase-3 activity in U87 and U251 cells with TUSC3 overexpression cultured in medium with or without 10 % FBS for 48 h. *$p < 0.05$ compared to vector control cells with FBS. #$p < 0.05$ compared to vector control cells without FBS

proliferation and invasion by TUSC3. We first examined the levels of phosphorylated Akt in U87 and U251 cells transfected with TUSC3 plasmids and found that overexpression of TUSC3 significantly inhibited Akt phosphorylation, indicating decreased Akt activity (Fig. 5a). We also examined the status of a variety of targets of Akt signaling, including BAD, caspase 9, p27, GSK3-β, and MMP9, all of which have been found to be involved in cancer cell malignancy [40, 41]. These targets were all consistently regulated by TUSC3 overexpression in both U87 and U251 cell lines (Fig. 5b).

To confirm that TUSC3 regulation of GBM cell proliferation and invasion involved the Akt signaling pathway, we blocked the Akt signaling pathway with a specific inhibitor, MK-2206, in GBM cells overexpressing TUSC3, then carried out a series of assays to examine the proliferation and invasion of these cells. Importantly, in the absence of MK-2206, overexpression of TUSC3 decreased levels of cell proliferation (Fig. 6a), survival (Fig. 6b), migration (Fig. 6c), and invasion (Fig. 6d). However, the presence of MK-2206 completely abolished the ability of TUSC3 to regulate cell proliferation and invasion in U87 and U251 cells. The fact that overexpression of TUSC3 no longer inhibited proliferation and invasion of GBM cells when the Akt signaling pathway was blocked strongly suggests that TUSC3 regulation of GBM cell proliferation and invasion occurs via the Akt signaling pathway.

