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ORIGINAL ARTICLE

MSI2-TGF-β/TGF-β R1/SMAD3 positive feedback regulation in glioblastoma

Xingjun Jiang[1] · Jun Tan[1] · Yin Wen[1] · Weidong Liu[2] · Shuyu Wu[1] · Lei Wang[2] · Siyi Wangou[1] · Dingyang Liu[1] · Can Du[1] · Bin Zhu[2] · Dan Xie[3] · Caiping Ren[2]

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Abstract

Purpose Glioblastoma is the most malignant glioma tumors with inevitable relapse and resistance to chemotherapy; however, the mechanisms driving chemoresistance remain to be fully elucidated. This study is to explore the molecular and cellular mechanisms involving in the chemoresistance of glioblastoma.

Methods The expression of musashi (MSI) RNA-binding protein in the tumor tissues and cells of glioblastoma was measured. The effects of MSI2 in epithelial-to-mesenchymal transition (EMT), resistance to temozolomide (TMZ), tumor cell invasion, migration, and proliferation and associated signaling were evaluated.

Results High MSI2 expression was observed in the glioblastoma tissues. Silencing or overexpression of MSI2 significantly affected tumor cells invasion, migration, and proliferation. Silencing of MSI2 expression significantly inhibited O_6-methylguanine-DNA methyltransferase (MGMT) expression and tumor growth, and reversed resistance to TMZ in xenograft tumor models. MSI2 expression regulated EMT through activating the transcription factors Snail and the TGFβ R1/SMAD3 signaling.

Conclusions Our study demonstrated a positive feedback loop of MSI2-TGFβ/SMAD3 signaling which activates the EMT and MGMT which may contribute to chemoresistance in glioblastoma. This study also highlights that MSI2 could be a new target for the therapy of glioblastoma.

Keywords Glioma · Glioblastoma · Chemoresistance · Epithelial-to-mesenchymal transition · TGFβ · SMAD3

Introduction

Malignant tumors are relatively uncommon in the brain and nervous system, but caused significant mortality. Gliomas are the most common tumors in nervous system, representing 81% of brain malignancies. Gliomas are classified into ependymoma, oligodendroglioma, and astrocytoma according to their tissue origin. Among them, 45% gliomas are astrocytomas which are subdivided into astrocytoma, pilocytic astrocytoma, anaplastic astrocytoma, and glioblastoma multiform with glioblastoma the most malignant [1]. Surgery and postoperative concomitant radiotherapy plus temozolomide (TMZ) improved overall survival of newly diagnosed glioblastoma in adult patients, but the highly invasive nature of glioblastoma impedes the surgical removal of all tumor cells, making relapse inevitable. Despite the advances in the surgical and non-surgical treatments of this disease, the arisen resistance to chemotherapy becomes a severe issue

✉ Xingjun Jiang
jiangxingjun@sina.com

[1] Department of Neurosurgery, Collaborative Innovation Center for Cancer Medicine, Xiangya Hospital, Central South University, Xiangya Road 87, Changsha 410008, Hunan, People's Republic of China

[2] Cancer Research Institute, Collaborative Innovation Center for Cancer Medicine, The Key Laboratory for Carcinogenesis of Chinese Ministry of Health and the Key Laboratory of Carcinogenesis and Cancer Invasion of the Chinese Ministry of Education, School of Basic Medical Science, Central South University, Changsha, Hunan, People's Republic of China

[3] State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong, People's Republic of China

[2]. However, the mechanisms of chemoresistance remain to be elucidated.

The epithelial-to-mesenchymal transition (EMT) allows epithelial cells to own a mesenchymal cell phenotype and subsequently obtains enhanced abilities in migration, invasion, and resistance to apoptosis. In addition, EMT exerts a wide effect in maintaining cancer stem cells and conferring radio- and chemoresistance [3]. More and more evidence suggests the involvement of EMT as a key regulator of this invasive state of glioblastoma [4]. For example, Tso et al.'s study identified the cellular and molecular characteristics of mesenchymal stem cells in primary glioblastoma tumor tissues [5]. A recent study demonstrated that EMT facilitates the chemoresistance of glioblastoma [6]. The EMT process is initiated by activation of several key transcription factors, including Snail and ZEB (zinc-finger E-box binding), which decrease the expression of epithelial proteins, such as E-cadherin, and increase the expression of mesenchymal cells specific proteins, such as vimentin. Thus, the decrease in E-cadherin and increase in vimentin expression are regarded as the biomarkers of EMT [7].

Two members of musashi (MSI) RNA-binding protein gene family (MSI1 and MSI2) have been identified with a high degree of sequence similarity in stem and early progenitor cells. These two members play a crucial role in regulating proliferation and differentiation of nervous and haematopoietic stem cells [8, 9]. MSI2 has two isoforms, the larger isoform (isoform 1) and a shorter isoform (isoform 2), and both isoforms have been revealed to be required for the self-renewal of embryonic stem cells, but only isoform 1 enhanced the cloning efficiency [10]. MSI2 has also been revealed to enhance invasion of hepatocellular carcinoma by inducing epithelial–mesenchymal transition [11]. However, it is unclear whether MSI2 is involved in EMT activation in glioblastoma. TGF-β is thought to be an important inducer of the EMT in various cancers through phosphorylation of SMAD proteins [12]. Moreover, TGF-β promotes EMT of glioblastoma cells by phosphorylating SMAD2/3 [13]. MSI2 plays an important regulatory role in homeostasis of hematopoietic stem cells through controlling TGF-β signaling [14] and contributes to invasive adenocarcinoma through essential support for TGF-β R1/SMAD3 signaling [15]. However, whether MSI2 regulates TGF-β signaling in the glioblastoma has not been reported.

Materials and methods

Specimens

The tumor tissues of 32 ependymomas, 38 oligodendrogliomas, 41 pilocytic astrocytomas, 28 astrocytomas, 33 anaplastic astrocytomas, and 52 glioblastomas were collected and paraffin-embedded from January 2012 to December 2016 at the Affiliated Xiangya Hospital, Central South University, and the use of tumor tissues was pre-approved by the Ethics Committee of Human Study of Xiangya Hospital. The information on the clinical classifications of glioma cancer patients was recorded. This study was approved by the Ethics Committee of the Affiliated Xiangya Hospital, Central South University.

Cell culture

LN-18, a human glioblastoma cell line and H4, a human neuroglioma cells line, were obtained from ATCC (American Type Culture Collection). Cells were cultured in Dulbecco's Modified Eagle's Medium-containing 10% fetal bovine serum (FBS) at 37 °C, 5% CO_2. H4 cell line was chosen for a control, because H4 cells have a low MGMT expression, which are less malignant and relatively sensitive to TMZ.

Calcium carbonate nanoparticles

Calcium carbonate nanoparticles were prepared mixing microemulsion A and B as previously described [15]. The $CaCO_3$ nanoparticles were examined by transmission electron microscopy [16]. The produced $CaCO_3$ nanoparticles were uniform with an averaged diameter of 49–59 nm and a positive surface charge of + 28.3 mV. The $CaCO_3$ nanoparticle–plasmid DNA or $CaCO_3$ nanoparticle–siRNA complex at a ratio of 2:3 in weight was directed used for in vitro and in vivo experiments.

Immunohistochemistry

The paraffin-embedded tumor tissues were sectioned at 4 μM thick. After deparaffinized, incubated with 3% H_2O_2 (15 min in dark), and performed for antigen retrieval with 10 mM sodium citrate, the sections were incubated with rabbit anti-human MSI2 antibody (detecting isoform 1) (Abcam, USA) for 1 h and then incubated with several drops of Solution A (HRP-conjugated secondary antibody) for 30 min. The sections were then treated with DAB staining, haematoxylin counter-staining, dehydration, soaking in xylene, and mounting with neutral balsam [17]. Five hundreds of cells were counted from several sections by two observers independently. The average of the percentages from these two observers was used for the final evaluation.

Real-time amplification of MSI2 gene

Total RNA was extracted from tumor tissues using Trizol reagent (Invitrogen, USA) by following the manufacturer's instructions. Reverse transcription was performed using One-Step cDNA Synthesis Kit by following the

manufacturer's instructions. Real-time quantitative PCR was performed using SYBR® Premix Ex Taq™ II (RAKARA, Japan). MSI2 gene (isoform 1) was amplified by forward primer: 5′-CAGGCACAGAGGGTTTGGC-3′ and reverse primer: 5′-GCGACAGGTCCTGGGCTGTTG-3′. The GAPDH was amplified as an internal control using forward primer: 5′-CTACATGGTTTACATGTTCC-3′ and reverse primer: 5′-GACACGGAAGGCCATGCCAGTG-3′ (Shanghai GenePharma Co., Ltd, Shanghai, China).

Western blot

Cells and tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer with proteases and phosphatases inhibitors (Sigma, USA) using sonication. Western blot was performed as previously described [18]. The antibodies for TGF-β receptor 1, phospho-SMAD2/3, Snail1, Snail2, E-cadherin, vimentin, MGMT, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The acquired images were analyzed with Photoshop software.

Overexpression and silencing of MSI2 gene

The open reading frame of MSI2 gene was amplified using forward primer: 5′-GATATGGAGGCAAATGGGAGC-3′ and reverse primer: 5′-GTAATTCCCCACTCCGGAGTC CTG-3′. The PCR product was purified and directly cloned into pcDNA3.3-TOPO vector (ThermoFisher Scientific, Grand Island, NY, USA) by following the user instruction. The target sequences that effectively silence MSI2 gene were 5′-GACCCAGCAAGUGUAGAUAAA-3′ [19] and 5′-CAAUGCUGAUGUUUGAUAA-3′ [11]. The non-silencing scramble sequence was used for control (5′-TTC TCCGAACGTGTCACGT-3′) (Shanghai GenePharma Co., Ltd, Shanghai, China).

Colony formation assay

The clonogenic cell survival was assayed using colony formation assay. Briefly, cells were treated with nanoparticles-DNA mixture or TMZ for 24 h and 1×10^5 cells were seeded on Matrigels-Matrix (Becton-Dickinson, Germany) precoated 6-well dishes. Fourteen days later, grown colonies were stained with 1 mg/ml of iodonitrotetrazolium chloride solution (Sigma-Aldrich; St. Louis, MO, USA) overnight, and colonies greater 50 cells were counted.

In vitro invasion and migration assay

The potential of invasion and migration of siRNA-transduced LN-18 and H4 tumor cells were assayed using Transwell assays (8-mm pore size, Corning Costar Corning Incorporated, NY, USA) pre-coated with and without Matrigel in a chamber. Cells (1×10^5) were suspended in 150 µl of DMEM and seeded on the upper chamber. Upper and lower chambers contained media with 10% FBS. After 48 h, noninvaded cells were wiped off the top surface of the membrane filter and filters were removed. The cells that migrated through the matrigel and adhered onto the lower chamber were fixed with 70% ethanol and 4% paraformaldehyde, stained with Coomassie blue and enumerated in four high powered fields. Cells were counted.

MTT assay

Cell proliferation was assayed using MTT Cell Proliferation Assay Kit (Ann Arbor, MI, USA). MTT is taken up by live cells and insoluble by intracellular NAD(P)H-oxidoreductases. The insoluble MTT was measured by absorbance at 570 nm.