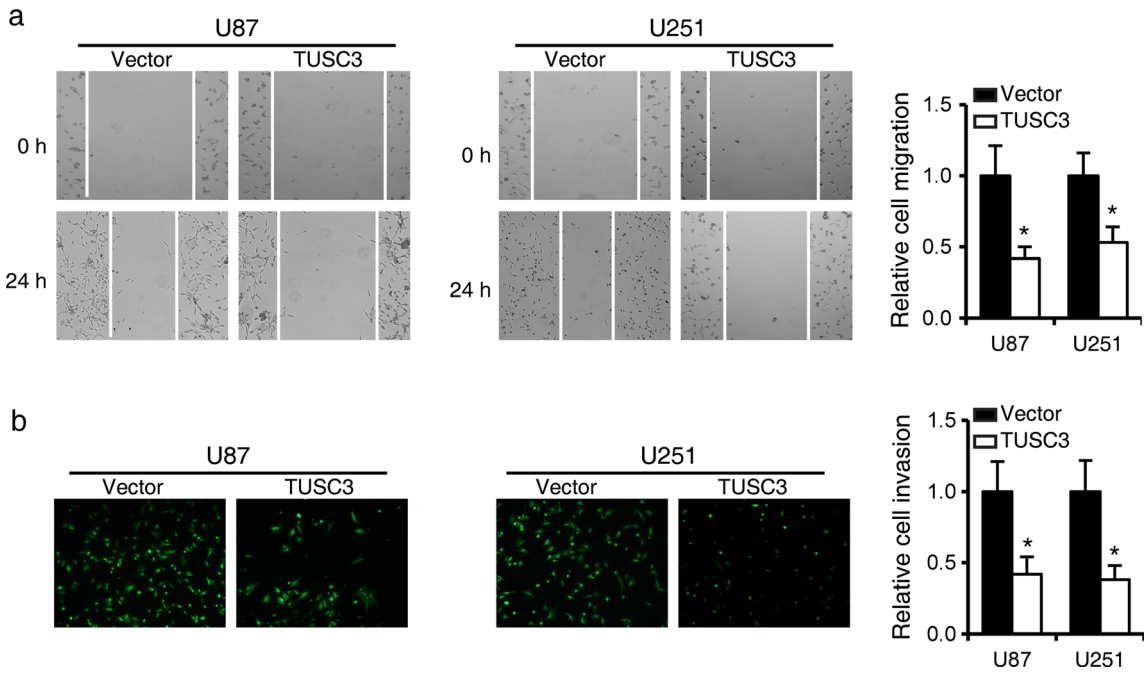

Fig. 3 TUSC3 suppresses GBM cell migration and invasion. **a** Representative image of wound healing assay in U87 and U251 cells with TUSC3 overexpression. Quantification of cell migration distance is shown in the *right panel*. *$p < 0.05$ compared to vector control cells. **b** Representative image of transwell invasion assay in U87 and U251 cells with TUSC3 overexpression. Quantification of invading cells is shown in the *right panel*. *$p < 0.05$ compared to vector control cells

Discussion

The involvement of TUSC3 in cancer development was first revealed by a series of genomic studies. TUSC3 was identified as a candidate tumor suppressor gene in ovarian cancer by screening for differentially regulated genes in chromosomal region 8p22 [24]. Homozygous deletions of the chromosomal region where TUSC3 resides have also been detected in

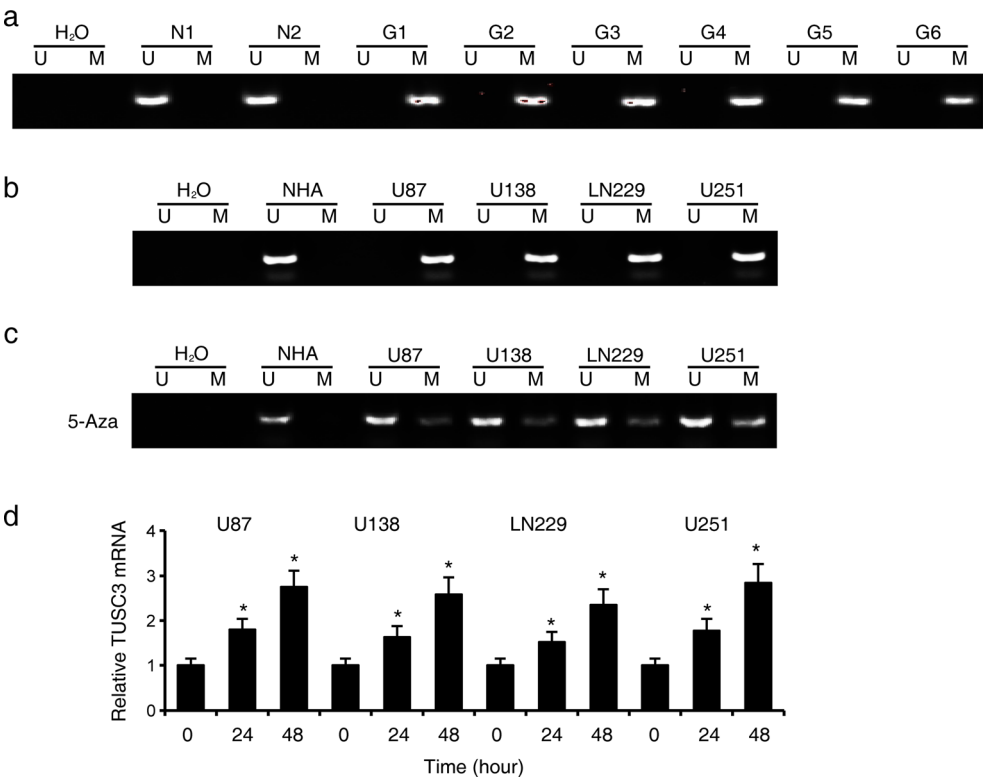

Fig. 4 The promoter of TUSC3 is methylated in GBM. **a** Methylation status of the TUSC3 promoter in GBM and normal brain tissues as determined by methylation-specific PCR analysis. **b** Methylation status of the TUSC3 promoter in normal human astrocytes (*NHA*) and GBM cell lines (U87, U138, LN229, and U251) as determined by methylation-specific PCR analysis. **c** Methylation status of the TUSC3 promoter in normal human astrocytes (*NHA*) and GBM cell lines (U87, U138, LN229, and U251) treated with 2.5 μM 5-Aza, as determined by methylation-specific PCR analysis. **d** Relative TUSC3 mRNA levels in four GBM cell lines treated with 2.5 μM 5-Aza for 24 and 48 h. *$p < 0.05$ compared to 0 h