Animal experiments

The immunocompetent male mice were provided by the Animal Facility of Central South University and animal protocol was pre-approved by the Animal Use Committee of Central South University. The mice were first injected with 1×10^6 LN-18 tumor cells into right flanks subcutaneously ($N = 40$). After tumors were grew up to 7–8 mm in diameter, mice were divided into four groups ($N = 10$). The control group received daily injection of blank nanoparticles (5 mg/kg) intratumorally and daily intraperitoneal injection of saline, the TMZ alone group received daily intraperitoneal injection of TMZ (50 mg/kg) and daily injection of blank nanoparticles (5 mg/kg) intratumorally, nano-siRNA group received daily injection of nanoparticles-MSI2 siRNA mixture (5 mg/kg) and daily intraperitoneal injection of saline, and the Nano-siRNA plus TMZ group received daily injection of nanoparticles–MSI2 siRNA mixture (5 mg/kg) intratumorally plus daily intraperitoneal injection of TMZ (50 mg/kg). Tumor size was measured every other day. Tumor volume was calculated using $V = a^2 \times b/2$ (a = length, b = width). Mice were killed and tumors were excised. The tumors were weighed. All animal experiments were approved by the Experimental Animal Ethics Committee of Central South University.

Statistics

Data were presented as mean ± standard error of mean (SEM) and analyzed using SSPE v17 (IBM, USA). Comparison of treatments was performed using one-way analysis of variance (ANOVA) with Newman–Keuls post-test. Differences were considered statistically significant at $P < 0.05$.

Results

High MSI2 expression implied a high malignancy in gliomas

Immunohistochemical staining showed that MSI2 expression mainly located in the nuclei of glioma tumor cells (Fig. 1a). Glioblastoma exhibited a highest percentage of positive MSI2 staining cells, followed by anaplastic astrocytoma, ependymoma, oligodendroglioma, pilocytic astrocytoma, and astrocytoma (Fig. 1b). Western blot showed a similar tendency of MSI2 protein expression found in immunohistochemical staining (Fig. 1c).

Silencing of MSI2 expression inhibited cell invasion, migration, and proliferation in human neuroglioma H4 and glioblastoma LN-18 cell lines

H4 and LN-18 cells were incubated with the complex of nanoparticle and MSI2 siRNA1 (Nano-MSI2-siRNA1), complex of nanoparticle and MSI2 siRNA2 (Nano-MSI2-siRNA1), nanoparticle control (NC), and blank control (BC). Real-time PCR (Fig. 2a) and western blot (Fig. 2b, c) showed that nanoparticles-delivered siRNA1 and siRNA2 of MSI2 gene significantly inhibited MSI2 mRNA and protein expression in LN-18 cells, respectively. Silencing of MSI2 gene expression significantly inhibited cell invasion (Fig. 2d), migration (Fig. 2e), and proliferation (Fig. 2f) in LN-18 cells. Similarly, nanoparticle-delivered siRNA1 and siRNA2 significantly inhibited MSI2 mRNA (Fig. 2g) and protein (Fig. 2h, i) expression in H4 cells. Silencing of MSI2 gene expression significantly inhibited cell invasion

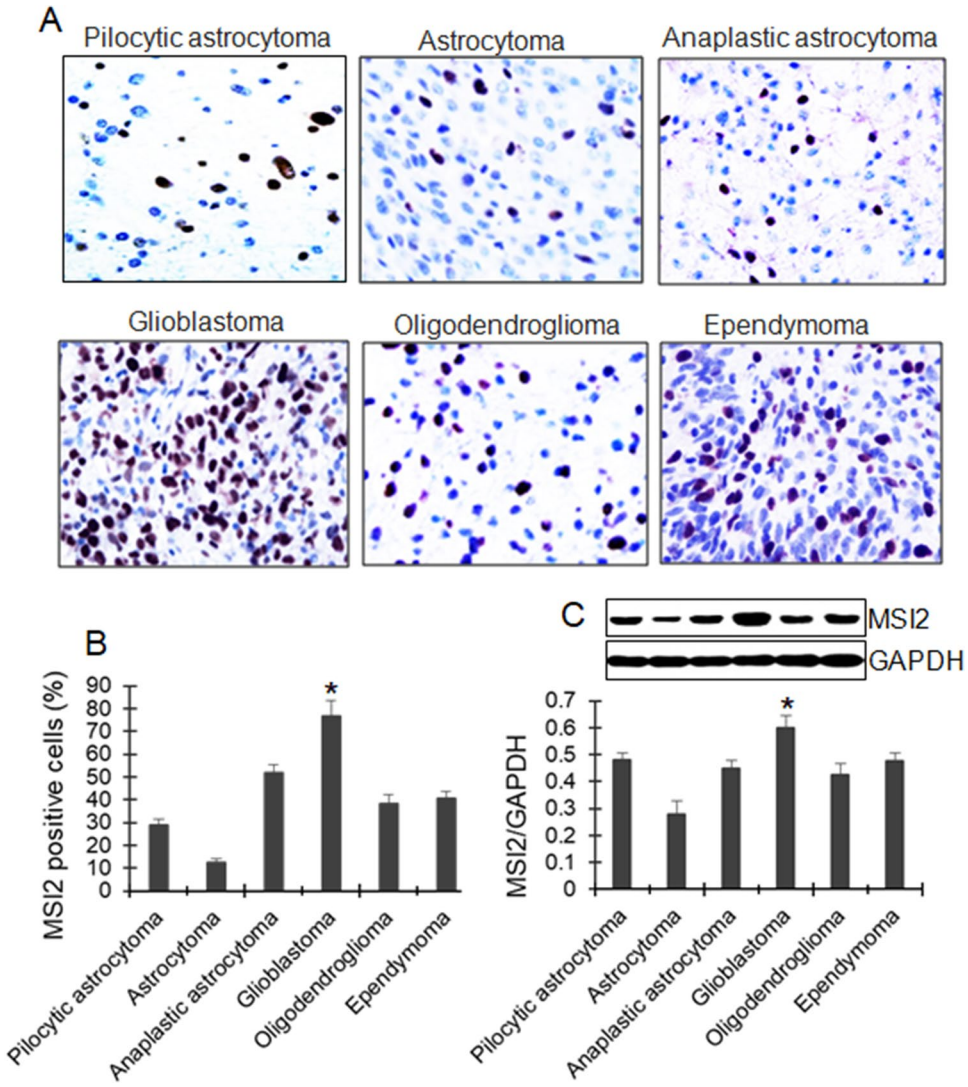

Fig. 1 MSI2 protein expression in tumor tissues. **a** Representative immunohistochemistry of MSI2 protein expression in different tumor tissues. Arrows indicated positive staining cells. **b** Comparison of percentages of MSI2 positive cells between tumor tissues. **c** Western blot of MSI2 protein expression. Top panel: representative western blots of MSI2 and GAPDH. Bottom panel: semi-quantitative analysis of MSI2 and GAPDH expression. $^{*}P < 0.01$ or < 0.001 vs. all other groups

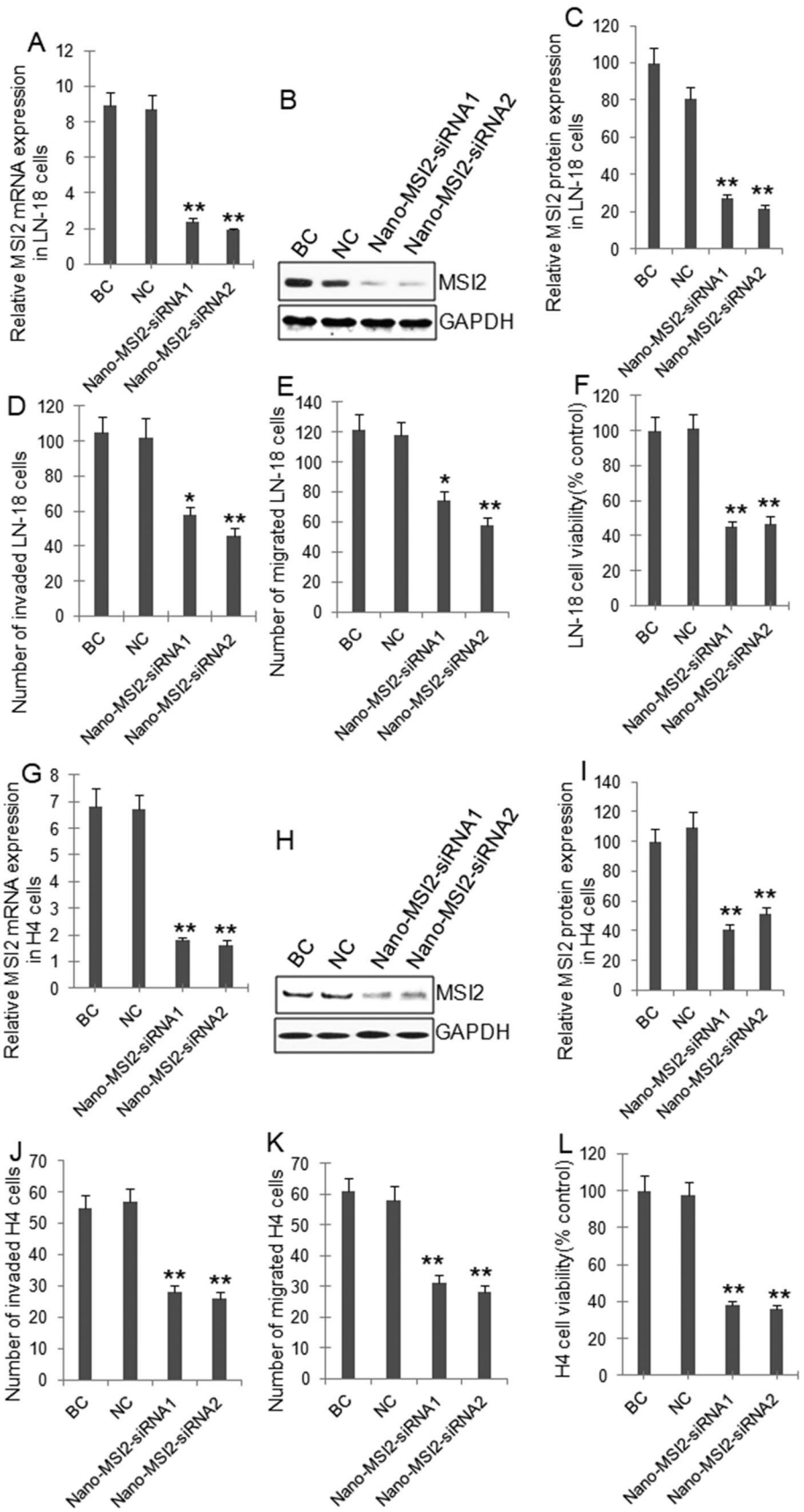

Fig. 2 Silencing of MSI2 expression inhibited cell proliferation, migration, and invasion in tumor cells. **a** Real-time PCR of MSI2 mRNA expression in LN-18 cells. siRNA significantly inhibited MSI2 mRNA expression. **b** Western blots of MSI2 and GAPDH protein expression in LN-18 cells. **c** Semi-quantitative analysis of MSI2 protein expression in LN-18 cells. miRNA significantly reduced MSI2 protein expression. **d** Invasion assay in LN-18 cells. Silencing of MSI2 expression significantly decreased cell invasion. **e** Migration assay in LN-18 cells. Silencing of MSI2 expression significantly decreased cell migration. **f** Cell proliferation in LN-18 cells. Silencing of MSI2 expression significantly decreased cell viability. **g** Real-time PCR of MSI2 mRNA expression in H4 cells. siRNA significantly inhibited MSI2 mRNA expression in H4 cells. **h** Western blot of MSI2 and GAPDH expression in H4 cells. **i** Semi-quantitative analysis of MSI2 protein expression in H4 cells. miRNA significantly reduced MSI2 protein expression in H4 cells. **j** Invasion assay in H4 cells. Silencing of MSI2 expression significantly decreased cell invasion in H4 cells. **k** Migration assay in H4 cells. Silencing of MSI2 expression significantly decreased cell migration in H4 cells. **l** Cell proliferation in H4 cells. Silencing of MSI2 expression significantly decreased cell viability in H4 cells. $N=4$. $^{*}P<0.01$, $^{**}P<0.001$ vs. BC

(Fig. 2j), migration (Fig. 2k), and proliferation (Fig. 2l) in H4 cells.