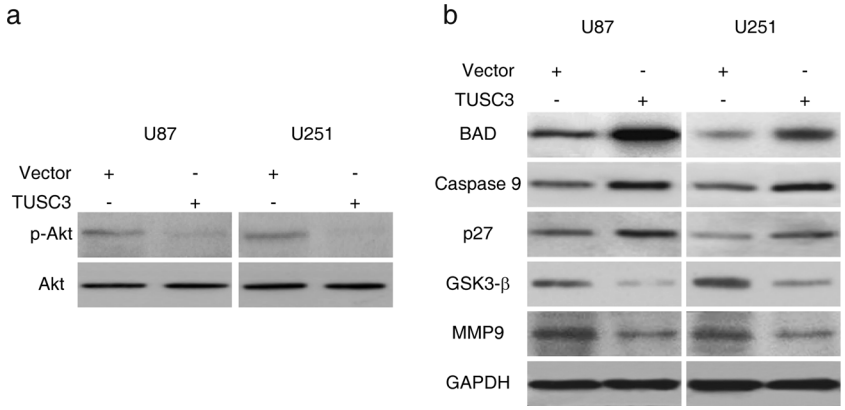

Fig. 5 TUSC3 regulates the Akt signaling pathway in GBM. **a** Protein levels of total and phosphorylated Akt in U87 and U251 cells with TUSC3 overexpression, as determined by Western blot analysis. **b** Protein levels of downstream targets of the Akt signaling pathway (BAD, caspase-9, p27, GSK3-β, and MMP9) in U87 and U251 cells with TUSC3 overexpression, as determined by Western blot analysis. GAPDH was used as the loading control

pancreatic cancer cell lines [21, 22]. However, subsequent mutational analysis in several cancer types failed to pinpoint any protein-disruptive mutation in the coding region of TUSC3. The focus of TUSC3 study in cancer biology has since been on epigenetic silencing of TUSC3 by promoter hypermethylation in various tumor entities, including colorectal [35], ovarian [27], and gastric cancers [36]. The involvement of TUSC3 in GBM, either genomically or epigenetically, has not been previously reported. Therefore, our study is the first to elucidate the function of TUSC3 in the development of GBM.

We found that the expression levels of TUSC3 were downregulated in GBM tissues and cells. The correlation between loss of TUSC3 expression and GBM occurrence suggests that TUSC3 plays important roles in suppressing tumor development under physiological conditions. In support of this hypothesis, we found that overexpression of TUSC3 inhibited GBM cell proliferation and invasion. Importantly, consistent with previous reports, we determined that the increased levels of methylation of the TUSC3 promoter were responsible for decreased expression of TUSC3 in GBM. Further investigation revealed