Silencing of MSI2 expression inhibited clonogenic cell survival and tumor growth and reversed resistance to TMZ

The expression of the DNA repair protein MGMT is associated with the resistance of tumor cells to TMZ [20]. The MGMT protein expression in LN-18 and H4 cells was measured by western blot and its expression was higher in LN-18 cells than in H4 cells (Fig. 3a). Thus, LN-18 cells were selected for the TMZ treatment. Colony formation assay showed that silencing of MSI2 expression significantly decreased clonogenic cell survival in LN-18 cells, but the combination of siRNA and TMZ treatment was more significant (Fig. 3b, c). The glioblastoma LN-18 cells were inoculated into the nude mice to establish the xenograft tumor models. Silencing of MSI2 expression significantly decreased tumor volume from day 15 compared to the control group, while the combination of siRNA and TMZ exhibited more significant inhibition on tumor growth beginning at day 24 than siRNA and TMZ alone group (Fig. 3d). The tumors were excised and weighed. Nanoparticles-delivered siRNA alone or combination with TMZ injection significantly decreased tumor weights compared to the control

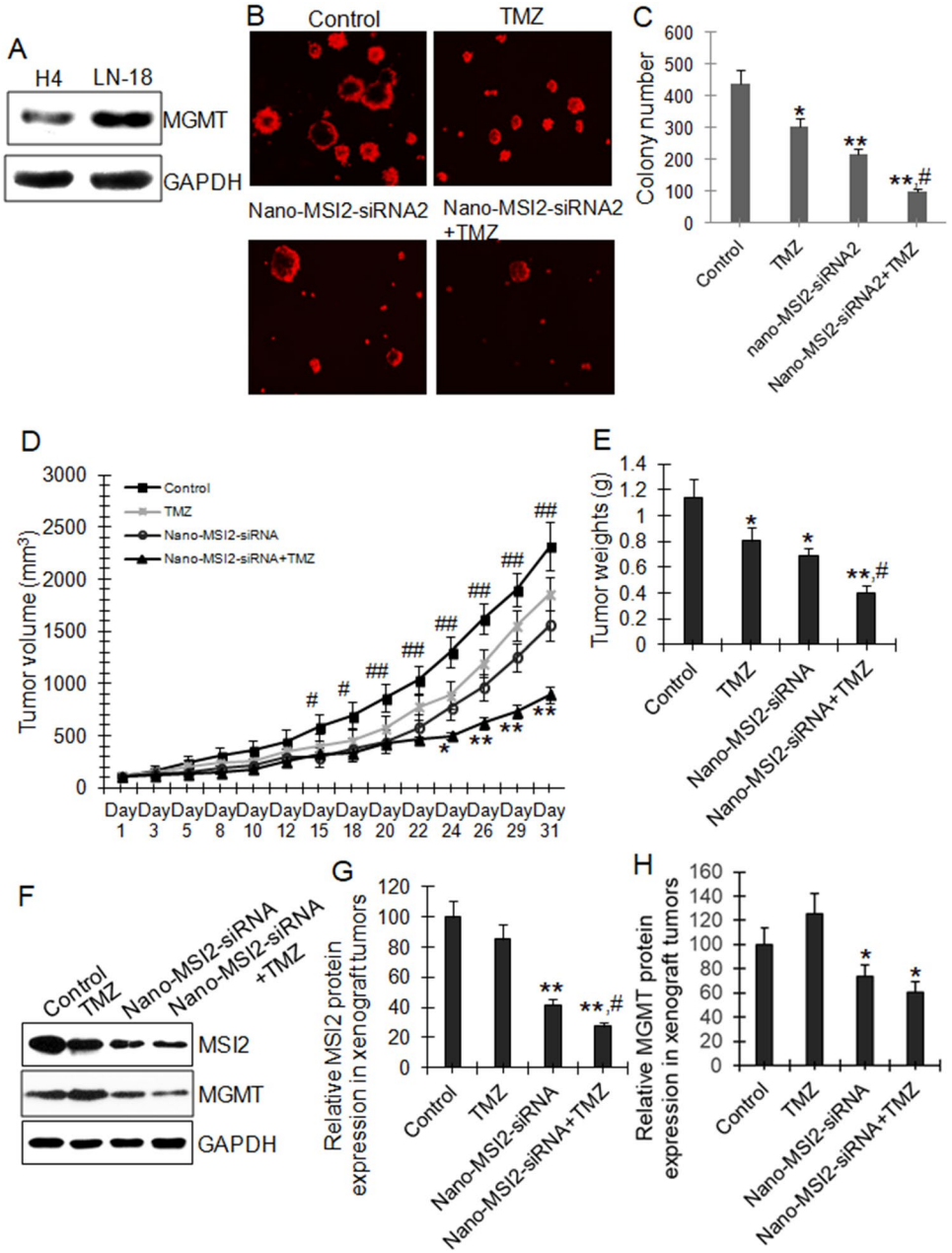

Fig. 3 Silencing of MSI2 expression inhibited tumor growth and resistance to TMZ. **a** Representative western blot of MGMT protein expression in H4 and LN-18 cells. **b** Representative images of clonogenic cell survival in LN-18 cells. **c** Counting of colony number in LN-18 cells. Both the nanoparticles-delivered siRNA and siRNA + TMZ treatment significantly decreased colony formation on Matrigels. $^{*}P < 0.05$, $^{**}P < 0.001$ vs. MSI^{+} group, $^{\#}P < 0.01$ vs. control group. **d** Tumor growth curves. Xenograft tumor models were established by subcutaneous injection of LN-18 tumor cells into right flanks of mice. Both the nanoparticles-delivered siRNA and siRNA + TMZ injection significantly inhibited tumor growth 22-day post-first injection. $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ between nano-siRNA and control group, $^{*}P < 0.05$, $^{**}P < 0.001$ between nano-siRNA alone group and nano-siRNA + TMZ group. **e** Tumor weights. $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control group, $^{\#}P < 0.05$ vs. nano-siRNA alone group. $N = 10$. **f** Representative western blots of MSI2, MGMT, and GAPDH protein expression in xenograft tumor tissues. **g** Semi-quantitative analysis of MSI2 protein expression measured by western blot. **h** Semi-quantitative analysis of MSI2 protein expression measured by western blot. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. control group, $^{\#}P < 0.05$ vs. nano-siRNA alone group. $N = 10$

group (Fig. 3e). The MSI2 and MGMT protein expression in xenograft tumors was measured by western blot (Fig. 3f). Nanoparticles-delivered siRNA with or without TMZ significantly inhibited MSI2 (Fig. 3g) and MGMT protein expression (Fig. 3h).

MSI2 regulated EMT and associated signaling in xenograft glioblastoma LN-18 tumors

Western blot was performed on all tumors, and then imaged, analyzed, and combined the results of all tumors to obtain p values. High TGF-β R1, Snail1, Snail2, and vimentin protein expression were observed in human glioblastoma tissues compared to human astrocytoma tissues (Fig. 4a). Western blots showed that injection of nanoparticles-siRNA, but not injection of TMZ, significantly inhibited TGF-β R1 (Fig. 4b, c), pSMAD3 (Fig. 4b, d), Snail1 (Fig. 4b, e), Snail2 (Fig. 4b, f), and vimentin protein (Fig. 4b, h), but significantly increased E-cadherin protein expression (Fig. 4b, g) in the xenograft LN-18 glioblastoma tumor tissues.

Overexpression of MSI2 expression increases invasion and migration in H4 cells through activation of EMT, and TGFβ1 regulates MSI2 expression

Western blot showed that overexpression of MSI2 obviously upregulated TGF-β R1, Snail1, Snail2, MGMT, and vimentin protein expression in H4 cells (Fig. 5a). Overexpression of MSI2 expression significantly increased invasion (Fig. 5b) and migration (Fig. 5c) of H4 cells. To investigate the feedback regulation of TGFβ on MSI2 expression, LN-18, and H4 tumor cells were treated with or without 20 ng/ml of TGFβ1 (Minneapolis, MN, USA) and/or 20 ng/ml of TGFβ receptor inhibitor SD208 (Selleckchem, Houston, TX, USA) for 48 h. Western blot showed that TGFβ1 significantly increased MSI2 (Fig. 5d, e), pSMAD3 (Fig. 5d, f), and MGMT (Fig. 5d, g) protein expression, but SD208 partially blocked the effects of TGFβ1 in LN-18 cells (Fig. 5d–g). Similarly, TGFβ1 significantly increased MSI2 (Fig. 5h, i), pSMAD3 (Fig. 5h, j), and MGMT (Fig. 5h, k) protein expression, but SD208 partially blocked the effects of TGFβ1 in H4 cells (Fig. 5g–i). These finding suggested that TGFβ signaling regulated MSI2 expression.

Discussion

Many factors can influence the resectability of glioblastoma and the unresectable glioblastomas were ordinarily treated with corticosteroids, radiotherapy, chemotherapy, or combined radio- and chemotherapy [21]. Temozolomide is the first-line treatment of malignant gliomas following surgery which can increase patients' median survival by 2–3 months [22]. However, drug resistance is a major obstacle in the treatment of glioblastomas [23]. Glioblastoma is the most malignant type of gliomas with a median survival time of about 10–15 months [24]. This study found that MSI2 is highly expressed in the glioblastoma cells. Moreover, MSI2 activated EMT through activating the transcription factors Snails and the TGF-β R1/SMAD3 signaling. At the same time, MSI2 may contribute to the TMZ resistance and could be an ideal target for developing therapeutic agents of glioblastoma.

Glioma stem cells have a capability of self-renewal, which support glioma genesis, therapeutic resistance, and recurrence [25]. MSI2 is identified a proliferation and differentiation factor of nervous stem cells [11, 12]. This study demonstrated that MSI2 is highly expressed in human glioblastoma tissues compared to other type of gliomas. Silencing of MSI2 expression significantly inhibited proliferation, invasion, and migration in human neuroglioma cells with high MSI2 expression and glioblastoma cell with low MSI2 expression. Moreover, overexpression of MSI2 increased the invasion and migration of neuroglioma cells. Therefore, high MSI2 expression is a crucial marker for the malignancy of gliomas, although it may not be a specific marker for glioblastoma. It is also not difficult to understand that MSI2 may be involved in the local relapse of glioblastoma. However, more studies on MSI2 expression in glioma cells regardless of mesenchymal versus proneural subtypes or in nodular cells versus invasive cells may provide more clear evidence.

Cancer stem cells have been widely revealed to confer drug resistance in a variety of tumors [26]. The activation of EMT can subsequently result in resistance to multiple drugs even they were initially sensitive and permit rapid progression of the tumors [27]. MSI2 has been identified a marker of cancer stem cells as described above and a molecule to induce epithelial–mesenchymal transition [11]. Drug resistance is broadly classified into primary and acquired based on tumor response to the initial therapy. While primary resistance exists before treatment, prolonged exposure to a single chemotherapeutic agent can develop multidrug resistance in some patients [28]. This study demonstrated that the $MSI2^{+}$ glioblastoma tumor cells with high MGMT expression were resistant to TMZ. In contrast, treatment with TMZ did not significantly upregulate MSI2 expression. However, silencing of MSI2 expression in $MSI2^{+}$ glioblastoma tumor cells significantly inhibited MGMT expression and tumor growth as well as sensitized the TMZ therapy in vivo. The effect of MSI2 on reversing TMZ resistance is associated with the inactivation of EMT. Therefore, high MSI2 expression confers the primary resistance of glioblastoma to TMZ associating with the activation of glioma stem cells and EMT.

TGF-β has been demonstrated to promote EMT in glioblastoma cells by inducing phosphorylated Smad2/3 [13].