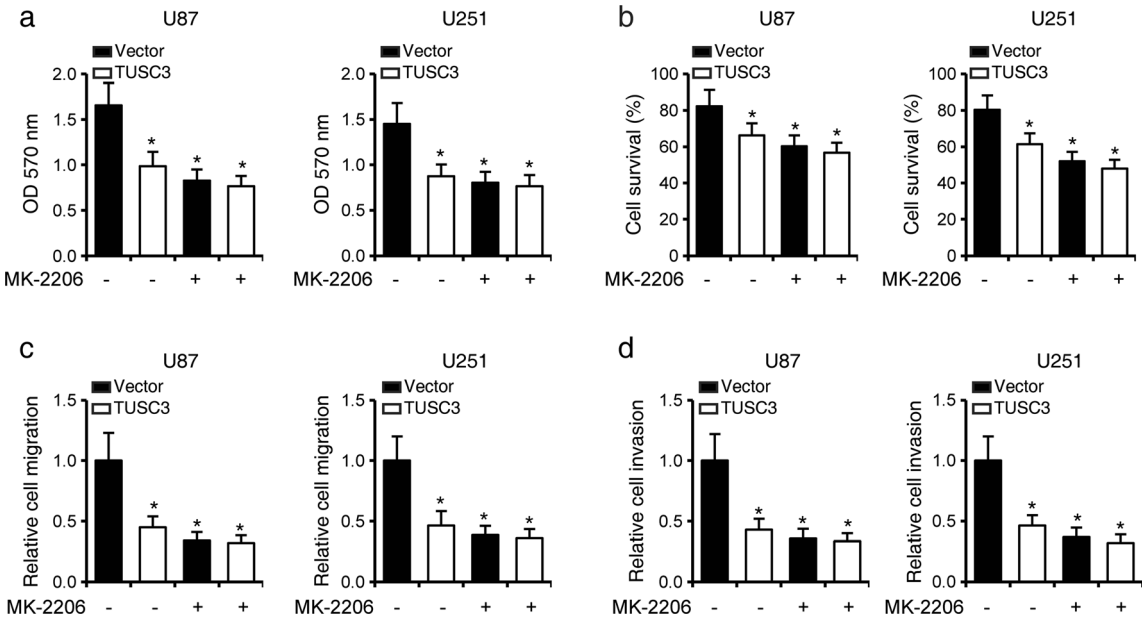

Fig. 6 TUSC3 regulates GBM proliferation and invasion through the Akt signaling pathway. **a** MTT assays in U87 and U251 cells with TUSC3 overexpression, in the presence or absence of 1 μM MK-2206. *$p < 0.05$ compared to vector control cells in the absence of MK-2206. **b** MTT assays in U87 and U251 cells with TUSC3 overexpression cultured in serum-free medium for 48 h in the presence or absence of 1 μM MK-2206. *$p < 0.05$ compared to vector control cells in the absence of MK-2206. **c** Cell migration distance in wound healing assays in U87 and U251 cells with TUSC3 overexpression, in the presence or absence of 1 μM MK-2206. *$p < 0.05$ compared to vector control cells in the absence of MK-2206. **d** Invading cells in transwell invasion assays in U87 and U251 cells with TUSC3 overexpression, in the presence or absence of 1 μM MK-2206. *$p < 0.05$ compared to vector control cells in the absence of MK-2206

that the functions of TUSC3 in inhibiting cell proliferation and invasion were dependent on the activity of the Akt signaling pathway, as blockage of Akt signaling abolished TUSC3 effects. Our results are in agreement with previous studies regarding the general functions of the Akt signaling pathway in cancer development. The Akt pathway plays important roles in regulating apoptosis [42]. Deregulation of apoptosis leads to expansion of neoplastic cells and accumulation of genetic mutations [43] and is therefore one of the major mechanisms for cancer development. The Akt signaling pathway was also demonstrated to be involved in many aspects of cancer development [44, 45], including cell growth [46] and mesenchymal transition [47]. In this context, the inhibition of proliferation and invasion of GBM cells by TUSC3 could be explained, at least in part, by downregulation of the Akt signaling pathway.

Details of the mechanism by which TUSC3 regulates the Akt signaling pathway are not clear and require further investigation. It is of interest to note that recent data have associated protein N-glycosylation with deregulated Akt pathways that affect tumor growth [31]. TUSC3 is the human homolog of *S. cerevisiae* Ost3p, a non-catalytic subunit of the oligosaccharyltransferase complex [7, 8]. Analyses of Ost3p demonstrated its function in regulating N-glycosylation efficiency [48]. Dysfunctional regulation of protein glycosylation has been found in various in vitro cancer models and human cancers, and many glycosylated epitopes have been shown to function as tumor-associated antigens [49, 50]. Although the data presented herein were primarily generated in in vitro cell culture systems, and therefore have intrinsic limitations, we believe that loss of TUSC3 and consequent aberrant N-glycosylation have much greater effects on GBM progression and metastasis. Considering recent data regarding N-glycosylation contributing to cancer progression by affecting the activity of the PI3K-Akt signaling cascade [31], our results further support the possible role of N-glycosylation events in GBM tumorigenesis.

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Compliance with ethical standards This study was approved by the institutional review board at Harbin Medical University.

Conflicts of interest None

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