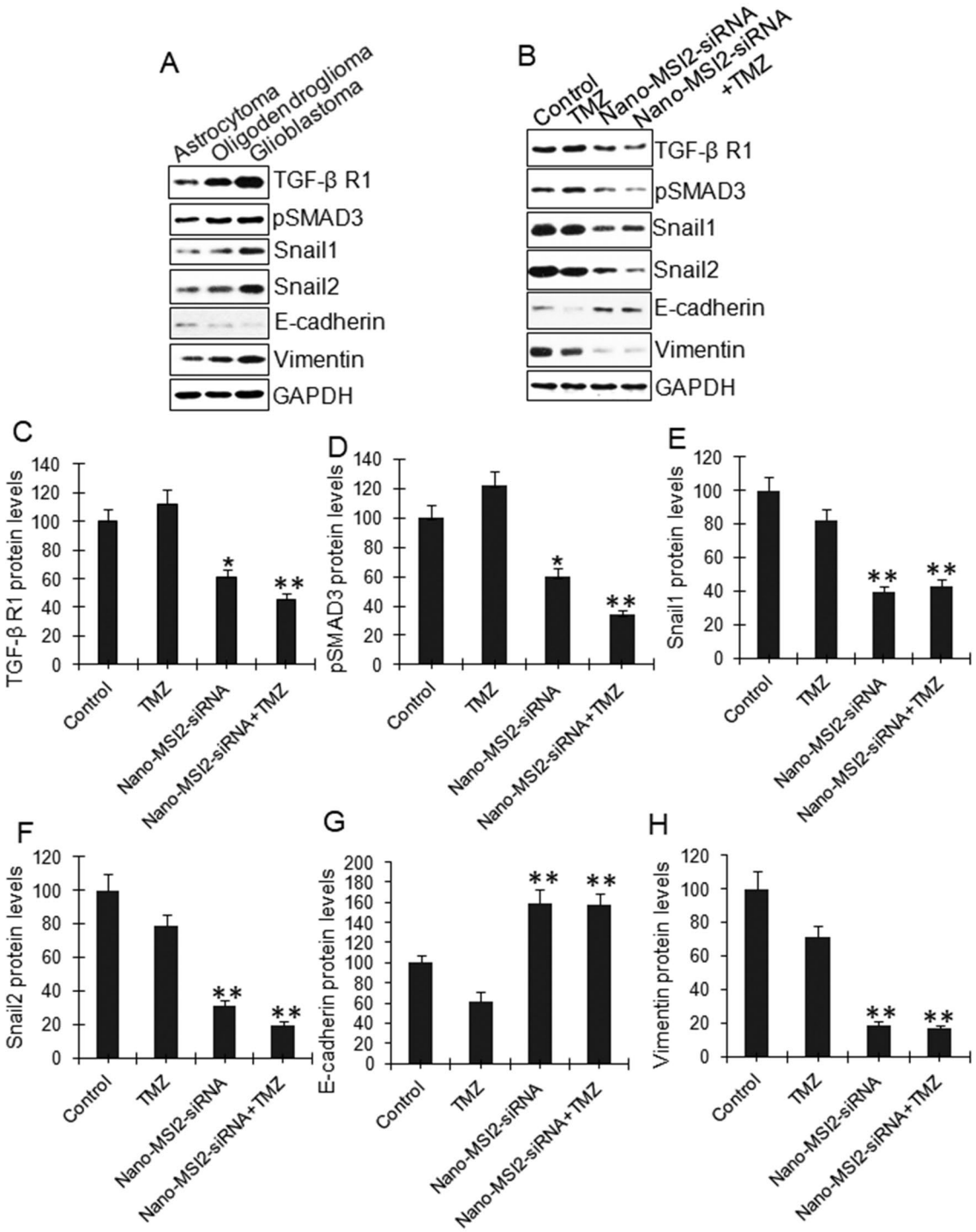

Fig. 4 EMT was activated in glioma tumors and xenograft LN-18 tumor tissues. **a** Representative western blots of EMT-associated proteins, TGF-β R1, and SMAD3 proteins in astrocytoma, oligodendroglioma, and glioblastoma tumor tissues. **b** Representative western blots of EMT-associated proteins, TGF-β R1, and SMAD3 proteins in xenograft LN-18 tumor tissues. **c** Bar diagrams describe quantitative estimates of TGF-β R1 protein in xenograft LN-18 tumor tissues. **d**–h Bar diagrams of quantitative estimates of pSMAD3, Snail1, Snail2, E-cadherin, and vimentin protein expression in xenograft LN-18 tumor tissues. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. control group. $N = 10$

In contrast, MSI2 has been demonstrated to regulate hematopoietic stem cells through controlling TGF-β R1/SMAD3 signaling [14, 15]. This study demonstrated that silencing of MSI2 gene expression significantly downregulated the TGF-β R1 protein expression and SMAD3 phosphorylation in glioblastoma tumor cells, whereas overexpression

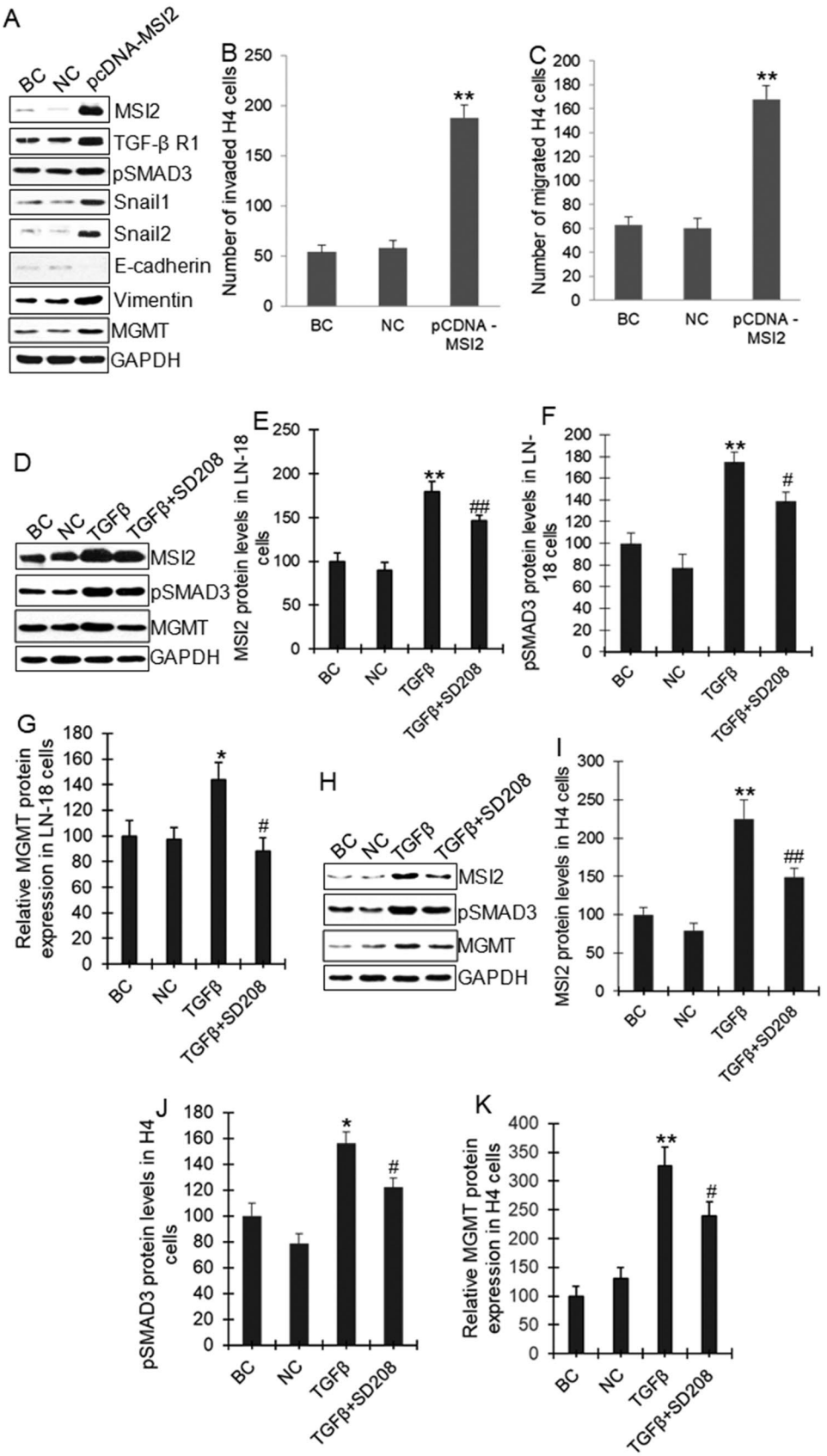

Fig. 5 The effect of MSI2 in glioma tumor cells and its regulation. The overexpression of MSI2 was established in H4 cells by transfection of pCDNA-MIS2 vector. **a** Representative western blots of changes of EMT-associated proteins, TGF-β R1, pSMAD3, and MGMT proteins in H4 tumor cells after overexpression of MSI2 expression. **b** Number of invaded H4 tumor cells. **c** Number of migrated H4 tumor cells. MSI2 overexpression increased the invaded and migrated H4 tumor cells. The LN-18 and H4 tumor cells were treated with or without 20 ng/ml of TGFβ1 and/or 20 ng/ml of TGFβ receptor inhibitor SD208 for 48 h. **d** Representative western blots in LN-18 cells. **e** Bar diagrams describe quantitative estimates of MSI2 protein in LN-18 cells from western blot analyses. **f** Bar diagrams describe quantitative estimates of pSMAD3 protein in LN-18 cells. **g** Bar diagrams describe quantitative estimates of MGMT protein in LN-18 cells. **h** Representative western blots in H4 cells. **i** Bar diagrams describe quantitative estimates of MSI2 protein in H4 cells from western blot analyses. **j** Bar diagrams describe quantitative estimates of pSMAD3 protein in H4 cells. **k** Bar diagrams describe quantitative estimates of MGMT protein in H4 cells. $^{*}P<0.01$, $^{**}P<0.001$ vs. BC group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. TGF β group. $N=4$

of MSI2 upregulated TGF-β R1 protein expression and SMAD3 phosphorylation in neuroglioma cells. The recombined TGF-β1 significantly increased MSI2 protein expression and SMAD3 phosphorylation, but TGF-β inhibitor SD208 partially blocked the effects of TGF-β1 in human neuroglioma cells with low MSI2 expression and glioblastoma cell with high MSI2 expression. These findings suggest that MSI2 and TGF-β signaling form a positive feedback which may regulate glioma stem cell growth and stimulate EMT activation in glioblastoma and subsequently increase MGMT expression and resistance of glioblastoma cells to TMZ.

The EMT process is initiated by activation of several key transcription factors. This study measured Snail1 and Snail2 expression. The MSI2 levels correlated with the Snail1 and Snail2 levels in all tests. The EMT activation is accompanied with decrease in E-cadherin and increase in vimentin gene expression [7]. This study showed that E-cadherin is expressed less abundant in glioblastoma tumor cells, but the regulatory effect of MSI2 on E-cadherin is still significant. In contrast, vimentin is highly expressed in glioblastoma tumor cells. Silencing of MSI2 expression downregulated, but overexpressing of MSI2 significantly upregulated vimentin protein expression. These findings suggest that MSI2 regulates the activation of EMT in glioblastoma.

In conclusion, this study found a positive feedback loop of MSI2-TGF-β/TGF-β R1/SMAD3 signaling which activates the EMT and may contribute to the invasion and chemoresistance of glioblastoma. This study also highlights a possible new target for the therapy of most malignant type of glioma. Introduction of siRNA or other agents into brain to target tumor cells is still limited by the blood–brain barrier. Nanoparticle-based delivery options may overcome this obstacle. However, glioblastoma is heterogeneity and the observations from cell lines may not provide enough supports for this conclusion of a new therapeutic target.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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